CONTINUOUS CHROMATOGRAPHIC BIOCHEMICAL REACTION - SEPARATION

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SUMMARY

Combined bioreaction separation studies have been carried out for the first time on a moving port semi-continuous counter-current chromatographic reactor-separator (SCCR-S1) consisting of twelve 5.4cm id x 75cm long columns packed with calcium charged cross-linked polystyrene resin (KORELA VO7C).

The inversion of sucrose to glucose and fructose in the presence of the enzyme invertase and the biochemical synthesis of dextran and fructose from sucrose in the presence of the enzyme dextransucrase were investigated. A dilute stream of the appropriate enzyme in deionised water was used as the eluent stream.

The effect of switch time, feed concentration, enzyme activity, eluent rate and enzyme to feed concentration ratio on the combined bioreaction-separation were investigated.

For the invertase reaction, at 20.77% w/v sucrose feed concentrations complete conversions were achieved. The enzyme usage was 34% of the theoretical enzyme amount needed to convert an equivalent amount of sucrose over the same time period when using a conventional fermenter. The fructose rich (FRP) and glucose rich (GRP) product purities obtained were over 90%.

By operating at 35% w/v sucrose feed concentration and employing the product splitting and recycling techniques, the total concentration and purity of the GRP increased from 3.2% w/v to 4.6% and from 92.3% to 95% respectively. The FRP concentration also increased from 1.82% w/v to 2.88% w/v.

A mathematical model was developed for the combined reaction-separation and used to simulate the continuous inversion of sucrose and product separation using the SCCR-S1.

In the biosynthesis of dextran studies, 52% conversion of a 2% w/v sucrose concentration feed was achieved. An average dextran molecular weight of 4 million was obtained in the dextran rich (DRP) product stream.

The enzyme dextransucrase was purified successfully using centrifugation and ultrafiltration techniques.

KEY WORDS:- Continuous chromatography, biochemical reaction-separation, sucrose inversion, dextran biosynthesis, enzyme purification.

Dedicated to

My Family

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CHAPTER 1

INTRODUCTION

1.0 INTRODUCTION

For many years the principle of chromatography has been applied to the separation of multi-component carbohydrate, hydrocarbon and essential oil mixtures amongst others. Chromatography has been used extensively in the analytical field and over the past twenty years, it has also found commercial large scale applications in the carbohydrate, pharmaceutical, petrochemical and cosmetic industries.

It was not until the early 1960's, that the application of chromatographic systems as combined reactor separators was investigated almost simultaneously by Magee [1] and Dinwindie [2] in the USA and Roginski et al [3] in USSR. Chemical reversible reactions were considered in all cases.

The chromatographic reactor was defined by Langer et al. [4] as the chromatographic column in which a solute or several solutes are intentionally converted either partially or totally to products during their residence in the column. In the chromatographic reactor-separator, both conversion to products and product separation take place simultaneously in the same colunn. The great promise of this innovation resulted in substantial research work being carried out as follows:

- In studying the reaction kinetics and mechanism of chemical reactions.
- In "shifting" equilibrium chemical reactions towards completion. For example in reactions such as:

$$A \rightleftharpoons B + C$$

the simultaneous removal of either B or C will cause a favourable shift in equilibrium.

The first industrial reactions performed in a chromatographic reactor were the dehydrogenation of butane and butenes [2], the water gas shift reaction and the Deacon reaction [1]. All these reactions were carried out on small scale batch chromatographic columns.

Although considerable work has been carried out in the chemical field, there has been no application in the biochemical field until the investigation of the work carried out by Barker and co-worker [5] which was initiated six years ago. In their work the biosynthesis of dextran in the presence of the enzyme dextransucrase was carried out on batch chromatographic reactor-separators varying in size from 0.97 to 5.4cm diameter and 30 to 200cm column lengths. By employing the simultaneous reaction and separation principle they obtained higher dextran yields. Characteristically at 20% W/V sucrose feed concentration, the amount of high molecular weight (over 157 000 daltons) dextran produced using the batch chromatographic bioreaction separator was almost twice as much as that produced at similar conditions using a conventional batch fermenter.

In the past thirty years, there has been an increased interest in the development of continuous counter-current chromatographic systems used in the separation field. This is mainly because the continuous counter-current system makes better use of the available mass transfer area, offers constant product quality and requires no product recycling. One of these systems is the semi-continuous chromatographic refiner (SCCR) which has been developed in this department.

The aim of this research work is to study the employment of such a semi-continuous counter-current chromatographic refiner as a combined biochemical reactor-separator. A semi continous chromatographic biochemical reactor separator (SCCR-S1) was therefore constructed for this work. It consisted of twelve columns, each of 5.4cm id x 75cm long and packed with calcium charged crosslinked polystyrene resin and having a size range between 150 to 360μm and an average particle size of 270μm.

The objectives were to study for the first time the continuous counter-current bioreaction separation by following more than one reaction, to optimise the equipment's performance by evaluating the effect of various operating parameters such as feed

concentration, eluent to feed rate ratio and feed to enzyme concentration ratio and finally to model its operation. The continuous conversion of the substrate and the production of product purities of 90% and above was aimed for. Improving the product concentration and reducing the enzyme usage was also of interest.

Two biochemical reactions were considered namely, the inversion of sucrose to glucose and fructose using the enzyme invertase and the biochemical synthesis of dextran and fructose from sucrose in the presence of the enzyme dextransucrase. The main emphasis of this work is mainly to carry out the inversion of sucrose. The dextran synthesis will be considered towards the later part of this work where the knowledge gained from the inversion studies will be utilised.

Fructose is known to be 1.8 times sweeter than sucrose in cold conditions [6]. There is also an increased demand for high fructose corn syrup (HFCS) as a sucrose substitute by the food and beverage industries. In the UK it sells for £1,200 per ton in its crystalline form. Dextran is a glucose polymer and for many years has been used in the field of medicine as a blood plasma volume expander, in the treatment of anaemia and for other industrial uses. In industry the production of large amounts of high molecular weight (HMW) dextran is preferred. During the biosynthesis of dextran, the fructose produced, acts as an acceptor molecule. Therefore, by removing fructose from the reaction mixture immediately as it is formed the dextran yield can be improved [5]. In the sucrose inversion reaction, the combined operation should be more economical and is expected to reduce problems related to substrate inhibition.

In carrying out these biochemical reactions, large amounts of enzymes are required. Although the invertase enzyme is commercially available in the purified form, the dextransucrase enzyme is not and for this work it had to be produced and purified in the department. To obtain enough dextransucrase enzyme for the research work, it was decided to investigate and select the appropriate separation processes that would purify

the crude dextransucrase enzyme produced by fermentation. The criteria for enzyme purification process selection were to be low enzyme denaturation, ease of operation, scaling up potential and good process integration.

Finally the mathematical modelling and computer simulation of the semi-continuous counter current bioreaction-separation was carried out by considering the sucrose inversion reaction.

CHAPTER 2

CHROMATOGRAPHIC PRINCIPLE

2.0 INTRODUCTION

Chromatography covers in general, a wide range of applications, from laboratory scale analytical techniques to large scale processes applied to food, chemical and biochemical industries. It is a unit operation where the separation of solutes occur due to their differential migration rates through a system of two phases, the mobile and the stationary phases.

The major contribution to the development of gas chromatography was by Martin and Sygne [7] whose work was recognised by the award of the Noble Prize. Giddings [8] later made a major contribution to the understanding of chromatographic operation through his theoretical work.

The use of a chromatographic system as combined reactor-separators in gaseous chemical application was first published simultaneously 27 years ago, both by Magee [1] and Dunwiddle [2] in the USA and Roginski et al. [3] in the Soviet Union. Since then a series of papers dealing with theoretical and experimental studies of chromatographic reactor-separator systems has been published.

This chapter gives a brief illustration of the principle, terminology and theories of chromatography, with a general introduction to the basic concept of chromatographic reactor-separators and their development.

2.1 PRINCIPLE OF CHROMATOGRAPHY

The principle of chromatography follows the mechanism that determines the regularities of the movement and the spreading of the chromatographic zone. This mechanism includes convective transportation, diffusion and sorption equilibration. However, the occurrence of differential migration along the column and hence separation is determined by the differences in magnitude of distribution coefficients of components in the column.

Geometrical irregularities of the chromatographic bed and the hydrodynamic properties of the fluid result in an uneven mobile phase flow velocity, hence the formation of velocity profiles.

The chromatographic efficiency therefore, depends on the uniformity of the bed, the mobile phase superficial velocity, the distribution coefficient differences, the speed in establishing a sorption equilibrium and on the rate of longitudinal diffusion of the solute in both phases.

2.2 CLASSIFICATION OF CHROMATOGRAPHY

The classification of chromatography into the types of phase employed and mode of operation have already been documented [9, 10]. Figure 2.1 shows the application of these groups.

The elution technique of discharging solutes from a chromatographic column can be classified into:

(a) Frontal analysis

(b) Displacement development

(c) Solution Development

Figure 2.2 gives a schematic representation of the three chromatographic techniques.

Frontal Analysis (Figure 2.2a)

When using this technique a sample is introduced into a column continuously. The less adsorbed Component A, migrates faster and is eluted pure until the accumulated strongly adsorbed Component B breaks through with newly introduced Component A.

Figure 2.1:- Classification of Chromatography

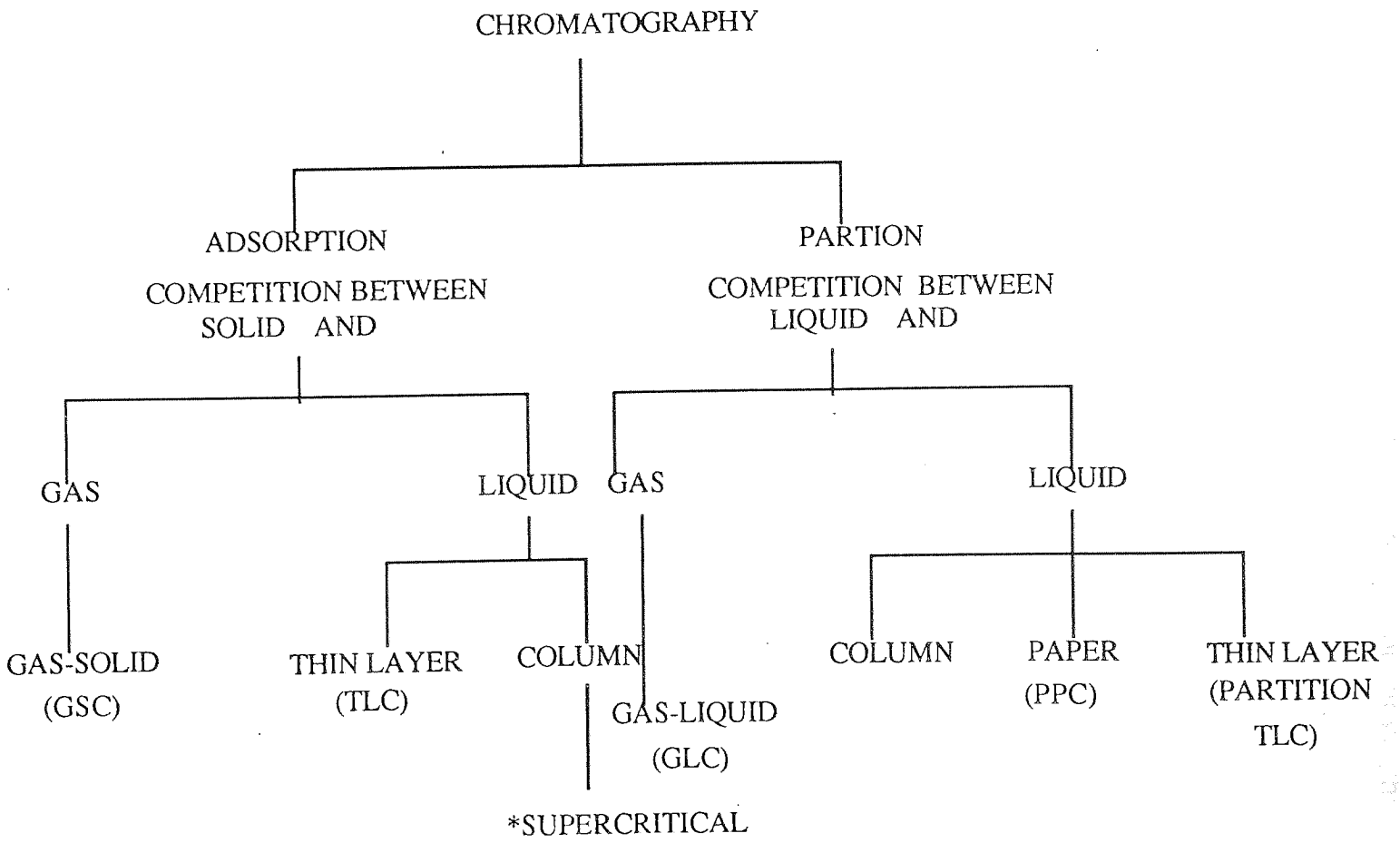
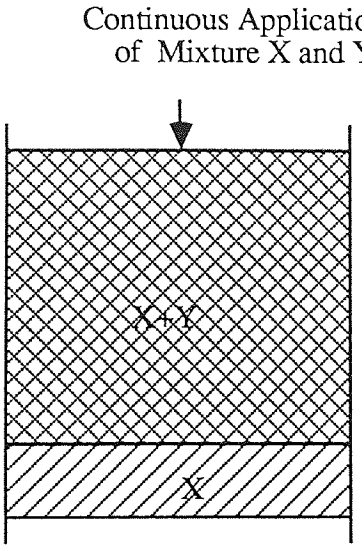
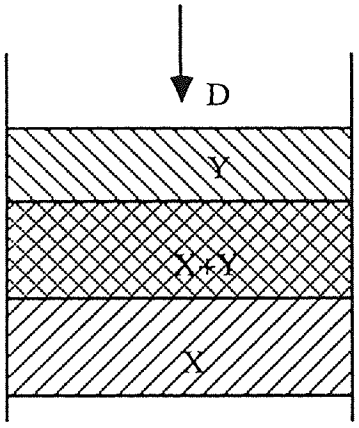


Figure 2.2:- Schematic Representation of the three Chromatographic Techniques



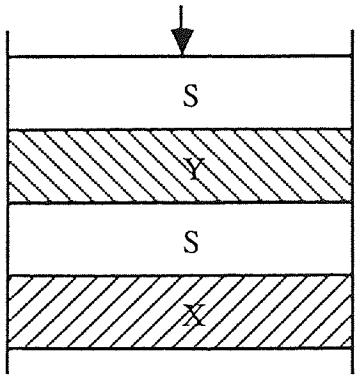
(a) FRONTAL ANALYSIS



(i) Mixture X and Y Introduced

(ii) Displacing solvent (D) Begins

(b) DISPLACEMENT DEVELOPMENT



(i) Mixture X and Y Intoduced

(ii) Solvent (S) Begins

(C) ELUTION DEVELOPMENT

Displacement Development (Figure 2.2b)

In displacement chromatography the mobile phase is more strongly retained by the stationary phase than the sample. The sample is therefore moved through the bed by the advancing mobile phase. Thus this technique is basically involved in the final stripping of a column or in the preliminary cleaning with a highly polar solvent.

Elution Development (Figure 2.2c)

In elution chromatography, the sample injection to a column is followed by the continuous flow of eluent. Development of component bands occur as the sample components migrate down the packed bed. The separation of the mixtures depend on the relative component distribution coefficients between the two phases. This technique is the most commonly used and the one employed for this research work.

2.3 CHROMATOGRAPHIC THEORY

When a sample is injected into a chromatographic column, the separation of its components is due to the relative affinities they exhibit for the column packing. By approximating the elution profiles to a Guassian distribution curve, the chromatographic separation can be illustrated by Figure 2.3.

The retention time (tr_A, tr_B, tr_C) is defined as the average time a component takes to be eluted from the column. It is measured to the midpoint of the elution curve.

The retention volume is the product of the retention time and the mobile phase flow rate. It is defined as the volume of the mobile phase required for complete elution of a compound.

The fundamental retention equation which relates the retention volume of component i, V_i, and the distribution coefficient, Kd_i, for any component i, is given by:

$$V_i = V_o + Kd_i V_s \qquad 2.1$$

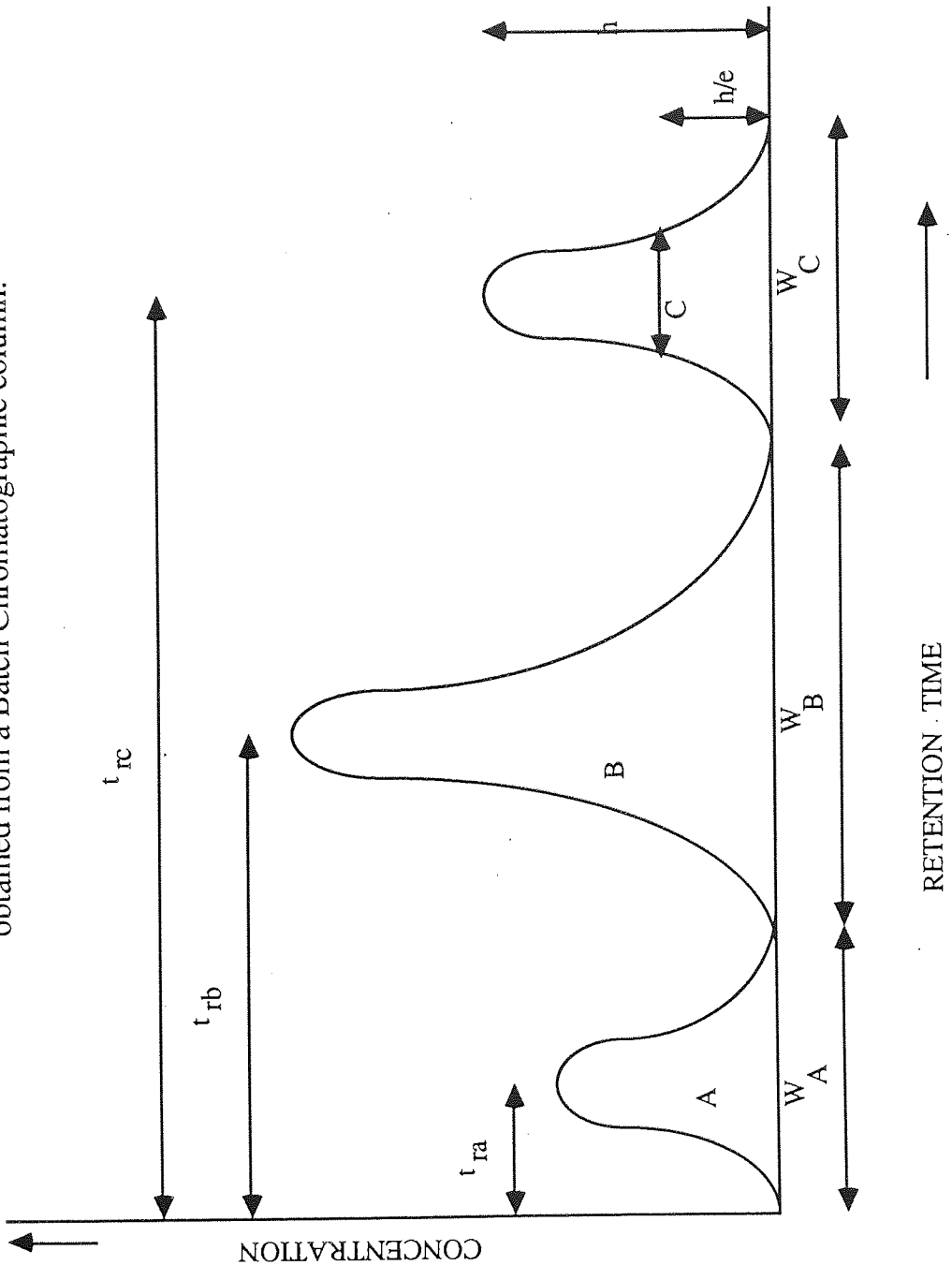


Figure 2.3:- A Typical Chromatogram for a three componet mixture obtained from a Batch Chromatographic column.

where

V_o = Total void volume in a column

V_s = Volume of stationary phase = $V_T - V_o$

V_T = Total empty column volume

This equation was originally defined by Kirkland [11] for gel permeation chromatography.

The capacity factor, another useful term in chromatographic terminology, is defined as the ratio of the amount of solute in the stationary phase to the amount in the mobile phase.

$$K_i = Kd_i \frac{V_s}{V_o} \qquad 2.2$$

The resolution of a chromatographic column is a measure of the degree of separation between two adjacent bands. It is defined as the distance between the peak maxima divided by the individual peak width.

$$R_s = \frac{2\,(tr_B - tr_A)}{W_B + W_A} \qquad 2.3$$

where tr_i = retention of component i

W_i = peak width at the base of elution curve of component i

By transforming Equation 2.3 to incorporate equilibrium distribution coefficients and the column dispersion factor, Purnell [12] showed that other parameters influence the resolution, i.e

$$R_s = 1/4 \left(\frac{\alpha - 1}{\alpha}\right) \left(\frac{K_B}{1 + K_B}\right) (N)^{1/2} \qquad 2.4$$

where

N = Number of theoretical plates

$\alpha = \frac{Kd_B}{Kd_A} = \frac{K_B}{K_A}$ = Separation factor

K_B = Capacity factor of component B

In normal chromatographic practice, a resolution of 1.0 is considered satisfactory, while values greater than 1.0 indicate better separation efficiencies.

2.4 CHROMATOGRAPHIC THEORIES ON BAND BROADENING

When a narrow band of feed is introduced into a chromatographic column it is subjected to non-uniformities of flow, diffusion of solutes in and out of the packing and mass transfer between the two phases, which result in band broadening.

Various researchers have shown that many factors affect band broadening and mathematical models developed to describe the band broadening have been reported by Giddings [8]. The most popular are the "theoretical plate concept" and the "rate theory".

2.4.1 The Theoretical Plate Theory

The theoretical plate theory has been used extensively in describing the separation performance of processes such as distillation and extraction operations and understandably Martin and Synge [7] followed a similar approach in quantifying the chromatographic column performance.

In a chromatographic column, equilibrium is difficult to establish due to the continuous motion of one of the phases. The column is visualised as being divided into zones or "plates" with each zone having such a length that complete equilibrium between the two phases can be assumed.

Mathematical models for using the theoretical plate theory in a chromatographic column have been developed [8].

The most common expression used is that developed by Glueckauf [13] when he related the elution time (tr_i) and variance to an "apparent number" of theoretical plates (N*) in the following form:

$$N^* = 8\left(\frac{t_{ri}}{W^h/e}\right)^2 \qquad 2.5$$

where $W^h/_e$ is the band width measured at a height equal to the peak height, h, divided by the base of the natural logarithm, e, Figure 2.3.

The major criticisms of the plate theory concept are that apart from its simplicity, it fails to account for the contribution of molecular structure, sorption phenomenon, temperature, molecular distribution, and flow pattern toward zone spreading.

2.4.2 Rate Theory

The foundation for this theoretical model which includes mass transfer and a longitudinal term, was first provided by Lapidus and Amundson [14]. The model was extended by van-Demeter et al [15] to include axial diffusion and finite rates of mass transfer contribution.

The form of the equation is:

$$H = A + \frac{B}{V} + Cm'V + Cs'V \qquad 2.6$$

where

H	=	Plate height
A	=	Eddy diffusion term
B	=	Longitudinal diffusion term

Cm', Cs'	=	Resistance to mass transfer in mobile and stationary phase respectively.
V	=	Mobile phase velocity

2.5 CHROMATOGRAPHIC REACTOR-SEPARATOR

Previous work in the field of chromatographic reaction separation has been limited to chemical gas applications.

Studies carried out on chromatographic reactors separators have been principally targeted on the kinetics and mechanism of chemical reactions for analytical purposes [16, 17], and improving the equilibrium conversion in reversible chemical reactions [17 - 20] Langer [16] and Haittori [21] used the chromatographic reactor to measure kinetic rates. Roginski and Coworker [17, 18] obtained a 20% improvement in conversion over equilibrium with the dehydrogenation of cyclohexane into benzene and butene into butadien.

As yet the potential of chromatographic reactor separators has still to be exploited in the biochemical reaction field. Based on the literature available, the only applications in the biochemical field are the work of Hashimoto et al [23] who devised a continuous process for high purity fructose production involving a moving bed system combining selective adsorption of fructose onto a cross-linked polystyrene resin in the Ca^{2+} form and an immobilised glucose isomerase reactor and of Barker et al [5] who studied the behaviour of a batch chromatographic reactor separator for the biosynthesis of dextran and fructose from sucrose in the presence of the enzyme dextransucrase. By use of a cross-linked polystyrene resin in the Ca^{2+} form and under specific operating conditions the fructose and dextran was separated.

2.5.1 Principle of Chromatographic Reactor-Separators

During the 1960's gas chromatographic reaction separation was the subject of many studies. This is partly due to the simplicity and attractiveness of its principle.

When a separation process such as chromatography and a chemical equilibrium reaction occur simultaneously, the quality and the amount of the reaction products are very different from those obtained when the reaction occurs alone at steady state.

Reaction can either take place in the mobile phase, similar to a packed bed reactor, with the stationary phase acting as an adsorber, or it could take place in the stationary phase which will act as a catalyst as well as an adsorber with the mobile phase being inert. In certain cases reaction in both phases is possible.

According to Langer [4] in an ideal chromatographic reactor:

- a non-steady state pulse reacts as it is swept through the column, with the resulting reaction products instantaneously separated from the reactant pulse, implying on infinite difference in retention volume.
- The column is homogeneous in composition; neither the mobile nor the stationary phases changes in composition except during passage of small concentrations of eluent. The mobile phase linear velocity is constant throughout the column.
- The height of a theoretical plate approaches zero. Peak spreading and axial diffusion are considered negligible.
- Reaction rates are chemically controlled with mass transfer or the adsorption rates not limiting.
- The distribution isotherms between mobile and stationary phases are linear.
- The column is isothermal and heat effects of both solution and reaction are negligible.

There are three major advantages in using a chromatographic reactor as opposed to a batch, plug flow or a stirred tank reactor.

(i) Inclusion of two unit operations into one would help in reducing both operating and capital cost.

(ii) The instantaneous separation of products and reactants allows greater conversions to be obtained with reversible reaction.

(iii) In some reactions, inhibitors are formed which prevent the reaction from proceeding further or unwanted by-products being formed. In a chromatographic reactor this problem can be overcome by separating the inhibitor from the reactants.

2.5.2 Reactions Under Chromatographic Conditions

Most of the chemical reactions studied to date on a chromatographic reactor-separator are reversible [4]. Matsen et al [26] presented conditions for operating a chromatographic reactor. They stated that:

(i) The reaction must be reversible, and the equilibrium constant must be small.

(ii) The separation of product should limit the reaction - i.e. reaction rates should be relatively high.

(iii) At least two chromatographically separable products must be formed.

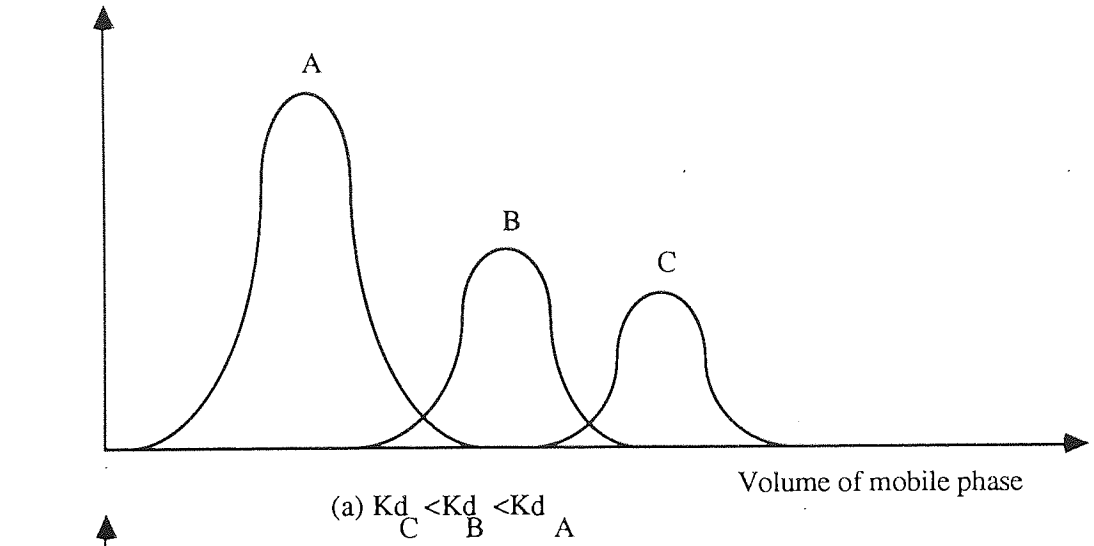
(iv) Reactants must not be separated in the reactor.

Under these conditions and with the objective of improving product yield, two types of chemical reaction could be considered.

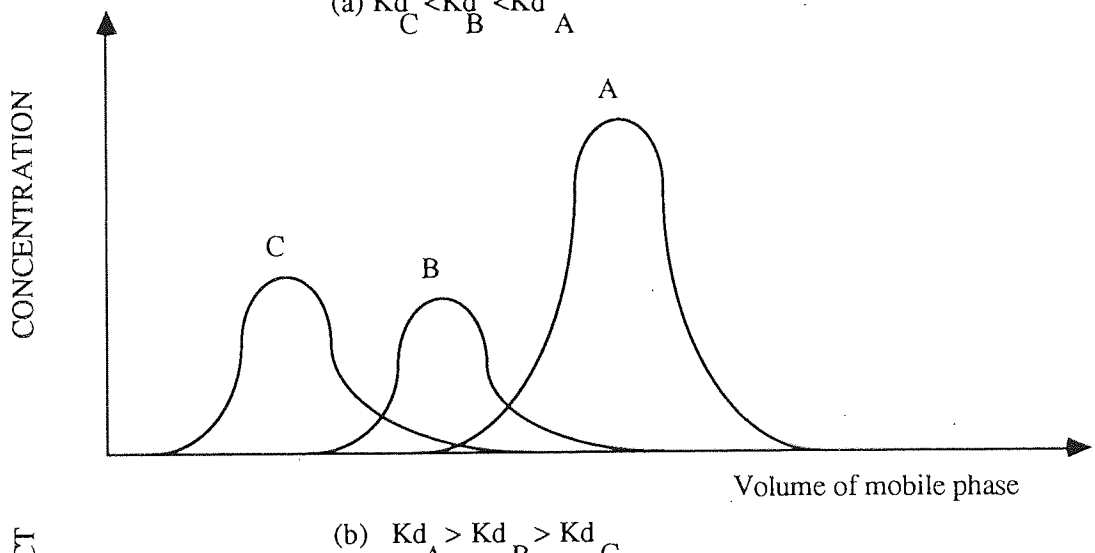
Type A Reaction:- $A \rightleftharpoons B + C$

For linear adsorption isotherms and instantaneous establishment of adsorption equilibrium, Figure 2.4 illustrates three possible relative arrangements of reactant A and products B and C on a chromatographic reactor-separator based on their relative distribution coefficients (Kd_i).

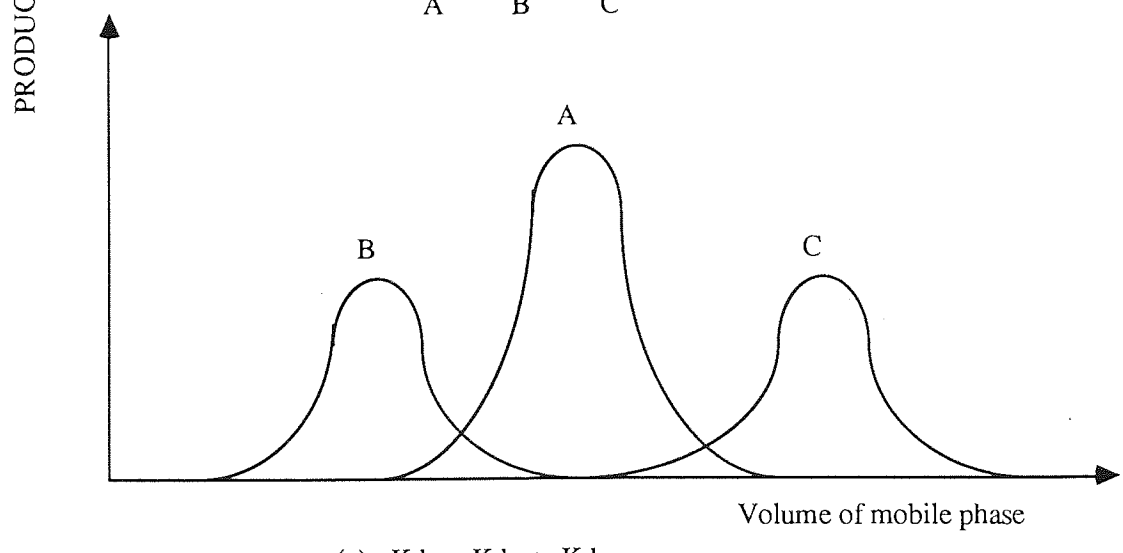
Figure 2.4:- Concentration Profiles when partial conversion is obtained under Pulse Chromatographic conditions.



(a) $Kd_C < Kd_B < Kd_A$



(b) $Kd_A > Kd_B > Kd_C$



(c) $Kd_C > Kd_A > Kd_B$

When the order of the relative values of the distribution coefficients are $Kd_C < Kd_B < Kd_A$ (Figure 2.4a), B and C will form tails at the rear of the unreacted band of A. Since this is an equilibrium reaction an overlap between B and C will occur. Thus A is regenerated which can result in poor purities of the product B and C. To obtain a high yield purity and conversion, one would require very large column lengths.

A similar result will occur when the distribution coefficient of reactant A is greater than both products - i.e. $Kd_A > Kd_B > Kd_C$ (Figure 2.4b).

An ideal situation is when the distribution coefficient Kd_A is greater than one of the products and lesser than the other - i.e. $Kd_C > Kd_A > Kd_B$ (Figure 2.4c). The products will move in an opposite direction to the reactant band, hence enabling higher conversions to be attained with high product purity [21].

Type B Reaction:- $A + B \rightleftharpoons R + S$

The requirements for this type of reaction are:

(i) Reactant A and B should have the same distribution coefficient ($Kd_A = Kd_B$), if the reaction is taking place in the mobile phase.

(ii) Product R must be separated from product S and from reactant A and B (i.e. $Kd_R \neq Kd_S \neq Kd_A = Kd_B$).

(iii) The rate of reaction must be high enough to promote the conversion of reactants during their transit in the column.

An example of this type of reaction is the esterification of ethanol by acetic acid catalysed by a strong acid ion-exchanger resin in the H^+ form [27].

The same combined chromatographic reaction-separation principle can also be applied to biochemical reactions. Biochemical reaction products tend to have toxic compounds, inhibitors and other unwanted impurities which prohibit high yield product

formation. Zafar [25] has shown that a higher yield molecular weight dextran, greater than that carried out in a conventional reactor, can be obtained when the biosynthesis of dextran from sucrose in the presence of the enzyme dextransucrase is carried out on a batch chromatographic reactor-separator.

2.5.3 Factors Affecting Chromatographic Reactor-Separator Performance

Researchers studying the chromatographic bioreaction-separation have identified the following factors that effect the extent of conversion and product yields:

(1) Pulse Size:- An increase in pulse size, with all other operating conditions constant, results in a decrease in the degree of conversion. This was experimentally verified by Matsen et al [26] when working on the dehydrogenation of cyclohexane to benzene on an alumina catalyst. Roginski et al [18] suggested that an absolute yield of product should reach a maximum after which it becomes independent of sample size. However, no maximisation of yield was reported in their work.

(2) Flow Rate:- At low flow rates the conversion of the reactant is independent of residence time. Matsen [26] found this was caused by a fast adsorption reaction and desorption process and that the extent of reaction was equilibrium limited. Mile et al [28] in their work found two features of interest; the occurrence of a maximisation of conversion products at certain flow rates and the fact that this maximum is well above the equilibrium conversion values (ca. 50%). These features are indicative of the displacement of equilibrium by the chromatographic process. It is expected that at high flow rates the conversion obtained will be low due to a drop in residence time. This will prevent higher than the equilibrium conversion being obtained in the case of equilibrium limited reactions.

(3) Reactor Length:- The degree of conversion is markedly increased with increased reactor length. This is because in equilibrium reactions or in product inhibited reactions peak overlap is greatly reduced. Increased bed

length can also result in an increase in surface area for the reaction and hence an increase in conversion [28].

(4) Repetitive Pulses:- When considering this effect one has to take into account the frequency at which pulses are injected into the column. Gore [29] in his work found that the maximum average conversion of a chromatographic reactor is rather sensitive to injection frequency. Matsen et al [26], observed an optimum frequency after which conversion decreases steadily.

Although in industrial applications it is important to employ repetitive pulsing, the frequency of pulsation needs to be controlled to maximise product yield and purity.

2.6 INDUSTRIAL APPLICATION OF CHROMATOGRAPHIC REACTOR-SEPARATORS

The potential applications of chromatographic reactor-separators on a batch scale were first recognised by Dinwiddie [2], Magee [1] and Gazier [30]. The Haber process reported by Unger [20] is a key example of a similar reaction that can be carried out industrially. The repetitive pulse mode operated on the Haber process, resulted in repurification of the hydrogen product produced. As a result the chromatographic reactor can only be used as a first stage in series with a conventional ammonia synthesis process.

Chromatographic reaction-separation on a continuous basis has received little attention compared to batch, even though it has obvious advantages with respect to throughput and purity.

2.6.1 Continuous Chromatographic Reactor System

A continuous chromatographic system can be described as the unit operation which enables reaction to take place while it endeavours to utilise the main features of the

chromatographic process for the instantaneous resolution of the reaction products in a continuous manner [31].

The employment of a repetitive feed injection technique as a means of increasing throughput on a production batch chromatographic system, requires considerable recycling of the partially purified product to increase purity.

Low throughputs due to recycling, directed interest towards the use of continuous chromatographic processes which have been found to offer higher throughput and uniform product quality.

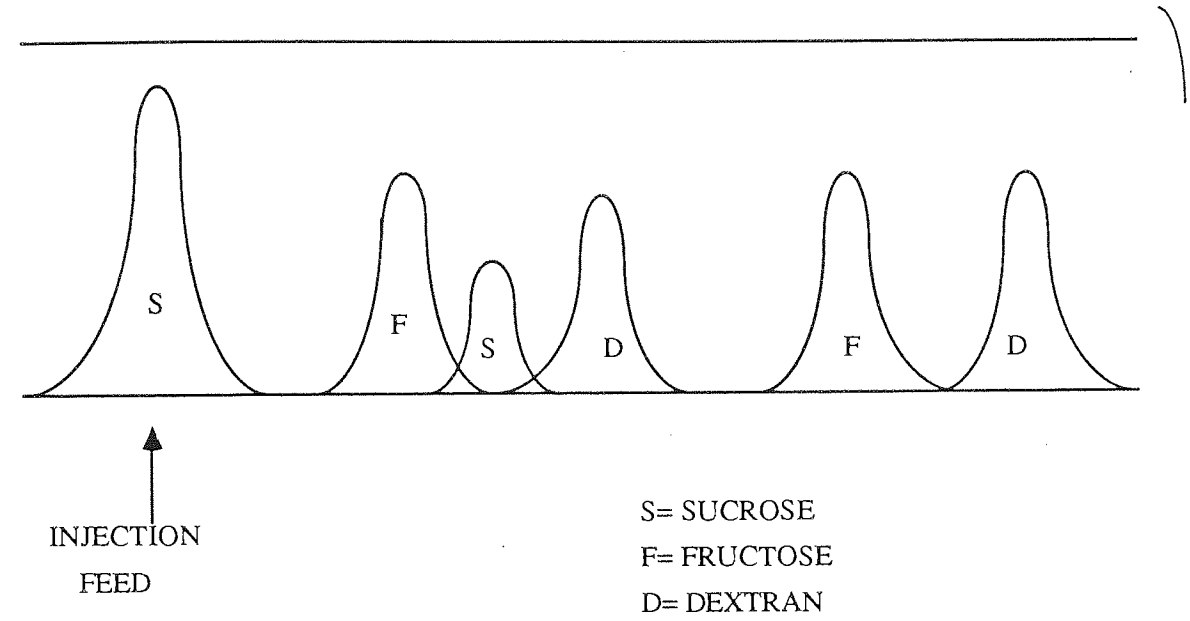
Continuous processes can be categorised into two groups, the counter-current and cross-current flow processes, defined according to the relative movement of the mobile phase to the stationary phases (Figure 2.5). Out of the two categories, the employment of counter-current flow for continuous chromatographic reactor is the most commonly used in a chromatographic reactor system.

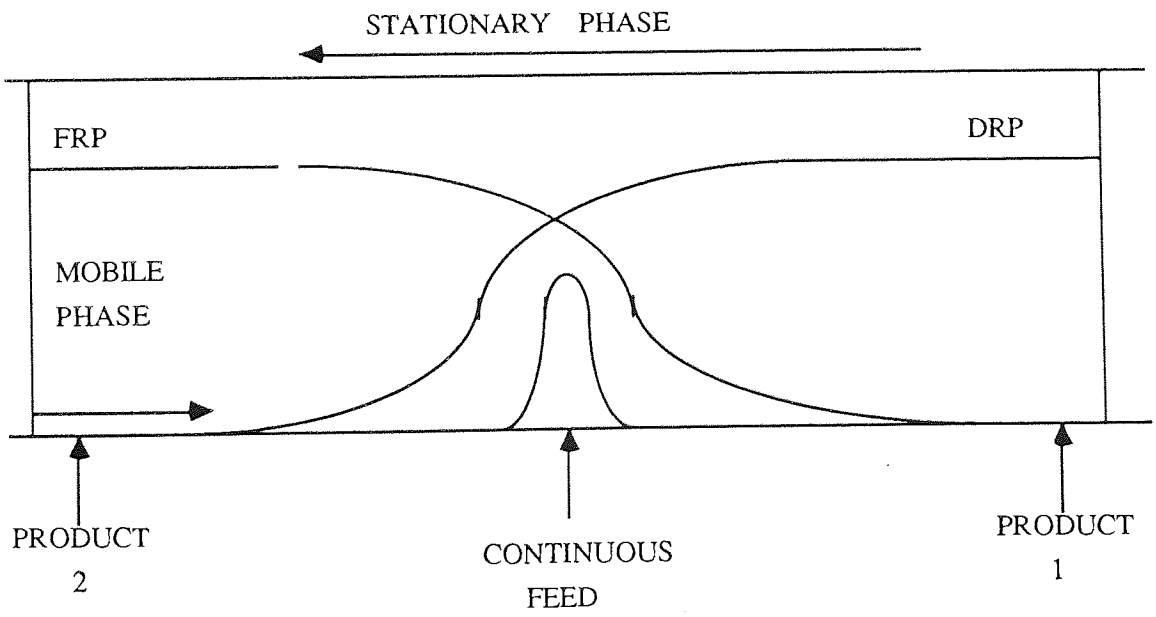
2.6.2 Cross-Current Flow Processes

In cross flow systems, the 'stationary' phase moves perpendicularly in the direction of the mobile phase. Cross-current flow processes can be categorised into two groups. The moving annulus system and the moving column-open end system.

As a chromatographic reactor-separator, only the annulus type systems have been employed. There are two types of chromatographic annulus reactors. The first one is the fixed feed with rotating bed type. In this case the reactant which is fed continuously from one stationary feed point at the top of the annulus is swept down the annulus by the eluent. Any unreacted reactants and products formed are separated in the column and leave with the eluent from the base of the vertical annulus. Due to the continuous rotation of the cylinder, there is both vertical as well as horizontal separation of the reaction mixture.

Figure 2.5:- Comparison of Cocurrent Process (a) and expected Countercurrent Flow Process (b).





The second type of reactor is where the cylinder is kept stationary but the feed and collection ports are rotated. Cho et al [32] used this type of reactor to study the acid catalysed hydrolysis of methyl formate. They also numerically simulated the reactor behaviour and obtained good agreement with the initial runs that were performed.

2.6.3 Counter-Current Flow Processes

Over the last 25 years in the separation field, counter-current chromatography has undergone three main stages of development, namely, moving bed, moving column and simulated moving bed.

In the field of reaction-separation, only the moving bed and the simulated moving bed have been employed.

2.6.3.1 Moving Bed Systems

These systems have been used in gas reaction-separation studies. In principle, chromatographic packings which also may act as catalysts flow under gravity counter-current to a stream of inert mobile phase in a vertical column.

The inert mobile phase contains reactants which are fed to the bottom of the reactor. Reaction and separation take place on the catalyst particles with the least strongly adsorbed products carried upwards, exiting from the top of the column, while the strongly adsorbed product is carried down the column where it is subsequently stripped from the packings by a heated stream of mobile phase.

Viswanathan and Aris [33] were the first ones to simulate a first order irreversible reaction (A -> B) in a falling bed reactor. The same type of reactor was investigated by Takeuchi et al [34 - 37] using a 14 mm diameter and 60 cm long system to study the catalytic oxidation of carbon-monoxide on activated alumina. The performance of a counter-current moving bed chromatographic reactor for reversible reaction was

examined theoretically by Petroulas and Carr [38]. They showed that reaction and separation can be achieved simultaneously in a reactor and under appropriate operating conditions, a reaction which favours thermodynamic equilibrium can lead to 100 percent product purity but with an overall conversion lower than a conventional fixed bed reactor.

Some of the problems highlighted in a counter-current moving bed column are:

- difficulties in achieving solid flow control when scaled up.
- Loss of mas-transfer efficiency due to comparatively uneven packing.
- Packing attrition due to the increased shear forces and packing entrainment.
- Mobile phase velocity limitations since it needs to be kept below the bed fluidisation velocity.

2.6.3.2(a) Moving Column Systems

To overcome the problems faced with moving bed systems, a number of equipments have been proposed based on the rotation of a closed circular column, packed with chromatographic packing and rotated past fixed inlet and outlet ports.

The objective was to make the packing and the column surrounding the packing move as one unit, thus avoiding the problem of attrition. Although a number of systems have been developed [39 - 43], scaling up is difficult because of the increased difficulties in achieving a reliable mechanical seal between the static ports and moving columns. There are two types of moving column systems. The moving feed point system and the simulated moving bed system.

The moving feed point system has only been used for separation and not reaction-separation studies. However, it has been found to be more efficient than batch processes when operated in a separation mode.

2.6.3.2(b) Simulated Moving Bed System

The potential of these systems is their ability to make better use of the available mass-transfer area and therefore better separation efficiencies and throughput can be achieved.

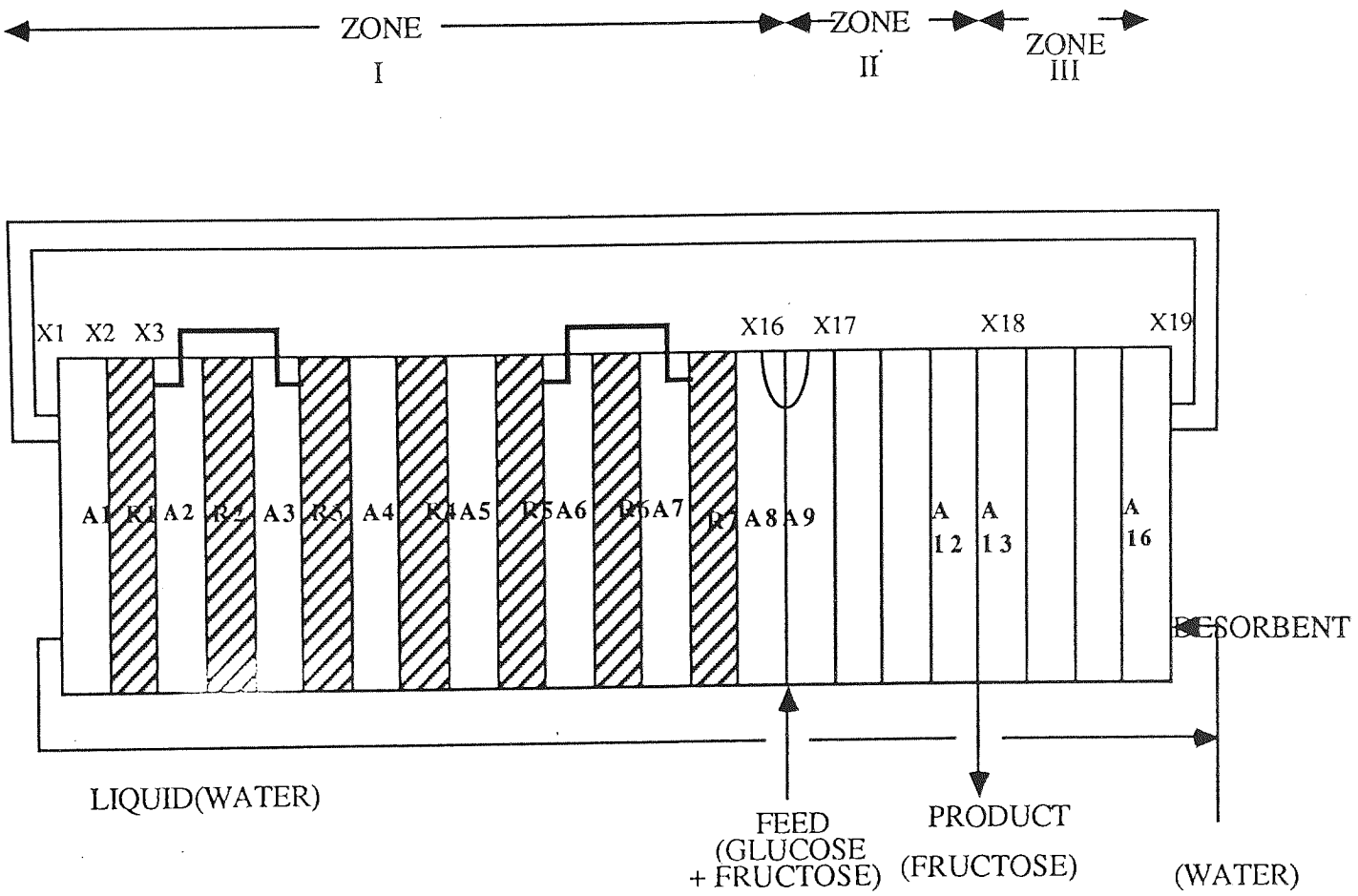
In a moving bed system as described previously, solid is moving in closed circuit past fixed inlet and outlet parts. The same effect can be achieved by holding the bed stationary and periodically altering the positions at which the feed and product streams enter or leave the system. Moving the inlet and outlet points in the direction of the main carrier fluid flow, the movement of solid in the opposite direction is simulated.

There are two design approaches to simulate moving bed systems. The Universal Oil Products (USA) Sorbex technique [44 - 46] and the semi-continuous chromatographic Refiners (SCCR) [47, 48].

In the Sorbex processes the chromatographic column is divided into compartments. Each compartment is connected to a specially designed rotary valve which simulates the counter current movement by operating on the principle of a multiport stop clock. For over a decade the Sorbex technique has been applied for separation only.

The Sorbex idea led Hashimoto et al [23] to develop a new continuous process for producing higher fructose syrup containing more than 50 percent fructose. The system comprises alternate columns containing cross linked polystyrene resin in the Ca^{2+} form and columns containing immobilized glucose isomerase. The system does not involve actual movement of the adsorbent particles thereby avoiding attrition problems. Its operation is best explained using Figure 2.6.

Figure 2.6:- Schematic representation of a system combining adsorption columns and Immobilised Glucose Isomerase reactors for producing HFCS (20)



The system is divided into 3 sections by the inlet and outlet points of the liquid streams. In section 1 the reactor and adsorbent columns are alternatively arranged, whereas section 2 and 3 contain only the adsorbent. Adsorbent particles move continuously to the right through the bed, skipping the reactors in section 1 and are returned to the left hand side of the apparatus by a mechanically rotating valve.

Some of the disadvantages of the Sorbex technique is the unreliability of large flat face moving seals associated with the rotary valve and the non-modular nature of the column compared to the SCCR system.

The experience gained through the long standing work in the development of chromatographic separators led Barker and co-workers to develop an alternative technique to simulate the counter current movement of the two phases. The "moving port" semi-continuous counter current refiners (SCCR) were developed whereby all face to face moving seals were eliminated by using valves of proven commercial reliability. The counter-current movement was simulated by simultaneous closing and opening of the appropriate valves connected to the inlet and outlet parts on each column, in the direction of the flow of the mobile phase.

These systems have been scaled up to ten columns, each of 10.8 cm id and 75 cm length. This type of system has been used in this research work and is described in detail in Chapter 5.

CHAPTER 3

ENZYME TECHNOLOGY

3.0 BACKGROUND HISTORY OF ENZYMES

Catalysis taking place in biological system was first recognised in the early 1800's from studies of the digestion of meat by secretions of the stomach and the conversion of starch into sugar by saliva and various plant extracts. Subsequently, a great many instances of biological catalysis, now known to be enzymatic have been recorded.

In the 1850's Louis Pasteur concluded that fermentation of sugar into alcohol by yeast is catalysed by "ferments" which were later named enzymes. He postulated that these enzymes are inseparable from the structure of living yeast cells. In 1897, Edward Buchner succeeded in extracting soluble active enzyme from yeast cells - the set of enzymes that catalyses the fermentation of sugar to alcohol. This was a major discovery in the history of enzymology, which proved that these important biocatalysts that catalyses a major energy yielding metabolic pathways, could still function when removed from the structure of living cells. It also encouraged biochemists to attempt the isolation of many different enzymes and to examine their catalyse properties.

3.1 STRUCTURE AND CHARACTERISATION OF ENZYMES

3.1.1 Structure and Properties

All enzymes consist primarily of proteins, moreover, their catalytic activity depends upon the integrity of their structure as proteins.

Structurally protein molecules can be divided into three classes; primary, secondary and tertiary structures. The primary structure consists of amino acids linked in linear sequences by peptide bonds due to condensation of the α - NH_2 and α - COOH groups of adjacent amino acids. Each specific protein has a single unique sequence of amino acids.

The secondary structure of proteins is a three dimensional arrangement of the amino acids in the peptide chain adjacent to one another. The conformation of the peptide chain depends on the bond length and angles formed along the backbone of the chain. X-ray diffraction studies established that two particular conformations are preferred, α - helix and β - pleated sheat. These conformations enable a maximum number of hydrogen bonds to be formed, resulting in maximum stability.

In addition to their secondary structure, proteins have a high order of structure due to interaction between side chains and between individual polypepticle chain. These interactions are capable of forming a range of non-covalent bonds such as hydrogen bonds, salt links, apolar bonds as well as covalent disulphide bridges.

Most enzymes have particular and specific conformation in their native and active states. Disruption of the specific conformation leads to a reduction or loss of biological activity. This loss in activity can be caused by exposing the enzyme molecule to heat, extreme pH changes, air-liquid interfaces, or by treatment with other denaturing agents.

Enzymes have molecular weights ranging from 12,000 to over a million [49]. They are therefore very large compared with the substrates or functional groups they act upon. The reacting site on the enzyme molecule also termed "active" site is a small part of the whole complex molecular structure. For activity, some enzymes require an additional chemical component called a cofactor. The cofactor may be either inorganic such as Fe^{2+}, Mn^{2+} or Zn^{2+} ions, on it may be a complex organic molecule called a coenzyme.

Enzymes accelerate specific chemical reactions without the formation of by-products and they function in dilute aqueous solutions under mild conditions of temperature and pH.

3.1.2 Enzyme Characterisation

To standardise the nomenclature of enzymes in a systematic manner, a commission was established under the auspices of the International Union of Biochemistry whose report was generally accepted as the basis for enzyme classification (49).

The classification of enzymes was based on the reaction they catalyse. Enzyme are grouped into six categories:-

Oxidoreductases	-	Transfer of electrons.
Tranferases	-	Group transfer reactions.
Hydrolases	-	Hydrolysis reaction (transfer of functional groups to water).
Lyases	-	Addition of groups to double bonds or the reverse.
Isomerases	-	Transfers of groups within molecules to yield an isomeric form.
Ligases	-	Formation of C-C C-S C-O and C-N bonds by condensation reactions coupled to ATP clearage.

Each of these categories are given an EC (Enzyme Commission) number and are then further subdivided into categories describing the substrate type and co-enzyme requirement. Finally a number refering to the individual reaction is assigned. Given a system of this flexibility any new enzyme can be classified once its catalytic properties are known. The non systematic names are still used to describe the commonly used enzyme (e.g. L-Lactate : NAD^+ oxido reductase; EC.1.1.1.27 is commonly known as Lactate dehydrogenase).

Industrially used enzymes are characterised not only by the nature of the reactions they catalyse but also by their activity. The activity gives an indication of how much enzyme should be used to achieve a desired product yield. It also serves as a basis for

the estimation of production costs and a guide in assessing investment and maintenance costs. In the 1960's, the international unit proposed by the commission on enzymes defined an enzyme activity unit (IU) as the amount of enzyme causing loss of 1 μ mol per minute under defined conditions. This definition of enzyme activity was based on a specified temperature (25°C) with other conditions (e.g. pH and substrate concentration) being optimal and defined. Although this definition is satisfactory in principle, industrially, it is hardly ever used due to the fact that in industries complex conditions have to be dealt with, some enzymes have optimum temperatures much higher than 25°C and sometimes economics prevent usage of enzyme at specified substrate concentrations. As a result industrialists tend to adopt a unit convenient for their own use.

3.2 ENZYME ASSAY

An idea of what activity an enzyme solution possesses can be established by carrying out an assay analysis. This analysis should be carried out under fixed and suitable conditions of pH, ionic strength and temperature. These conditions are governed by stability and reaction rate. For instance at low temperature (<4°C) enzymes tend to be inactive and stable, whereas at higher temperatures they are very active but unstable. The assay technique used, therefore, should take into account the conditions of reaction and be capable of being accurately monitored. A variety of detection procedures can be used of which some are discussed below:

(a) Manometer method:- Used to investigate where a gas is consumed or produced.

(b) Electrochemical method:- Based on measurement of change in potential as the reactions proceeds.

(c) Spectrophotometric method:- Based on following the colour change of the reaction mixture.

(d) HPLC method:- Is based on following the concentration of the reactant or product using high performance liquid chromatographic columns.

(e) Radiochemical method:- involves the use of a radioactively labelled substrate.

3.3 ENZYME RECOVERY AND PURIFICATION

Enzymes are present in all living things and if sufficient care is taken to protect them, they can be recovered and purified from any organism. The great bulk of enzymes used in industry are microbial in origin, but there are exceptions, such as plant and animal proteases.

The first stage of purification is to release the enzymes into solution from the cells which produce them. Due to the variety of different proteins in individual cells and the very wide range of concentrations found, recovery and purification present formidable problems. This is exacerbated by their general instability and their interaction with other proteins and cellular components. Since the feedstock concentrations are often relatively low (<10% w/v), a major problem is water removal. Furthermore, it may be necessary for high degrees of purification to minimise the possibility of toxic effects from contaminants.

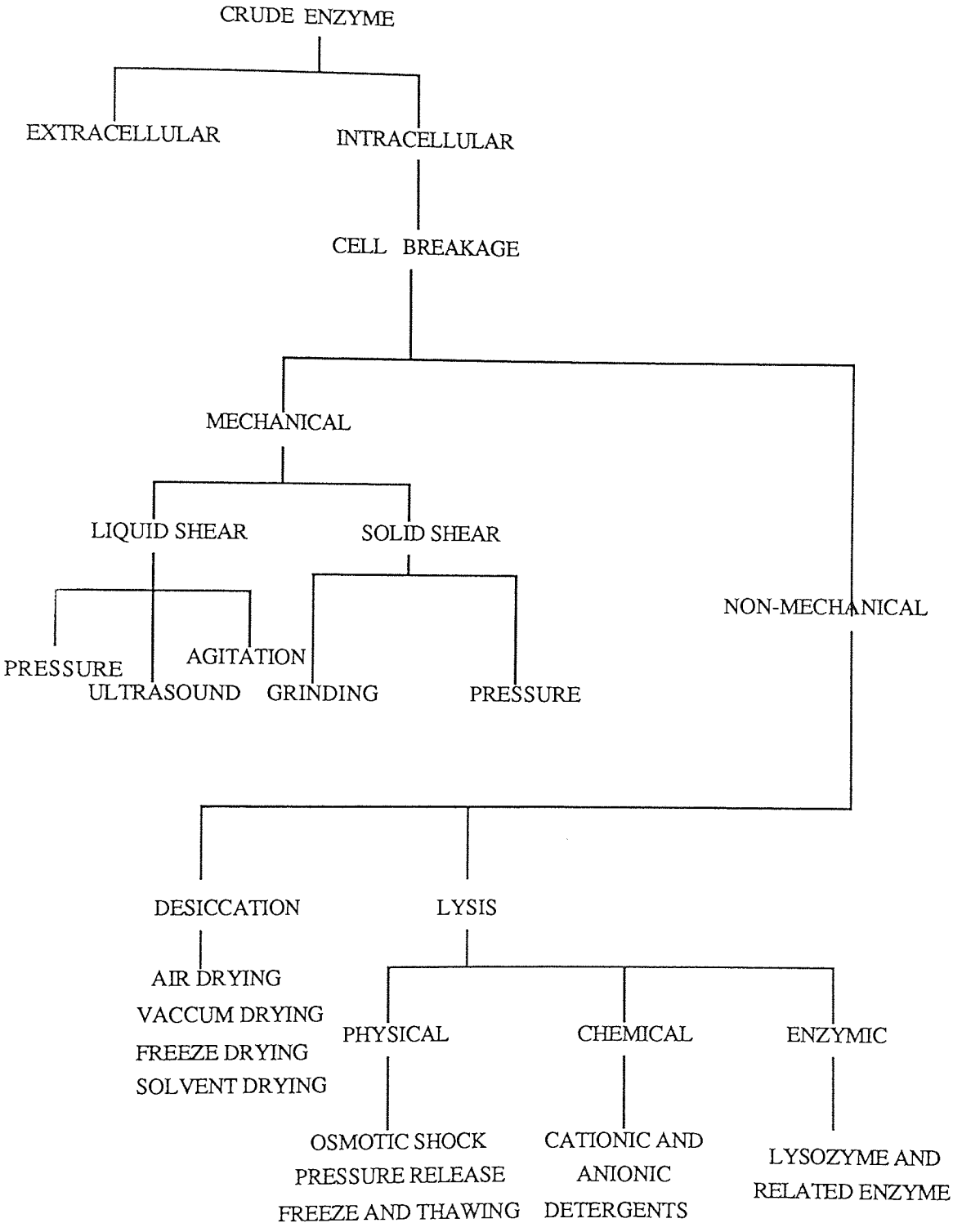
The different techniques used for recovering enzymes are outlined in Figure 3.1. A brief description of the purifying methods are described below.

3.3.1 Centrifugation

This is at present one of the elemental separation techniques that compose biotechnological downstream processing. It has distinct advantages (speed of separation, containment) which will always be at a premium.

There are wide varieties of separators available. These can be characterised by their mode of action; batch, semicontinuous or continuous discharge. The semicontinuous discharge is most frequently used commercially.

Figure 3.1:- Enzyme Recovery Techniques



For centrifugation to maintain its dominant role, improvements in efficiency and cost effectiveness are required. Efficient operation is favoured greatly by large particle diameter, large density differences between particle density and fluid density and low fluid viscosity. In practice, particles of biological material are often small and of low density, while fermentation broths are often viscous and occasionally of fairly high density. It is therefore unlikely that improvement will be forthcoming via the use of expensive alloys for bowl construction, but rather from a deeper understanding of the separation phenomena occuring in the bowl.

3.3.2 Filtration

The performance of classical "dead end" filtration equipment has radically changed but with relatively few changes taking place in the character of the feedstock (whole broth). Two types of dead end filters are predominantly used; rotary vacuum drum and filter presses. The smaller the size of the cell debris, the more troublesome the filtration operation. The addition of filter aids for broth filters is still necessary to obtain economic filtering output. Indeed, a precoat is usually necessary in order to prevent the clogging of a filter cloth. The use of body feed or precoat may be regarded as a form of broth conditioning. The objective is straight forward however. The factors involved that lead to a successful enzyme recovery operation are numerous and complex.

3.3.3 Cross-Flow Membrane Filtration

This is referred to as microfiltration. It may be thought of as a special form of static filtration where the aim is to prevent cake formation at the filter surface by "sweeping" the particles off with tangential shearing forces. A problem very similar to the plate and frame filter press.

Cross-flow membrane filtration processes are increasingly gaining popularity and application in biotechnological downstream processing. They are used for cell removal,

selective separation of various soluble components in fermentation broth and the concentration of final enzyme mixture.

The membrane filtration processes are classified according to the range of size of particles the membranes reject and also by the driving force (pressure) exerted on each membrane.

- Microfiltration:- these membranes are used to filter out particles greater then 0.1 μm with very low pressure (< 10 psi).
- Ultrafiltration:- these membranes are often used to retain macromolecules (10^3 - 10^6 MW). The operating pressure is slightly higher.
- Reverse Osmosis:- are capable of rejecting salts ion (Ÿ 10Å) with very high operating pressure.

3.3.4 Precipitation

Precipitation is one of the most widely used methods for enzyme recovery. Protein aggregation can be achieved by changing the environment thus making the inter-protein attraction greater than that with a solvent. Such changes are brought about by changes in ionic strength of solution, pH and dielectric constant.

One of the most interesting developments in the area of enzyme fractionation has been the use of high molecular weight polymers as precipitants. Stenberg and Hershberger (50) have recently described the use of polyacrylic acids as a precipitant and Ogston and his colleagues (51) have developed an extensive theory for the phase equilibria of aqueous polymer mixtures which embraces both precipitation and formation of two or three liquid phases. The transposition of this physical biochemistry into biochemical engineering practice does not require extensive plant facilities and should provide a basis for the wider use of polymers in enzyme fractionation.

3.3.5 Chromatography

To be able to achieve high levels of purity traditional purification techniques such as precipitation technology are unable to accomplish this task. Therefore, ideas have been focussed on chromatography. Its effectiveness has been proved both at the research and production level [52].

Chromatographic purification techniques use different molecular properties, such as size, charge, structure, polarity and isoelectric point and can easily be integrated with separation methods, particularly filtration and ultrafiltration. Chromatography, therefore, provides the biotechnology industries with reliable and efficient methods of purification that leads directly to the commercialisation of end products.

There are different chromatographic techniques that are used for purifying enzymes based on different molecular parameters:

Gel Filtration:- is limited mainly to the fractionation of macromolecules by the exclusion principle. As an initial method of precipitant removal and buffer exchange it is comparable to high performance liquid chromatography (HPLC) in speed and efficiency.

Ion-Exchange Chromatography:- is a powerful purification technique, due in part to the high protein capacity which can be used and in part to the variation in elution procedure that is possible i.e. combination of pH and ionic-strength and continuous and stepwise gradient. Many types of ion-exchange materials are available. The most common are the cellulose ion-exchangers, diethyl-aminoethyl (DEAE) and Carboxymethyl (CM) cellulose, anion and cations. Ion-exchange resins are water soluble polymers containing anionic or cationic groups such as $-SO_3^-$, $-COO^-$ or $-NH_3^+$.

Affinity Chromatography:- This technique is a highly specific technique and is equally applicable to large scale use and laboratory research. It relies on biospecific interaction

and has been most commonly used in situations such as enzyme antibody, antigen nucleic acid, hormone and vitamin isolation. Enzymes are capable of binding to their substrates, inhibitors and cofactors. If one of these are covalently linked to supports suitable for use in chromatography, it will bind specifically to the appropriate enzyme. The binding tends to be reversible, to enable the release of the enzyme usually by change in elution pH, ionic strength or composition. Affinity chromatography is clearly entering the field of commercial enzyme purification via its use in removing unwanted but low concentration contaminating enzymes. A comprehensive review of affinity separation has been carried out by Chase (53).

Chromatofocussing:- differs from ion-exchange in that proteins are separated according to their iso-electric points in a pH gradient, rather than by their overall charge. The pH gradient is created by titrating the gel with a specially prepared buffer, a poly buffer. It offers very high resolution, equivalent to that obtained by iso-electric focussing. In addition the gels have very high capacities for proteins and can tolerate very high flowrates which makes them very suitable for large scale use.

Hydrophobic Interaction Chromatography (HIC):- This is a recent development that offers good resolving power. It is particularly suitable when the sample is in the presence of high salt concentrations such as a precipitation step with ammonia sulphate. HIC is useful for purifying a number of important enzymes and other substances including interferons.

Fast Protein Liquid Chromatography (FPLC):- is used for products that are extremely potent in small quantities e.g. hormones, peptides and monoclonal-antibodies. FPLC can be used to assess the progress of a separation as well as for the final quality control and will certainly be a very important purification tool in biotechnology.

Although all these techniques are widely used for biological molecule purification, when scaling-up economy becomes very important and thus optimal performance becomes important.

3.4 ENZYME KINETICS

Kinetic studies represent just one of a range of tools available for investigating the structure and function of enzymes, and a range of complex mechanistic models have been developed [54]. Most enzyme kinetics follow the Michaelis-Menton model (Equation 3.1 and Figure 3.2)

$$\upsilon = \frac{V_{max}[S]}{K_m + [S]} \qquad 3.1$$

where

υ = Initial reaction velocity

V_{max} = maximum velocity

K_m = Michaelis constant

[S] Substrate concentration

When considering the commercial suitability of an enzyme catalyst there are three major criteria that must be considered:

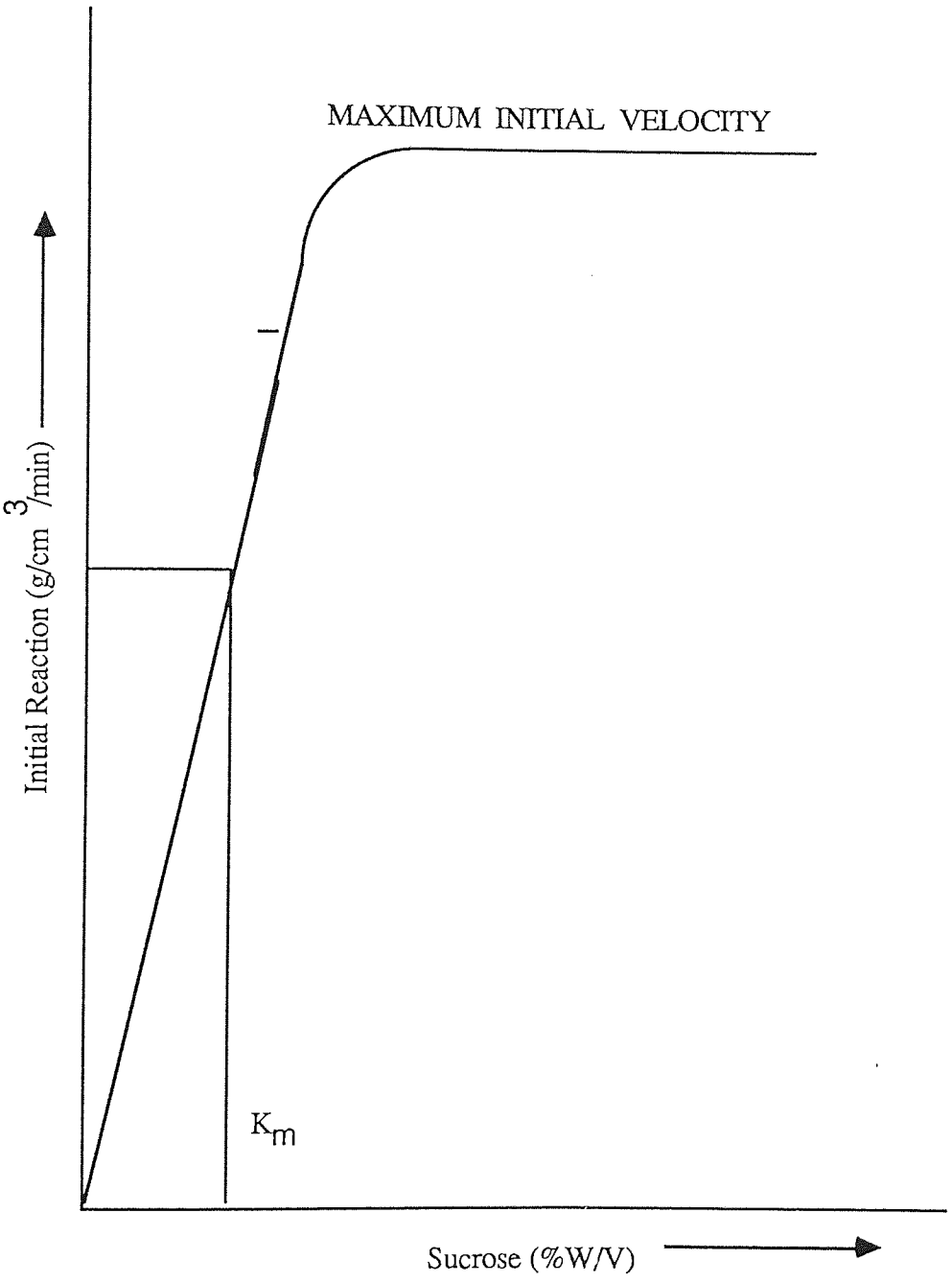
(i) The rate of reaction (catalytic activity).

(ii) The extent of reaction (equilibrium constant).

(iii) The duration of usable activity (stability).

The relative importance of these criteria will vary with the application envisaged. Enzymes as catalysts, promote chemical reactions but are not irreversibly modified themselves. Rate enhancements are attributed to lowering of the activation energy of the reaction.

Figure: 3.2 Initial Reaction Velocity vs Substrate Concentration



3.5 APPLICATION OF ENZYMES

With the understanding of the nature of enzymes and their catalytic potential, the use of enzymes has gradually been extended in a variety of fields, such as food production, brewing, pharmaceuticals, textiles and detergents.

The use however, of all known enzymes in industrial application, has been limited to only a few because most enzymes are relatively unstable, the isolation and purification costs are still high and health implications must be first overcome. In addition to the above, recovering active enzymes from the reaction mixtures after completion of catalytic processes is difficult and expensive. These factors restrict the use of soluble enzyme to batch operational use.

Over the past two decades, there have been rapid development in the use of immobilised enzymes as catalysts for industrial, analytical and medical purposes.

Enzyme immobilisation has already provided industry with major biocatalytic processes, such as the production of isosyrup for food use and the production of fine chemicals, for example resolved amino acids and penicinallic acid, a key pharmaceutical intermediate. The main requirement for industrial production is the conversion of large volumes of substrate to various products in a controlled manner at low cost.

The largest merit of immobilised enzymes is that a high productivity can be obtained by repeated use of the enzyme. Other advantages include the continuous control of processes, effluent problems and material handling are minimised and in some cases enzyme properties such as activity and stability are enhanced. Their limitations include deactivation, mass transfer, restriction during operation and poor discrimination between catalyst and fluid.

3.6 INVERTASE

Invertase (β-D-Fructofuronosidase; EC 3.2.1.26) is an intracellular heterogeneous enzyme. It can be produced from yeast (Saccharomyces spiceses) and the Fungus Neurospora crassa.

The enzyme hydrolyses sucrose into a fructose/glucose mixture (invert sugar). It breaks the C-O-C glycosidic bond in the sucrose molecule with the C-O group remaining with the glucose half of the molecule and the C-group with the fructose half of the molecule. The H-O- part of the water then links with the fructose part to give a fructose molecule and the -H part with the glucose part to give a glucose molecule as shown in Figure 3.3.

The ability of invertase to invert sugar and its ease of production makes it commercially viable. It has applications in the sugar, confectionary and dairy industries.

3.6.1 Invertase Properties

The molecular weight of invertase enzyme varies from 112, 000 MW to 270,000MW [54]. It has an iso electric point of 6.9 [55]. The pH activity curve (Figure 3.4) has a broad optimum at pH 4.5 - 5.5 [55]. This is due to the heterogeneous nature of invertase. An optimum temperature of 20 - 55°C has also been reported (Figure 3.5).

Yeast invertase is very stable with a half life of 48 hrs. It is however, inhibited by zinc (Zn^{2+}) and Mercury (Hg^{2+}). Reversible inactivation is immediate by applying urea which does not produce major changes in physical properties.

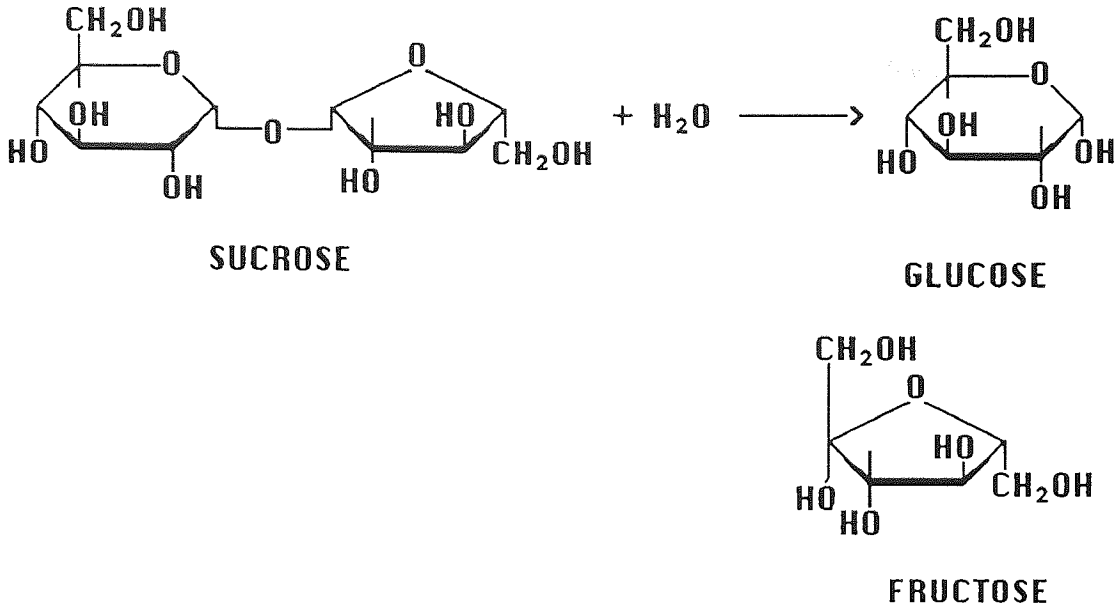


Figure 3.3: The inversion of sucrose using invertase enzyme

3.6.2 Mechanism of Invertase Action

In the past invertase enzyme was thought to be a hydrolase which performed a simple hydrolysis of sucrose to glucose and fructose. However, in 1950 it was shown simultaneously by Bacon and Edelman [56] and Blanchard and Albon [57] who described a product fraction containing trisaccharides being produced and that glucose and fructose were not the only products observed during the enzyme catalysed process.

The hypothesis put forward by Fischer et al [58] is that the mechanism of hydrolysis by invertase on sucrose is a two step transfer reaction. The first step is the formation of an enzyme fructose complex and the liberation of glucose and the second step is the transfer of the fructosyl group from the active complex to water or sucrose, forming a trisaccharides.

This hypothesis was later confirmed qu...titatively by Anderson et al [59] where he described the two step mechanism as:

I GLU-FRU + E $\rightleftharpoons$ FRU-E + GLU

II FRU-E + HOH $\rightleftharpoons$ E + FRU

Figure 3.4: Effect of pH on Invertase Activity at 55 deg.C

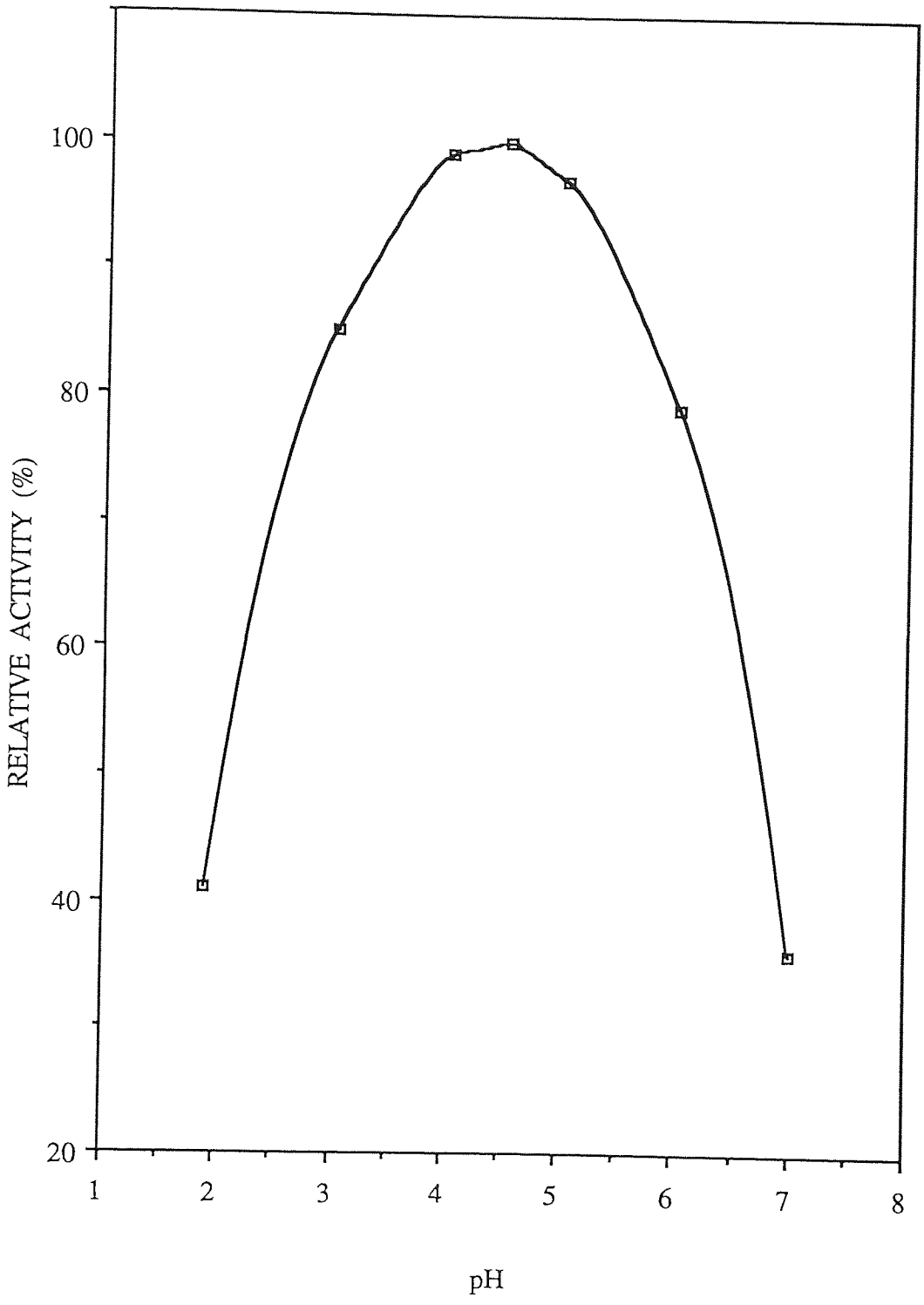
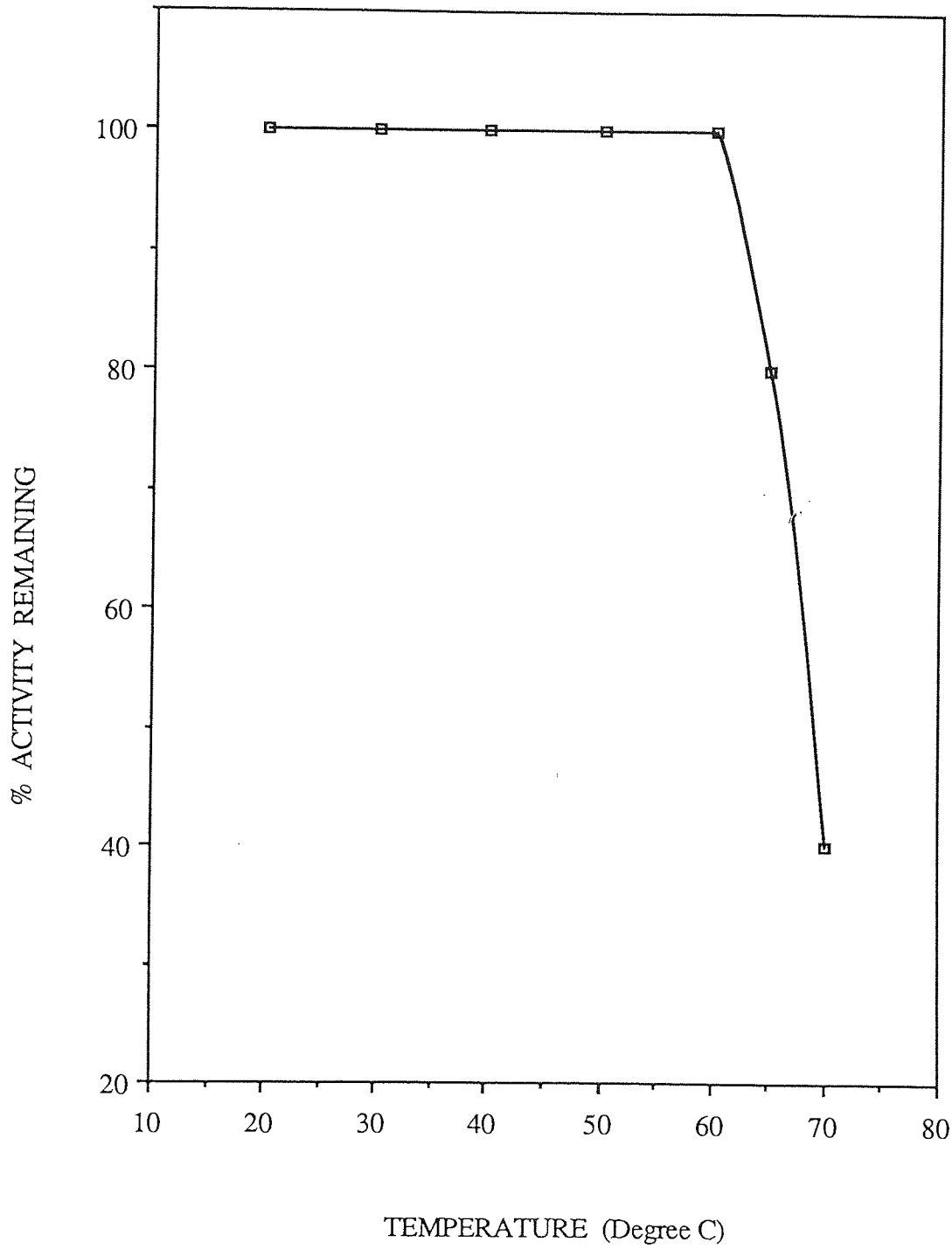


Figure 3.5: Effect of Temperature on Invertase Activity at pH 5.2



III FRU-E + GLU-FRU ⇌ FRU-FRU-GLU + E

where GLU-FRU stands for sucrose, GLU and FRU for glucose and fructose respectively and FRU-FRU-GLU for any receptor sugar.

Reactions I and II are responsible for the ultimate hydrolysis whereas reactions I and III give the intermediate oligosaccharides.

The oligosaccharides formed can be reducing disaccharides, trisaccharides or tetrasaccharides depending on the degree of reaction. The reducing disaccharides are hydrolysed exclusively to glucose and fructose or fructose only depending on the type formed and the trisaccharides are hydrolysed to glucose and fructose in 1 : 2 proportion. Analysis of the amount of glucose and fructose present in the hydrolysis of tetrasaccharides by paper chromatography, gave 1 part glucose to 3 parts fructose.

Reactions carried out at a pH of 4.75 and at 25°C showed that initially, more trisaccharides are produced than reducing disaccharides, since the trisaccharides are formed from sucrose, whereas the disaccharides are formed from the reaction products glucose and fructose. Only moderate amounts of tetra saccharides occur and they are formed late in the reaction.

According to Anderson et al [59] the affinity of the enzyme invertase to fructose by transfering fructose residue from sucrose to any carbohydrate receptor molecule and also to water in the reaction mixture does produce an erroneously low reaction rate.

3.7 DEXTRANSUCRASE

Dextransucrase (sucrose 1,6 α-D glucan 6 α glucosyl transferase E.C 2.4.1.5) is the name given to the enzyme or group of enzymes (glucosyl transferases) responsible for the synthesis of dextran from sucrose.

Although, many different species of the genera Leuconostoc, Lactobacillus, and Streptococcus are known to synthesise extracellular dextransucrase under appropriate growth conditions (i.e. pH and temperature), the dextran product and hence the enzyme are species specific. By far the most extensively studied dextransucrase is that elaborated by Leuconostoc mesenteriode strain NRRL B512 F [60]. This enzyme is responsible for the conversion of sucrose to dextran and fructose according to the following equation:

$$\underset{\text{Sucrose}}{nC_{12}H_{22}O_{11}} \xrightarrow{\text{Dextransucrase}} \underset{\text{Dextran}}{(C_6H_{10}O_5)_n} + \underset{\text{Fructose}}{nC_6H_{12}O_{16}}$$

The dextran is soluble with only 5% branching (α-1,3-bonds) and the other 95% being linear α (1,6) bonds. It is an inducible enzyme with only sucrose required for its induction. The mechanism of reaction in described in the next Chapter.

3.7.1 Dextransucrase Properties

The enzyme dextransucrase has been reported to have molecular weights ranging from 100,000MW up to 300,000MW [61, 62]. The isoelectric point was estimated as 4.1[60]. Kaboli and Reilly [63] studied the effect of pH and temperature on enzyme activity and showed an optimum pH of 5.2 (Figure 3.6) and an optimum temperature of 30°C (Figure 3.7). These values were also confirmed by Kobayashi and Matsuda [60]. The enzyme is thermally stable up to 35°C. The decay of activity deviates from first order kinetics at all temperature above 35°C. A half life of 13.5min at 30°C and 3.28 min at 35°C during the first order decay segment was reported [61]. Kobayashi [61] reported a stability range in pH at 4°C of 6.0 - 9.0 with dextran and pH 7.0 - 9.0 without dextran. This effect of using dextran to stablise the enzyme on storage was confirmed by Robyt and Walseth [64].

Dextransucrase is a glycoprotein containing mannose. Its activity can be inhibited by the addition of ethyl dimethyl tetra amine (EDTA) [64]. Inhibition of up to 50% is possible due to the strong chelate complexes formed by EDTA at pH > 5.0. The

Figure 3.6: Effect of pH on activity of soluble dextransucrase at 30 deg.C

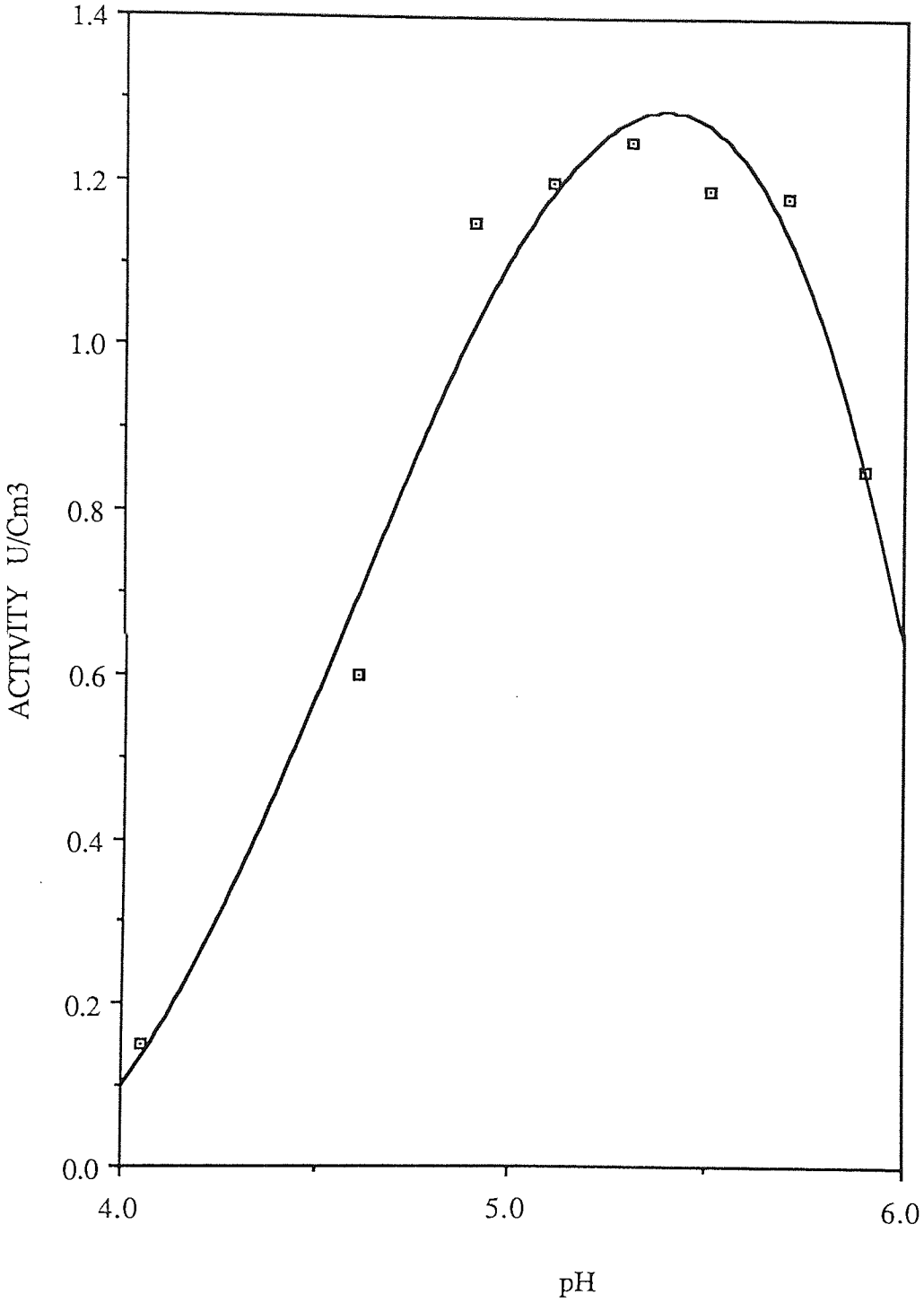
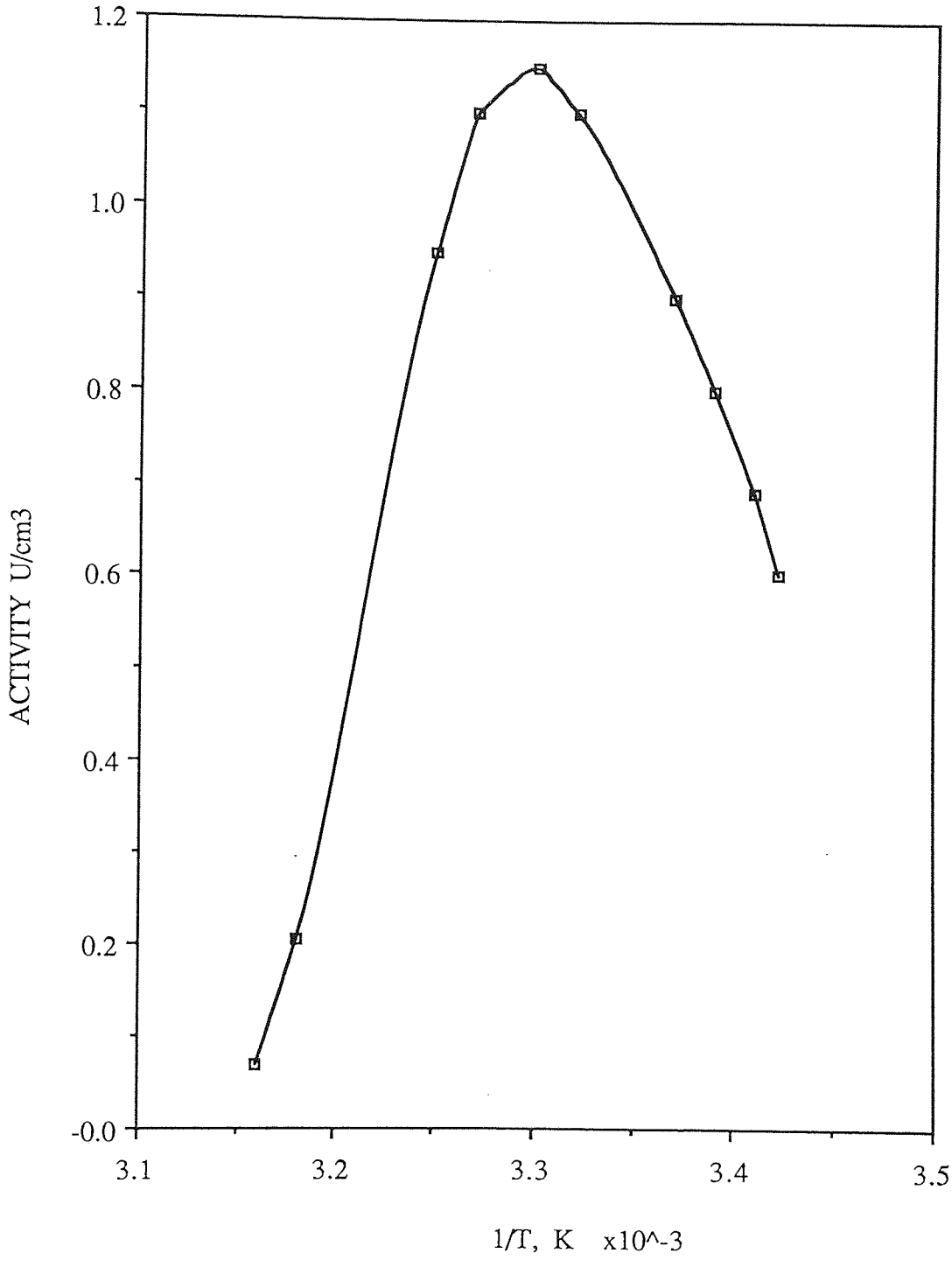


Figure 3.7: Effect of Temperature on soluble dextransucrase at pH 5.2



addition of metal ions to EDTA treated enzyme shows that calcium is the only ion that can completly restore the activity, (Table 3.1). Thus dextransucrase can be regarded as a calcium metalloenzyme.

Dextransucrase is very difficult to immobilise unlike invertase which is very commonly used in industry. From the thirteen different carriers, surveied by Kaboli et al [63], only alkylamine porous silica allowed more than 10% retention of activity. Immobilised dextransucrase shows a similar trend of properties to the soluble form. The activation energy was found to be lower than the soluble enzyme. According to Kaboli et al [63], immobilisation did not substantially increase dextransucrase stability.

Table 3 : The Effect of Divalent Metal Ions on 5 mM EDTA - Treated, Purified Dextransucrase [64]

METAL ION ADDED (5 MM)	RELATING ACTIVITY (%)
None	56
Ca^{2+}	99
Mg^{2+}	40
Mn^{2+}	40
Sr^{2+}	68
Ba^{2+}	65
Zn^{2+}	-
Ca2+	-
Ni^{2+}	-
Cd^{2+}	-
Fe^{2+}	-
Hg^{2+}	-
Cu^{2+}	-
Pb^{2+}	-

CHAPTER 4

CARBOHYDRATES

4.0 CARBOHYDRATES

Carbohydrates represent a major part of the total caloric intake for humans, for most animal life, as well as for many micro-organisms. They are also central in the metabolism of green plants and other photosynthetic organisms. Carohydrates have very important biological functions. Starch and glycogen serve as temporary stores of energy, while some carbohydrates (insoluble polymers) serve as structural or supportive elements in the cell walls of bacteria, plants and in connective tissue and cell coats of animal organisms.

Generally, carbohydrates are polyhydroxy aldehydes or ketones. The name carbohydrates owes its origin to the fact that most substances of this class have empirical formulars suggesting they are carbon "hydrates" in which the ratio of carbon to hydrogen to oxygen atom is 1 : 2 : 1.

Carbohydrates are usually classified as monosaccharides, oligosaccharides and polysaccharides (the word "saccharide" is derived from a greek word meaning sugar). Monosaccharides, or simple sugars consist of a single polyhydroxy aldehyde or ketone unit. The most abundant monosaccharide in nature is the 6-carbon sugar D-glucose.

Oligosaccharides (greek oligos, "few") consist of short chain monosaccharide units joined together by covalent bonds. The most abundant type are the disaccharides which have two monosaccharides units. Typical is sucrose or cane sugar which consist of the 6-carbon sugars D-glucose and D-fructose joined in covalent linkage.

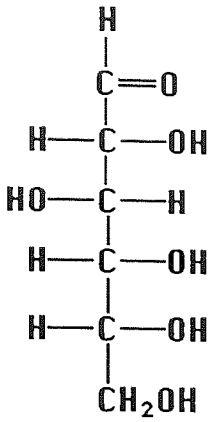
Polysaccharides consist of long chains having hundreds or thousand of monosaccharide units. Some have linear chains (cellulose) while others such as glycogen have branch chains. Dextrans have a mixture of linear and branched chains depending on their synthesis route.

4.1 FRUCTOSE

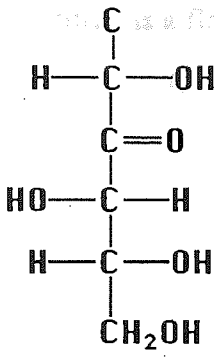
Fructose, a monosaccharide is sweet and colourless and is freely soluble in water but insoluble in non-polar solvents. These characteristics apply to most monosaccharide carbohydrates. The common form of fructose in nature (D-fructose) is optically active and is laevorotatary with a specific rotation of $[\alpha]_D^{20} = -92.4°$. Likewise D-glucose the common form of glucose is dextrorotatary ($[\alpha]_D^{20} = +52.7°$).

The backbone of monosaccharides is an unbranched single bonded carbon chain. One of the carbon atoms is double-bonded to an oxygen atom to form a carbonyl group, each of the other carbon atoms has a hydroxyl group. If the carbonyl group is at the end of the carbon chain, the monosaccharides is an aldehyde and is called an aldose. If the carbonyl group is at any other position, the monosaccharides is a ketone or ketose. Monosaccharides are named according to the number of carbon atoms in their backbones. Each of these monosaccharides exist in two series; aldo and keto - form. The six carbon atoms (hexoses) have two series an aldohexose (D-glucose) and ketohexose (D-Fructose) (Figure 4.1).

Fructose occurs in α and β anomeric forms. In these compounds the hydroxyl group on the carbon atom 5 reacts with the carbonyl group at carbon 2, forming a five membered furanose ring. D-fructose forms two different furanoses (Figure 4.2), the more common form is β - D - fructofuranose.

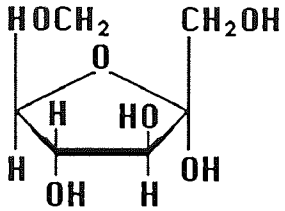


D-Glucose, an aldohexose

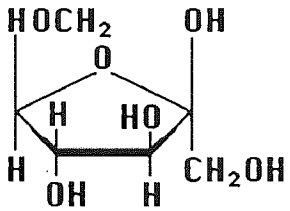


D-Fructose, a ketohexose

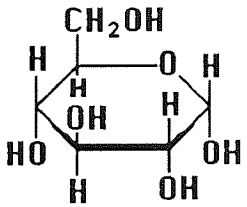
Figure 4.1 Two Common Hexoses



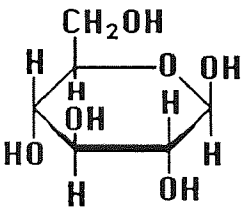
α-D-Fructofuranose



β-D-Fructofuranose



α-D-Glucopyranose



β-D-Glucopyranose

Figure 4.2: Pyranose Form of D-Glucose and Furanose Form of D-Fructose

4.1.1 Fructose as a Sweetner

Fructose, after the reshaping of the USA sugar industry in the 1970's, has become established as a sweetner substitute to sucrose in beverages and various dietetic food products. In Finland and Sweden it is used in baby foods and in some medicinal

preparations intended for children. Fructose can be used in small quantities as a flavour enhancer in meat dishes and as a raw material in the industrial manufacturing of flavours. It enhances the inherent aroma of fruits, berries and vegetables more than any other sugar and its uses in juices, jams and desserts offers particular advantages.

Fructose is a natural sugar without toxic properties. Its calorific value is slightly lower than sucrose (i.e. 3.7 and 4 k cal kg^{-1} respectively), but it is 1.8 times sweeter than sucrose in cold solution [6, 65, 66]. It is 15% more soluble in water than sucrose. Fructose has been found to overcome the bitter after-taste of saccharin and a 99.7 : 0.3% fructose : saccharin mixture has been found to be 3 to 4 times sweeter than pure sucrose solution without any distinguishing difference in taste. A similar synergistic sweeting effect is exhibited with sucrose.

The β-D-fructopyranose is believed to be the sweetest fructose form and the solution sweetness depends on the equilibrium between the above form and the β-D-fructofuranose. At high temperature (50°C), the fructose sweetness is reduced to that of sucrose. The equilibrium is also affected by concentration, pH and time.

Although fructose is not expected to substitute completely sucrose its usage is expanding rapidly and it would become even more favourable when new and more economical production methods have been developed and utilised. The separation efficiency and low energy intensiveness of chromatography favours this area of work and a large initiative has been commenced by the Scandinavian countries in particular.

4.1.2 Production of Fructose

The industrial production of fructose can be classified as follows:

- Hydrolysis of inulin
- Sucrose hydrolysis
- Enzymatic isomerisation

4.1.2.1 Hydrolysis of Inulin

Inulin is a soluble polysaccharide consisting of approximately thirty D-fructofuranose units which occurs in many plants as a stored food. It can be easily hydrolysed to yield comparatively pure fructose. This was the earliest method of producing fructose but it was found to be economically unviable [67].

4.1.2.2 Sucrose Hydrolysis

Sucrose is a disaccharide carbohydrate consisting of one fructose and one glucose molecule. The hydrolysis (or inversion) of sucrose can be achieved either by acid hydrolysis, hydrogen ion resin treatment or by the enzyme invertase.

For acid hydrolysis, the solution is treated with dilute acid and for hydrogen ion resin treatment the hydrolysis is carried out on a cation exchange resin in the H^+ form. The hydrolysis product mixture results in less than 50% fructose with some sucrose also present due to the difficulty of achieving 100 percent inversion. Lauer et al [68 - 70] have patented a process that inverts sucrose and separates the glucose - fructose mixtures in the same column which was packed with calcium charged Dowex WX4 resin.

The inversion reaction of sucrose with invertase does give a 100 percent product mixture and this reaction has been used for this research work.

4.1.2.3 Enzymatic Isomerisation

The isomerisation of glucose into fructose was first achieved as far back as 1895 when Bruyn and Van Eckenstien [71] used an alkaline catalyst at elevated temperature. The first enzyme discovered to isomerise glucose to fructose was xylose isomerase by Marshall and Kooi in 1957 [72, 73]. This enzyme production was however expensive and poisonous arsenate and flouride were present. Other alternatives of enzyme isomerases (e.g. glucose isomerase) produced by micro-organisms have now been discovered

without the poisonous components, resulting in stability improvement of the enzyme and thus can be utilised effectively by immobilising the enzyme on the cells [74 - 77].

The USA company Standard Brands Inc., immobilised glucose isomerase by adsorption on DEAE cellulose packing based on the work carried out by Takasaki et al [78]. The isomerisation was carried out in reactors having shallow beds of immobilised isomerase. In 1975 an immobilised enzyme under the trade name Sweetzyme was used for continuous on-column isomerisation by Novo Industrial (Denmark) [79]. The typical composition of the isomerisation product mixture is 42% fructose, 52% glucose with the balance incorporating maltose and other oligosaccharides.

4.2 PRODUCTION OF HIGH FRUCTOSE CORN SYRUP (HFCS)

In the nineteenth century, starch was the major source of sugar. Commerical sources of starch are corn (maize), wheat, barley, potatoe, sago, cassava and rice.

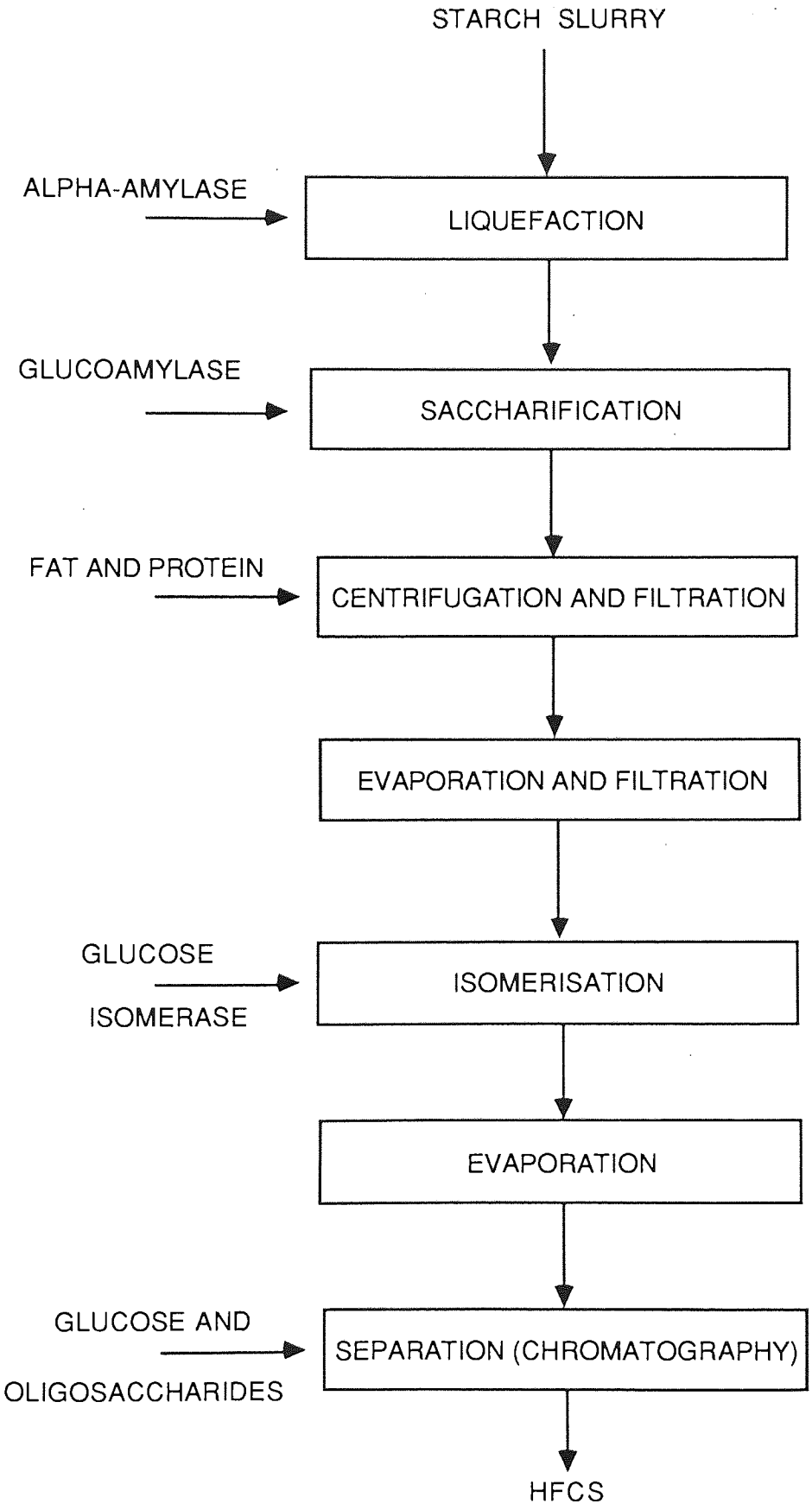
Starch consists of amylose, a linear glucose polymer containing 1, 4-α-glucocidic links and a branch polymer, amylopectin, in which linear chains of 1, 4-α-glucose residues are interlinked by 1, 6-α-glucocidic bonds. Starch hydrolysis is carried out using alpha amylase enzymes.

An aqueous starch slurry obtained by liquefaction of cereal or root crops, results in maltodextrins being produced. Further treatment of the slurry by a "Saccharification process" produces syrups containing glucose, maltose and other oligosaccharides. The "saccharification step" is carried out in a batch process in the presence of the amyloglucosidase enzyme. The glucose in the product mixture is then isomerised to fructose using glucose isomerase enzyme and concentrated to around 70% w/v by evaporation.

DDS Kroyer (Denmark) have built a 150 ton/day plant of 42% fructose syrup from 130 tons of broken rice, by employing immobilised glucose isomerase enzymes in Pakistan [80]. A 400 ton/day maize processing plant to produce 150 ton/day of 42% fructose syrup has been reported to be in operation in Hungary [81]. The isomerisation is carried out continuously in columns filled with immobilised glucose isomerase.

High fructose corn syrup is produced by separating the fructose from the isomerase mixture by application of chromatographic columns packed with an ion-exchange resin. A typical flowsheet of a HFCS producing process is shown in Figure 4.3.

Figure 4.3 A Typical Flowsheet for the Production of HFCS



The market specification of HFCS is a mixture with a fructose content of greater then 50%. Due to the relative low price of sucrose, the HFCS processes have become more economical in order to compete in the sugar market. The share of HFCS in sugar is increasing worldwide notably in Japan and the Scandinavian countries. This has allowed researchers to investigate different methods of improving separation efficiencies, and throughputs by utilising chromatography. Hashimoto et al [23] developed a process to produce a high fructose syrup, containing more then 50% fructose. The moving column continuous chromatographic system employed involves combining the selective adsorption of fructose on Ca^{2+} ions on an ion-exchange resin and an immobilised glucose isomerase reaction.

4.3 DEXTRAN

The term "dextran" was coined by Scheibler [82] when he found, in 1874, that the mysterious thickening of cane and beet sugar juices was caused by a carbohydrate of empirical formula $(C_6H_{10}O_5)_n$ having a positive optical rotation.

Dextran (or dextrans) is the name collectively given to a large class of extracellular bacterial polysaccharides composed almost exclusively of the monomeric unit α-D-glucopyranose linked mainly by 1-6 bonds. The formation of dextran was recognised as the result of the transformation of sucrose solution into viscous solutions, gels and precipitated floculent material.

The synthesis of dextran from sucrose by a cell-free bacterial culture was first demonstrated in 1940 [83]. The two principal genera of bacteria that produce the enzymes that synthesise dextrans are Leuconostoc and Streptococcus. These genera are gram positive, facultatively anaerobic cocci closely related to each other. The Leuconostoc species require sucrose in the culture medium as an inducing agent while the Streptococcus species do not (constitutive).

The isolation of Leuconostoc mesenteroides B-512F strain by the United States department of Agricultures Northern Regional Research Laboratory (NRRL) is now the organism of choice for the commercial production and study of dextran.

4.3.1 Dextran Structure

The approximate structure of the dextrans was determined in 1954 [84]. At that time, however, the nature of the branch linkages, their distribution and the length of the branch chain had not been definitely determined. Later, methylation analysis of Leuconostoc mesenteroides B-512F dextran showed that it contained 95% α-1-6-linear linkages and 5% α-1-3 branch linkages.

Dextrans can be divided into three classes based on structural fractures.

Class 1 Dextrans:- contains a main chain of consecutive α-1-6 linked glucosyl residue, with branching at position 2, 3, or 4.

Class 2 Dextran:- contains non consecutive α-1-3 and α-1-6 linkages and α-1-3 branch linkages.

Class 3 Dextrans:- contains consecutive α-1-3 linkages and α-1-6 branch linkages.

Leuconostoc mesenteroides B-512F dextran is in the Class 1 category containing a high percentage (95%) of consecutive α-1-6 linkages and a relatively low percentage (5%) of α-1-3 branch linkages and is the only strain approved medically worldwide for intravinous injection.

4.3.2 Mechanism of Dextran Biosynthesis

Mechanistic studies have shown that B-512F dextran is synthesised from sucrose by dextransucrase enzyme that form covalent glucosyl intermediates. Figure 4.4 illustrates the utilisation of sucrose by the enzyme to synthesise the α-1-6 linkages of the main chain.

The glucosyl moieties are transferred to the reducing end of a growing glucosyl chain, which is covalently linked to the active site of the enzyme [85]. The proposed mechanism for the synthesis of a sequence of α-1-6 linked glucose residues in B-512F dextran involves two nucleophiles at the active site, which attack sucrose and displace fructose to give two β-glucosyl intermediates. The C_6- OH group of one of the glucosyl residues attacks C_1 of the other to form a α (1-6) linkage and one glucosyl residue is transferred to the other. The freed nucleophile attacks another sucrose molecule forming a new enzyme glucosyl intermediate. The C_6- OH of this new glucosyl intermediate attacks C_1 of the isomaltosyl unit (the growing dextran chain), which is in effect transferred to the glucosyl residue. The glucosyl and dextranosyl units are alternatively transferred between the two nucleophiles as the dextran chain is elongated at the reducing end (Figure 4.5). The elongation is terminated and the chain released by acceptor reaction.

4.3.3 Mechanism of Dextran Biosynthesis Termination

When other carbohydrates besides sucrose are added to dextransucrase - sucrose digests, the glucosyl moiety of sucrose is diverted from the synthesis of dextran and is added to the carbohydrate [86], called an acceptor. Many different carbohydrates act as acceptors (Table 4.1).

Figure 4.4 : Reaction of Sucrose to give ∝- (1-6)- Glucan and D-Fructose

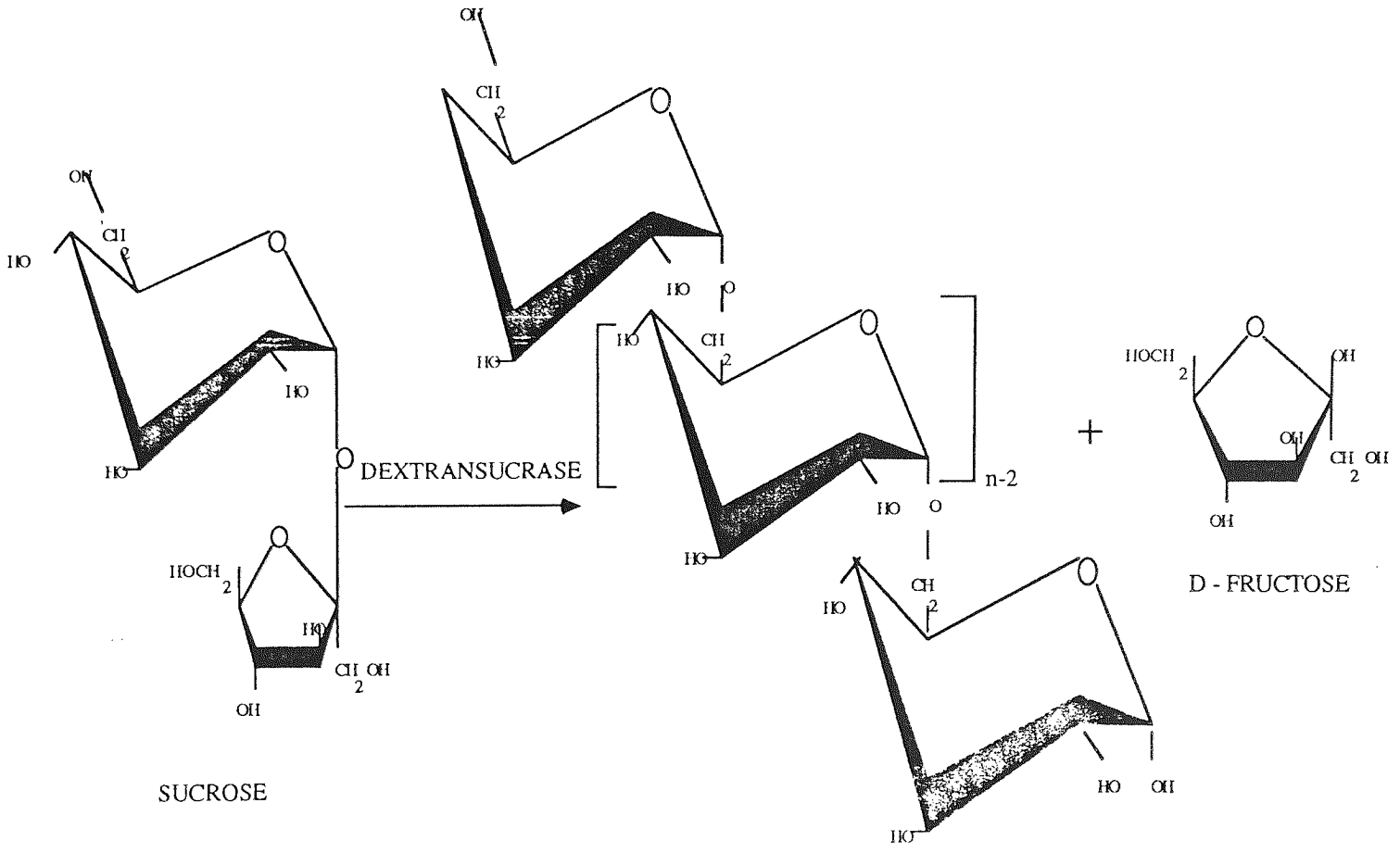
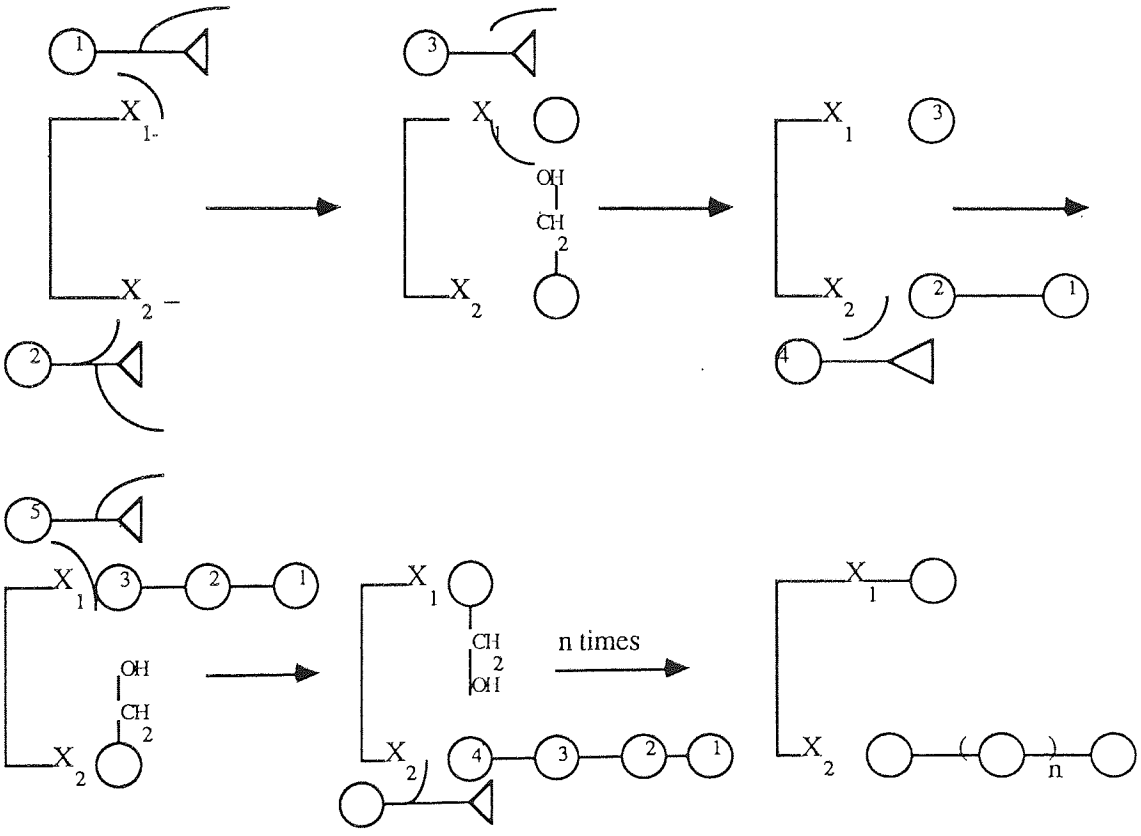


Figure 4.5: Mechanism for Synthesis of α- (1-6)- Glucan by B-512F Dextransucrase .
X1 and X2 Represent nucleophiles at the active site:


= Sucrose ; O-CH $_2$- OH = Glucose;


= Fructose and - O - O - = Two Glucose Residues linked - (1-6)



Mechanistic studies show that the acceptor interacts with the covalent enzyme - glucosyl or enzyme - dextranosyl intermediates to release oligosaccharide or dextran from the enzyme active site. The acceptor subsequently forms a covalent linkage at the reducing end with the glucosyl or dextranosyl chain. When the acceptor displaces the dextran from the active site, the polymerisation of the dextran chain is terminated.

ACCEPTORS	PRODUCTS
D-Glucose	Isomaltodextran series
α-methyl-D-glucoside	α-methyl-isomaltoside series
D-Fructose	Leucrose and isomaltilose
D-Mannose	α-D-glucopyranosyl-β-D-mannopyranoside
D-Galactose	α-D-glucopyranosyl-β-D-galactofuranoside
Isomaltose	Isomaltodextran series
Maltose	Panose and higher isomaltodextrin homologues
Cellubiose	2–α-siomaltodextrinyl-cellobiose series
KOJIBLOSE	2-α-isomaltosyl-D-glucose
NIGEROSE	3-α-isomaltosyl-D-glucose
Lactose	2 GLC-α-D-glucopyranosyl-Lactose
Raffinose	2 GLC-α-D-glucopyranosyl-raffinose
B-512F Dextran	α-1,3-dextranosyl-dextran or α-1,3-glucopyranosyl-dextran

Table 4.1: Observed Acceptors and their Products in the Reaction of Dextransucrase and Sucrose [86]

4.4 PRODUCTION OF DEXTRAN

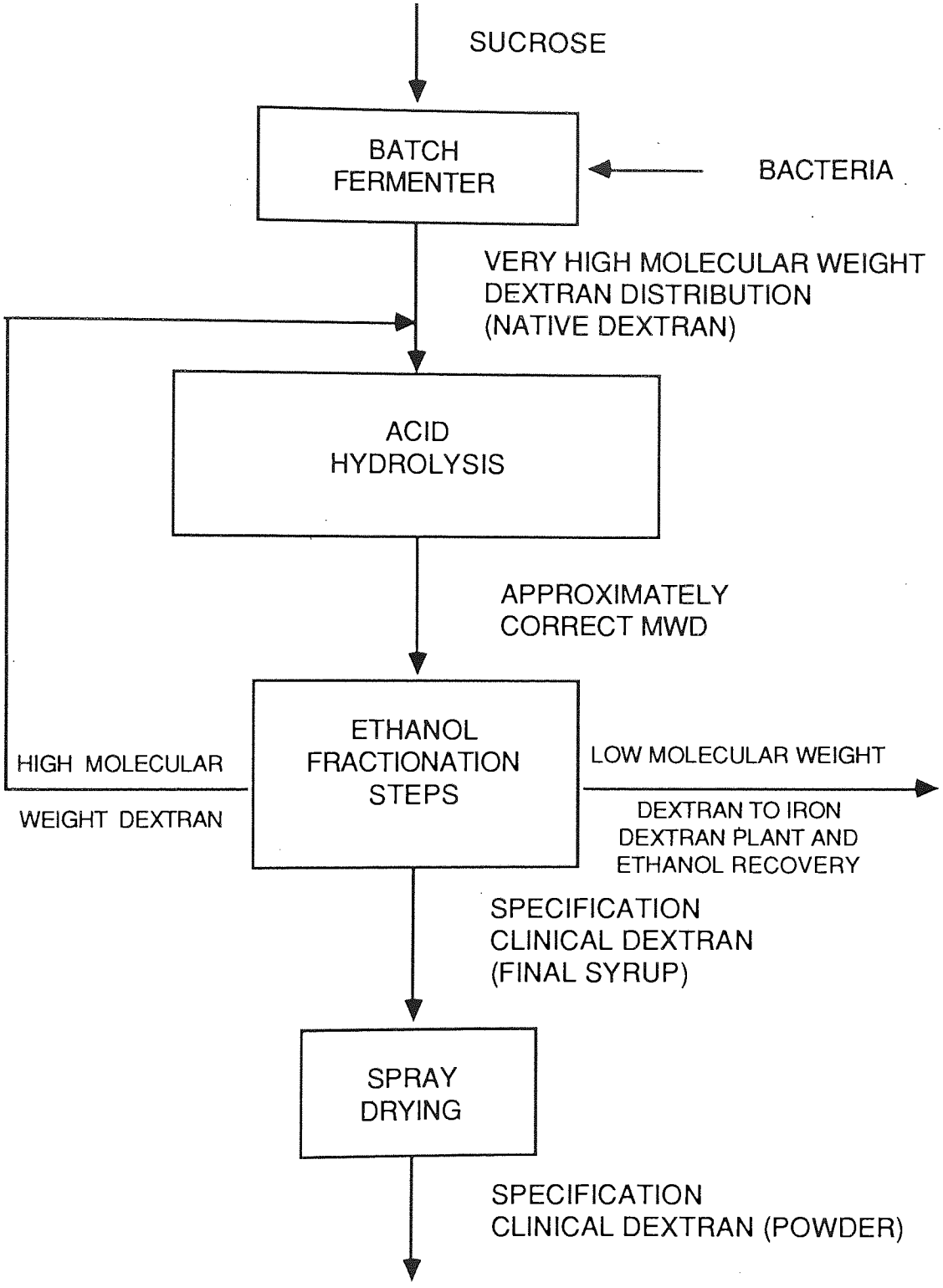
Traditionally dextran production was carried out in sucrose rich growing culture of Leuconostoc mesentenodes. The growing cells secrete dextransucrase enzyme into the medium which converts excess sucrose to dextran. The optimum conditions of synthesis are pH 5.0 - 5.3 and a temperature of 25 - 30°C. Alsop [87] reported an optimum sucrose concentration of 17.9% w/v. This method of production is still prominent in industry.

The dextran produced called 'native' dextran has a high molecular weight distribution (MWD). For clinical use, the dextran undergoes a lengthy isolation and purification stage. This includes acid hydrolysis and ethanol fractionation. Figure 4.6 illustrates the different stages required to produce clinical dextran. Acid hydrolysis breaks the dextran molecules to smaller units and ethanol fractionation separates the smaller molecules from the larger molecules. The fructose produced contains impurities and is usually sold well below its market value.

The traditional process was described by Jeanes [88] as presenting the disadvantages of propagating the cells, producing the enzyme and synthesising dextran under a single set of conditions which changes during the course of fermentation and therefore, does not allow correct optimisation of dextran due to lack of proper control of the process parameters (pH, temperature). By correct control of the enzyme producing stage of the fermentation, the activity of the enzyme dextransucrase can be increased ten fold from that obtained by conventional methods. Tsuchiya [89] obtained an activity up to 70 DSU/mcm^3 compared with an activity of 9 DSU/cm^3 obtained by traditional methods. On a larger scale, Alsop [87] obtained an activity of 300 DSU/cm^3 while Ajongwen [90] in his work achieved an activity of 450 DSU/cm^3. These high activities of enzyme will enable a fast conversion time to be achieved and a more rapid plant turnround time.

Another area of improvement is the process for producing clinical dextran. Alsop et al [87, 91-92], Barker et al [5, 93], Poland [94] and Till [95] have described methods of characterising the MWD of dextran. They have also described processes for producing clinical dextran as well as dextrans of different MWDs. These involved the combined use of gel chromatography, ultrafiltration, and ion exchange chromatography.

Figure 4.6 Industrial Manufacture of Dextran



4.5 USES OF DEXTRAN

Some of the potential uses for native dextran are [87].

- Oil drilling muds
- Stabilisation of sud aggregates
- Protective coating for seeds
- Deflocculant in paper products
- Metal plating processes
- Surgical sutures
- Food use - stabilising and imparting viscosity to syrups.

Low molecular weight dextrans have their biggest outlet in the pharmaceutical industry where they have been used as blood plasma extenders (70 000 MW) and blood flow improvers (40 000 MW).

Some other uses of dextran are as a blood substitute when complexed with haemoglobin and in the treatment of iron deficiency anaemia in both human and veterinary medicine. It is also used in the manufacture of molecular seives.

CHAPTER 5

EQUIPMENT DESCRIPTION, CHARACTERISATION AND OPERATION

5.0 INTRODUCTION

Ideally one would prefer to carry out the reaction-separation studies in a chromatographic system with a suitable biocatalyst immobilised on the stationary phase. Unfortunately this has not been possible in this research work because the enzymes (invertase and dextransucrase) were not immobilisable on the resin used (KORELA VO7C).

Attempts to immobilise dextransucrase on different solid supports have been made by Monsan and Lopez [96,97] who showed that only 10% of the initial activity of the enzyme could be retained on the media. The different types of media used for this work (e.g. alkylamine porous silica) are not suitable for chromatographic separation.

Similarly, although invertase has been successfully immobilised on a commercial scale, in its immobilised form it does not have the chromatographic characteristics required for separation purposes.

The method which Zafar [25] and Ganetsos [9] used in their work on a batch chromatographic reactor separator (i.e. carrying out reaction in the mobile phase and using the stationary phase as adsorber) with the enzyme added into the eluent stream at the required strength has been similarly adopted in this work.

A continuous counter-current chromatographic refiner was employed in this research work and the resulting on-column profiles are anticipated to be of a form shown in Figure 5.2. The advantages of continuous chromatographic processes over batch processes, led Barker and co-worker [47, 98] into developing the semi-continuous counter current chromatographic refiners (SCCR) which have been used successfully in gas and liquid separations. These systems have been scaled up to column diameters of 10.8 cm [100]. Such a system has been modified, rebuilt and used in this work.

Figure 5.1: Concentration Profile of a Batch Co-current Reaction Separation

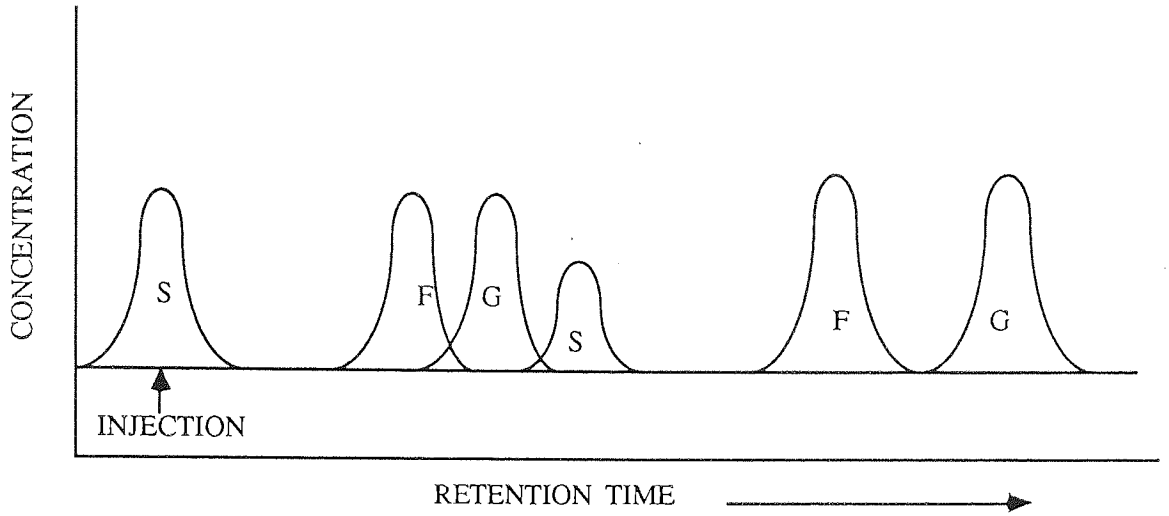
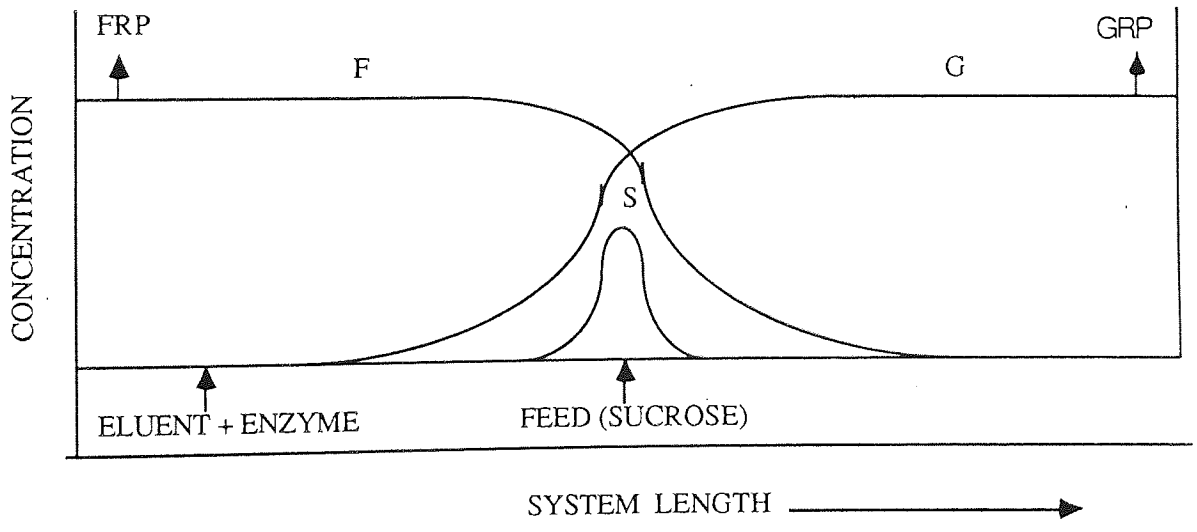


Figure 5.2: Concentration Profile of a Continuous Countercurrent Reaction - Separation.



KEY: F: Fructose; FRP: Fructose Rich Product; G: Glucose; GRP: Glucose Rich Product; S: Surose

5.1.1 SCCR-S1 Principle of Operation

The counter-current operation mode of the SCCR-S1 system is demonstrated in Figure 5.3. The system is made up of twelve 5.4 cm id x 75 cm long stainless steel columns connected at the top and bottom to form a closed loop. Six pneumatic poppet valves are associated with each column namely the feed, eluent and purge inlet valves, the glucose rich (GRP) and fructose rich (FRP) product valves and the transfer valve to the next column.

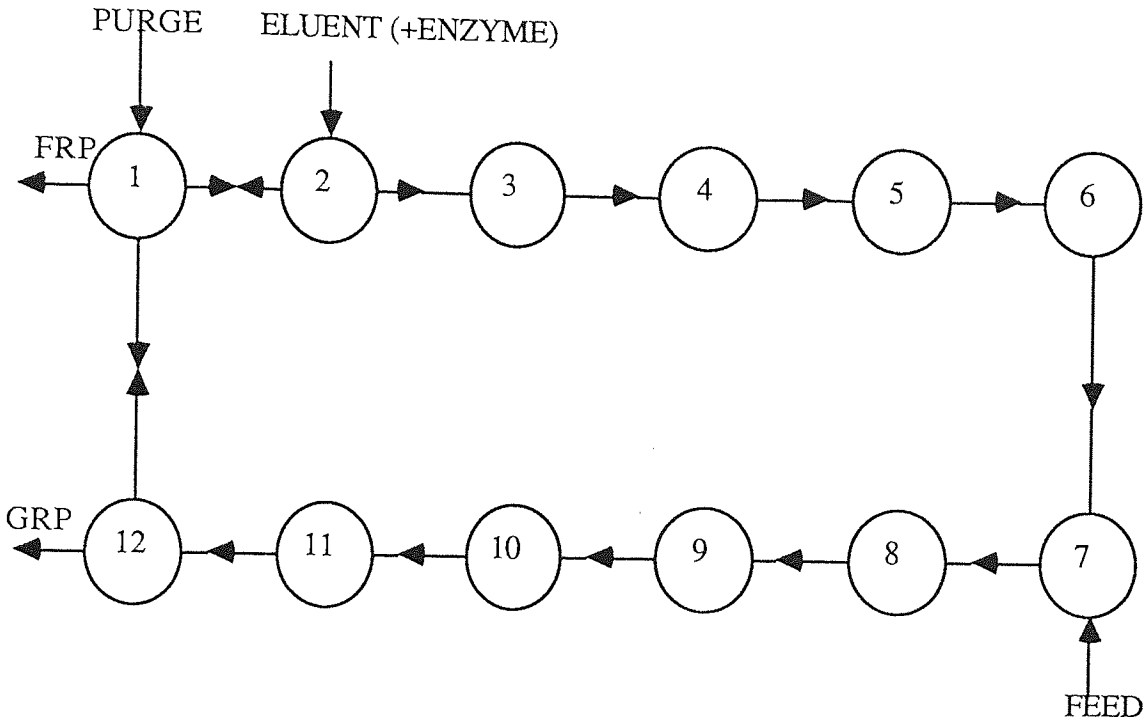
The principle of SCCR.S1 system operation is shown Schematically in Figure 5.4 where the whole system is illustrated as a closed loop. The feed is fed at port F and the less strongly adsorbed glucose product moves preferentially with the mobile phase towards the glucose rich product off-take P1. A section of the loop is isolated at any one time by the two locks V1 and V2 and an independent purge fluid stream (deionised water) enters at port PU, strips the adsorbed fructose product and exists from port P2 as the fructose rich product.

Figure 5.4(a) represents the component distribution within the system soon after "start-up". In Figure 5.4(b), all the port functions have been advanced by one position in the direction of the mobile phase flow. This port advancement results in a simulated movement of the stationary phase counter-current to the direction of the mobile phase. To achieve separation of the reaction products and hence to enriched product streams, the rate of port advancement must be greater than the fructose migration velocity through the bed and lower than the glucose and unreacted sucrose migration velocity. Figure 5.4(c). The frequency with which this port advancement occurs represent the "switch time".

Figure 5.3(a) represents the first switch period where column 1 is isolated and purged to give the FRP product. Feed and eluent enters columns 7 and 2 respectively and the GRP product is eluted from column 12. In the next switch period, Figure 5.3(b),

Figure 5.3:- Sequential Operation of the SCCR.S1 System

(A) SWITCH ONE



(B) SWITCH TWO

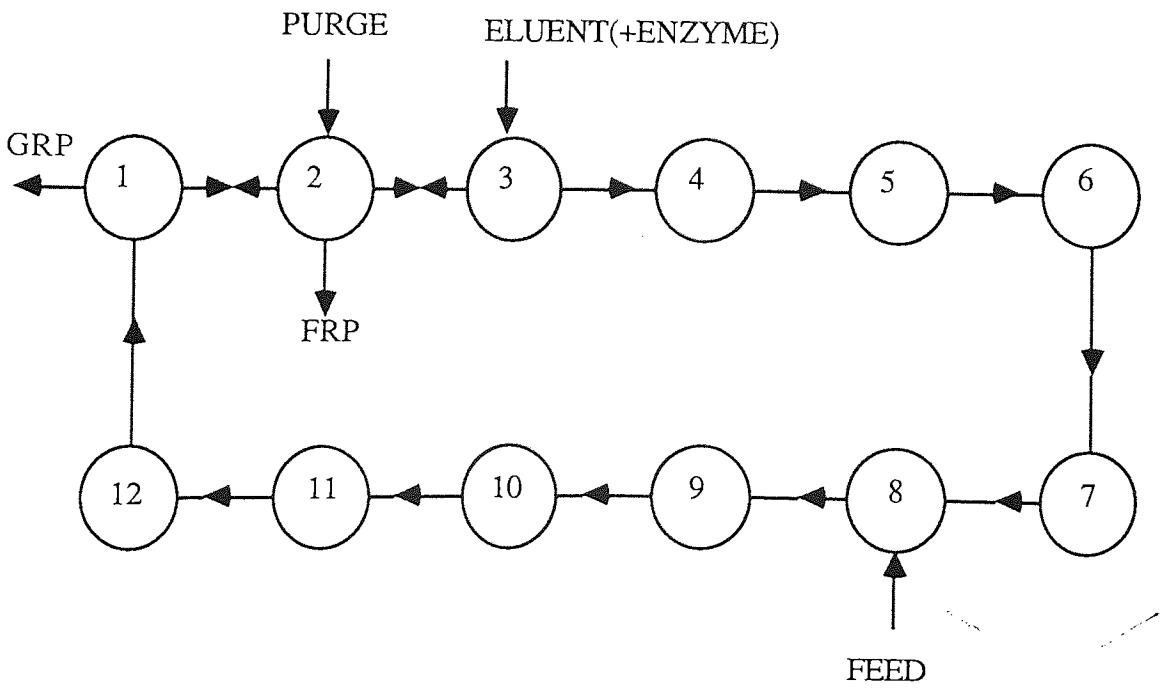
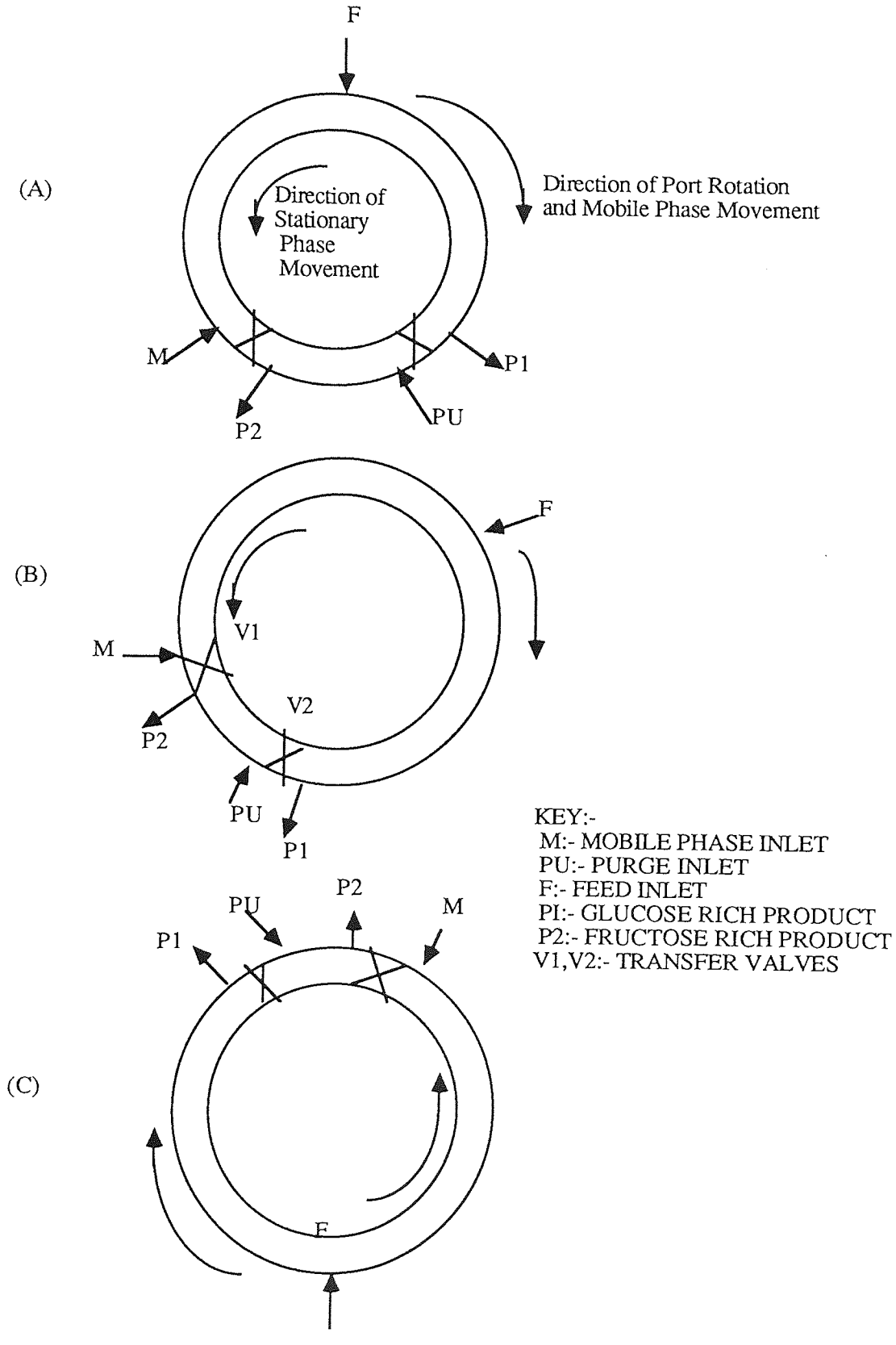


Figure 5.4:- Principle of Operation of the SCCR-S1 System



all ports are advanced by one position, then column 2 is purged, feed and eluent enters column 8 and 3 respectively and the glucose rich product exits at column 1. After twelve such advancements the "cycle" is completed.

5.1.2 Idealised Operating Conditions

During the inversion reaction of sucrose by invertase and the separation of glucose and fructose products on the counter-current semi-continuous system, glucose and unreacted sucrose move with the mobile phase and the fructose with the stationary phase. An idealised model can be constructed relating mobile and stationary flow rates, reaction rates and component separation. With reaction taking place only in the mobile phase a material balance on the glucose product about the feed point (Figure 5.5) gives:

$$Le \cdot Y_g + P \cdot X_g + V_1\gamma = O$$

where V_1 = mobile phase volume (cm^3)

Le = mobile phase flow rate ($cm^3\ min^{-1}$)

P = stationary phase effective flow rate ($cm^3\ min^{-1}$) = total system volume/(total number of columns x switch time)

γ = reaction rates ($g \cdot cm^{-3}\ min^{-1}$)

Y_g = glucose concentration in mobile phase ($g \cdot cm^{-3}$)

X_g = glucose concentration in stationary phase ($g.cm^{-3}$)

For glucose molecules to move preferentially with the mobile phase and where the reaction rate (γ) is predominatly taking place in the mobile phase

$$(Le\ Y_g + V_1\gamma) > P \cdot X_g \qquad 5.1$$

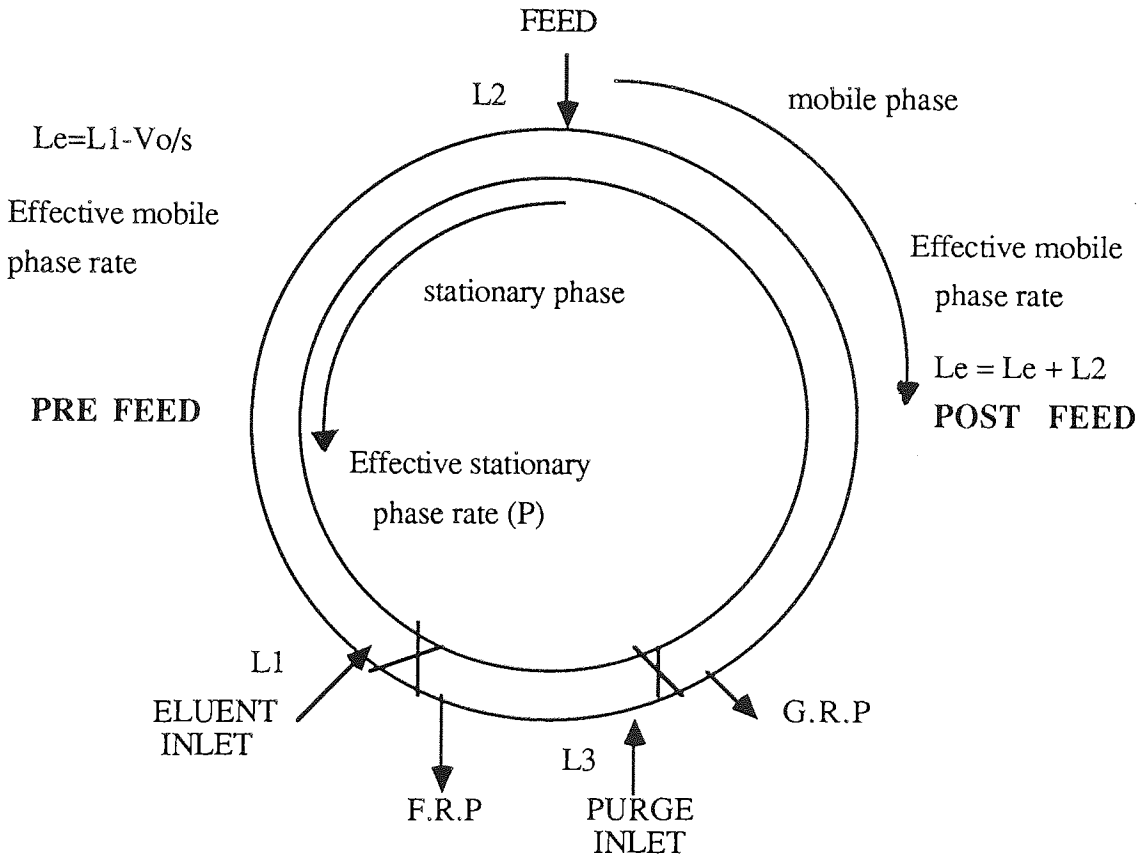
dividing P and Y_g and rearranging

$$\frac{Le}{P} + \frac{V_1}{P} \cdot \frac{\gamma}{Y_g} > \frac{X_g}{Y_g} \qquad 5.2$$

and since by definition

$$Kd_g = \frac{X_g}{Y_g} \qquad 5.3$$

Figure 5.5:- Diagramatic Representation of the Semi-Continuous Principle of Operation.



G.R.P: GLUCOSE RICH PRODUCT

F.R.P: FRUCTOSE RICH PRODUCT

Then

$$\left(\frac{Le}{P} + \frac{V_1 \gamma}{P.Y_g}\right) > Kd_g \qquad 5.4$$

where

$\left(\frac{Y_i . \gamma}{P . Y_g}\right)$ is a constant dependent on reaction rate (γ).

For sucrose to move with mobile phase:

$$\left(\frac{Le}{P} - \frac{V_1 \gamma}{P.Y_S}\right) > Kd_s \qquad 5.5$$

Similarly for fructose to move with the stationary phase

$$\left(\frac{Le}{P} + \frac{V_1 . \gamma}{P.Y_f}\right) < Kd_f \qquad 5.6$$

The theoretical limits of the mobile and stationary phase to give separation of fructose and glucose by combining Equation 5.4 and 5.6, i.e.

$$Kd_g < \left(\frac{Le}{P} + \frac{V_1 , \gamma}{P}\left(\frac{1}{Y_g} + \frac{1}{Y_f}\right)\right) < Kd_f \qquad 5.7$$

As each column contains eluent phase in the void volume, V_O, the effective mobile phase flowrate is reduced to:

$$Le = L_1 - \frac{V_O}{S} \qquad 5.8$$

where L_1 = mobile phase inlet flowrate ($cm^3\ min^{-1}$)

S = Switch period (min)

Because of the feed flowrate L_2, the effective mobile flowrate is different before and after the feed point. That means the effective mobile phase rate in the post feed section Le, becomes

$$Le' = Le + L_2 = (L_1 + L_2) - V_{O/S} \qquad 5.9$$

Therefore Equation 5.7 becomes:

$$Kd_g < \left(\frac{Le}{P} + \frac{V_1 \cdot \gamma}{P} \left(\frac{1}{Y_g} + \frac{1}{Y_f} \right) \right) < \left(\frac{Le'}{P} + \frac{V_1 + \gamma}{P} \left(\frac{1}{Y_g} + \frac{1}{Y_f} \right) \right) < Kd_f \qquad 5.10$$

and this equation now gives the true theoretical limits.

The purging flowrate L_3 in the isolated column is also governed by:

$$\frac{L_3}{P} \quad >> Kd_f \qquad 5.11$$

5.2 DESCRIPTION OF THE SCCR-S1 SYSTEM

5.2.1 Overview

The SCCR-S1 system consists of 12 stainless steel columns, 75cm long and 5.4cm internal diameter. The columns were packed with a calcium charged 7% cross-linked polystyrene resin supplied by Finn-Sugar Engineering (Finland), under the trade name KORELA VO7C and had a particle size range between 150 and 300 μm.

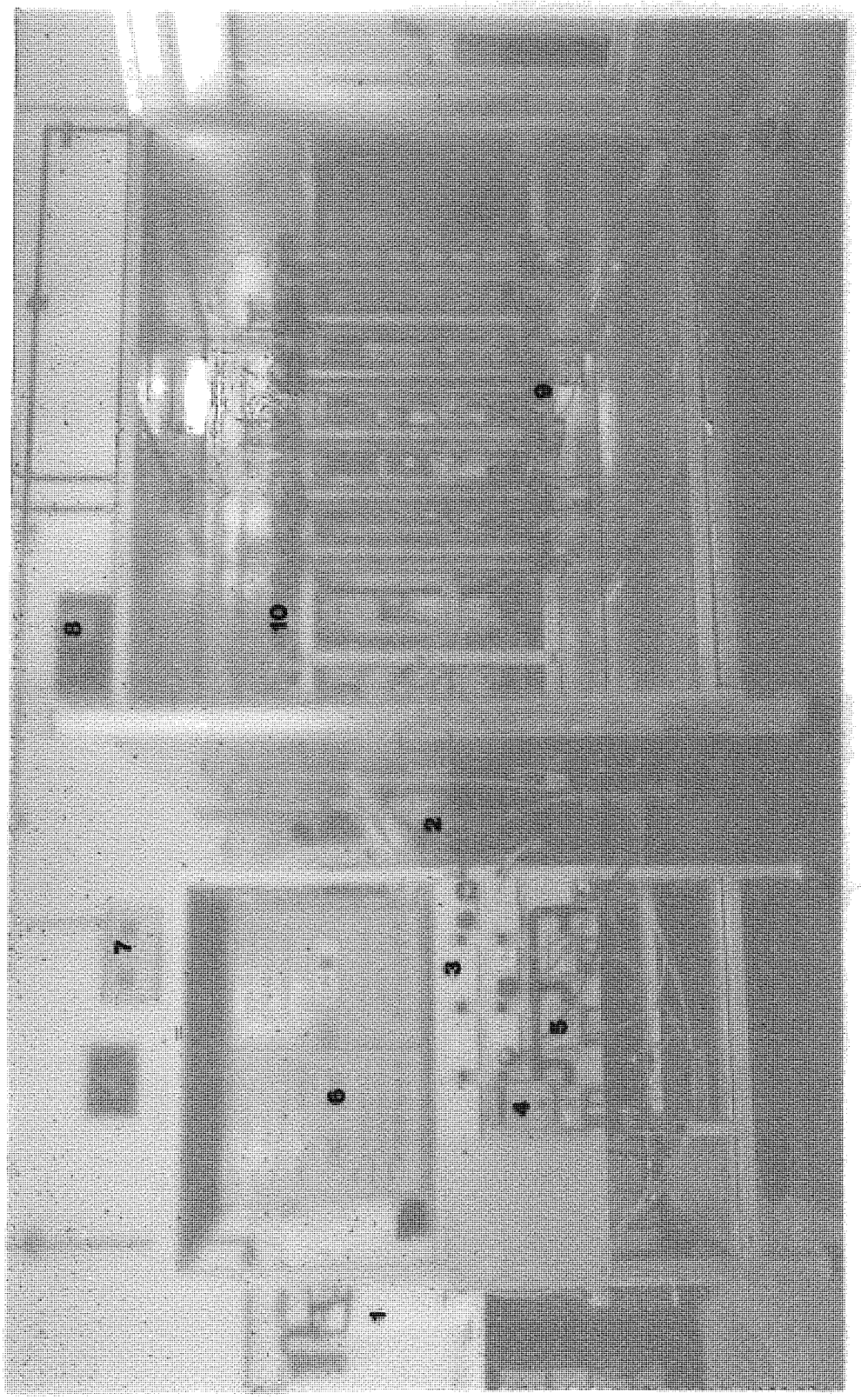
The equipment was originally constructed by Ching [100] for the separation of carbohydrate compounds and later modified by Chuah [101], Abusabah [99] and Ganetsos [9] for their research work. The SCCR-S1 has now been remodified to allow reaction and separation to take place. Figure 5.6 is a photograph of the apparatus used for this work.

The equipment layout is shown in Figure 5.7. The columns are mounted on a mobile frame and are enclosed in a constant temperature enclosure. The columns are linked by a ring distribution network connected to the supply pumps and product

Figure 5.6 : Picture of the SCCR-S1 System

KEY:

1. Feed Tank
2. Heater aspirator for the eluent and purge
3. Mains distribution board
4. Automatic cut off
5. Pressure sensors
6. Pneumatic controllers
7. Product splitting timers
8. Temperature controller
9. The SCCR-S1
10. Poppet valves



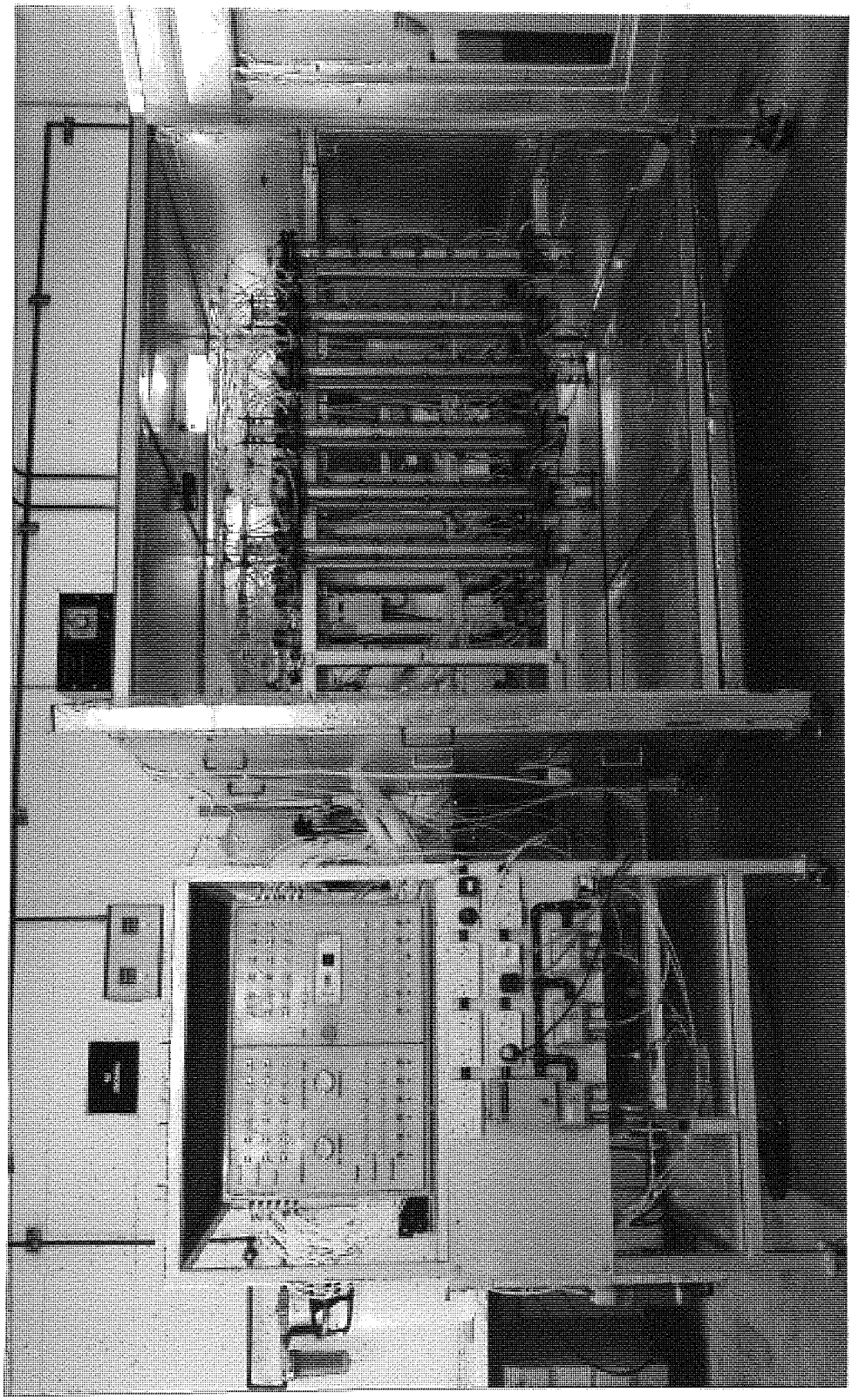
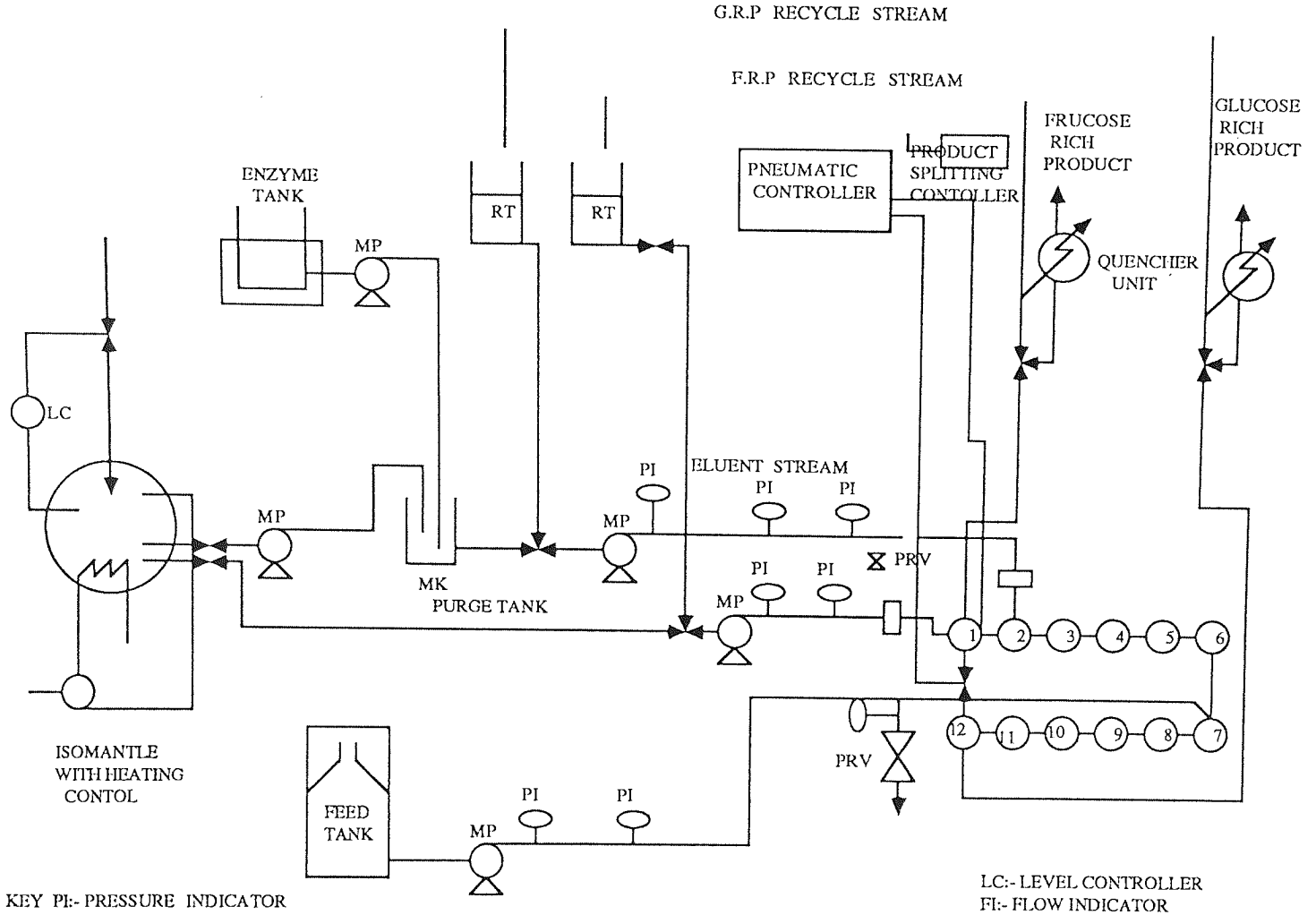


Figure 5.7:- Equipment Layout



collection devices. The sequential functioning of the poppet valves is governed by a pneumatic controller. A description of the units of the equipment is given in the following section.

5.2.1 The Columns and Fittings

The SCCR-S1 columns are fabricated from type 321 seamless stainless steel tube and designed according to BS5500 : 1976 for pressure vessels. Each column is 5.4 cm id and 75 cm long and fitted with ten 20 mm x 1/2 BSP sampling points. These sampling points are 2 cm long stainless steel rods welded to the columm with 2mm id holes. They are plugged with silicone rubber septa which are held in position by a simplifix nut. Samples are taken by inserting hypodermic needles through the septa into the column. At each end of the columns there is a 130 x 10 mm mild steel flange fitted and a 2 mm thick neoprene gasket used to seal the flanges to the column.

The column inlet assembly consisted of a 65 x 40 mm polypropylene inlet head and a compression plunger made of 130 x 19 mm stainless steel rod and a 54 x 50 μm polypropylene piston. A polypropylene ring was fitted at the piston's end and it retained a 100 μm polypropylene mesh and distributor used to ensure an even velocity profile. Two Dowty O-rings, no 20-830-4470 were fitted around the piston to ensure perfect sealing. A length of a column's wall on the inlet side was accurately machined to obtain a good seal with the dowty O-rings. Five 12.7 mm x 1/4 BSP ports were taped on the inlet head to accommodate CK - 1/4 PK4 - KU type Festo plastic connectors for feed, eluent, purge and transfer lines and the piston rod 12 mm x 2 mm thick neoprene gaskets of 6 mm diameter central hole was placed inside these ports to prevent leakages. Liquid channels of 3 mm diameter were drilled through the head, the plunger and the piston.

The outlet assembly consisted of a stainless steel packing support and a 70 x 38 mm polypropylene head machine to a T-shape to form a bottom 70 x 10 mm flange. The packing support consisted of two meshes of different sizes sandwiched together. The top

mesh is a 50 μm mesh to prevent fine resin particles falling through and blocking the liquid ducts and the bottom mesh is a 100 μm mesh used for the bed support.

Three 12.7 mm x 1/4 BSP ports were tapped on the outlet head and fitted with the same Festo fitting as the inlet head. The ports were connected to the fructose rich and glucose rich product lines and the transfer line. A 3° cone was turned on the inside face of the outlet bolck to ensure uniform flow of liquid out of the column. A 2 mm thick neoprene gasket was placed on each side of the support mesh to prevent leakages.

5.2.3 Pneumatic Poppet Valves and Controller

The flow of liquid in and out of the SCCR-S1 system was pneumatically controlled by operating 72 double acting poppet valves. Some of the advantages of these type of valves are their high degree of reliability, smoother operation and application for a wide range of operation [98, 100, 102]. These poppet valves constructed by Aston Technical Services Ltd, have a minimum internal volume, thus minimising the system dead volume. The switching sequence of these valves during a cycle has been illustrated by Ganetsos [9].

Compressed air supplied by the Department's main air compressor was used to operate the valves. A pressure relief valve, set at 700 kNm^{-2}, was fitted on the main air supply line. The air supply was divided into bias and actuating streams whose pressures were controlled to 240 kN m^{-2} and 550 kN m^{-2} respectively by a pressure regulator. The air supply lines were made from Festo PP3 polyamide tubing with a 3mm internal diameter. In a closed position the bias pressure was applied to the lower side and in the open position, the actuating pressure was applied to the upperside of the poppet diaphram. A locking nut was incorporated with the valves to give a proper seal and prevent any leakages.

The pneumatic controller, which governs the poppet valves operation, consists of a number of solenoid valves activated individually by a rotating Festo type PN-20B camshaft mechanism. There are twelve solenoid valves, each associated with a particular column. Each solenoid valve directly activates the appropriate transfer valves to isolate the corresponding column. The solenoid valves were each linked to double acting return valves which activated the appropriate feed, eluent and purge inlet and the product outlets. A digital timer programmable down to 10 seconds was incorporated to control the rotating mechanism which in turn opened and closed the solenoid valves at a fixed time period. Any change to the sequence of operation of the poppet valves can be achieved by altering the relative positions of the coloured "programme carriers" on the rotating mechanism. The timer was set at a preselected fixed time period which corresponded to the switch time, and at the end of that period an electrical signal was triggered, activating the electric motor to rotate the Camshaft Unit to the next position.

5.2.4 Fluid Delivery

Deionised water was used for the eluent and purging streams for the SCCR--S1 system. An Elgastat B224 deioniser was used to produce the deionised water. The deionised water was then stored in two large stainless steel tanks with a combined capacity of 750 litres. The water from the tanks was then passed through a Gelman Acroflow II cartridge of 1.2 μm pore size and a Millipore carbon cartridge in order to remove any impurities and ions present and then stored finally for use in an elevated $0.12m^3$ polypropylene reservoir. The need for a polypropylene reservoir was to enable the control of the pH of the water fed into the system and to minimise any possible hazard during overnight operation. The deionised water was then fed from the reservoir by gravity through 4 mm i.d Festo tubing and a solenoid valve into a 7.5×10^{-2} m^3 heated glass aspirator. The temperature and the water level inside the aspirator was controlled by a CAL 9000 temperature/level controller supplied by RS component Ltd. The water from the aspirator was then divided into eluent and purge streams. The purge stream was

pumped through a flow measuring device directly into the SCCR-S1 system. The eluent stream was pumped through a flow measuring device into a mixing tank.

The feed once prepared was stored inside an elevated 50 litres polypropylene container and then fed through a flow measuring device into the SCCR-S1.

The enzyme was stored inside an elevated 10 litre glass aspirator which was kept in an ice bath at around 4°C, thus reducing the loss of enzyme activity. The enzyme was then fed via a flow measuring device into the mixing tank where it was mixed with the eluent deionised water to the required strength and then fed into the SCCR-S1 system via another flow measuring device.

The set of pumps used during this research are the E-range metering pump series supplied by MPL pumps Limited. Loading valves were fitted on all pump lines to control the flow of liquid through the pump and to prevent flowrate fluctuations due to pressure differences in the columns.

All liquid supply lines were made of Festo PP4 and PP6 polyamide tubing. All lines were colour coded. In the SCCR-S1 System the columns were mounted in such a way that for two adjacent columns, the inlet of one was at the top and the inlet of the other at the bottom. This arrangement allows the system dead volume to be reduced. It was necessary to have an upper and lower pipe ring network. A 5% total dead volume of the system was estimated. The products were collected in plastic containers.

5.2.5 Pressure Control

In the case of pressure increase or decrease in the system, four Beta presure sensors (Loba Ltd, Reigate, Surrey) were fitted to the system, two on the eluent line inlet, one on the purge inlet and one on the feed line inlet stream. These sensors were connected to the power supply board and in the case of a malfunction they could switch

the system off. The high pressure sensor settings are, 345 kN m^{-2} on the purge stream and 483 kN m^{-2} on the eluent stream. The low pressure sensors on the feed and on the eluent lines are 28 kN m^{-2}.

Two pulsating dampeners were incorporated in the eluent and feed line in order to smooth out liquid flow. The dampeners are made of stainless steel with nitrile rubber diaphragms.

5.2.6 Heating Control

The deionised water used was heated in a 0.75 m^3 glass aspirator. The aspirator was electrically heated with 2 kW and 5 kW heaters. During start-up both heaters were used to boost the temperature to the required value. After which the 5 kW heater was switched off and the 2 kW heater was used to control the temperature.

The SCCR-S1 system was enclosed inside a heated enclosure constructed from galvanised steel sheets lagged with 50 mm thick glass fibre pads, and covered by aluminium foil. The enclosure was heated by a U-shaped 5 kW finned air heater supplied by Elton Ltd and was controlled by a diamond DH 82 type temperature controller. Additional safety devices were incorporated which would cut-off the electrical supply if the temperature inside the enclosure exceeded 70°C. The hot air was circulated by an electrical fan placed at the centre of the enclosure.

A set of Nickel-chrome thermocouples were used to measure the temperature on the column surface, in the liquid lines (purge and eluent) and in the product lines. The thermocouples were connected to a 12 way - type K selector switch box (RS components Ltd), which digitally displayed the temperature reading.

A quenching unit was installed at the product outlet lines to denature the enzyme during product collection. It consisted of two glass heat exchangers - one for the fructose

rich product line and the other for the glucose rich product line - connected to a heater/circulator unit supplied by Techne Ltd.

5.2.7 Product Splitting and Collection

To increase the product concentrations and to minimise enzyme usage, an attempt was made to separate the dilute product fractions coming out of the SCCR-S1 system. A splitting device was incorporated into each of the product lines. Each device consisted of a RS346-390 digital timer and two 3-way solenoid valves supplied by AJ Foster Ltd., (Manchester).

After a preselected time the solenoid valves were switched from one position to the other allowing the splitting of the corresponding product into two separate fractions. The more diluted fraction can be recycled back into the system, thus minimising the amount of enzyme and water used.

5.3 COLUMN CHARACTERISATION

5.3.1 Introduction

Characterising a chromatographic column, enables one to investigate the separating capacity of the packing material. This is normally carried out on a batch scale. The packing performance can be studied by varying the operating temperature and flowrates.

The methods used for the packing and characterisation of the columns were similar to those employed by previous workers [9, 10]. This allowed a direct comparison with previous work. Column characterisation was carried out at infinite dilution conditions i.e. very low sugar concentrations. Although, in practice, high sugar concentrations are encountered, correlations have been developed [9] to accommodate for this effect.

5.3.2 Ion Exchange Resins

5.3.2.1 Properties

Ion exchange resins are particles consisting of porous matries having electrically charged functional groups which are covalently bonded. Four main types exist:

(i) Synthetic resins

(ii) Cellulose ion exchangers

(iii) Ion-exchange polydextran (Sephadex)

(iv) Inorganic exchangers - based on alluminium silicate

Synthetic ion exchangers are most commonly, used in chromatographic processes.

The functional groups that are covalently bonded to the exchangers can be in the form of cation, anion, chelating, amphoteric and dipolar and selective ion-exchangers.

In this research work, synthetic cation exchangers were used and the mechanism of operation is as shown:

$$R - X^+ + Y^+ \rightleftharpoons R - Y^+ + X^+$$

The forward process is called adsorption and the reverse desorption. Equilibrium is governed by the concentration of the solute ions (Y^+) and the relative affinities of the ions for the exchangers. The synthetic resins are solid insoluble high molecular weight polyelectrolytes consisting of a three dimensional matrix with larger numbers of attached ionizable groups. These matrices are produced by polymerization of styrene cross-linked with itself and with divinyl-benzene (DVB).

The degree of cross-linking is very important in chromatography and it defines the average porosity of the exchangers. The process of cross-linking is easily controlled.

The more cross-links present the less an exchange swells. Swelling is known to disturb the chromatographic operation especially when resins of low cross-linking are used. In addition, although the lower cross-linked resin exchange ions more rapidly, they are less selective.

5.3.2.2 Particle Size of the Resin

The resin used for this research work was the same as that used by Ganetsos [9] and is the KORELA VO7C cationic package. The resin was 7% cross-linked polystyrene, and it was supplied in the calcium form by Finn-Sugar Engineering, Finland.

A particle size analysis was carried out using BS410 meshes and the results are shown in Table 5.1. Over 99% was within the 150 - 300 μm size range, with over 94% within 212 to 300 μm. These results were in agreement with the quoted value from the manufacturer, i.e. 95% with 40% of a mean (270 μm).

To remove the fines present in the resin and to avoid any blockages in the valves and column inlet and outlet ports, a settling technique was adopted. Two volumes of water were added to one volume of resin. The mixture was shaken vigorously and then left to settle. The top layer was decanted to remove the fine particles.

Table 5.1 : Sieve Analysis Results of the Korela VO7C Packing

Size Range μ m	Weight Collected %	Average Mass Fraction
>355	0.45	0.0045
300 - 355	0.8	0.008
250 - 300	72.0	0.72
212 - 250	22.1	0.22
180 - 212	3.55	0.0355
150 - 180	0.6	0.006
125 - 150	0.35	0.0035
<125	0.15	0.0015
TOTAL	100	1

5.3.2.3 Column Packing Technique

The old packing in the columns were removed and new neoprene gaskets were fitted. Before packing the column with a fresh resin material, each column outlet was connected to a vacuum system. They are then filled with deionised water.

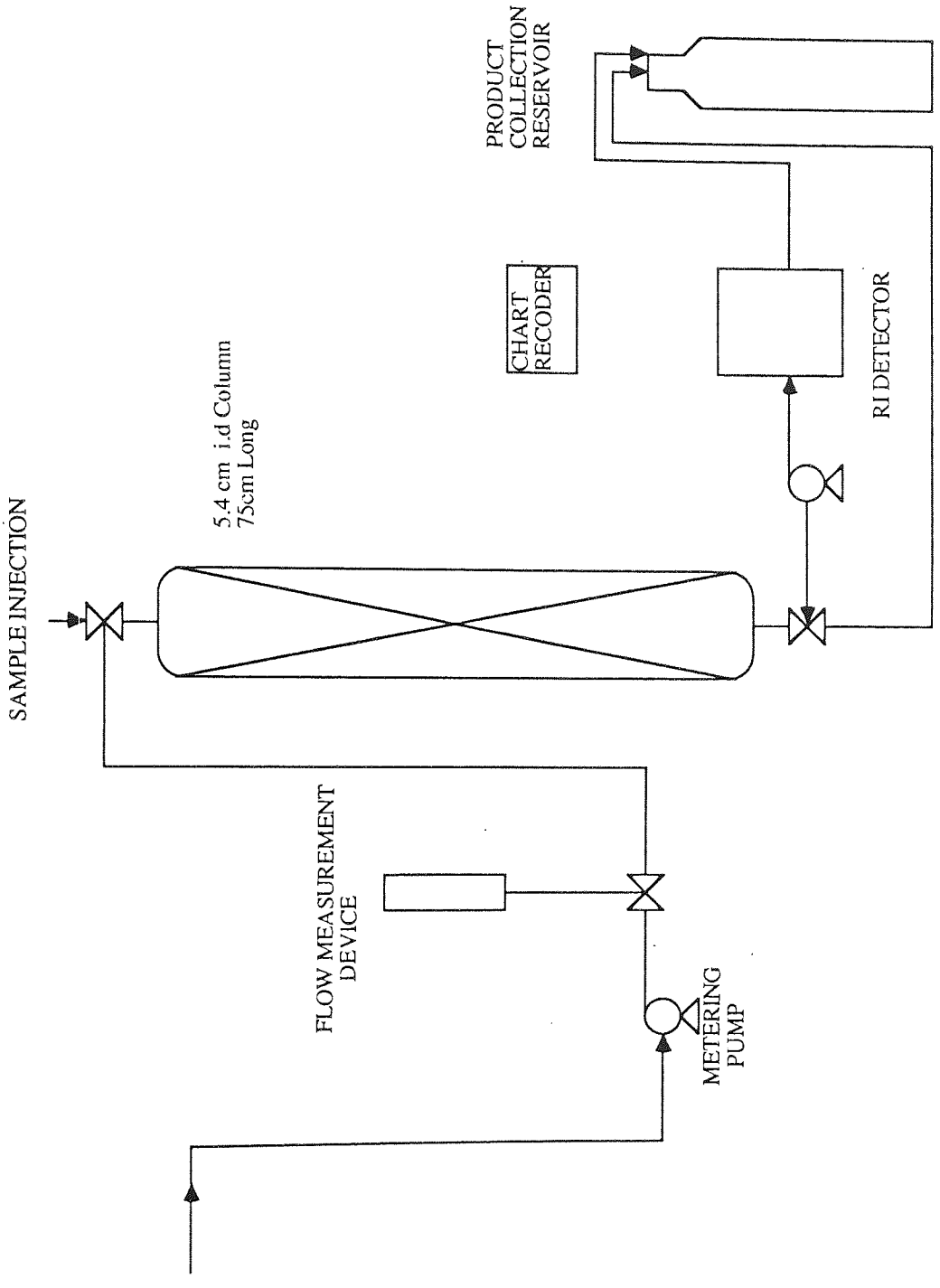
A 50% slurry of resin and deionised water was added to the column at the same rate that the water was removed by the vacuum suction. This method was used to minimise the segregation of the particles apparent in gravitational settling. During the packing procedure deionised water was added continuously and the column tapped at random. When the column packing height had reached approximately 66 cm, the procedure was stopped, the inlet plunger fitted and the column reassembled. Deionised water was pumped through the packed column for 45 min to remove any remaining fine particles.

5.3.3 Experimental Technique Used for Characterising the Columns

Each packed column in the SCCR-S1 system was individualy linked to the eluent delivery pumphead and a sample detection system as shown in Figure 5.8. A T-piece was connected as close as possible to the column inlet with a silicon rubber septum fitted to one of the T-piece inlets. The sample was loaded into the column by inserting a needle through the septum.

A similar T-piece was connected at the column outlet. A sampling needle was inserted into the rubber septum at the outlet T-piece allowing a small stream of the outlet stream to bypass through the sample detection system which consisted of a Technicon auto analyser, a proportioning pump, a Jobling Laboratory refractive index detector linked to a venture servoscribe Type 2 chart recorder. The refractometer's outlet stream was connected to a product collection reservoir together with the mainstream outlet from the column.

Figure 5.8: Equipment set up for Column Characterisation



Eluent flowrates of 25 cm^3 min^{-1} and 40 cm^3 min^{-1} were used. The 25 cm^3 min^{-1} flowrate was chosen to make a comparable performance evaluation of different size columns to the one used by previous workers [9, 101, 103] and the 40 $cm^3 min^{-1}$ flowrate was used because this was approximately the operating flowrate of the mass transfer section of the SCCR-S1 system. The proportioning pump drew a steady continuous stream of 1 cm^3 min^{-1} from the outlet stream. Separate 10% W/V solutions of dextran T70, glucose, sucrose and fructose were made and a 10 cm^3 slug of each of these solutions was injected through the inlet rubber septum. A peak was produced on the chart recorder as each sugar was eluted. The chromatograms produced were analysed and the method of determining the column parameters is described in the next section. All experiments were carried out at ambient temperature (20°C).

5.3.4 Column Parameter Calculations

The elution profiles of dextran, sucrose, glucose and fructose were similar to those shown in Figure 2.3, and were used in determining the column parameters. The elution volumes, V_i, of the individual components were calculated by multiplying their respective retention times, tr_i, with the eluent flowrate.

As the dextran T7 molecules are too large to enter the inter-particle volume and thus travel only through the interstitial or void volume, the elution volume of the dextran T70 was used as the column's void volume, V_O. The individual column volumes were expressed as voidage or void fraction i.e. Column Void Volume to total empty column volume. A measure of the column's separation potential is the separation factor, α, and for a sucrose - fructose mixture is:

$$\alpha = \frac{K^{\infty} d_F}{K^{\infty} d_S} \qquad 5.12$$

where:

$K^{\infty} d_F$ = distribution coefficient of fructose at infinite dilution.

$K^{\infty} d_S$ = distribution coefficient of sucrose at infinite dilution.

The distribution coefficients were determined using the fundamental retention equation (2.3). By rearranging Equation (2.3) in terms of the distribution coefficient gives:

$$Kd_i = \frac{V_i - V_O}{V_S} \qquad 5.13$$

Or for infinite dilution coefficient

$$K^{\infty} d_i = \frac{V_I - V_O}{V_T - V_O} \qquad 5.14$$

where i is the solute component.

The number of plates with respect to the individual components were calculated from the corresponding chromatogram and the Glueckauf equation (Equation 2.11).

The results for the individual columns and average values are shown in Table 5.2 and 5.3.

5.3.4.1 Discussion and Conclusion

The theoretical value for the voidage of a bed of spherical particles is reported to be 0.4 [104]. The average column voidage for the SCCR-S1 system was 0.38. The different packing geometry and the non-uniformity of the packing material was the cause of this low voidage value. The smaller particles were dispersed and filled the empty space between the larger ones. This was also enhanced by the compression exerted by

the piston plunger. The packing of the columns for the SCCR-S1 system was reasonably consistent with a voidage variation of ± 15% about the mean value.

The dextran molecules were totally excluded and were not delayed. The sucrose molecules diffuse slightly into and out of the intra-particle space and hence were delayed slightly with respect to the dextran molecules. The glucose molecules, due to their small sizes compared to sucrose molecules, diffuse more into and out of the intra-particle space and hence were delayed more than the sucrose molecule. The result of this was a larger elution volume for glucose compared to sucrose. For the fructose molecules, they were retained further due to chemical complex formation with the calcium ions on the resin thus giving even high elution volumes. The average elution volumes for sucrose, glucose and fructose were 670 cm^3 821 cm^3 and 1145 cm^3 compared to the average column void volume of 566 cm^3.

With an eluent flowrate of 25 cm^3 min^{-1}, the average separation factor for a sucrose and fructose mixture was found to be 5.6 and for a glucose and fructose mixture it was 2.27. The average number of theoretical plates per column was 33 with respect to fructose and sucrose and 46 with respect to glucose. This gave an average HETP value of 2 cm for fructose and sucrose and 1.46 cm for glucose. These values are in agreement with the results reported by Ganetsos [9] in his work with a similar system.

With a high eluent flowrate (40 cm^3 min^{-1}), a separation factor between sucrose and fructose was found to be 6.7 and 2.3 between glucose and fructose. Average HETP values of 3.13 cm, 2.19 cm and 2.79 cm were calculated for fructose, glucose and sucrose respectively. The difference in the column characterisation parameters (i.e. HETP and separation factor) between the eluent flowrates was expected.

The higher the eluent rate the less efficient the chromatographic column performs.

TABLE 5.2 : SCCR-S1 Column Characterisation for a 25 cm^3 min^{-1} Eluent Flowrate

Column No.	Bed Height (cm)	Void Volumes (cm^3)	Voidage	ELUTION VOLUME (cm^3)			DISTRIBUTION COEFFICIENT			NUMBER OF THEORETICAL PLATES			HETP'S (CM)		
				SUC	GLU	FRU	Kd_S	Kd_G	Kd_F	SUC	GLU	FRU	SUC	GLU	FRU
1	65.5	505	0.336	647.5	790	1130	0.14	0.283	0.62	28	35	30	2.34	1.87	2.18
2	66.0	557.5	0.369	695	847.5	118.5	0.144	0.304	0.658	25	41	29	2.64	1.61	2.27
3	66.0	527.5	0.348	640	775	1115	0.114	0.25	0.597	29	41	30	2.27	1.61	2.27
4	65.5	547.5	0.365	620	772.5	1065	0.76	0.236	0.543	35	41	32	1.87	1.42	2.04
5	65.7	570	0.379	650	832.5	1166	0.08	0.267	0.6	29	44	33	2.26	1.49	1.99
6	65.5	650	0.433	747.5	885	1225	0.115	0.276	0.676	34	58	40	1.92	1.13	1.64
7	65.6	562.5	0.375	700	865	1167.5	0.146	0.32	0.64	35	42	36	1.87	1.56	1.82
8	65.5	617.5	0.41	722.5	882.5	1195	0.119	0.3	0.654	45	47	43	1.45	1.39	1.52
9	65.8	625	0.415	690	827.5	1160	0.073	0.229	0.606	34	47	31	1.93	1.4	2.12
10	66.2	537.5	0.354	700	835	1160	0.166	0.304	0.636	34	51	37	1.95	1.3	1.84
11	65.6	592.5	0.394	710	865	1190	0.13	0.299	0.657	46	59	32	1.43	1.11	2.05
12	65.9	522.5	0.348	642.5	782.5	1120	0.123	0.266	0.61	26	40	30	2.53	1.65	2.2
Average	65.7	568	0.377	680.4	830	1156.5	0.119	0.278	0.624	33	45	34	2.04	1.46	1.99

TABLE 5.3 : SCCR-S1 Column Characterisation for a 40 cm^3 min^{-1} Eluent Flowrate

Column No.	Bed Height (cm)	Void Volumes (cm^3)	Voidage	ELUTION VOLUME (cm^3)			DISTRIBUTION COEFFICIENT			NUMBER OF THEORETICAL PLATES			HETP'S (CM)		
				SUC	GLU	FRU	Kd_S	Kd_G	Kd_F	SUC	GLU	FRU	SUC	GLU	FRU
1	65.5	516	0.344	604	776	1084	0.088	0.26	0.57	25	28	17	2.66	2.35	3.9
2	66	600	0.397	696	856	1178	0.105	0.28	0.634	22	30	19	3.07	2.18	3.55
3	66	564	0.346	644	820	1140	0.084	0.27	0.608	22	32	20	3.0	2.08	3.3
4	65.5	532	0.354	588	776	1080	0.058	0.252	0.566	20	22	19	3.21	3.03	3.45
5	67.8	512	0.34	588	776	1080	0.058	0.252	0.566	20	22	19	3.21	3.03	3.45
6	65.5	656	0.43	728	900	1236	0.085	0.289	0.687	28	35	20	2.37	1.85	3.36
7	65.6	648	0.43	680	844	1144	0.04	0.23	0.58	28	35	20	2.35	1.88	3.36
8	65.5	612	0.408	692	868	1188	0.09	0.288	0.648	35	47	29	1.89	1.41	2.29
9	65.8	608	0.404	680	828	1164	0.08	0.246	0.622	22	28	21	3.02	2.36	3.18
10	66.2	588	0.42	720	880	1240	0.142	0.314	0.702	20	26	24	3.38	2.5	2.79
11	65.6	612	0.407	712	888	1210	0.112	0.31	0.674	22	39	21	3.02	1.7	3.2
12	65.9	520	0.346	644	784	1100	0.126	0.269	0.592	22	28	31	3.0	2.36	2.10
Average	65.7	580.6	0.385	664.6	832.6	1154	0.09	0.27	0.619	24	31	22	2.79	2.19	3.13

It was observed that on average there was not too much difference in the distribution coefficient (Kd_i) of the components with the two eluent flowrates at low sugar concentration and ambient temperature. However, previous workers [10, 105], have shown that eluent rates, background concentration and temperature do have an effect on the distribution coefficient. The effect of eluent rate on the distribution coefficients can be observed in Tables 5.2 and 5.3 when the distribution coefficients of each column are compared at the different eluent flowrates.

Variation in voidage, distribution coefficient and HETP values from column to column are due to the non-uniformity of packing and the different degree of calcium charging of the individual columns. Ideally all these column parameters should be constant in order to ensure similar migration velocities for the individual components through successive columns. However, these variations can be tolerated in the SCCR-S1 system because eleven columns are serially linked at any one time and continuous cyclic operation ensures comparatively constant component migration rates through the system.

5.4 EXPERIMENTAL OPERATION OF THE SCCR-S1 SYSTEM

5.4.1 Synthetic Feedstock Preparation

Fructose was purchased in a pure food grade crystalline form from Roche Products Ltd (Dunstable, Beds). Glucose was purchased from Sigma Chemical Co Ltd (Poole, Dorset) in an anhydrous crystalline form and sucrose was purchased from British Sugar (Kidderminster). Dextran was obtained from Fisons PLC (Holmes Chapel, Cheshire).

The feed solution of the required concentrations was prepared by dissolving the calculated weight of the sugars in deionised water. For high concentration of sugar, solubility is difficult to achieve. The glucose and dextran had to be dissolved with continual stirring of the solution to prevent lumps being formed. The fructose and sucrose are easily dissolved.

The quantity of feedstock required for a complete run was prepared in one batch. To prevent any microbial growth in the feed storage tank, tubings or in the columns, a solution of 0.02% w/v sodium azide, NaN_3, was used. This was particularly necessary at ambient temperature and low concentration runs.

The prepared feedstock was analysed daily using HPLC to ensure that the feed composition and concentration did not change.

5.4.2 Enzyme Preparation

The invertase was purchased from Biocon Ltd (Tenbury, Wells) in liquid form under the trade name of Bioinvert. It is a clear concentrated solution and is produced by the extraction and purification of the enzyme from a selected strain of Saccharomyces cerevisiae. It had an activity of 11,600 U/cm^3 and a storage life of 3 months when stored at 20°C.

The enzyme dextransucrase was used for the biosynthesis of dextran from sucrose. The enzyme was produced from the strain NRRL-512F of Leuconostoc mesenteroides at Fison (Holmes Chapel) and at Aston University. It was purified using ultrafiltration methods as described in Chapter 9. Dextransucrase is very unstable and needs to be kept in cold storage (4°C).

For each run, the required activity of the enzyme being used is made up and put in the enzyme storage tank which is kept in an ice bath. The activity of the enzyme is measured regularly to check if there is any loss of activity during the run.

5.5 PRELIMINARY CHECKS AND START-UP PROCEDURES

For safe operation of the system, the following procedures were carried out before commencing a run. The feed and enzyme had to be prepared as described in Section 5.4 and placed in their respective tanks which are connected to the equipment. These tanks hold enough capacity for half a run and needed to be refilled regularly.

The desired temperature settings were selected and the column oven heater and the heater heating the deionised water tank switched on. The air circulating fan, in the oven was also switched on to distribute the heat evenly in the oven.

The air supply to the system was turned on and the pneumatic controller was set to the required switch time for the run.

All inlet and outlet valves were opened and the product collecting containers were positioned. In the latter part of the research work when the automatic product splitting system was used the appropriate timers were set to the proper values.

The eluent, feed, enzyme and purge pumps were switched on and set to the required flowrates.

When the correct operating pressure was reached the automatic shut off mechanism was activated and all controllers were checked.

5.6 PROCEDURES DURING AN EXPERIMENTAL RUN

During any run, the eluent, feed, enzyme and purge flowrates were checked frequently to ensure constant operating conditions. The flowrates were measured at the inlet using the calibrated measuring device installed on each line. The pressure drop in the streams and the temperature inside the enclosure were recorded during each cycle.

At the beginning of a cycle, the quencher unit installed on the product lines was switched on and set to 75°C to deactivate the enzyme in the product.

Half way through a switch a sample was withdraw from the same sample point on the same column and then analysed to produce the on-column concentration profiles of each cycle. This was possible because each column served a different function every switch i.e. as a feed, eluent entry, purge column or any other column of the separating length.

At the end of each cycle the product collecting vessels were weighed and analysed and a mass balance carried out. When a pseudo-equilibrium was reached, usually after 9 or 10 cycles, the results of the last two cycles were used to indicate the system performance. The run was usually continued to cycle 16, to ensure that a pseudo-equilibrium had been reached.

At the end of the run, all the pumps were switched off simultaneously. The controller was then reset to a new purging period (i.e. 20 minutes). The purge pump was switched on and the flowrate increased to 120 cm^3 min^{-1}. The products were collected in a separate container, thoroughly mixed and analysed. The procedure was repeated until all columns were purged out. The plotting of the analysed data gave the purging concentration profiles and represented the average carbohydrate concentrations in each column for the particular operating conditions. When the SCCR-S1 system was operating as a bioreactor-separator, the purging concentration profiles did not represent the average concentration since reaction was still occurring inside the system after switching off. As a result, the purge concentration profiles were of limited value in the bioreaction-separation runs.

The operating conditions and results of each experimental run were presented in a codified form and the run was defined with a set of six figures. For example, the set 20-9-31.5-30-60-55 corresponded to a run having the following experimental conditions:

20	=	feed concentration (% w/v solids)
9	=	feed flowrate ($cm^3\ min^{-1}$)
31.5	=	Eluent flowrate ($cm^3\ min^{-1}$)
30	=	Switch time (min)
60	=	Enzyme activity (Ucm^{-3}) $DSUcm^{-3}$)
55	=	Temperature (°C)

Since the SCCR-S1 system operated in a semi-continuous mode, only a "pseudo-equilibrium" state could be achieved and not a "true" equilibrium like in other counter-current mass transfer processes such as continuous distillation. When the on-column concentration profiles of two consecutive cycles were identical, within experimental accuracy, the "pseudo-equilibrium" state was deduced to have been reached (Figure 5.9 and 5.10). The reference points were the relative concentrations in the corresponding columns and the position of the "cross-over" point, i.e. the point of intersection of the glucose and fructose profiles.

5.7 SHUT-DOWN PROCEDURE

To shut down the SCCR-S1 system all pumps were switched off followed by the switching off of the enclosure heater, quencher, deionised water heater and the air fan. The product lines were then blocked to prevent the packing from drying out.

The pneumatic controller and the main valve in the deionised water supply line was also closed. The compressed air supply was turned off and the electrical power to the system was switched off.

When the SCCR-S1 system was not in use for long periods, 0.02% w/v sodium azide was pumped through to prevent any bacterial growth.

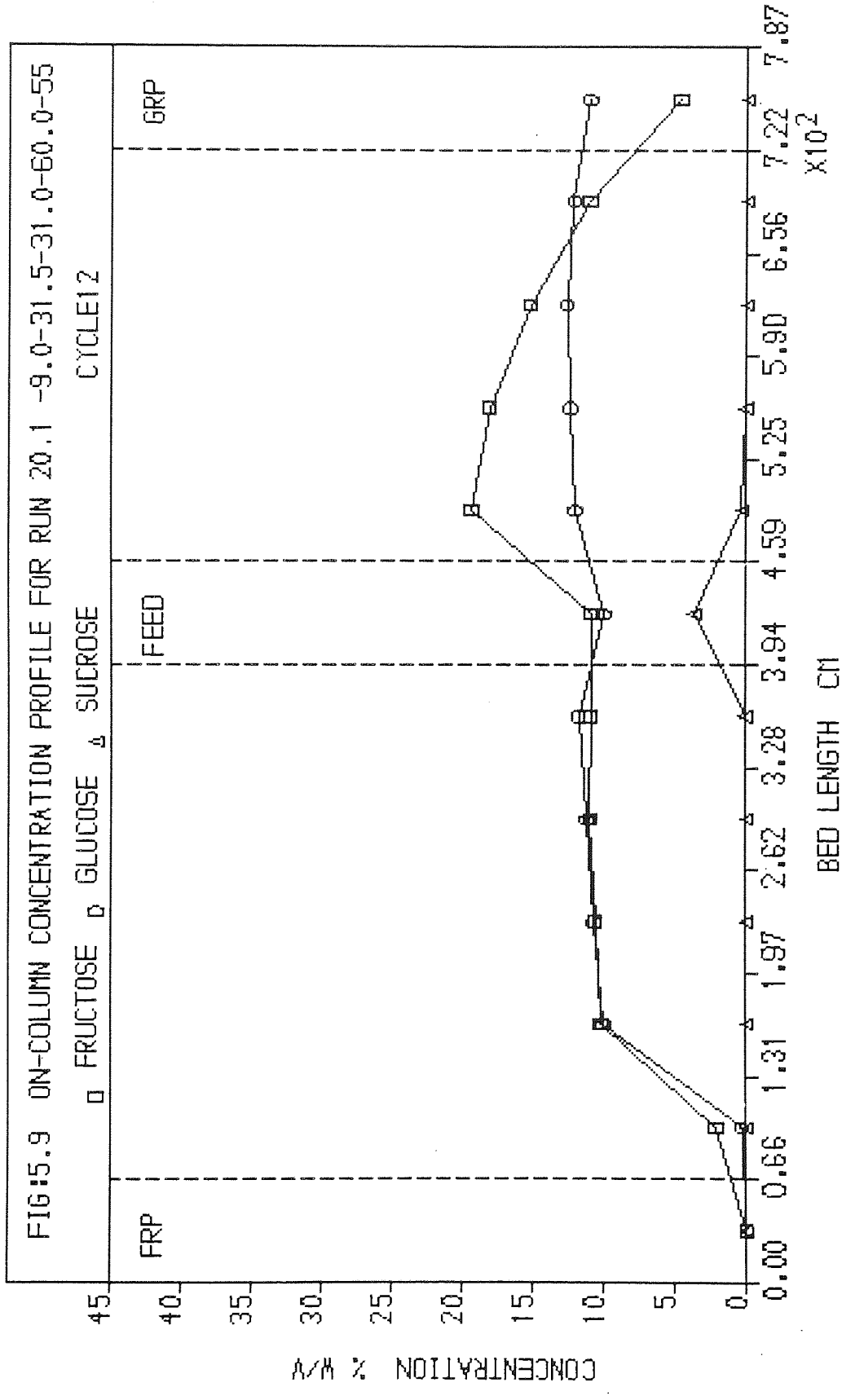


FIG:5.9 ON-COLUMN CONCENTRATION PROFILE FOR RUN 20.1 -9.0-31.5-31.0-60.0-55
□ FRUCTOSE ○ GLUCOSE △ SUCROSE CYCLE12
FRP
FEED
GRP
CONCENTRATION % W/V
BED LENGTH CM
X10²
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
0
5
10
15
20
25
30
35
40
45

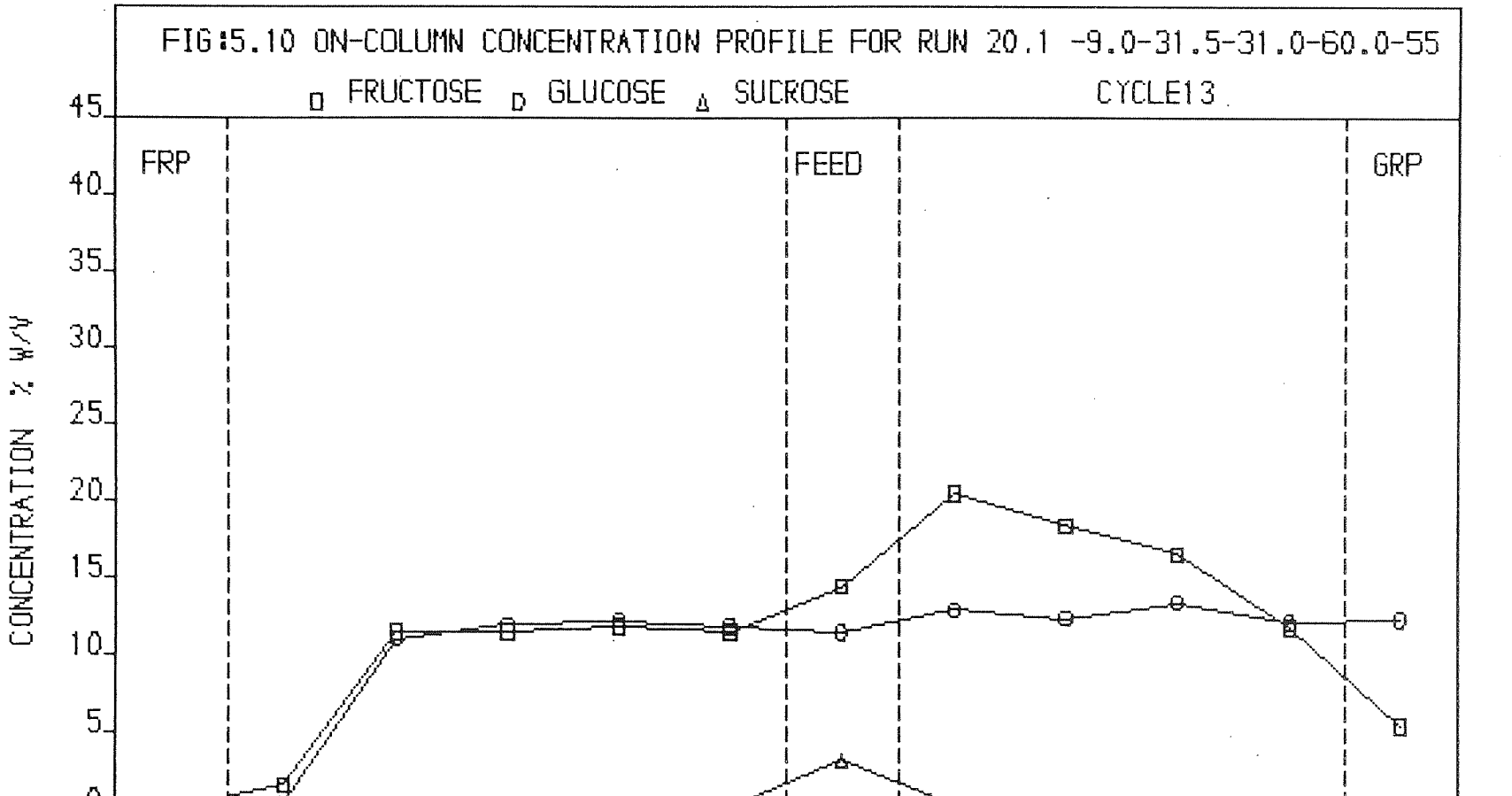


FIG:5.10 ON-COLUMN CONCENTRATION PROFILE FOR RUN 20.1 -9.0-31.5-31.0-60.0-55
FRUCTOSE
GLUCOSE
SUCROSE
CYCLE13
FRP
FEED
GRP
CONCENTRATION % W/W
45
40
35
30
25
20
15
10
5
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10^2
BED LENGTH CM

CHAPTER 6

ANALYTICAL EQUIPMENT AND ENZYME ASSAY TECHNIQUES

6.0 INTRODUCTION

The analytical techniques used throughout the course of this work were HPLC, GPC and gel electrophoresis. HPLC was used to measure reactant and product concentrations in the chromatographic bioreaction - separation studies and also used in work related to enzyme assay and purification.

GPC was used to obtain the MWD of dextran produced from the biosynthesis reaction and gel electrophoresis was employed to determine the proteins present in the enzyme solution and in estimating the molecular weight of the enzyme used.

Enzyme assay techniques were also used to measure enzyme activity. The invertase assay technique was used to calculate the activity of the invertase enzyme and the Hostettler method [106] used in finding the activity of the enzyme dextransucrase.

6.1 THE ANALYTICAL HPLC SYSTEM

6.1.1 System Description

An isocratic High Performance Liquid Chromatography (HPLC) system was used for the analysis of the samples generated during the experiments. It consisted of :

- a 30 x 0.78 cm AMINEX HPX - 87C calcium charged BIO-RAD column.
- a BIO-RAD 1330 Pump (BIO-RAD UK Ltd; Watford). The pump employed a two piston arrangement which operated out of phase by 180° thus minimising flow pulsations;
- a BIO-RAD column block heater;
- a BIO-RAD refractive index monitor model 1750;
- a SPECTRA - PHYSICS SP4270 Integrator (St Albans, Herts),
- and a TALBOT AS1-4 Auto-sampler (Alderley Edge, Cheshire);
- The eluent was effectively degassed by heating it to 80°C. This was achieved using an isomantle and a Baird and Tatlock (Atherstone Warwickshire) temperature controller.

- an Anachem (Luton, Bedfordshire) debubbler was used to remove any bubbles that found their way inside the eluent delivery pipe.
- An eluent flowrate of 0.5 cm^3 min^{-1} was used and the pressure drop of the system was approximately 6900 kNm^{-2}.

6.1.2 System Maintenance

The HPLC column used is a microparticulate gel ion exchange bed designed for chromatographic analysis of sugars. The resins although they are suitable for separating certain solutes, they are also capable of retaining other impurities - thus resulting in a decrease in column efficiency. To minimise this occurring the following precautions were taken:

- distilled deionised water, filtered using a 10μm slip-on filter, was used as eluent;
- the eluent was heated to 80°C to prevent bacterial growth;
- 0.02gL^{-1} calcium acetate was added to the eluent to replace any calcium ions lost from the resin due to the presence of other ions and to prevent any on-column sucrose inversion;
- the column was flushed with 0.02% W/V sodium azide when not used for long periods;
- the colunm was reversed after every 1-2L of eluent had passed through the bed;
- when a reduction in performance was noted, the columns were cleaned with a 30% acetonitrile solution and then regenerated using 0.1M calcium acetate. The use of calcium chloride, calcium nitrate or any other calcium salts formed from a strong acid was avoided as this led to column corrosion and irreversible bed poisoning [107].
- a guard column was used as a pre-column to filter any isoluble matter present in the system. Hibar-Lichocart 4-4 cartridges (BDH Chemicals Ltd,

Atherstone, Warwickshire) were used and replaced regularly as they have a tendency to clog easily.

- reduced sensitivity of the system was corrected by cleaning the refractometer with 6M nitric acid.

By operating at a pH of around 7, approximately 2200 samples were analysed on this column and it is in regular use.

6.1.3 System Operating Procedure

The analysis sample was filtered using a 0.45μm GELMAN filter (Gelman Ltd, Brackmills, Northampton) and was introduced either manually or via the autosampler into a 20μL injection loop in the injection valve. The sample was then injected and the integrator was activated simultaneously. The results were interpreted as areas under plotted peaks on the integrator and these were compared to areas corresponding to standard solutions, i.e. solutions of known concentration. The retention times of the component are shown in Table 6.1.

6.1.4 System Reliability

The column is the heart of any HPLC system and therefore reflects the reliability of the system as a whole.

The AMINEX BIO-RAD column, has a good resolution for monosaccharide separation. However, the detection of high molecular weight dextran (> 100,000 MW) was very poor. This poor resolution of dextran can be attributed to the dextran molecules being adsorbed on the resin, thus causing high pressure in the system.

Table 6.1 : Retention Times of Sugars

Component	Retention Time (min)
Dextran	7.5
Sucrose	9.97
Glucose	12.23
Fructose	15.73

6.2 ANALYTICAL GPC SYSTEM

6.2.1 Equipment Description

The GPC System consists of an eluent reservoir, an isomantle linked to a thermal cut out unit, a microfilter, a pump, a sample injection valve, a fractionating column (Lichro sphere Diol Columns), column heater, chart recorder and a PET computer.

The eluent used was deionised water which was degassed in the isomantle by raising its temperature to 80°C.

The eluent was pumped by a dual piston, low pulsation pump (model 1330, Bio-Rad Labs). The samples were filtered with an 0.45μm mesh acrofilter and injected using a Rheodyne valve (Bio-Rad Labs) fitted with a 20μl sample loop. The GPC columns were enclosed in a column heater (Bio-Rad Labs) and maintained at the required temperature.

The fractionated dextran from the GPC columns may then pass through a differential refractometer (model R401, Waters Associates Ltd, London), where it was compared with a standard reference of deionised water. The refractometer output was recorded by a PET computer (model CBM 4032, 32k bytes, Commodore, UK) which

was programmed to store and print heights such that they could be used to obtain the MWD of dextran.

The resulting voltage output from the refractometer was also registered on a chart recorder (Venture Servoscribe, RE 541.2, Smith Ltd) which produced the corresponding chromatogram.

6.2.2 Data Aquisition

The height values of the chromatogram of samples at set time periods can be obtained by the use of the PET computer with the associated Commodore printer (model CBM 4022P). From these heights a program was developed to assess the calibration of the GPC column and calculate the MWD of dextran. The listing of the program used is given in Appendix A.

This program recorded and printed the heights in the following way:

- Immediately after the sample was injected the program was initiated and readings from the refractometer are recorded via the multiplexer. At this point also, the computer internal clock is set to zero.
- Every two seconds the program reads from the refractometer. This procedure is repeated until five readings have been taken. These five readings are then averaged to give one reading which corresponds to an average height every 10 seconds of the chromatogram.
- The program enters a loop during the calculation of the heights. Moreover, to help the operator, these heights are continually displayed on the computer's VDU.
- The program comes out of the loop when the time has reached its maximum setting.

Once the sample elution has finished the recorded heights along with the sample times are printed out. The printout is used to calculate the MWD by the use of a calibration method.

6.2.3 Calibration of the GPC Columns

Before any analysis of samples can be carried out, the columns need to be calibrated using several Pharmacia dextran T-fraction [108] whose weight average molecular weights Mw, had been measured previously by light scattering.

A variety of methods can be used for calibrating the GPC columns [109-118], however, detailed investigation by Vlachogiannis [120] and Bhrambra's [119] work showed that the most convenient method was that devised by Nillson and Nillson [121]. The molecular weight (M) is expressed as a five term polynomial, where:

$$M = B_5 + \exp\left(b_4 + b_1\,(kd) + b_2\,(kd)^2 + b_3\,(kd^3)\right) \qquad 6.1$$

b_1 - b_5 are constants

kd is the distribution coefficient, and is determined from Equation 2.3.

$$Kd = \frac{V_r - V_o}{V_1} \qquad 6.2$$

$$V_1 = V_t - V_o \qquad 6.3$$

V_1 is the internal (pore) volume of the column.

V_t is the total liquid volume of the column.

V_o is the void volume of the column.

The values of V_t and V_o can be found experimentally by using a glucose and a very high molecular weight dextran standard respectively. The glucose molecules penetrates the pores easily, so it can be used to give V_t. The high molecular weight

dextran is totally excluded hence it gives the interstitial volume V_0. The calibration limits depend on the molecular weight of matierals which freely enter the pores and those which are totally excluded. Calibration profiles follow a characteristic S shape. Figure 6.1 shows the relationship between molecular weight and elution volume (V_r) on a semi-logarithmic scale.

In an ideal situation, the GPC column should only be calibrated and used within the linear section c-d, however, this is not possible. By using a series of dextran T fractions it is possible to calibrate over the whole fractionation range. Once the elution profiles from each T fraction was obtained, the calibration constants b_1 - b_5 were found using an optimisation program which was the Hertley modification of the Gaussian-Newton method [121]. It requires inputs of the heights, elution volumes (converted to kd values), and light scattering MW values of the T-fraction chromatograms with initial guesses of the b_1 - b_5 constants. The program produces a comparison MW values obtained from light scattering and the GPC system (Table 6.2). To check the accuracy of the calibration the standard deviation between the two sets of values were calculated. A standard deviation of less than 5% was found acceptable.

Figure 6.1:- A Typical Calibration Curve For A GPC System

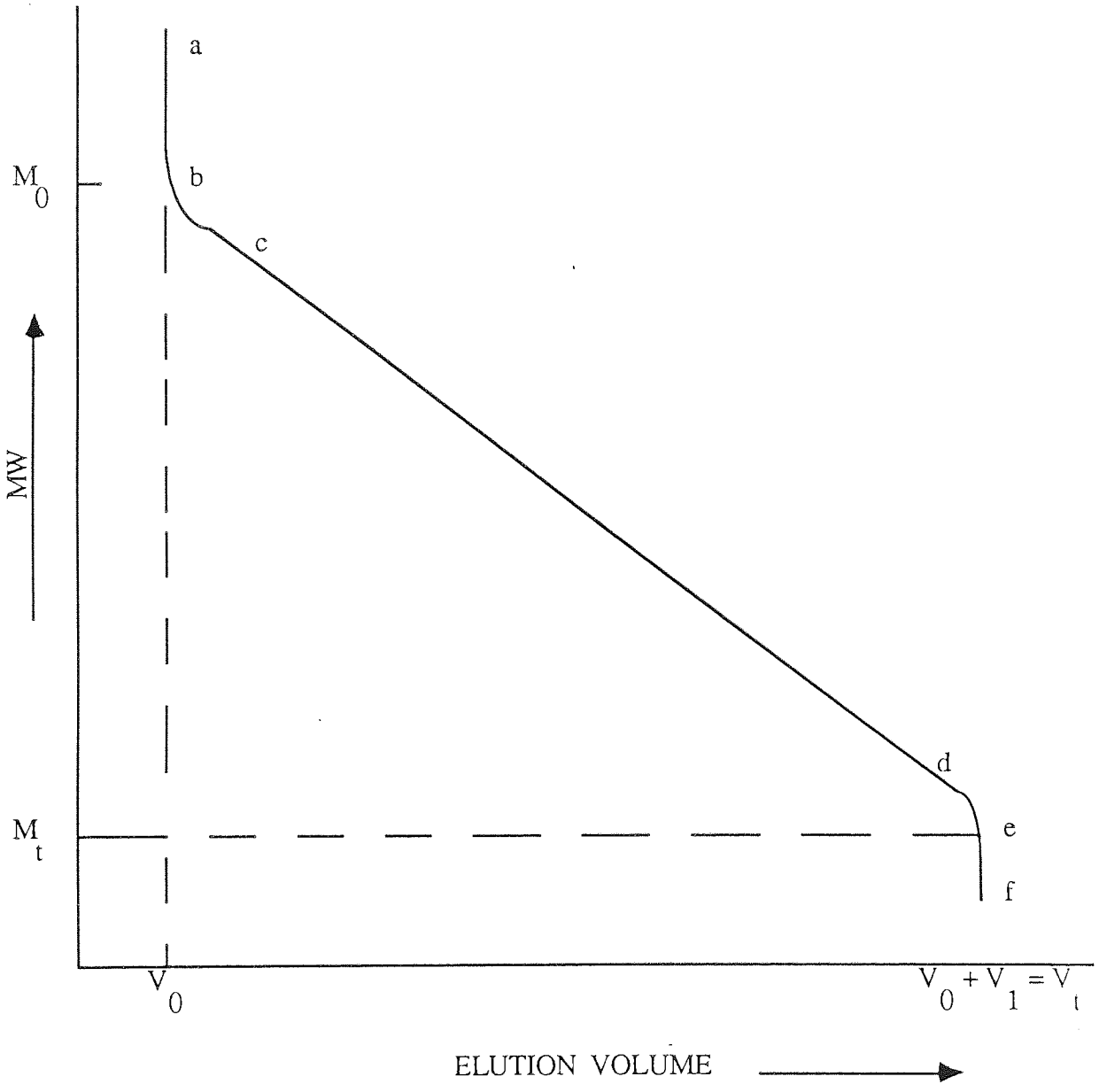


Table 6.2 : Calibration of the Diol Columns

Batch Number	Weight Average Molecular Weights		GPC x 100 LS
	Light Scattering (LS)	GPC	
PT3636	239825	243473	101.5
PH1076	14960	147934	101.12
PG7427	104450	101024	103.39
PF1601	73625	74824	98.39
PB5227	42150	40304	104.57
PE5382	21975	23877	92.03
PA0094	11500	11232	102.38
JD2985	8825	8546	103.26
DX8868	5250	5174	104.7
PD235	4100	4205	97.5
GLUCOSE	180	180	100

Calibration Constants

$b_1 = -13.795$; $b_2 = 14.649$; $b_3 = -5.346$; $b_4 = 15.498$; $b_5 = -59961.678$

6.2.4 Determination of Molecular Weight Distribution and the Molecular Weight Average

The peak heights were calculated from the beginning to the end of the elution profile. Using these peak heights and equation 6.1, the molecular weights were then calculated. The weight fraction of each component was found using the chromatographic height (hi).

$$\text{Weight fraction} = \frac{\text{chromatographic height, hi}}{\text{sum of chromatographic heights } \Sigma hi} \qquad 6.4$$

Due to the polydisperse nature of dextran it is not normally possible to characterise it by a single molecular weight. To overcome this problem various averages can be used. The most common are:-

$$\text{the weight average, } Mw = \frac{\Sigma hi\ Mi}{\Sigma hi} \qquad 6.5$$

$$\text{and the number average, } Mn = \frac{\Sigma hi}{\Sigma hi/Mi} \qquad 6.6$$

For a polydisperse polymer Mw is always greater than Mn. The values are the same for a monodisperse polymer.

Polydispersity is often used to describe the breadth of molecular weight distribution. The polydispersity (D) is defined as:

$$D = \frac{Mw}{Mn} \qquad 6.7$$

The polydispersity ratio is 1.0 for monodisperse samples and for commercial polymers a typical ratio of 2-20 are common.

6.3 ENZYME ASSAY TECHNIQUES

To measure the activity of invertase enzyme and dextransucrase enzyme, the invertase assay and the Hostettler methods were used respectively.

6.3.1. Invertase Assay

This assay involves measurement of the rate of production of total reducing sugars (glucose and fructose) by the action of the invertase on its substrate, sucrose. The enzyme activity established by the assay was expressed as micromoles of reducing sugars formed/minute/cm^3 of concentrated invertase solution.

The reducing sugars were determined by reaction with 3, 5 - dinitrosalicylic acid (DNS) to give a product that adsorbs strongly at an optical density of 540nm [123].

0.1cm^3 of enzyme solution was added to 1.4cm^3 of distilled water and 0.5cm^3 of acetate buffer (0.05M sodium Acetate pH 4.7), and then incubated for 10 mins at 55°C. 1cm^3 of 0.3M sucrose was then added and incubated for a further 10 mins. Reaction was stopped by addition of 2 cm^3 of chilled (4°C) DNS solution (1 gram DNS in 20 cm^3 of 2M NaOH and 30 g potassium sodium tartrate in 50cm^3 distilled water made up to 100cm^3) and then boiled for 10 mins. The solution was then cooled down and 15cm^3 of distilled water added. The OD_{540} was read against a reagent blank and the micromoles of reducing sugar was obtained from a total reducing sugar standard (0.01M) curve [124]

The absorbance was measured on a Pye Unicam SP1800 ultraviolet spectro-photometer.

The activity of the enzyme invertase was measured in U/cm^3 (or μmol/min/cm^3) which is defined as the amount of enzyme that will convert 1μmol of sucrose in one minute at pH 5.2 and 55°C. The activity is calculated as follows:

$$\text{Activity } (\mu\text{moles/min/cm}^3) = \frac{\mu\text{moles of reducing sugars (From standard curve)} \times t \times d}{20} \quad 6.8$$

where

t = incubation time

d = dilution factor

6.3.2 Hostettlers Method

This method was used in determining the dextransucrase activity by measuring the fructose released from the biosynthesis reaction.

1cm^3 of enzyme containing 30-50 DSU cm^3 was added to 4cm^3 of 6.25% W/V sucrose in 0.1M sodium acetate buffer at pH 5.2. Immediately 0.5cm^3 of this mixture was taken out and the rest incubated in a water bath at 25°C. The 0.5cm^3 sample was added to 1cm^3 of Sumner reagent (10g 3-5-dinitrosalicylic acid, 300g potassium-sodium-tartate dissolved in 1 litre of 0.4µm NaOH), placed in boiling water for about 5 minutes, cooled and the volume made up to 12.5cm^3 with distilled water. This provided the unincubated sample. Every 5 to 10 minutes, samples were taken from the reaction mixture and treated in a similar way for the next 20 minutes. The optical densities of the samples were read at 530nm against a blank solution on the same spectrophotometer used for the invertase assay. The enzyme activity was then calculated from the following formula:

$$\text{Activity } (\text{DSU cm}^3) = \frac{(\text{ODi} - \text{ODu}) \times d \times 60 \times 2}{\text{ODs} \times 0.52 \times 0.2 \times t} \quad 6.9$$

where ODi = Optical density at 530nm of incubated sample

ODu = Optical density at 530nm of unincubated sample

d = Dilution factor of enzyme solution

t = Incubation time (min)

ODs = The optical density of a 2g/L fructose standard solution

One dextransucrase unit (DSU) is defined as the amount of enzyme which will convert 1 mg of sucrose to dextran in 1 hour at 25°C and pH 5.2.

6.3.2.1 HPLC Method of Dextransucrase Activity Assay

Alternatively the HPLC technique can be used in assaying enzyme activity by following the sucrose consumption or fructose production or both. The activity analysis was carried out on a 2% W/V sucrose solution and was based on a 2 hours conversion time. The expected theoretical yield after 2 hours is 1.052% W/V fructose and 0.948% W/V dextran.

The procedure was as follows:

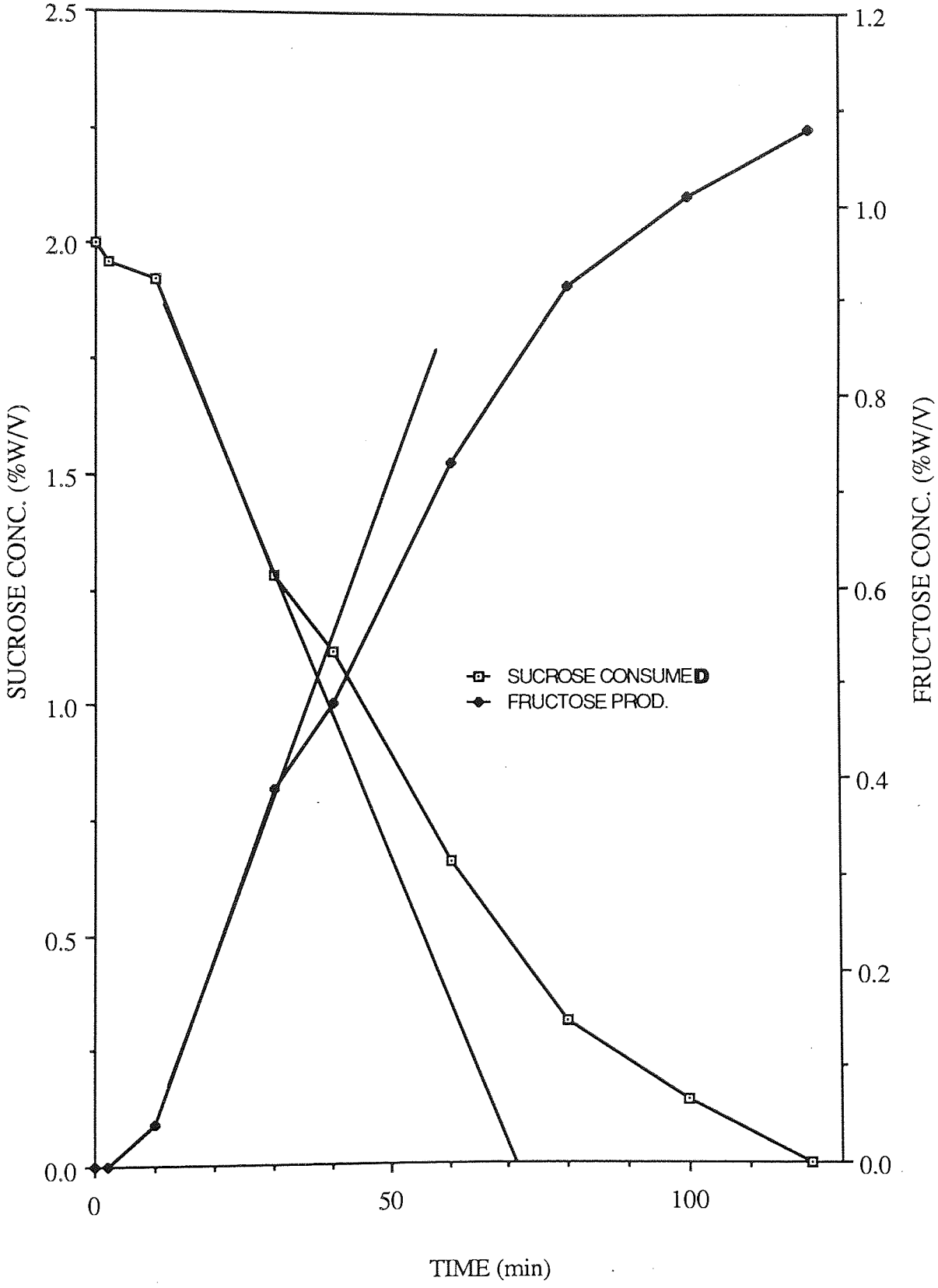
$20cm^3$ of enzyme containing approximately 50 DSU cm^3 of activity were added to $80cm^3$ of a solution containing 2.0g of sucrose at pH 5.2. The mixture was shaken and a sample was taken every 10 or 20 minutes over a period of 2 hours. The samples were filtered and injected into the HPLC System. The reaction mixture was maintained at 25°C in a water bath. The samples were heated immediately in boiling water for about 5 minutes to denature the enzyme and stop further reaction.

The sucrose, fructose and dextran compositions present in the samples at the time of assay, were calculated by comparing the chromatogram area with those of a standard solution. Figure 6.2 shows a plot of the residual sucrose concentration in the reaction mixture during a typical assay. The activity was calculated from the following equations:

Activity based on sucrose consumption

$$DSU/cm^3 = \frac{M_1}{1} \times 50 \times d$$

Figure 6.2:- Activity determination using HPLC technique



Activity based on fructose produced

$$DSU/cm^3 = \frac{2 \times M_2}{1.052} \times 50 \times d$$

where M_1 and M_2 are the initial slopes for the sucrose and fructose curves respectively, expressed in % W/V h^{-1}, and d, the enzyme dilution factor.

The HPLC method for dextransucrase activity analysis has the advantage that it allows both the sucrose and fructose concentrations to be followed. This facilitates a double check on enzyme activity. However, it suffers from the disadvantage that the time involved in carrying out this technique is considerable when compared to the Hostettler's method. A 90% agreement between the two methods was achieved.

The Hostettler's method was mainly used during the biosynthesis of dextran work on the SCCR-S1 system because of its relative speed and simplicity.

6.4. PROTEIN DETERMINATION

Any proteins present in the purified dextransucrase enzyme and invertase enzyme were detected by the following methods: The gel electrophoresis method and the Lowry method.

6.4.1. Sodium Dodecylsulphate - Polyacrylamide Gel Electrophoresis (SDS - PAGE)

The gel system described by Lugtenberg et al [124] was used for SDS - PAGE. The system consisted of a stacking gel and a running gel in which proteins were electrophoretically separated according to molecular weight. Optimum separation of proteins was achieved using 12% W/V acrylamide in the running gel. Formulae for the stacking and running gels are shown below:

12% Running Gel		Stacking Gel	
Stock 1	15.00cm^3	Stock II	5cm^3
SDS 10% W/V	1.50cm^3	SDS 10% W/V	0.3cm^3
1.5M Tris pH 8.8	18.50cm^3	0.5M Tris pH 6.8	7.5cm^3
Distilled water	23.75cm^3	Distilled water	16.0cm^3
TEMED	0.14cm^3	TEMED	0.08cm^{13}
AMPs	0.20cm^3	AMPs	0.10cm^3

where

Stock I = 44% W/V acrylamide (Sigma) and 0.8% W/V Bis (N, N' methylene bisacrylamide; Sigma) in water.

Stock II = 30% W/V acrylamide and 0.8% BIS in water.

TEMED = N, N, N, N' tetramethylethylene diamine (BDH).

AMPs = ammonium persulphate (BDH).

(Volumes shown are sufficient for one 20cm x 25cm x 0.1cm gel)

Solutions for the separating gel were added, as shown above, to a 250cm^3 beaker and stirred with a magnetic stirrer. Polymerisation was initiated upon the addition of TEMED and AMPs. The running gel was cast by pouring the solution between two glass plates separated with plastic spacers (Bio-Rad Mine Protean II gel apparatus, gel dimensions 10cm x 7cm x 0.75mm). After the running gel had set the stacking gel was prepared in a similar way and cast above the running gel. Wells were formed in the stacking gel by the insertion of a teflon comb (1, 10 or 15 wells per comb). The depth of the stacking gel was at least 5mm greater than that of the wells.

Samples of the enzyme solution were added to an equal volume of sample buffer, which consisted of the following:

Sample Buffer, pH 6.8

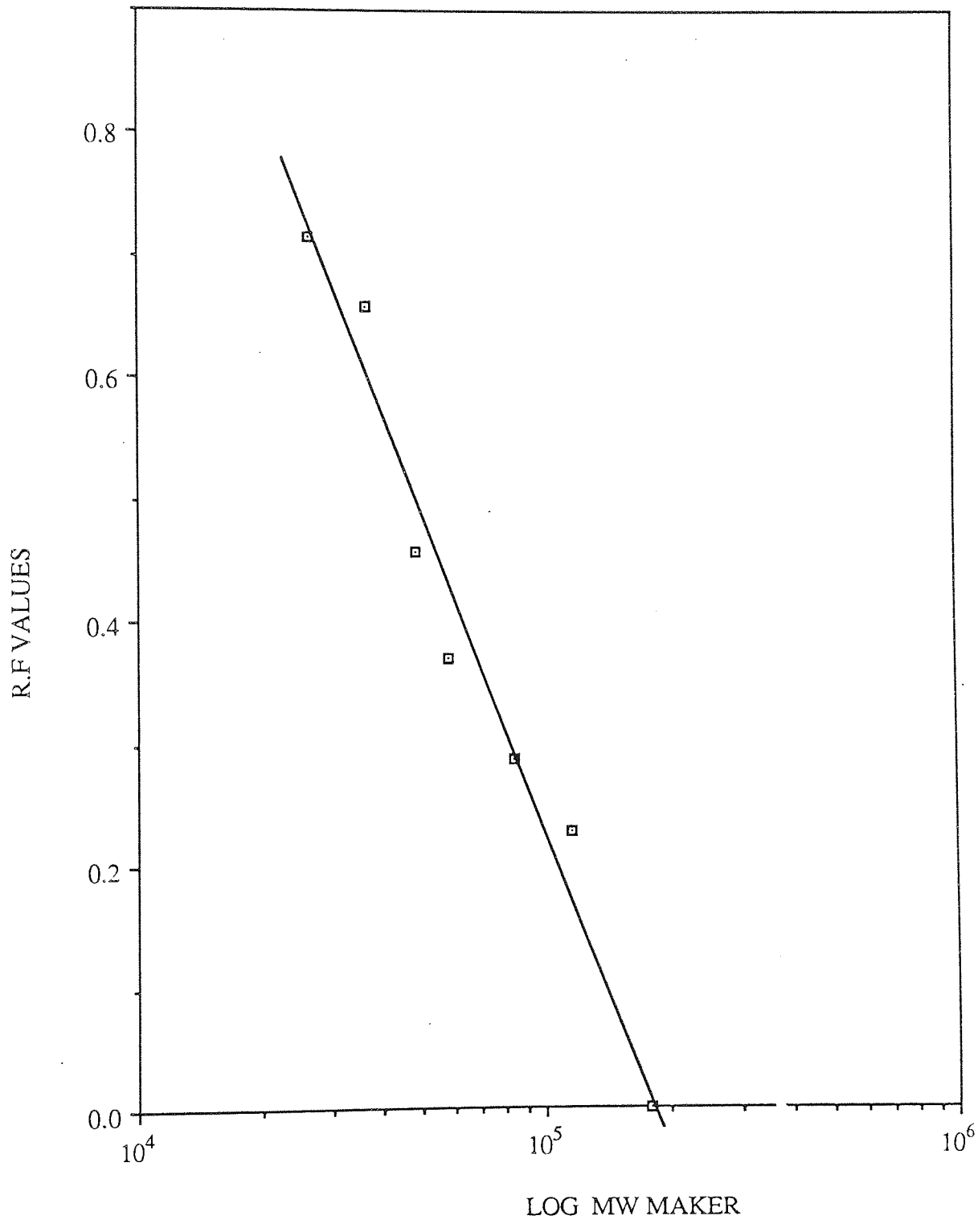
0.5M Tris pH 6.8	$5.0cm^3$
SDS 10% W/V	$10.0cm^3$
Mercaptoethanol	$0.5cm^3$
Glycerol	$5.0cm^3$
Distilled water	$10.0cm^3$
Bromophenol blue 0.5% W/V	$0.4cm^3$

The samples were denatured by boiling with an equal volume of sample buffer for 10 minutes and applied to the wells in the stacking gels. The electrophoretic separation was carried out at room temperature in an electrode buffer (containing per 2L distilled water: Tris 6g; glycine 28.8g; and $20cm^3$ SDS 10% W/V) at a constant voltage of 200V for approximately 1 hour (Bio-Rad Mini Protean II gel apparatus). The gels were stained overnight for protein with coomassie brilliant blue R-250 in methanol 50% W/V, glacial acetic acid 10% W/V in water and then destained with methanol 5% W/V glacial acetic acid 10% W/V in water.

To determine the molecular weight of proteins separated by SDS-PAGE a calibration curve was constructed [125]. Proteins of known molecular weight (commercially available molecular weight markers; sigma) were subjected to SDS - PAGE and their RF values were determined (Rf value - distance moved by protein through the running gel divided by distance moved by bromophenol blue marker dye through the running gel). A plot of Log_{10} (molecular weight) against Rf value gave a calibration curve (Figure 6.3) from which the molecular weight of unknown proteins were determined.

The proteins present in the dextransucrase enzyme solution and invertase solution are shown on the gel pictures Figure 6.4 and 6.5 respectively. The molecular weights

Figure 6.3:- Gel Electrophoresis Protein Determination



calculated are approximately 160,000MW for dextransucrase and 60,000MW for invertase.

6.4.2 Lowry's Method

Lowry's method is used to assay the amount of total protein in an enzyme solution.

Bovin serium albumin (BSA) standards, in the range of 10 to 100$\mu g/cm^3$ were prepared. 0.1cm^3 of the standards and enzyme solution were made up to 0.5cm^3 with distilled water. 0.5cm^3 of 1M NaOH was added and the mixtures boiled at 100°C for 5 minutes.

After cooling down the mixture to room temperature, 2.5cm^3 of a solution made by mixing 2 cm^3 of 5%W/V sodium carbonate, was added to the enzyme sample and the standards. After 10 min at room temperature, 0.5ml of the folin ciocalteau reagent (Sigma) was added resulting in the development of a blue colour. The colour was allowed to develop for 30 minutes before the optical density reading was taken against a blank at an absorbance of 750mm. An estimation of the total protein constant of the enzyme fraction was taken from the calibration curve of albumin concentration against optical density 750mm.

Figure 6.4:- Gel electrophoresis picture for dextransucrase enzyme solution

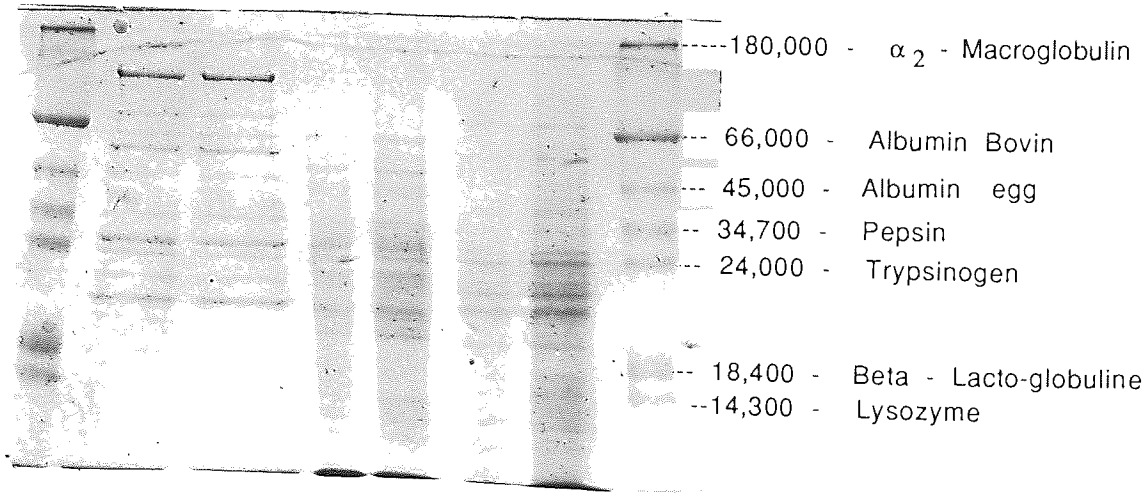
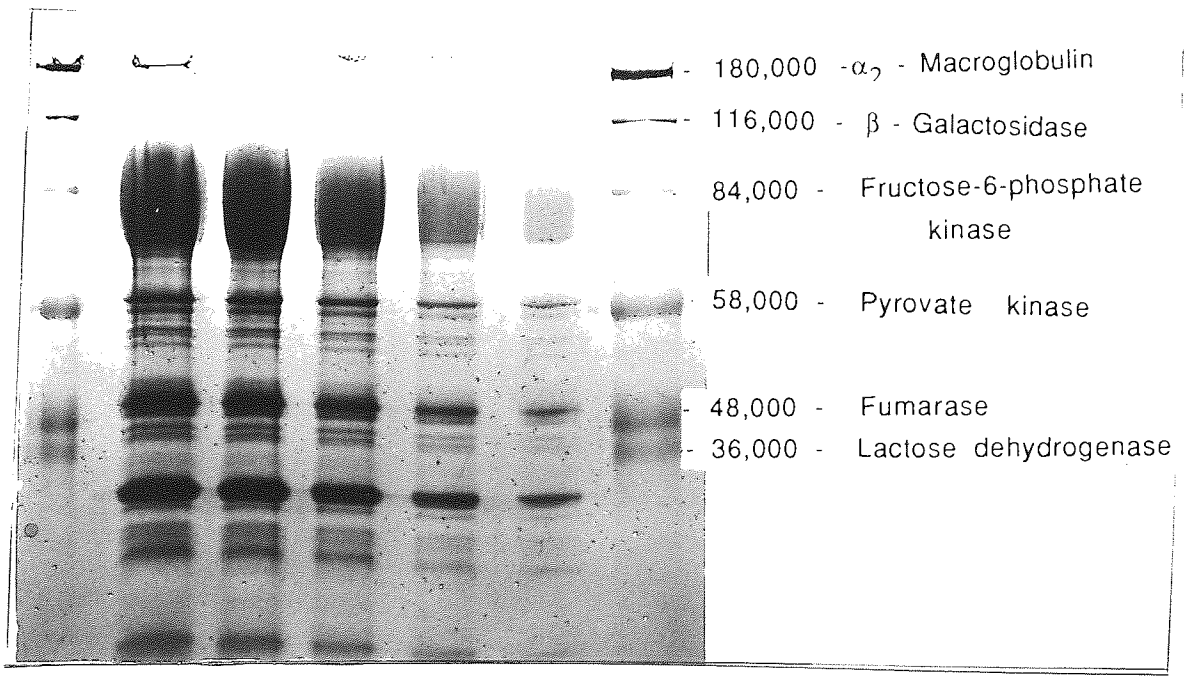


Figure 6.5:- Gel electrophoresis picture for Invertase enzyme solution



CHAPTER 7

OPERATION DEVELOPMENT AND PRELIMINARY EXPERIMENTS ON THE SCCR-S1 IN THE SEPARATION MODE

7.0 PRELIMINARY EXPERIMENTS USING A SCCR-S1 EQUIPMENT IN A SEPARATION MODE

7.1 INTRODUCTION

The SCCR system has been developed and applied to the separation of carbohydrate mixtures by previous researchers [9, 10, 101, 105] especially in the production of high fructose corn syrup (HFCS). As a result, the operating parameters have been well investigated.

These parameters will be looked at in relation to the SCCR-S1 system to establish the operating conditions required to study its performance as a separator, to make some comparisons with previous SCCR systems and to provide a basis for the reaction-separation work.

To this effect, synthetic carbohydrate mixtures of two components (fructose and glucose), of a three component mixture namely, sucrose, dextran and fructose feed and finally an inverted sucrose feed were used.

7.1.1 Selecting The Operating Parameters

Previous workers [9, 10, 98] have identified factors that affected the separating potential of the SCCR system.

These factors are:-

- The effect of solute concentration.
- The variation of solute migration velocity due to variations of temperature, pressure gradients and solute concentration.
- System characteristics, namely semi continuous operation mode and limited length of the separation section.

By taking these factors into consideration, the basic design equation (Equation 5.10) can be modified for a separation process to give:-

$$(Kd_g + \delta_{1g} + \delta_{2g}) < \frac{Le}{P} < \frac{Le + Lf}{P} < (Kd_f - \delta_{1f} - \delta_{2f}) \qquad 7.1$$

or

$$(Kd_g + \delta_{1g} + \delta_{2g}) < \frac{Lm}{P} < (Kd_f - \delta_{1f} - \delta_{2f}) \qquad 7.2$$

where

$$\frac{Lm}{P} = \frac{(\frac{Le}{P} + \frac{Le + Lf}{P})}{2} \qquad 7.3$$

and δ_{1g} and δ_{1f} - fractional changes due to the effect of concentration on the distribution coefficients. δ_{2g} and δ_{2f} - fractional changes due to the effects of system characteristics on distribution coefficients [24].

7.1.2.1 Effect of Concentration

Previous research work [98, 105] on the SCCR system has shown that as the feed concentration to the system was increased, the on-column concentration also increased. The profiles became broader, the "cross-over" point moved towards the FRP and the product purities, especially the GRP purities were reduced. This was a direct result of the effect of increased background concentration on the distribution coefficients.

The background concentration effects on the distribution coefficients have been correlated for glucose, fructose, dextran mixtures by Ganetsos [24] and the resulting expressions are as follows:-

- For a glucose background concentration

$$K^{G}dg = (320.9 \times cg + 102.91) \times K^{\infty}dg/100 \qquad 7.4$$

$$K^{G}df = (173.1 \times cg + 95.71) \times K^{\infty}df/100 \quad 7.5$$

- For a fructose background concentration

$$K^{F}dg = (198.3 \times cf + 107.43) \times K^{\infty}dg/100 \quad 7.6$$

$$K^{F}df = (19.45 \times cf + 101.62) \times K^{\infty}df/100 \quad 7.7$$

- For a dextran background concentration

$$K^{D}dg = (627.4 \times cd + 111.6) \times K^{\infty}dg/100 \quad 7.8$$

$$K^{D}df = (289.11 \times cd + 112.2) \times K^{\infty}df/100 \quad 7.9$$

where K^{∞} is the infinite dilution coefficient which has been calculated during the column characterisation (Section 5.3).

7.1.2.2 Effect of Operating Temperature

Ching [100] and Chuah [101] have shown that by increasing the temperature from ambient to 60°C the on-column concentration profiles are altered and the 'cross-over' point moved progressively towards the glucose rich product resulting in reduced GRP purities. An investigation of the individual concentration profiles [9] reveals that the glucose profile remains unaffected while the fructose profile shifts towards the GRP. The explanation for this effect was partly due to the retention volume generally being reduced with an increase in temperature and as a result the solute bands are brought nearer to each other resulting in a greater overlap. In addition, β-D-fructopyranose is the sweetest fructose tautomer and is the only known form of fructose that forms a complex with the calcium ions. The proportion of this tautomer at equilibrium to other forms is a function of temperature and it decreases with increasing temperature as shown in Table 7.1 [144]. This reduces the fraction of fructose available for complexing and increasing the amount of fructose moving with the mobile phase.

Table 7.1 : Equilibrium Concentration of 20% Fructose at Different Temperatures [7]

Temperature ℃	α-D-Fructo Furanose %	β-D-Fructo Furanose %	β-D-Fructo Pyranose %
0	4	18	78
22	6	21	73
67	8	28	64
77	12	31	57

The amounts of keto-fructose and α-D-Fructopyranose present are negligible.

An expression to relate the effect of temperature on the distribution coefficients was developed by Ganetsos [9] in which the changes in the Kd_f values were expressed as a percentage, where the $^{25}Kd_f$ value at 25°C was taken as 100%. By assuming a linear change in Kd_f a regression line provides the equation:

$$Kd_f = (114.55 - 0.563 \times TEMP) \times {}^{25}Kd_f/100 \qquad 7.10$$

The glucose distribution coefficient had been found not to be affected by temperature Thus, for each system its fructose distribution coefficient value (Kd_f), derived at 25°C and at infinite dilution can be modified using Equation 7.10 to give a better estimate of the actual Kd_f value for the particular operating temperature.

7.1.2.3 Effect of Switch Time and Flow Rates

The switch time and flow rates are directly related (Equation 7.1), i.e. to maintain the Lm/P ratio constant, an increase in switch time (i.e. reduced P) will require a decrease in the operating flowrates. Throughout the development of the SCCR systems, the eluent, feed and purge flowrates were selected for an arbitrary chosen switch time, by using the constrains imposed by Equations 7.1 and 5.11.

A reduction in the switch time will result in an increased throughput which in turn will cause a reduction in the fructose rich product (FRP) purity. A compromise between the switch time and flow rates must therefore be made.

It has been found that an increase in the eluent flow rate reduces the system separation potential [101] because of its resulting effects on the distribution coefficients. By expressing the eluent flowrate in terms of linear velocity (flow rate over column cross sectional area) and expressing the Kd variation in percentage terms, linear expressions were derived relating the effect of flow rates on the distribution coefficients for any chromatographic SCCR system used for carbohydrate separations [24], i.e.

for glucose

$$Kd_g = (111.71 - 10.631 \times \text{Linear Velocity}) \times K^*d_g/100 \qquad 7.11$$

and for fructose

$$Kd_f = (112.68 - 11.593 \times \text{Linear Velocity}) \times K^*d_f/100 \qquad 7.12$$

where

K*di = distribution coefficient of component i calculated for the particular system, at infinite dilution, at 25°C and at 1.09 (cm min^{-1}) linear velocity.

Ching [101] in his work found that the separation performance is also affected by changes in the eluent to feed flow ratios. A decrease in this ratio from 6 : 1 to 2 : 1 resulted in shifting the "cross-over" point towards the GRP and in decreasing the glucose rich product purities. Subsequent work by other workers has shown that an eluent to feed ratio of 3 : 1 is the most favourable, and this ratio was used in this work.

For comparison purposes with previous work on the SCCR7 unit [9] and bearing in mind the restriction of Equation 7.1, the following operating conditions were used in this work to obtain an experimental run which will form a basis for the experimental programme, i.e.

Eluent flowrate :- 30cm^3/min

Feed flowrate :- 9cm^3/min

Switch time :- 30 min

The purging flowrate was about twice the eluent flowrate (75cm^3/min). This was chosen to increase the concentration of the fructose rich product and to be very close to the condition set by Equation 5.11. In the past purging flowrates as high as 3 to 50 times the eluent [99, 101, 105] have been used which have resulted in very dilute fructose rich products.

An analysis carried out by Ganetsos [9] on the fructose rich product stream with a purging rate twice the eluent flow rate have proved that this value (i.e. 2.1) is sufficient .

7.1.2.4 Improvement of Product Concentration

The product concentrations obtained from the SCCR systems are very low. To increase these concentrations, two technqiues can be employed namely: product splitting and recycling of the dilute fraction.

Product Splitting

It has been found that most of the fructose rich product is removed at the early part of the purging period (Figure 7.1) [9]. An analysis of the GRP product stream, carried out by Ganetsos, has shown that most of the glucose is eluted towards the end of a switch (Figure 7.2). Therefore, by dividing the GRP and FRP product collection period into two fractions, the concentrations of the desired products can almost be doubled without any significant decrease in purity. FRP product concentration increases from 5.07% w/v to 5.79% w/v was reported [9]. Thawait's [105] results also confirm the use of this technique. In her work, FRP concentration increases from 1.74% w/v to 3.4% w/v were obtained.

The SCCR-S1 system was modified to allow such an operation (Chapter 5).

Figure 7.1:- FRP Concentration Profile during Purging (9)

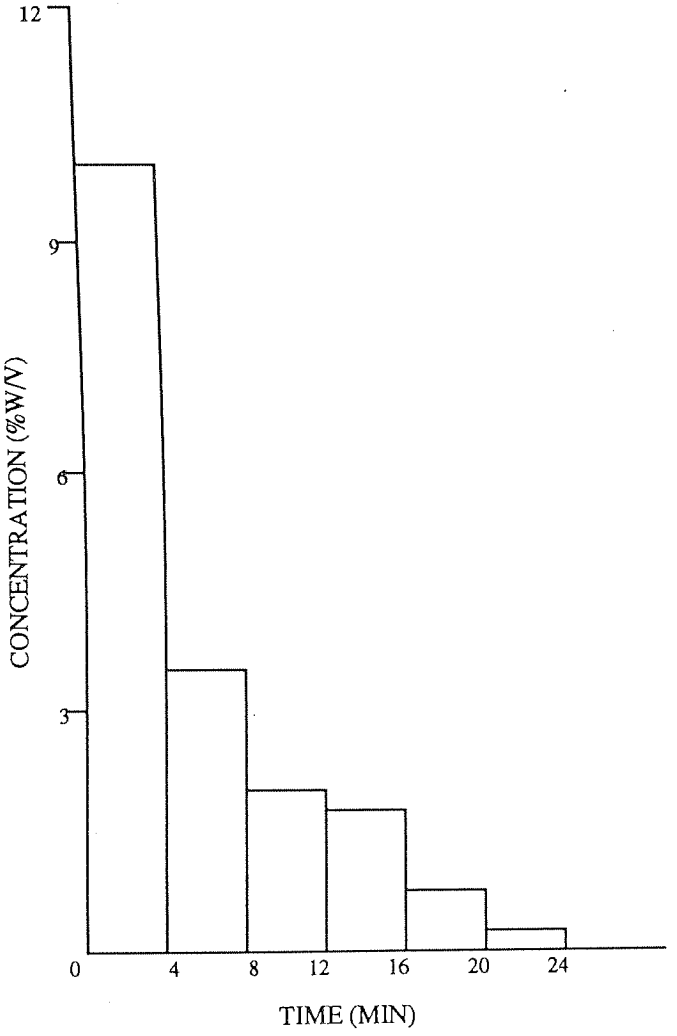
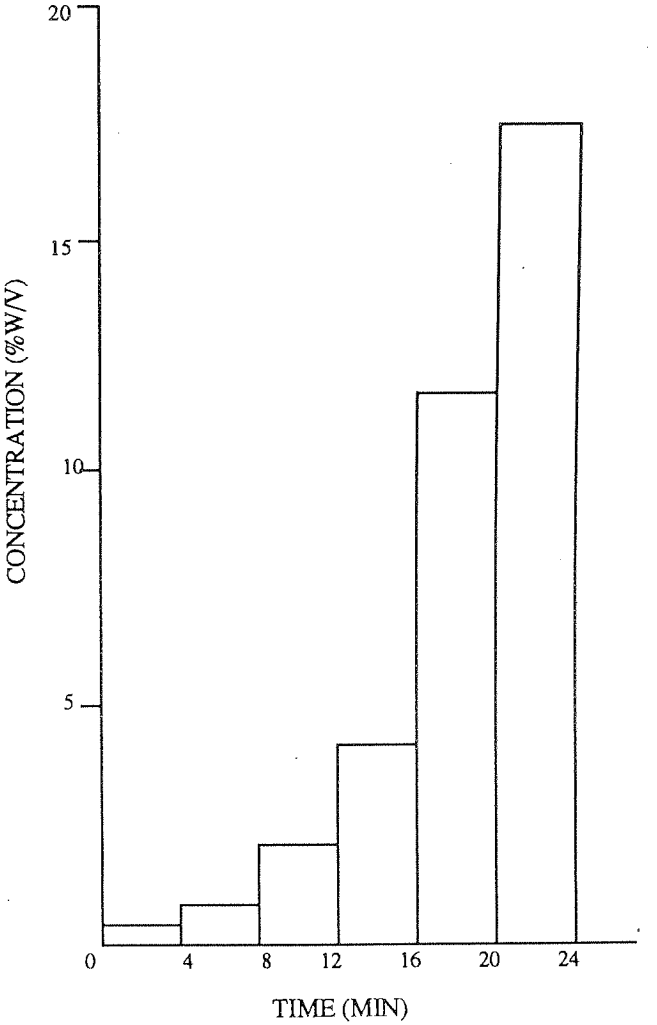


Figure 7.2:- GRP Concentration Profile during a switch (9)



Recycling Technique

Recycling of the dilute and concentrated fructose rich products have been employed on the SCCR system. On the SCCR 6 system [105], concentrated FRP was recycled. The product concentration increased from 1.74% w/v to 9.4% w/v, but the purity was reduced from 82% to 72%. Due to the loss in purities and reduced throughputs this technique was not used.

Recycling of the diluted FRP and GRP fractions in the SCCR7 system [9] as purge and eluent water respectively, increased both the product purities and concentrations. There was also a high recovery of sugar solids.

7.2 COMMISSIONING OF SCCR-S1

After the construction and characterisation of the SCCR-S1 system, all the ancillary equipment associated with it were tested. The pumps were checked for flowrate accuracy and for any leakages and the pneumatic controller was checked to verify its operating sequences.

The temperature/level controller on the aspirator was checked for accuracy and all air lines and liquid delivery lines were checked to see if they were correctly connected.

A pressure test was then carried out. This was done by pressurising the system with water by closing the outlet product lines, and letting the pressure build up to about 345 kNm^{-2}. The system was then held at this pressure for 30 minutes.

The operating conditions for the separation of feed components were then chosen to correspond to similar runs carried out on the SCCR7 System [9]. Each experimental run in the separation studies is represented in a codified form similar to the one employed by Ganetsos in his work on the SCCR7 system. For example, the set 18.6-9-30-30-25

corresponds to feed solid content (% w/v), feed flowrate (cm^3min^{-1}) eluent flowrate (cm^3 min^{-1}), switch time (min) and temperature (°C) respectively.

7.2.1 Initial Commissioning Runs Using Synthetic Feedstocks

The commissioning runs on the SCCR-S1 were carried out with synthetic feeds of fructose/glucose under the following conditions:

- Feed concentration 18.6% w/v total sugar solids consisting of a 55 : 45 ratio of glucose to fructose.
- Feed flowrate 9 cm^3 min^{-1}
- Eluent flowrate 30 cm^3 min^{-1}
- Purge flowrate 76 cm^3 min^{-1}
- Temperature 25°C
- Switch time 30 min

The aim was to compare the results with those of Ganetsos [9]. Two sets of runs were carried out, the results of which are shown in Tables 7.2 and 7.3 and Figures 7.3 and 7.4. For the first run, a high purging rate (154 cm^3 min^{-1}) was used resulting in a very dilute FRP concentration.

The operating conditions of the second run (Run:- 19.12-9-30-30-25) were similar to the one carried out on the SCCR7 system (Run:- 18.6-9-30-30-20) [9]. From the results shown in Tables 7.2 and 7.3, 81.3% w/w fructose was achieved in the FRP and 99% w/w glucose in the GRP for the second run. These results deviated from the ones obtained previously on the SCCR7 system (i.e. 99.9% w/w on both streams) [9]. The on column profile (Figure 7.4) shows that the "cross-over" point is very close to the FRP. This indicates that the relative movement of the stationary phase to the mobile phase is too fast, causing some of the glucose to be eluted in the fructose rich products. On the SCCR7 system, the "cross-over" point was further to the right and closer to the feed point, indicating a more slower movement of the effective stationary phase giving better product purities in both streams. The different results achieved in both systems can be

attributed to their respective column characterisations. The glucose and fructose infinite dilution distribution coefficients (Kd_1^{∞}) obtained previously on the SCCR7 [9] operating under the same conditions were 0.215 and 0.472 respectively. The respective values for the SCCR-S1 system used in this work were 0.278 and 0.624 (Section 5.3). Therefore from the criterion of separation a slightly different switch time should have been used to give better purities in both product streams.

7.3 OPTIMISING THE SEPARATION OF SYNTHETIC FRUCTOSE AND GLUCOSE FEED

7.3.1 Experimental Results and Discussion

The commissioning runs on the SCCR-S1 have shown that separation of a two component feed is possible on the system.

The effect of switch time on the separation performance of the SCCR-S1 system was studied by using a 19% w/v synthetic glucose and fructose feed, having a weight ratio of 42:58 (fructose : glucose) mixture. By using a high feed concentration and a reduced feed fructose to glucose ratio the on-column concentrations increased, with the glucose concentration increasing relatively more, due to the greater amounts of glucose present. This is supported by the fact that the distribution coefficients increased linearly with concentration, and also their rate of change increased more with increasing glucose background concentration than fructose (Section 7.1.2.1). The experimental conditions and results are shown in Tables 7.4 and 7.5.

For the first run (19.14-9-30-30-25), the switch time of 30 minutes was chosen to correspond to the switch time used by previous workers [9, 101]. From Table 7.5 there is a general improvement in the FRP product purities. For run (19.14-9-30-30-25) and run (19.96-9-30-30.167-25), an increase in switch time by 10 seconds brought about a drop in the FRP product purities from 73 to 70%. Since one would expect an increase in the

FRP purity when the switch time is increased, this slight drop in purity is attributed to experimental errors.

A further increase to 32 minutes (19.21-9-30-32-25) slightly improved the fructose purity in the FRP (80%). A good separation in both streams was achieved when the switch time was set at 33min (Run:- 18.6-9-36-33-25). Product purities over 95% w/v were obtained in both streams.

Taking into consideration the effect of eluent and background concentration on the distribution coefficient and by applying the correlations Ganetsos [24] has proposed, average Kd values were obtained for fructose and glucose (Table 7.4). The constraint in Equation 7.1 was applied and the Lm/P ratio was found to be outside the range. However when the infinite dilution coefficients ($K^{\infty}d_g = 0.278$ and $K^{\infty}d_F = 0.625$) were used instead of the average weighted distribution coefficient the Lm/P ratios were within the constraint.

The first two sets of runs (19.14-9-30-30-25) and (19.96-9-30-30.167-25) show that there is no difference in the Lm/P ratio. This was reflected in Figures 7.5 and 7.6 where there was no shift in the position of the "cross-over" point. The broader profile of the glucose concentration is due to the relatively high glucose to fructose concentration ratio present in the feed. Run (19.21-9-30-32-25) showed a slight shift in the "cross-over" point (Figure 7.7) while in Run (19.6-9-30-33-25) there was a sharp movement of the "cross-over" point from column 2 to column 5 (Figure 7.8) which accounted for the high product purities achieved in both the FRP and GRP. The low fructose product purities obtained from the first three runs was a result of the "cross-over" point being very close to the FRP.

7.3.2 Conclusion

Using a two component synthetic feed of glucose and fructose at a concentration of about 20% w/v it is possible to achieve over 95% product purities in the GRP and FRP stream by varying the switch time. The experimental results and a close investigation of the concentration profiles (Figures 7.5 - 7.8) verified clearly this effect.

The variation of the switch time as the controlling parameter in the system's operation means that no hardware modification was needed. This therefore allowed for easy control. An approximate value of the switch time, for any particular operating conditions can be obtained from the criterion of separation (Equation 7.1) and by the use of the correlations proposed by Ganetsos [9] and stated in Section 7.1.

7.4 SEPARATION OF SYNTHETIC SUCROSE, FRUCTOSE AND DEXTRAN *FEED*

The separation performance of the SCCR-S1 was also investigated with a multi-component synthetic feed. A 20% w/v concentration synthetic feed containing equal amounts of dextran, sucrose and fructose was used. The choice of this particular feed was to observe the dextran and sucrose concentration profiles and their effect on product purities. It would also give an indication of the expected on-column concentration profiles for the dextran bioreaction-separation studies to be carried out later on.

7.4.1 Experimental Results and Discussions

Figures 7.11 and 7.12 show two identical profiles for two consecutive cycles (9 and 10) under the same conditions. Tables 7.6 and 7.7 contain the experimental condition and results.

The switch time was varied between 28-33 minutes. The dextran profile for all the runs was consistently the same (Figures 7.9 to 7.15). The dextran feed had an average molecular weight of 101,000 MW and was eluted with the mobile phase, hence the

dextran profile was more pronounced at the GRP end and had no significant effect on the switch time. The "cross-over" point between the dextran profile and the fructose profile was at the same point.

There was quite a shift however in the cross-over point between the sucrose profile and the fructose profile as the switch time was varied. For Run (19.38-9-30-28-25) (Figure 7.9), the "cross-over" point is on column 3 nearer the FRP product end. This shift in "cross-over" point is due to the relative motion of the mobile phase which controls the sucrose profile to the stationary phase which controls in turn the fructose profile. The shift towards the FRP product end is because the slower motion of the mobile phase relative to the stationary phase. This indicated that the FRP product stream was contaminated and was a reflection in the low FRP fructose purity (85%) (Table 7.7). As the switch time was increased the "cross-over" point shifted towards the DRP (Figures 7.9, 7.10, 7.11, 7.13 and 7.14) thus also improving the fructose product purities in the FRP.

However, when the switch time was increased to 33 minutes (Run:- 20-9-30-33-25 and Figure 7.15), the fructose product purity in the FRP was reduced and the sucrose profile started to overlap with the dextran profile forming a second "cross-over point. A similar effect was observed with Figure 7.9 (Run:- 19.38-9-30-28-25) where the sucrose profile overlapped with the dextran profile resulting in reduced FRP purities.

At the 33 minute switch time the effective mobile phase flowrate is too fast that the sucrose molecules do not have enough time to diffuse into the resin and as such are eluted with the dextran molecule. Similary at 28 minutes switch time the effective movement of the stationary phase is so fast resulting in some contamination in the FRP.

Applying the criterion of separation (Equation 7.1), the Lm/P ratios were within the defined constraint, when the infinite dilution distribution coefficient of sucrose ($K^{\infty}d_s$

= 0.119) and fructose ($K^{\infty}d_F = 0.625$) were used. However it is impossible to apply this criterion to dextran since its infinite dilution distribution coefficient is zero indicating that it is eluted with the eluent flow with no adsorption on the resin particle.

7.4.2 Conclusions

Working within the criterion (Equation 7.1) and with the right operating switch time, approximately 100% equimolar dextran and sucrose DRP product can be obtained while the FRP purity is 97% W/W. Switch time variation showed the system's sensitivity. An increase from 28 minutes to 29.83 minutes shifted the "cross-over" point from Column 3 (Figure 7.9) to Column 6 (Figure 7.10). This had an improved effect on the product purities (Table 7.8).

7.5 SEPARATION OF INVERTED SUCROSE

The separation performance of the SCCR-S1 system was further tested using a different feedstock (inverted sucrose). The use of this feedstock also allows a comparison to be made with the work carried out previously on SCCR6 system [10] and provides a basis for the sucrose inversion studies to be carried out in this work.

Sucrose was inverted outside the system to glucose and fructose by the enzyme invertase. The inversion reaction was carried out at a pH of 5.2 and 22°C. From the definition of the enzyme activity given in Chapter 6 and the activity of invertase purchased (11,600 $U.cm^3$), the amount of enzyme required to convert 20% W/V sucrose per run in one hour was calculated. Since all the sucrose was not consumed after the reaction time, the reaction was left to proceed overnight. The analysis of the mixture after approximately 17 hours showed complete inversion with 51% glucose and 49% fructose present. This feedstock was then used in the SCCR-S1 equipment.

7.5.1 Experimental Results and Discussion

Tables 7.8 and 7.9 show the conditions and results obtained from the separation of inverted sucrose feedstock. Run (19.65-9-30-33-35) was carried out using the completely inverted sucrose feed and in Run (18.5-9-30-33-35) a modified inverted sucrose feed was used where the ratio of fructose to glucose was reduced from 49 : 51 to 45 - 55. In Run (19.83-9-30-33-25) a synthetic glucose and fructose feed was used. All operating conditions were kept constant.

From Table 7.9, it can be observed that there was a reduced fructose purity in the FRP with the inverted sucrose feed and the modified sucrose feed compared to the synthetic feed. It was expected that the modified inverted sucrose feed (18.15-9-30-33-35) and the synthetic feed (19.83-9-30-33-35) should have approximately the same product purities since the ratios of fructose to glucose in the feeds are very similar. However, this proved not to be the case. Although its on-column concentration profiles (Figures 7.16, 7.17 and 7.18) look very much similar, the "cross-over" point is slightly shifted even at the same switch time. When comparing the profiles of the synthetic feed with the modified inverted sucrose feed (Figures 7.17 and 7.18) the "cross-over" point had shifted by one column towards the FRP. Hence the drop in fructose purity in the FRP. With the inverted sucrose feed (Run 19.65-9-30-33-35) profile (Figure 7.16) the "cross-over" point is similar to that of the synthetic feed (Figure 7.17), although with a slight reduced fructose purity product in the FRP.

A possible explanation for this effect can be found from the tautomeric equilibria of the fructose formed. The β-D-fructopyranose is the only tautomer which forms a complex with calcium. If more of this tautomer was available during the enzyme inversion, more fructose would be retained on the resin and less will travel with the mobile phase thus increasing the distribution coefficient of fructose. With the modified inverted sucrose, glucose was added to the original inverted feed to reduce the fructose to glucose ratio. This might account for the broader glucose concentration profile (Figure

7.18) and resulted in a shift towards FRP. In this case the distribution coefficient of glucose will increase.

7.6 EFFECT OF CALCIUM ION ON GLUCOSE

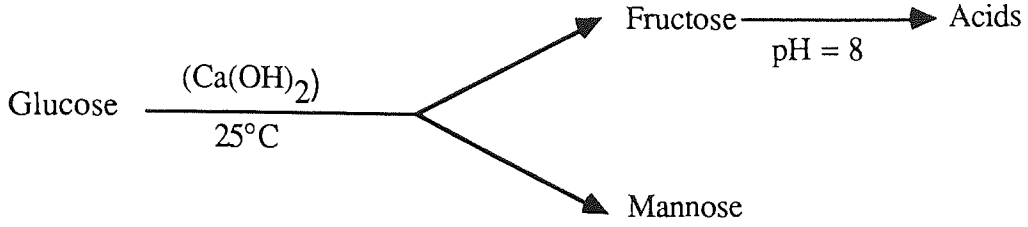
Attempts to achieve 99.9% purities in both the glucose rich product (GRP) and fructose rich product (FRP) streams proved very difficult. The maximum separation achieved was 97% GRP and 90% FRP (Table 7.9). This pattern of results was very consistent with all the runs carried out with the exception of the synthetic glucose and fructose feed runs (Table 7.5), which were the first set of runs carried out on the system. The variation of the switch time did not improve the GRP product purity and values greater than 97% could not be obtained.

To examine the reason for this a test run was carried out in a 1.97cm id x 1.7m long batch chromatographic column packed with a similar calcium charged resin used in the SCCR-S1 system. With an eluent rate of $5cm^3\ min^{-1}$ going through the column 10% W/V glucose was charged onto the column. The conditions of the test run were similar to runs carried out on the SCCR-S1 system. The results obtained from the test run are outlined as follows:-

Conc. of glucose added	:	10% W/V
Volume of glucose added	:	$5cm^3$
Conc. of glucose collected	:	0.48% W/V
Conc. of fructose collected	:	0.014% W/V
Total conc. of product	:	0.494%W/V
Ratio of glucose : fructose formed	:	97:3

From this result the presence of fructose at the column's outlet shows that the glucose is partially isomerised within the column. Work carried out by Barker et al. [145] has shown that calcium salts have the tendency to isomerise glucose to various degrees depending on the operating conditions.

From their work, calcium hydroxide ($Ca(OH)_2$) isomerised glucose to fructose and mannose at room temperature. The fructose formed can subsequently be converted to acids at a pH of around 8.



A solution of calcium chloride ($CaCl_2$) derived by dissolving anhydrous $CaCl_2$ which is slightly acidic was also found to convert 35% glucose to fructose with no mannose or acids being formed. A solution of calcium chloride of the crystal form ($CaCl_2$ $10H_2O$) was said not to have any effect on the isomerisation reaction. However, when the hydrate was heated, water and some hydrochloric acid were removed forming a catalyst called calcium hydroxy chloride (Ca (OH) CL). It is only this form that can actually catalyse the isomerisation reaction. All the isomerisation reactions that occurred with calcium salts are said to be irreversible reactions.

From these findings, there is a good indication that calcium ions do affect the isomerisation of glucose to a certain extent. On the SCCR-S1 system and on the batch chromatographic columns, calcium nitrate was used in recharging the columns when the system was not in use. Although the effect of calcium nitrate was not studied [145], the presence of fructose at the column exit when glucose was injected to the calcium charged columns with no mannose or acid detected shows that the combination of calcium ions present on the resin and the operating conditions pH (5.2-6) and temperature (25°C) are possible causes for not attaining the degree of separation expected. This therefore indicates that the system's separation efficiency is better than that indicated by the FRP and GRP purities, since the additional GRP product contamination is due to the partial isomerisation of glucose.

Table 7.2 : Operating Conditions Used In The Commissioning Runs

Experimental Run	Average Flowrates $cm^3\ min^{-1}$			Feed Concentration % W/V			F:G ratio in feed	Switch time run	Cycle
	Feed	Eluent	Purge	G	F	Total			
18.58-9-30-30-25	9	30	154	10.17	842	18.58	45 : 55	30	9
19.12-9-30-30-25	9	30	75	11.46	7.7	19.12	40 : 60	30	8
* 18.6-9-30-30-20	9	30	75	10.78	7.82	18.6	42.58	30	7

G : glucose; F : fructose; * Run from SCCR7 [9]

Table 7.3 : Experimental Results from Commissioning Runs

Experimental Run	Feed throughput (sugar solids kgh^{-1})	GLUCOSE RICH PRODUCT					FRUCTOSE RICH PRODUCT				
		Glucose Purity %	% of glucose in feed recovered	Total product conc. % W/V	Impurities % F	Others	Fructose Purity %	% of Fructose in feed recovered	Total Product conc. % W/V	Impurities % G	Others
18.58-9-30-30.25	0.111	98.8	83.5	2.34	1.2	-	98.3	78	0.41	1.7	-
19.12-9-30-30-25	0.115	99.2	87.4	2.48	0.73	-	81.3	84.7	1.04	18.7	-
* 18.6-9-30-30-20	0.1	99.9	97.1	2.12	0.1	-	99.9	98.4	0.93	0.1	-

* Run from SCCR7 [9].

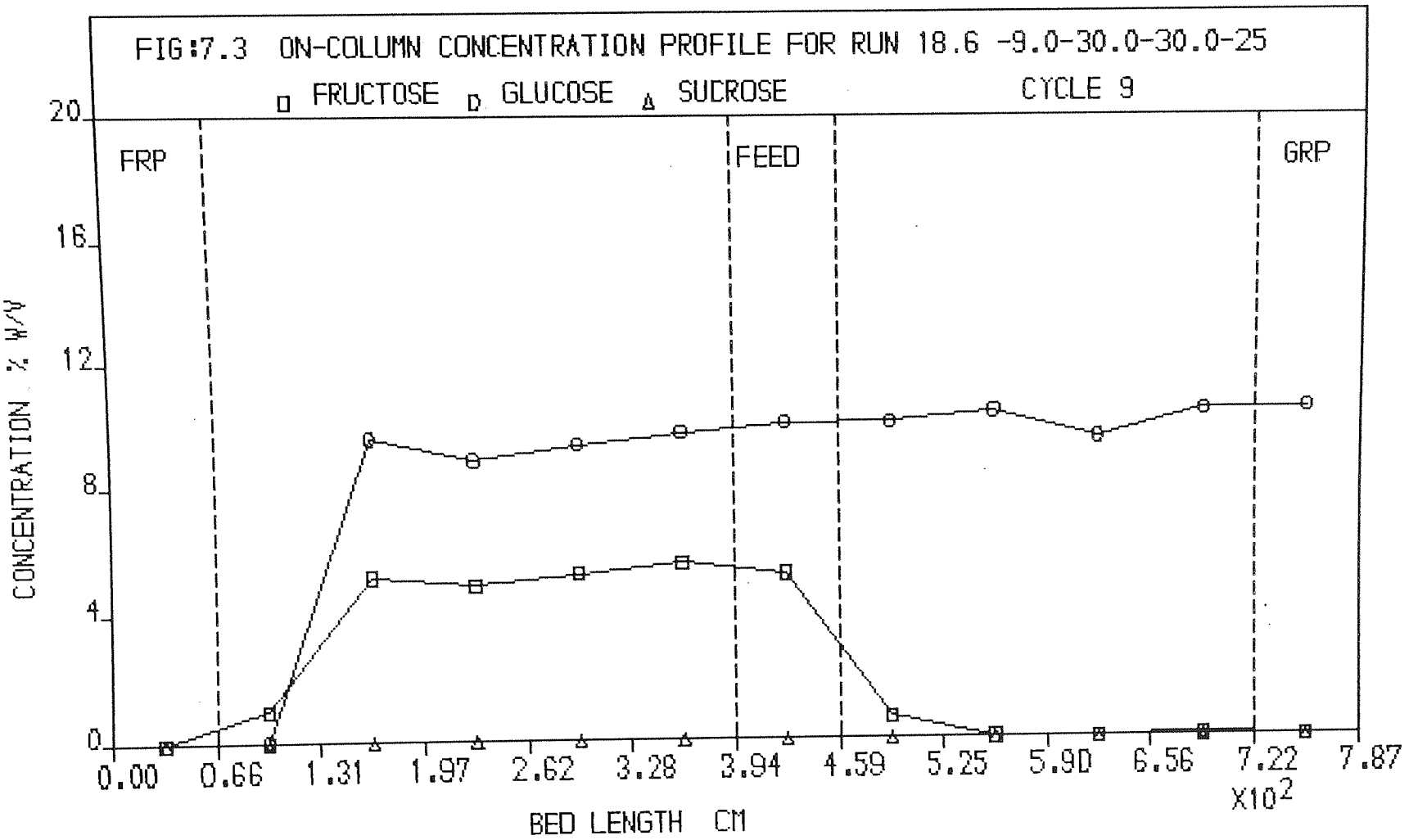


FIG:7.3 ON-COLUMN CONCENTRATION PROFILE FOR RUN 18.6 -9.0-30.0-30.0-25
FRUCTOSE
GLUCOSE
SUCROSE
CYCLE 9
FRP
FEED
GRP
CONCENTRATION % W/V
20
16
12
8
4
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10^2
BED LENGTH CM

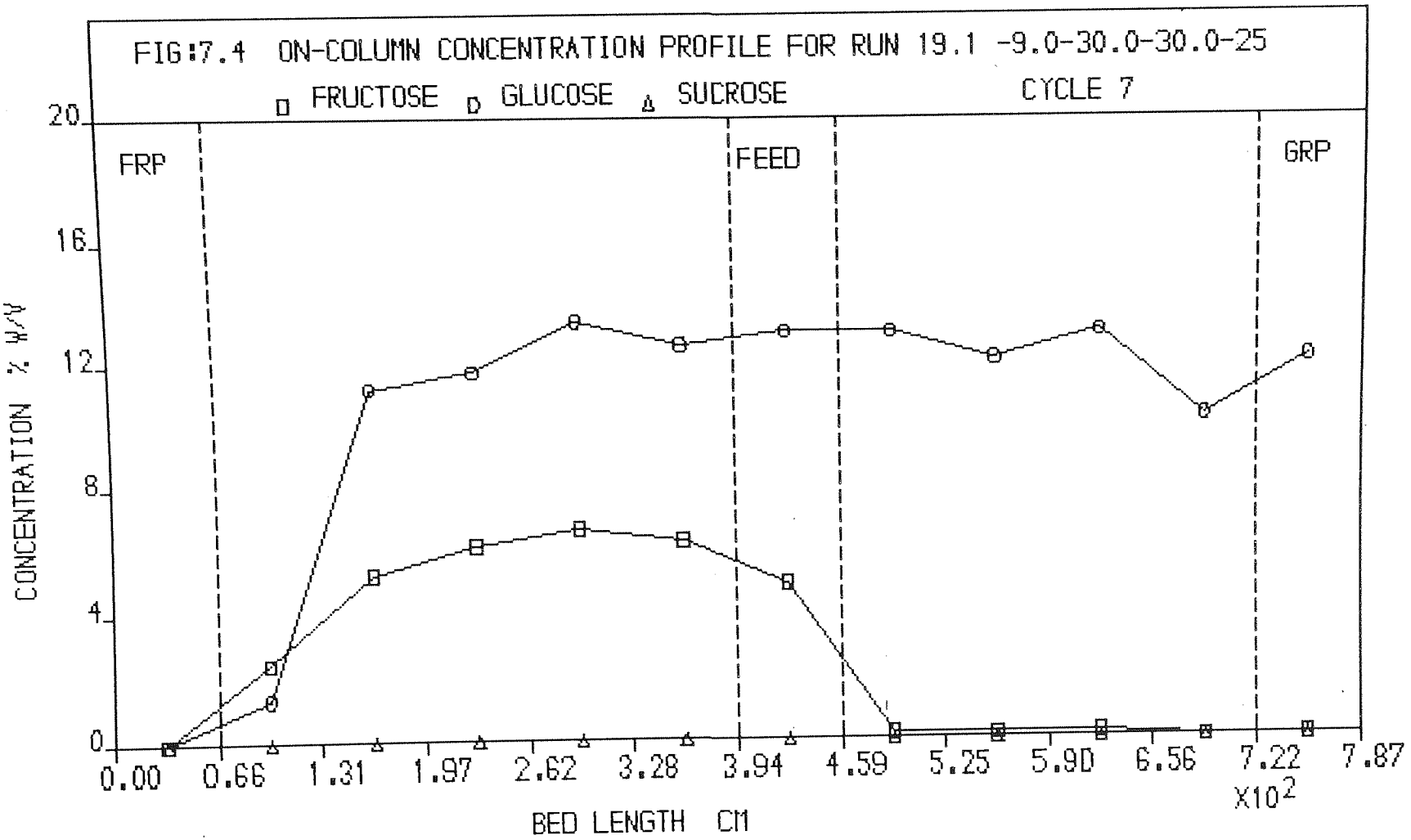


FIG:7.4 ON-COLUMN CONCENTRATION PROFILE FOR RUN 19.1 -9.0-30.0-30.0-25
□ FRUCTOSE ○ GLUCOSE △ SUCROSE
CYCLE 7
FRP
FEED
GRP
CONCENTRATION % W/W
20
16
12
8
4
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10²
BED LENGTH CM

Table 7.4 : Effect of Switch Time on Product Purities - Operating Conditions

Experimental Run	Average Flowrates $cm^3\ min^{-1}$			Feed Concentration % W/V			F:G ratio in feed	Switch time run	Average K^d Values inc. the effects of temp, flow rates & conc.		Lm/P ratio	Cycle
	Feed	Eluent	Purge	G	F	Total			Kd_g	Kd_f		
19.14-9-30-30-25	9	30	75	11.28	7.86	19.14	41 : 59	30	0.429	0.66	0.285	+7
19.96-9-30-30.167-25	9	30	75	11.48	8.48	19.96	42.5 : 57.5	30.167	0.431	0.662	0.289	9
19.21-9-30-32-25	9	30	75	10.86	8.35	19.21	43.5 : 56.5	32	0.429	0.658	0.328	8
18.6-9-30-33-25	9	30	75	10.79	7.81	18.6	42 : 58	33	0.427	0.658	0.349	+3

G - glucose; F - fructose;

+ indicates the number of additional cycles carried out after making a change in the operating condition without purging the system.

Table 7.5 : Effect of Switch time on Product Purities - Experimental Results

Experimental Run	Feed throughput (sugar solids kgh^{-1})	GLUCOSE RICH PRODUCT					FRUCTOSE RICH PRODUCT				
		Glucose Purity %	% of glucose in feed recovered	Total product conc. % W/V	Impurities % F	Others	Fructose Purity %	% of Fructose in feed recovered	Total Product conc. % W/V	Impurities % G	Others
19.14-9-30-30-25	0.103	100	73.3	1.965	-	-	73.3	91	1.18	26.7	-
19.96-9-30-30-167.25	0.108	100	72.8	2.0	-	-	70.17	964	1.418	29.83	-
19.21-9-30-32-25	0.104	100	70	1.93	-	-	80	84	1.15	20.0	-
18.6-9-30-33-25	0.1	97.8	109.5	2.31	2.2	-	96.7	94	0.82	3.26	-

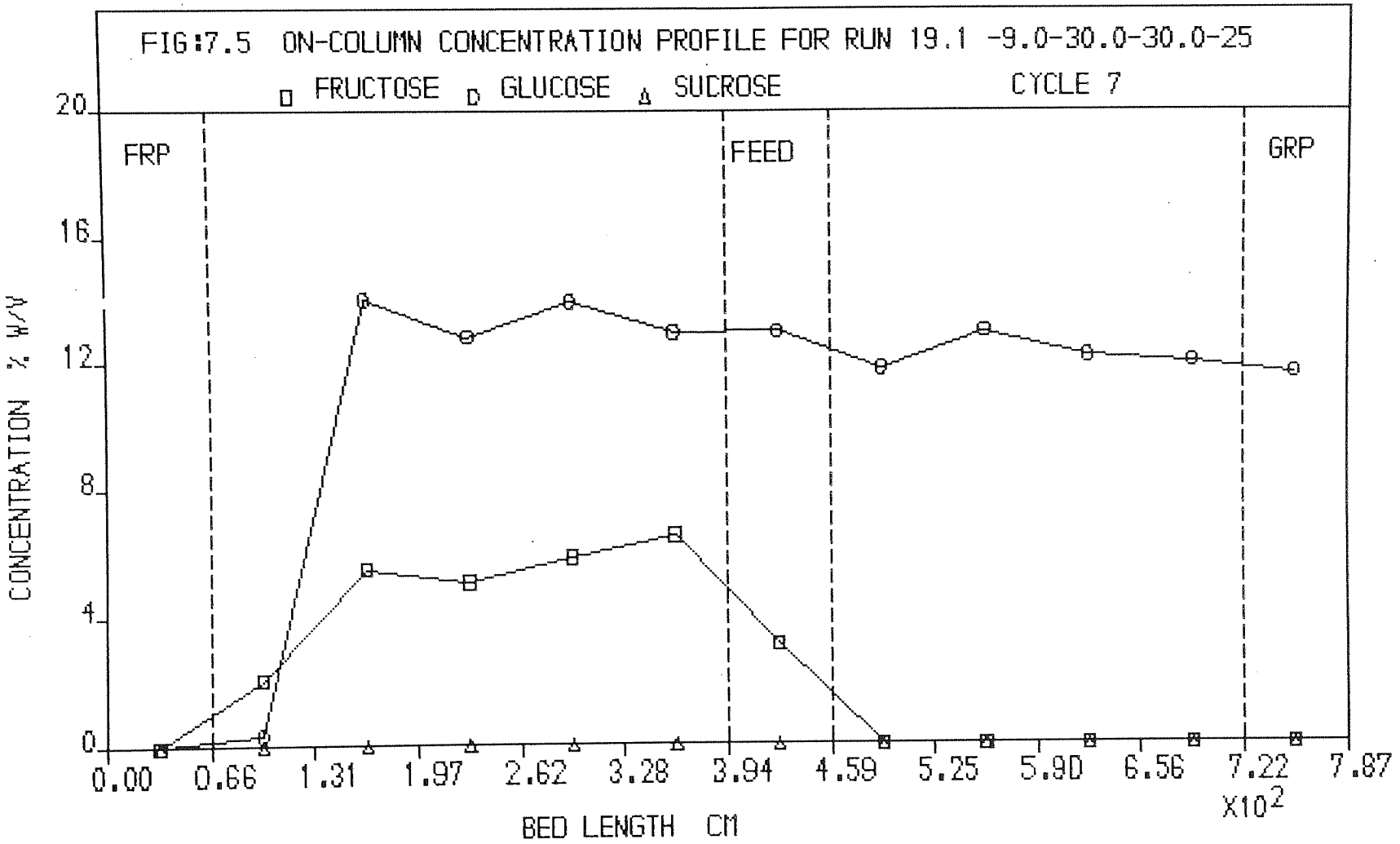


FIG:7.5 ON-COLUMN CONCENTRATION PROFILE FOR RUN 19.1 -9.0-30.0-30.0-25
□ FRUCTOSE ▫ GLUCOSE △ SUCROSE
CYCLE 7
FRP
FEED
GRP
CONCENTRATION % W/V
20
16
12
8
4
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10²
BED LENGTH CM

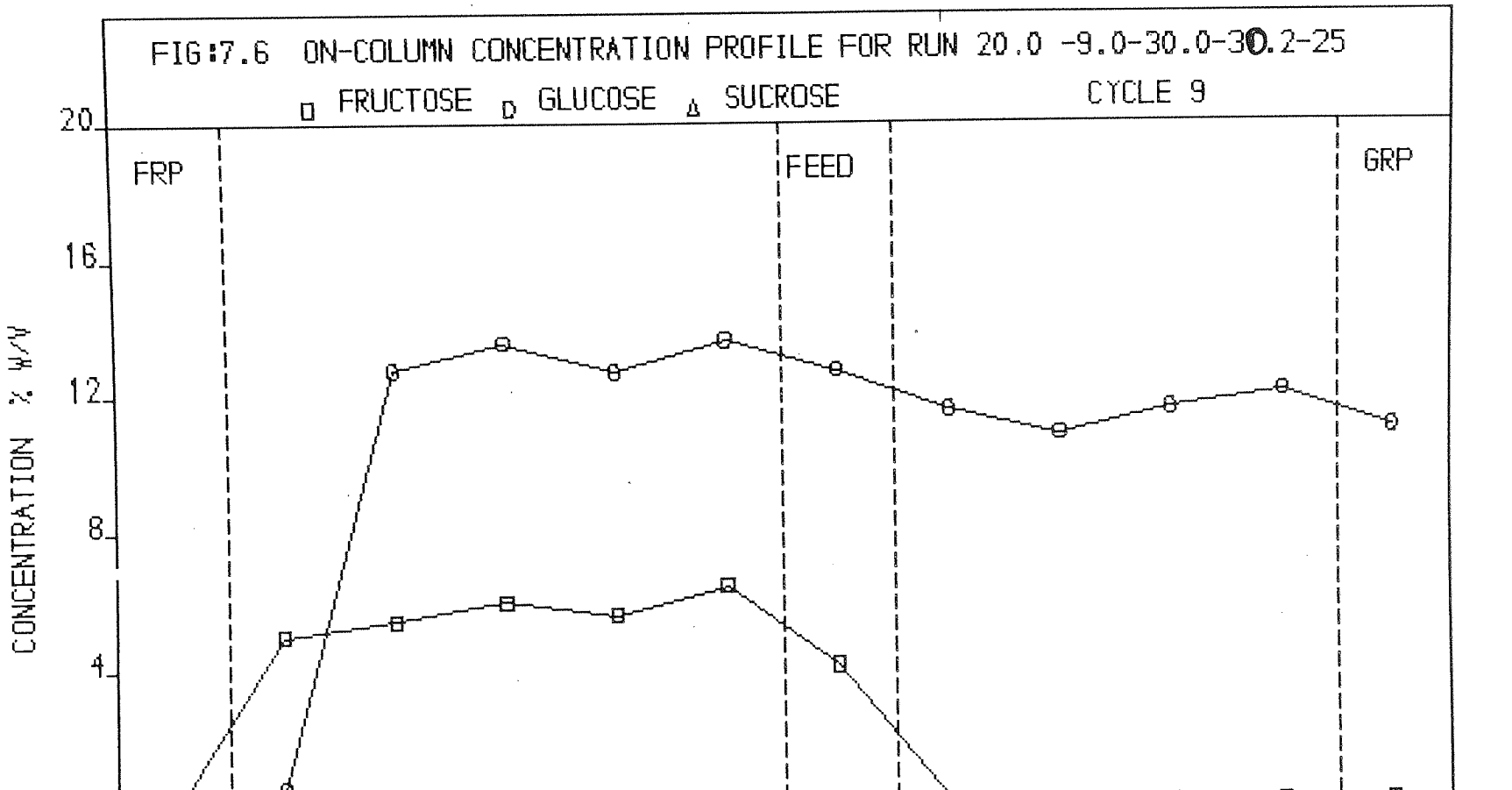


FIG:7.6 ON-COLUMN CONCENTRATION PROFILE FOR RUN 20.0 -9.0-30.0-30.2-25
FRUCTOSE GLUCOSE SUCROSE
CYCLE 9
FRP
FEED
GRP
CONCENTRATION % W/W
20
16
12
8
4
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10^2
BED LENGTH CM

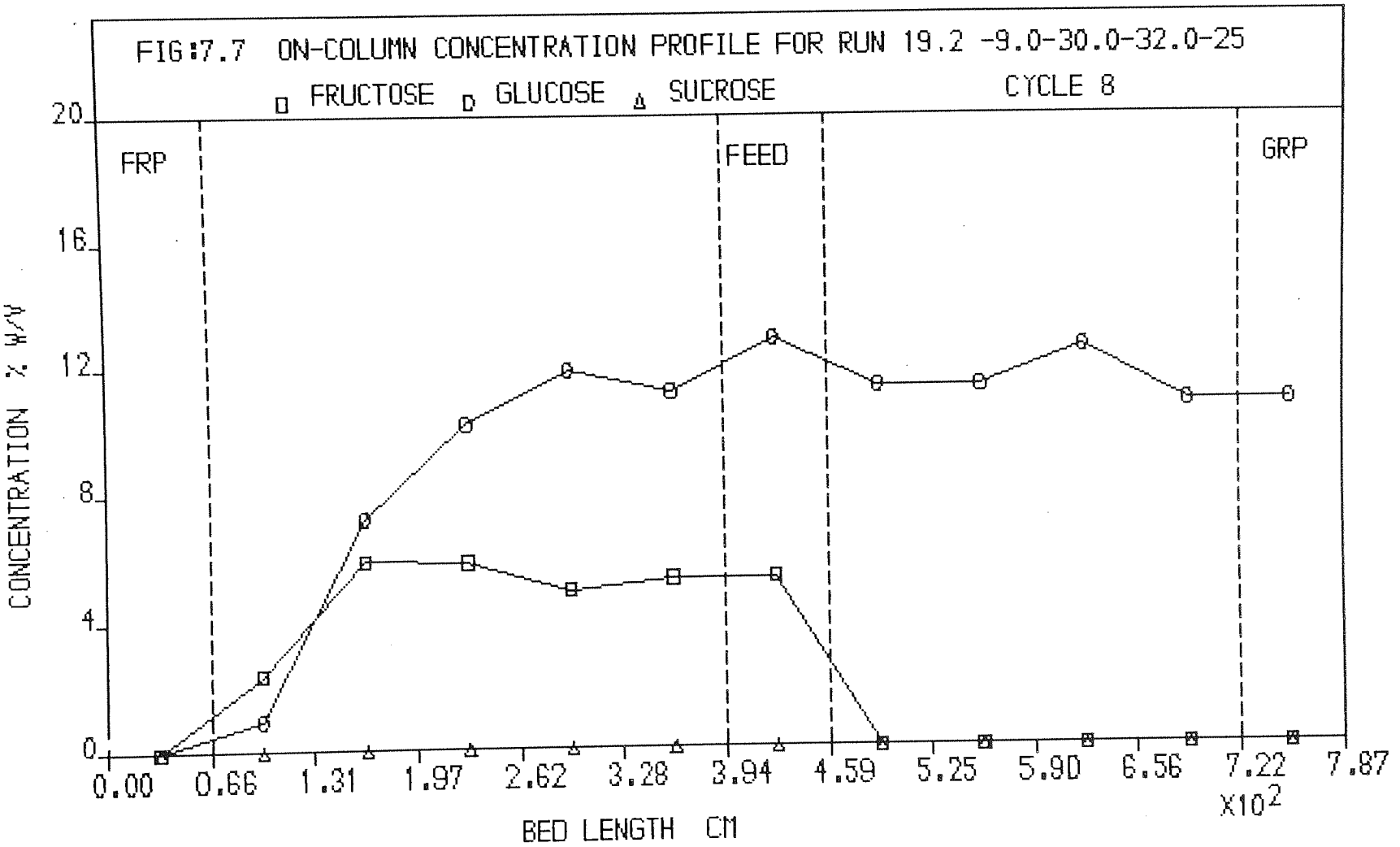


FIG:7.7 ON-COLUMN CONCENTRATION PROFILE FOR RUN 19.2 -9.0-30.0-32.0-25
FRUCTOSE
GLUCOSE
SUCROSE
CYCLE 8
FRP
FEED
GRP
CONCENTRATION % W/W
20
16
12
8
4
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10^2
BED LENGTH CM

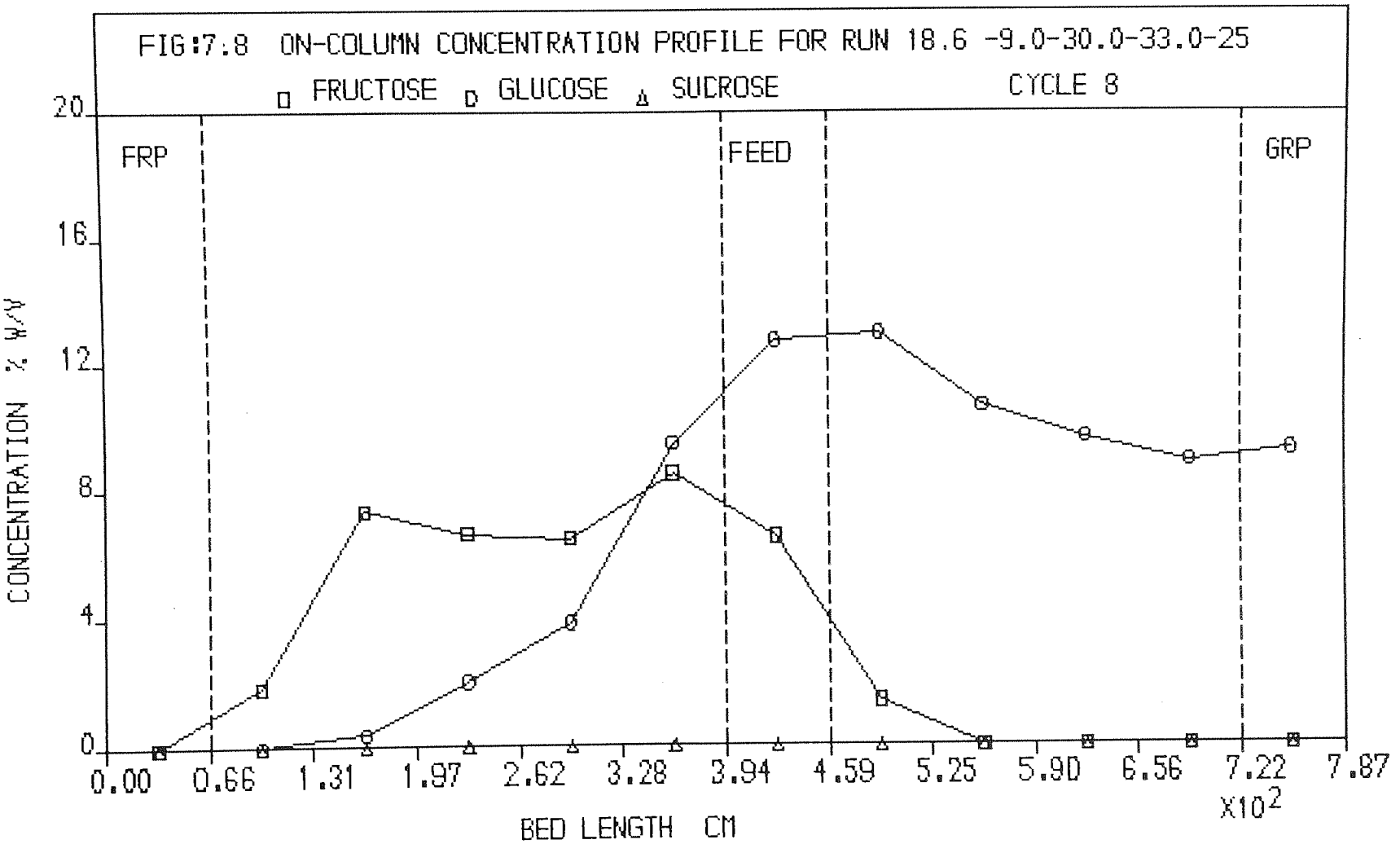


FIG:7.8 ON-COLUMN CONCENTRATION PROFILE FOR RUN 18.6 -9.0-30.0-33.0-25
□ FRUCTOSE ▫ GLUCOSE △ SUCROSE
CYCLE 8
FRP
FEED
GRP
CONCENTRATION % W/W
20
16
12
8
4
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10²
BED LENGTH CM

Table 7.6 : Effect of Switch Time on Product Purities on the Separation of the Three Component Feed - Operating Conditions

Experimental Run	Average Flowrates $cm^3\ min^{-1}$			Feed Concentration % W/V				D:S:F ratio in feed	Switch time (min)	Lm/P ratio	Cycle
	Feed	Eluent	Purge	D	S	F	Total				
19.38-9-30-28-25	9	30	76	6.45	6.76	6.17	19.38	1: 1: 1	28	0.243	9
19.25-9-30-29.833-25	9	30	76	6.53	6.88	5.83	19.25	1: 1: 1	29.833	0.282	9
20.88-9-30-30-25	9	30	76	7.39	7.23	6.24	20.88	1: 1: 1	30	0.285	9
21.67-9-30-30.5-25	9	30	76	7.29	7.50	6.87	21.67	1: 1: 1	30.5	0.296	9
20.63-9-30-31-25	9	30	76	7.43	7.12	6.08	20.63	1: 1: 1	31	0.307	8
20-9-30-33-25	9	30	76	7.0	6	7	20	1: 1: 1	33	0.349	8

D - Dextran
S - Sucrose
F - Fructose

Table 7.7 : Effect of Switch Time on Product Purities on the Separation of the Three Component Feed - Experimental Results

Experimental Run	Feed throughput (sugar solids kgh^{-1})	DEXTRAN RICH PRODUCT						FRUCTOSE RICH PRODUCT					
		Dextran Purity %	% of Dextran in feed recovered	Total product conc. % W/V	Impurities % S	F	Others	Fructose Purity %	% of Fructose in feed recovered	Total Product conc. % W/V	Impurities % D	S	Others
19.38-9-30-28-25	0.015	61.73	99.6	3.09	38.2	-	-	85	107	1.05	27	12.3	-
19.25-9-30-29.83-25	0.104	48.6	94	2.95	51.4	-	-	96	95	0.7	-	4	-
20.88-9-30-30-25	0.113	50.4	94.5	3.2	48.6	0.9	-	97.3	91.15	0.7	-	2.7	-
21.67-9-30-30.5-25	0.117	49.85	100	3.4	49.06	1.07	-	97.6	97	0.82	-	2.37	-
20.63-9-30-31-25	0.111	51.6	96	3.22	47.3	1.11	-	97.8	97.3	0.73	-	2.2	-
20-9-30-33-25	0.108	56.1	97	3.0	41.8	2.02	-	93	106	0.98	1.55	5.35	-

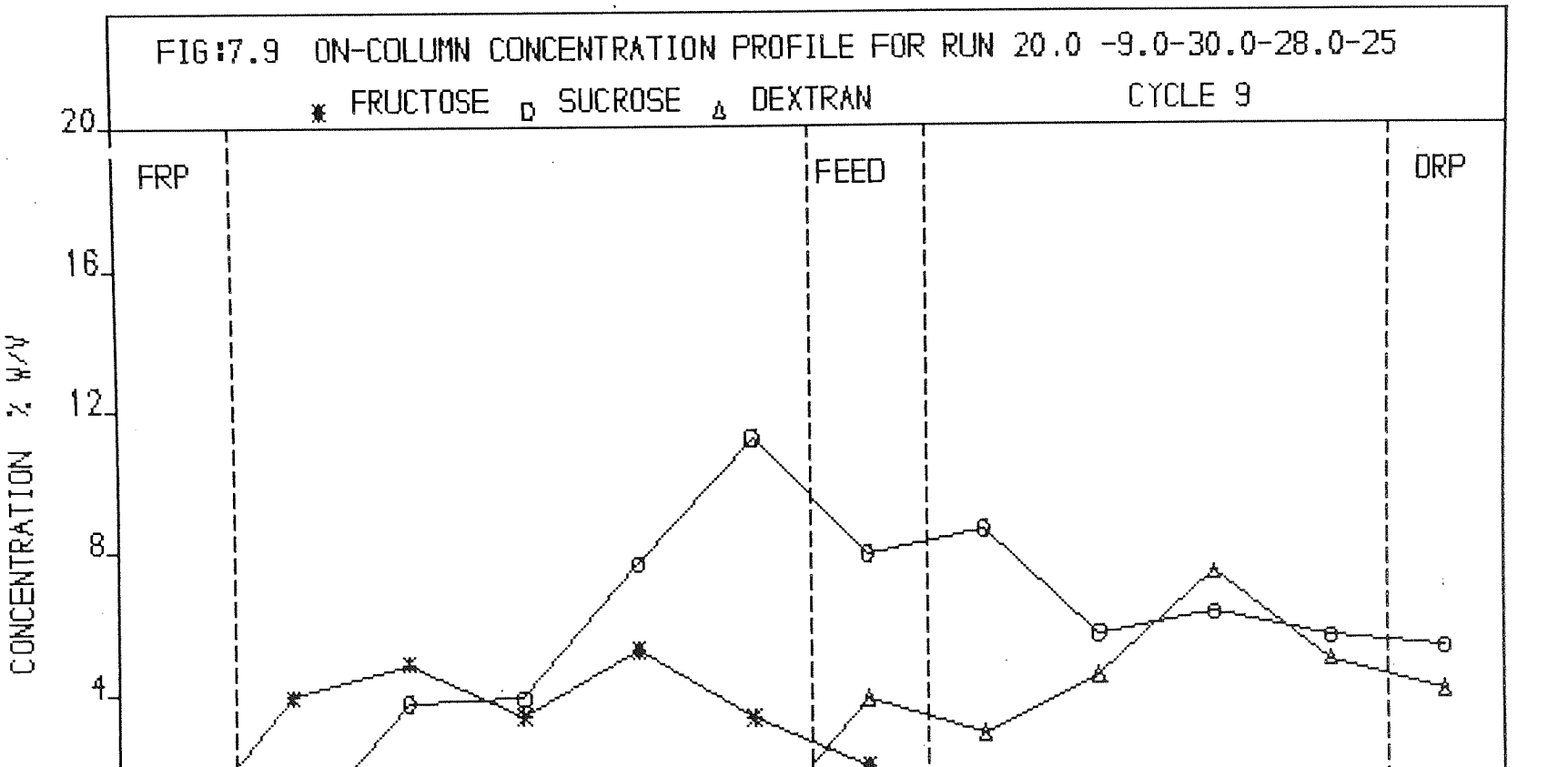


FIG:7.9 ON-COLUMN CONCENTRATION PROFILE FOR RUN 20.0 -9.0-30.0-28.0-25
* FRUCTOSE □ SUCROSE △ DEXTRAN
CYCLE 9
FRP
FEED
DRP
CONCENTRATION % W/V
20
16
12
8
4
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10^2
BED LENGTH CM

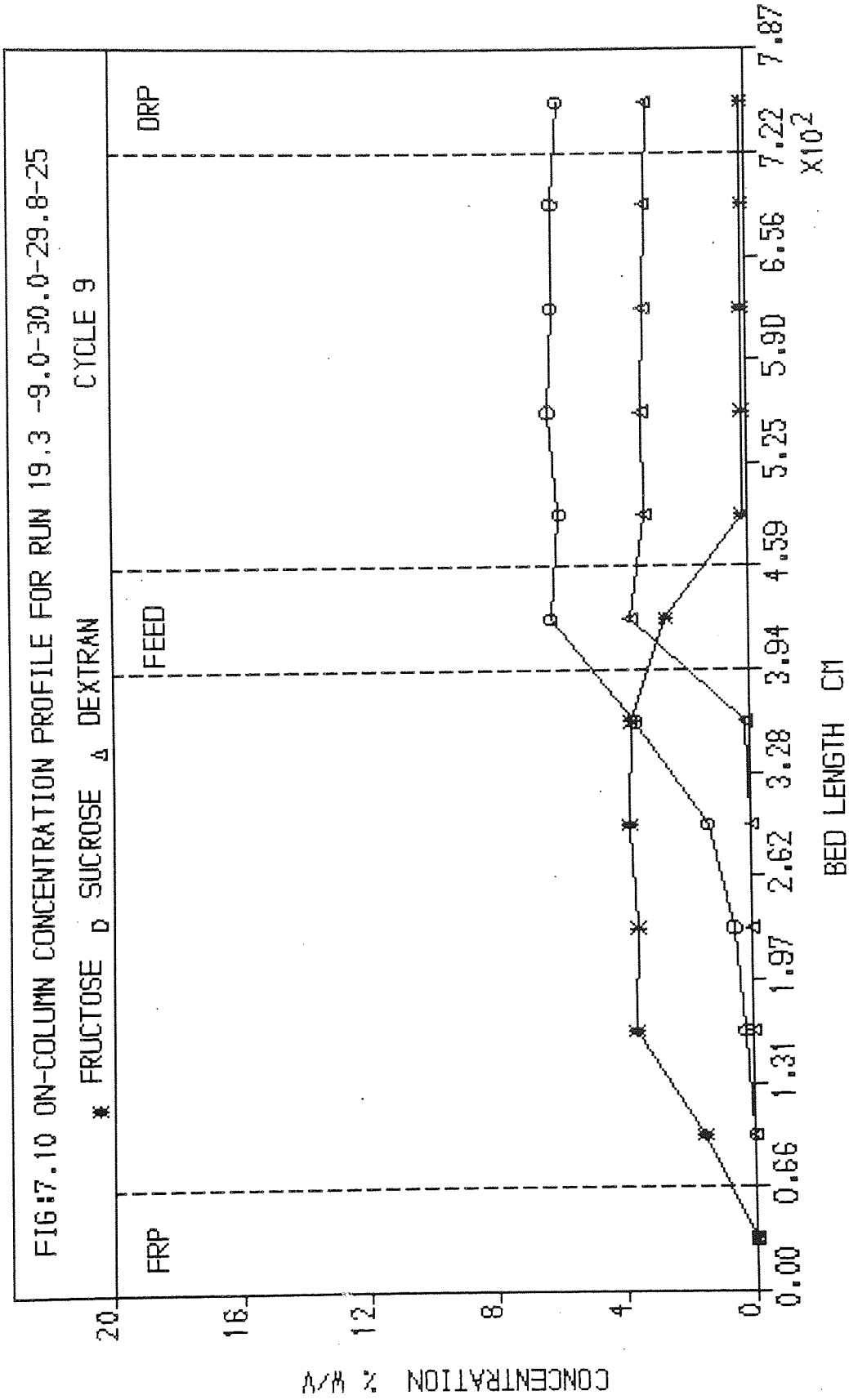


FIG:7.10 ON-COLUMN CONCENTRATION PROFILE FOR RUN 19.3 -9.0-30.0-29.8-25
CYCLE 9
* FRUCTOSE □ SUCROSE △ DEXTRAN
FRP
FEED
DRP
CONCENTRATION % W/V
20
16
12
8
4
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10²
BED LENGTH CM

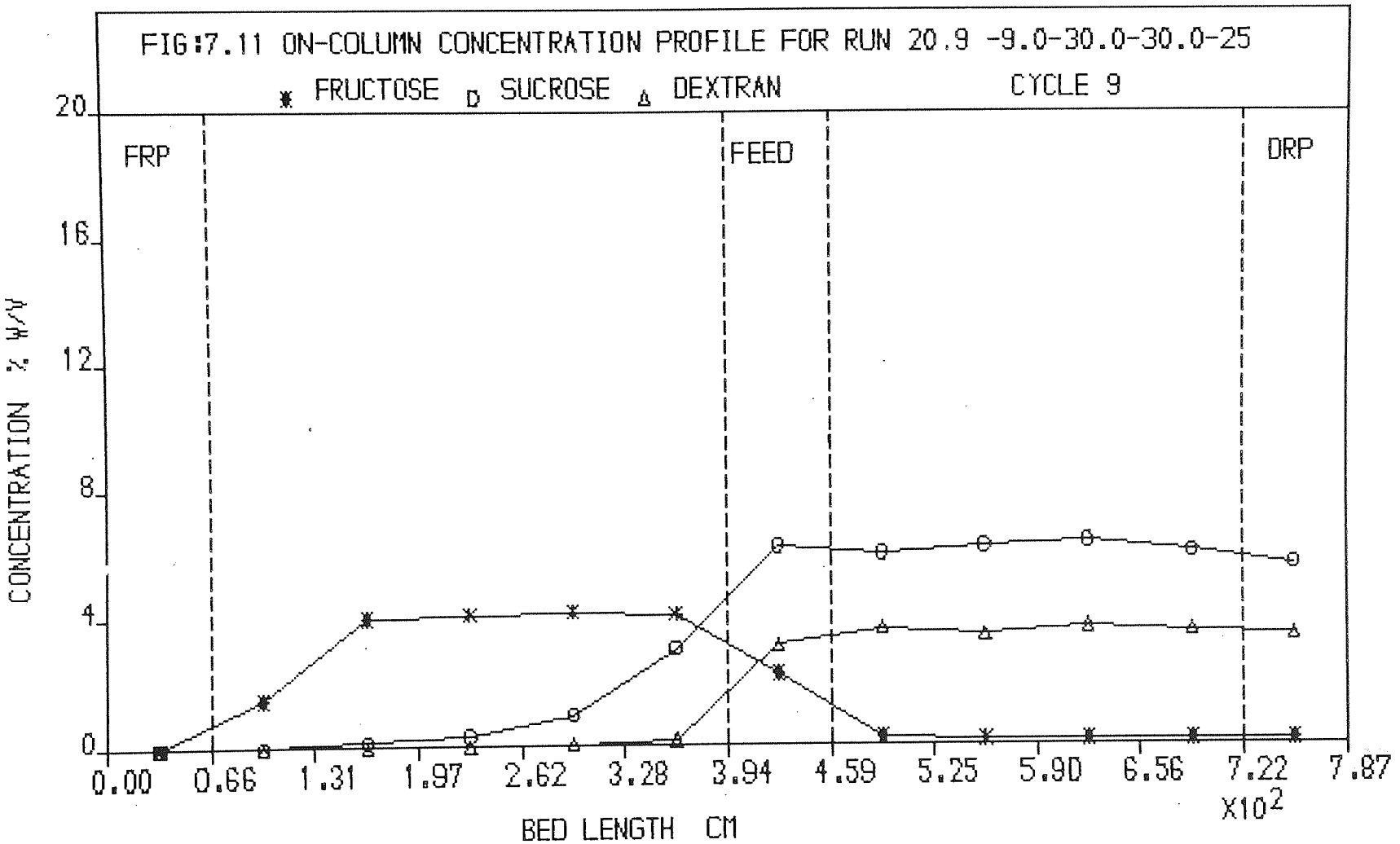


FIG:7.11 ON-COLUMN CONCENTRATION PROFILE FOR RUN 20.9 -9.0-30.0-30.0-25
* FRUCTOSE □ SUCROSE △ DEXTRAN
CYCLE 9
FRP
FEED
DRP
CONCENTRATION % W/W
20
16
12
8
4
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10^2
BED LENGTH CM

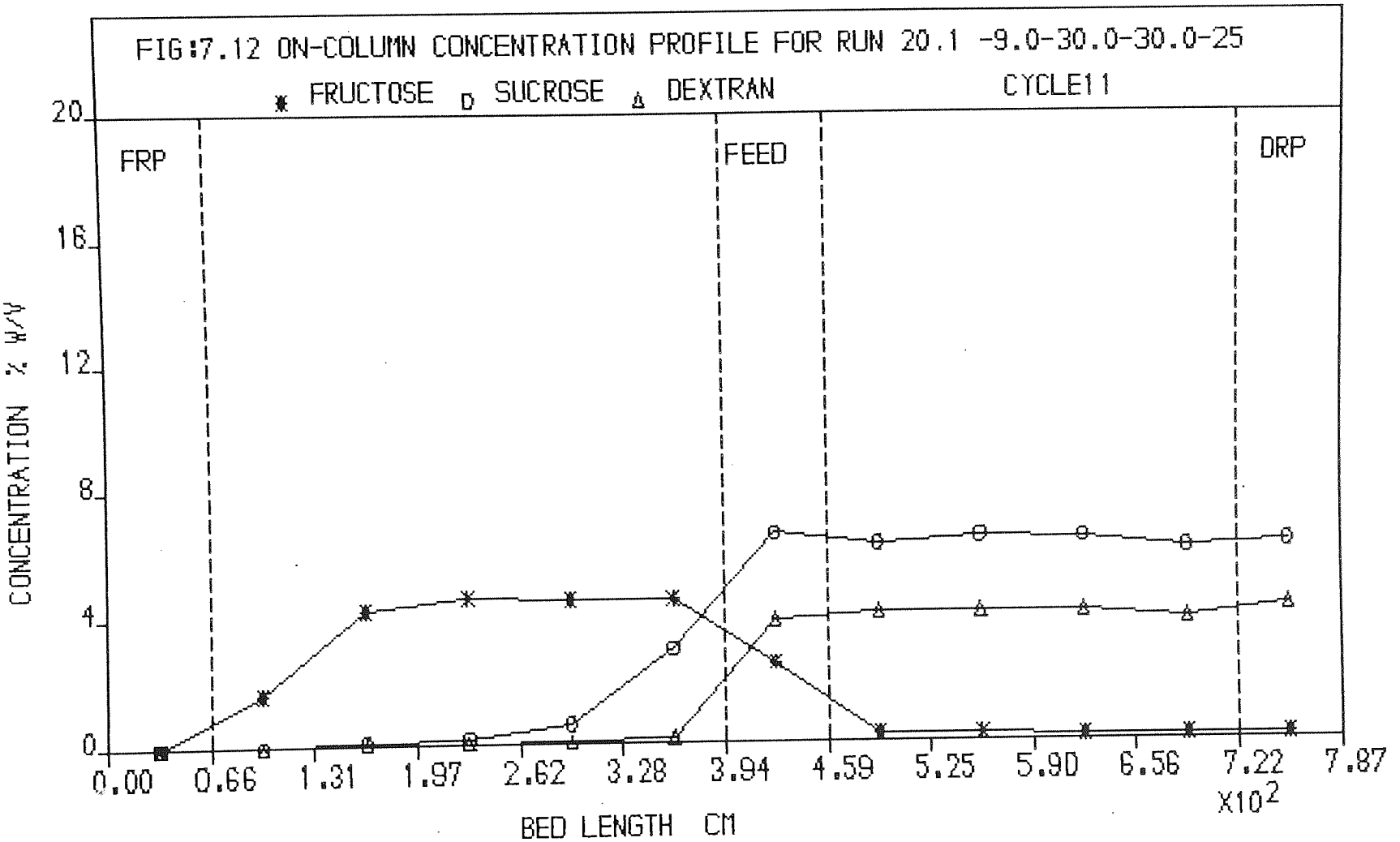


FIG:7.12 ON-COLUMN CONCENTRATION PROFILE FOR RUN 20.1 -9.0-30.0-30.0-25
* FRUCTOSE D SUCROSE Δ DEXTRAN
CYCLE11
FRP
FEED
DRP
CONCENTRATION % W/V
20
16
12
8
4
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10^2
BED LENGTH CM

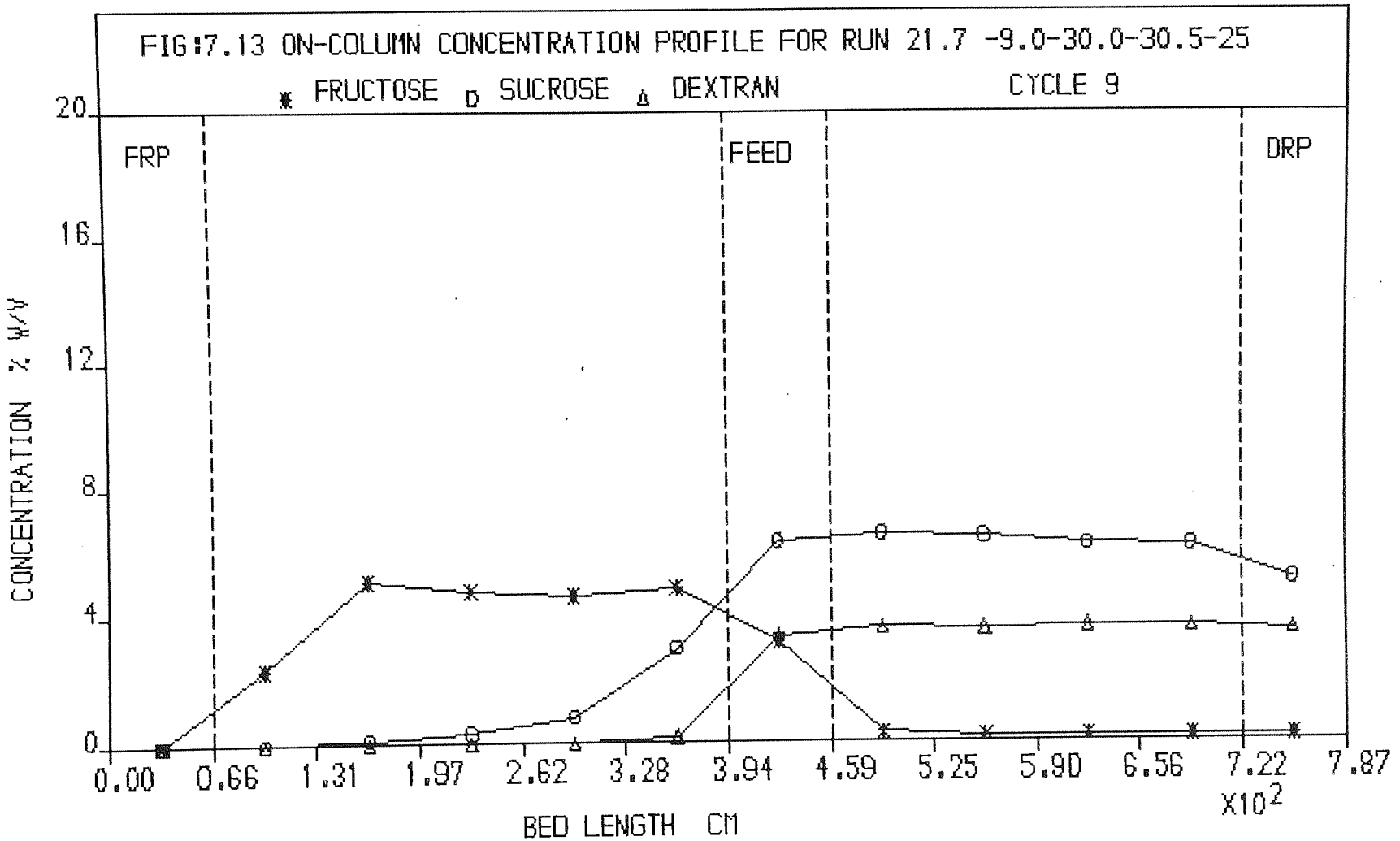


FIG:7.13 ON-COLUMN CONCENTRATION PROFILE FOR RUN 21.7 -9.0-30.0-30.5-25
* FRUCTOSE D SUCROSE Δ DEXTRAN
CYCLE 9
FRP
FEED
DRP
CONCENTRATION % W/W
20
16
12
8
4
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10^2
BED LENGTH CM

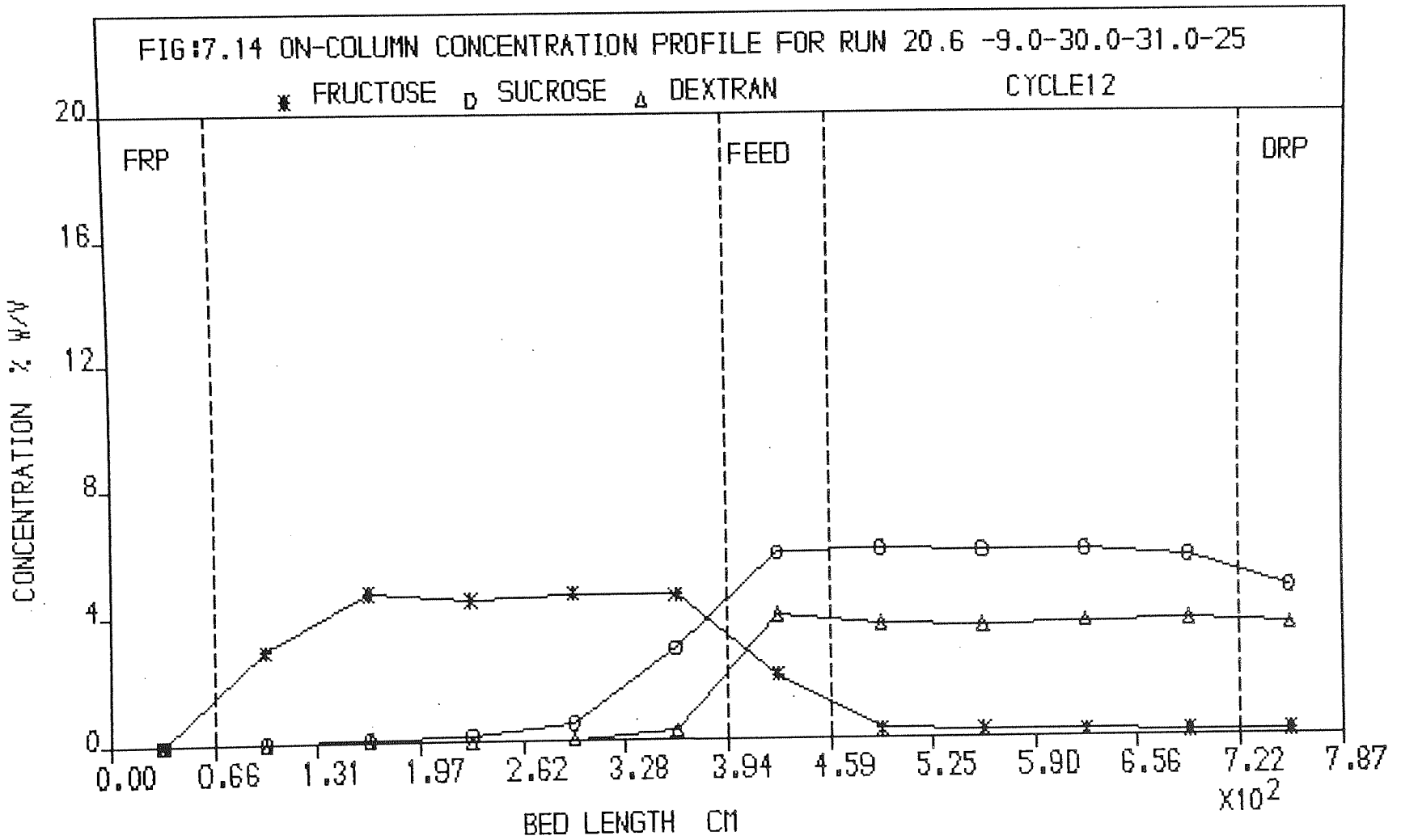


FIG:7.14 ON-COLUMN CONCENTRATION PROFILE FOR RUN 20.6 -9.0-30.0-31.0-25
* FRUCTOSE D SUCROSE Δ DEXTRAN
CYCLE12
FRP
FEED
DRP
CONCENTRATION % W/V
20
16
12
8
4
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10^2
BED LENGTH CM

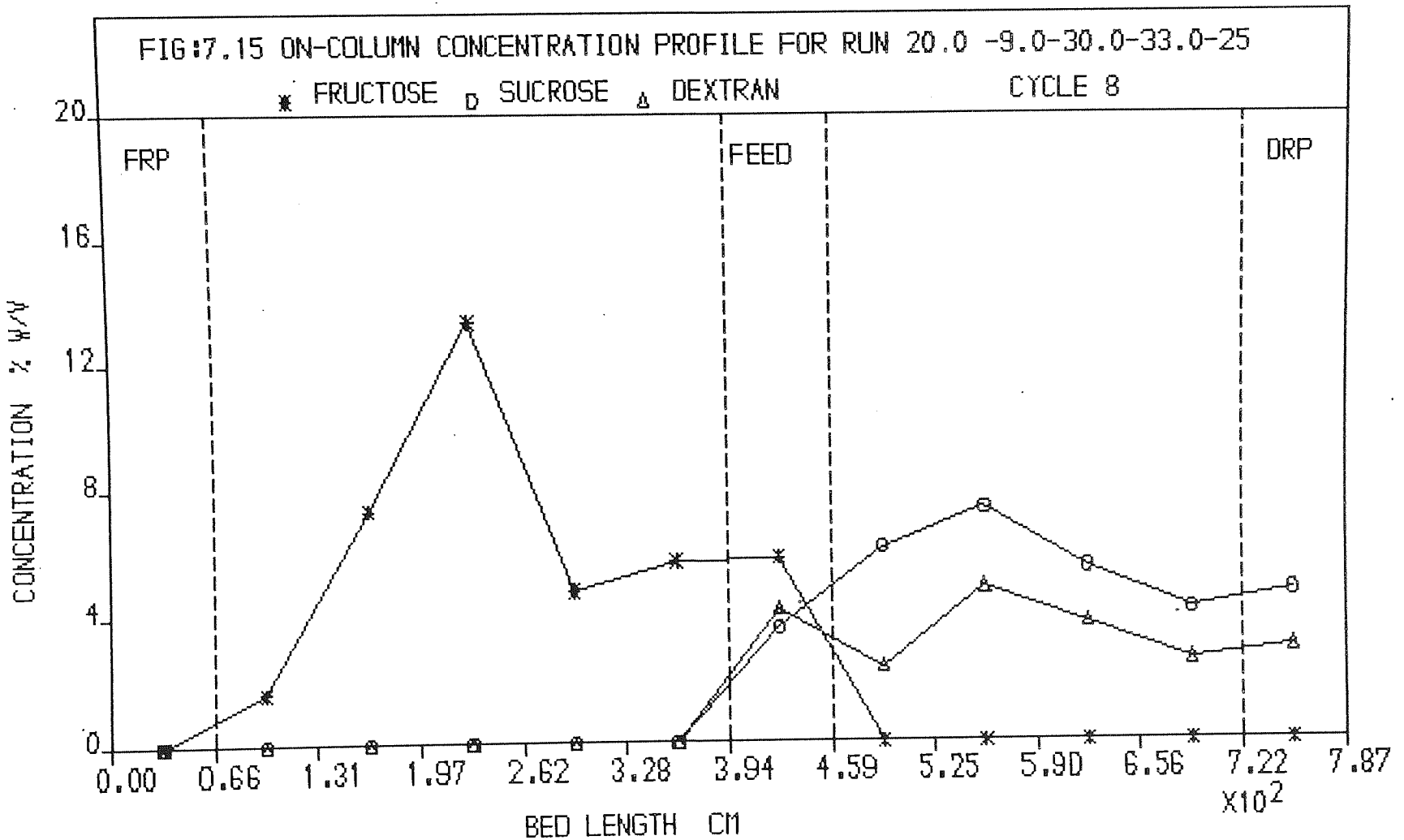


FIG:7.15 ON-COLUMN CONCENTRATION PROFILE FOR RUN 20.0 -9.0-30.0-33.0-25
* FRUCTOSE D SUCROSE Δ DEXTRAN
CYCLE 8
FRP
FEED
DRP
CONCENTRATION % W/W
20
16
12
8
4
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10^2
BED LENGTH CM

Table 7.8 : Separation of an Inverted Sucrose Feed - Operating Conditions

Experimental Run	Average Flowrates $cm^3\ min^{-1}$			Feed Concentration % W/V			F:G ratio in feed	Switch time (min)	Average K^d Values inc. the effects of temp, flow rates & conc.		Lm/P ratio	Cycle
	Feed	Eluent	Purge	G	F	Total			Kd_g	Kd_f		
19.65-9-30-33-35	9	30	76	9.98	9.67	19.65	49:51	33	0.429	0.624	0.349	+8
19.83-9-30-33-35	9	30	76	11.47	8.36	19.83	42:58	33	0.431	0.631	0.349	8
18.15-9-30-33-35	9	30	76	10	8.15	18.15	45:55	33	0.425	0.623	0.349	+7

G : Glucose
F : Fructose

+ indicates the number of additional cycles carried out after making a change in the operating condition without purging the system.

Table 7.9 : Separation of an Inverted Sucrose Feed - Experimental Results

Experimental Run	Feed throughput (sugar solids kgh^{-1})	GLUCOSE RICH PRODUCT					FRUCTOSE RICH PRODUCT				
		Glucose Purity %	% of glucose in feed recovered	Total product conc. % W/V	Impurities % F	Others	Fructose Purity %	% of Fructose in feed recovered	Total Product conc. % W/V	Impurities % G	Others
19.65-9-30-33.35	0.106	97	100	2.49	3.1	-	88	85.3	1.1	11.8	-
* 19.83-9-30-33-35	0.107	97.16	85.2	2.44	2.87	-	90	100	1.1	10.1	-
18.15-9-30-33.35	0.098	97	85.6	2.14	3.1	-	80	107	1.31	20	-

* Synthetic Feed

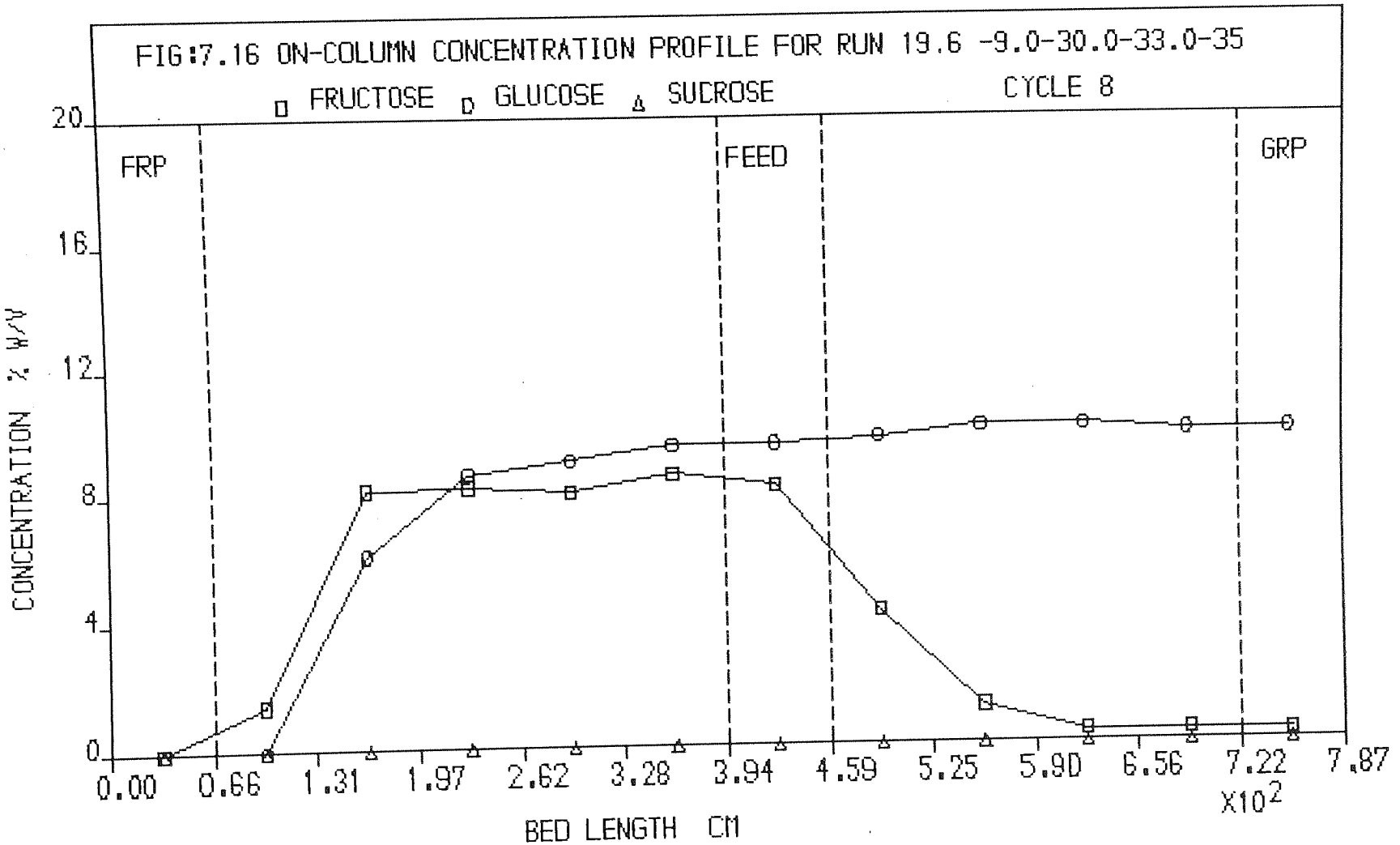


FIG:7.16 ON-COLUMN CONCENTRATION PROFILE FOR RUN 19.6 -9.0-30.0-33.0-35
FRUCTOSE
GLUCOSE
SUCROSE
CYCLE 8
FRP
FEED
GRP
CONCENTRATION % W/V
20
16
12
8
4
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10^2
BED LENGTH CM

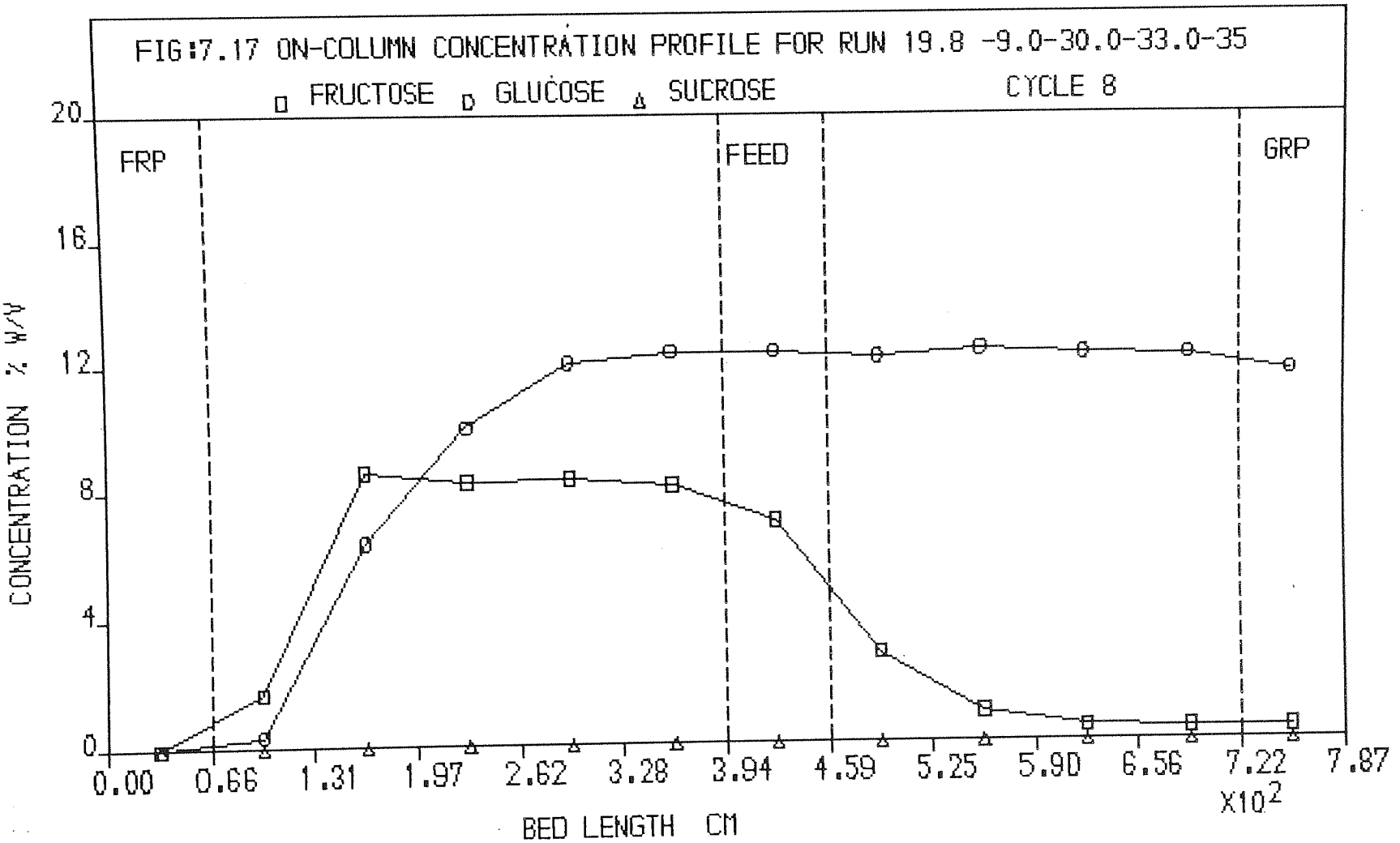


FIG:7.17 ON-COLUMN CONCENTRATION PROFILE FOR RUN 19.8 -9.0-30.0-33.0-35
FRUCTOSE
GLUCOSE
SUCROSE
CYCLE 8
FRP
FEED
GRP
CONCENTRATION % W/V
20
16
12
8
4
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10^2
BED LENGTH CM

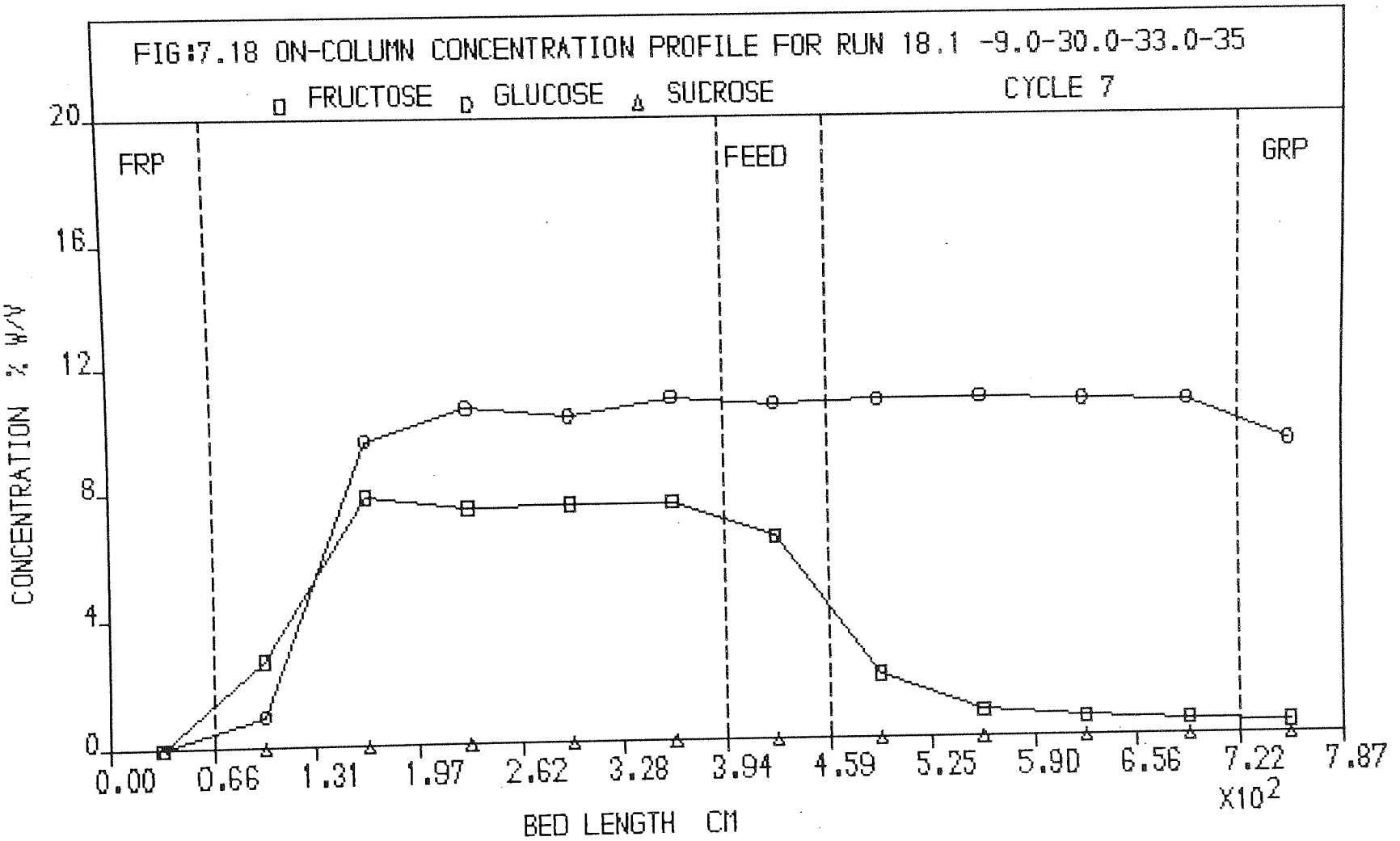


FIG:7.18 ON-COLUMN CONCENTRATION PROFILE FOR RUN 18.1 -9.0-30.0-33.0-35
FRUCTOSE
GLUCOSE
SUCROSE
CYCLE 7
FRP
FEED
GRP
CONCENTRATION % W/V
20
16
12
8
4
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10^2
BED LENGTH CM

CHAPTER 8

STUDY OF THE SCCR-S1 AS A COMBINED BIOCHEMICAL REACTOR-SEPARATOR USING A SUCROSE INVERSION REACTION

8.0 CONTINUOUS REACTION AND SEPARATION OF SUCROSE INVERSION PRODUCTS ON THE SCCR-S1 SYSTEM

8.1 INTRODUCTION

The inversion of sucrose is a very simple reaction with a very high reaction rate. Sucrose is hydrolysed by the enzyme invertase to yield fructose and glucose. The invertase is produced from the fermentation of yeast and was available commercially (Chapter 5).

One of the conditions stated by Matsen [26] in his chemical reaction-separation studies was that a fast reaction rate is required so that the reaction rate is not limited during a combined reaction-separation process. This condition was investigated on the SCCR-S1 system using the sucrose inversion reaction. A study of the reaction kinetics of invertase was carried out to give an idea of the reaction rate and some insight into the reaction mechanism.

The operating parameters of the system were selected from the knowledge gained from earlier separation studies (Chapter 7).

Initial runs were carried out to commission the system and to give an indication of the system's behaviour. Twenty five successful runs were carried out and the appropriate ones have been selected to investigate the effect of the following parameters on the performance of the SCCR-S1 as a combined reactor-separator:

- Switch time
- Feed concentration
- Enzyme activity
- Eluent-to-feed rate ratio
- Constant enzyme-to-feed concentration ratio

Product purities of both the GRP and FRP stream's of approximately 90% were aimed for.

8.2 ENZYME KINETIC STUDIES

A necessary first step towards the goal of good utilisation of enzyme in the process industries is the correlation of enzyme function with enzyme structure. An explanation of the enzyme function begins with the formulation of a kinetic model which is capable of describing the observed data.

The study of the rate of invertase action on the inversion of sucrose was investigated by measuring the initial reaction rate for a range of initial sucrose concentrations. This study was carried out in a conventional batch reactor at constant temperature (55°C) and constant pH (5.0). Using the invertase assay method (Chapter 6). The activity of invertase was 60 Ucm^{-3}. Samples were removed from the reaction vessel at 2min intervals and placed into a test tube immersed in a 100°C bath. Invertase denaturation was complete after one minute. The samples were then assayed for reducing sugar.

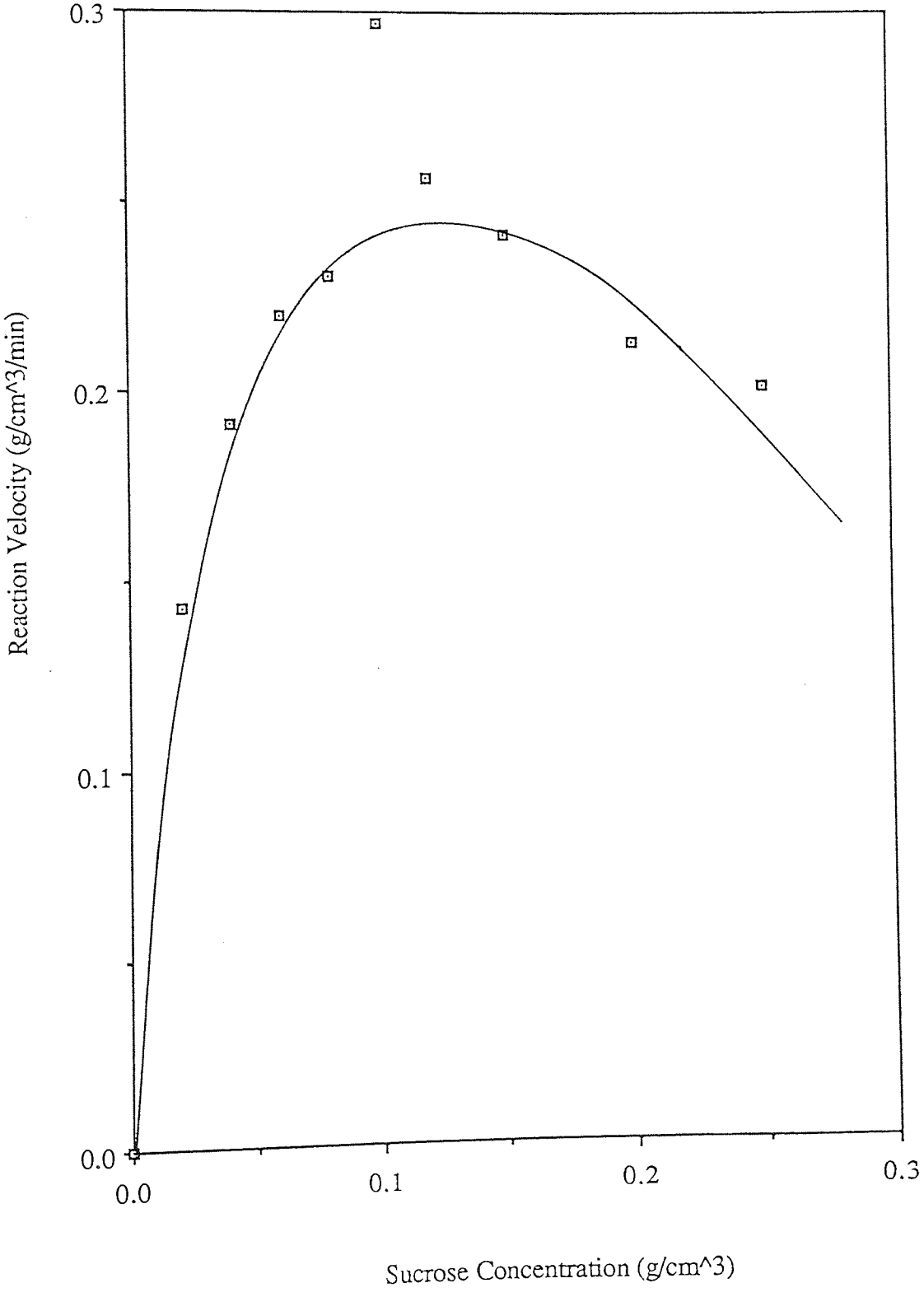
The reaction rate data presented in Table 8.1 and plotted in Figure 8.1 demonstrate the kinetics of the hydrolysis of sucrose to glucose and fructose. The maximum reaction velocity occurred at 9.75% W/V (0.285M) sucrose concentration, which is in close agreement with the work carried out by Bowski et al. [146]. Using this type of method of determining initial reaction rates is more accurate than the use of polarimetric measurements which assume equal amounts of glucose and fructose neglecting the intermediate oligosaccharide formation by the transferase action of invertase [59, 147].

The results obtained do not fit the Michaelis-Menten kinetic model (Equation 3.1) at high sucrose concentrations (greater than 9.75% W/V) where zero order was predicted (Figure 3.2). The modified Michaelis-Menten model incorporating the substrate inhibition effect is written as follows:

Table 8.1: Sucrose Inversion Rates as Determined by an Invertase Assay

SUCROSE CONCENTRATION (g/L)	(Moles/Litre)	REACTION VELOCITY g/cm^3 min
20	0.058	0.143
40	0.117	0.191
60	0.175	0.22
80	0.234	0.23
100	0.292	0.297
120	0.351	0.256
150	0.438	0.241
200	0.585	0.212
250	0.731	0.2

Figure 8.1:- Reaction Velocity vs Sucrose Concentration using Invertase Enzyme



$$u = \frac{V_{max}\,[s]}{K_m + [s] + \frac{[s]^2}{K_{si}}} \qquad 8.1$$

where u = the initial reaction velocity

V_{max} = the maximum reaction velocity

K_m = the Michaelis Menten constant

K_{si} = the substrate inhibition constant

$[s]$ = Sucrose concentration.

This fits reasonably well with the experimental results.

The kinetic constants (V_{max} and K_m) were calculated from the linear section of the initial velocity vs sucrose concentration graph (Figure 8.1) using the Cornish-Bowden plot [158] (Figure 8.2) which was derived from the Michaelis Menten model as:

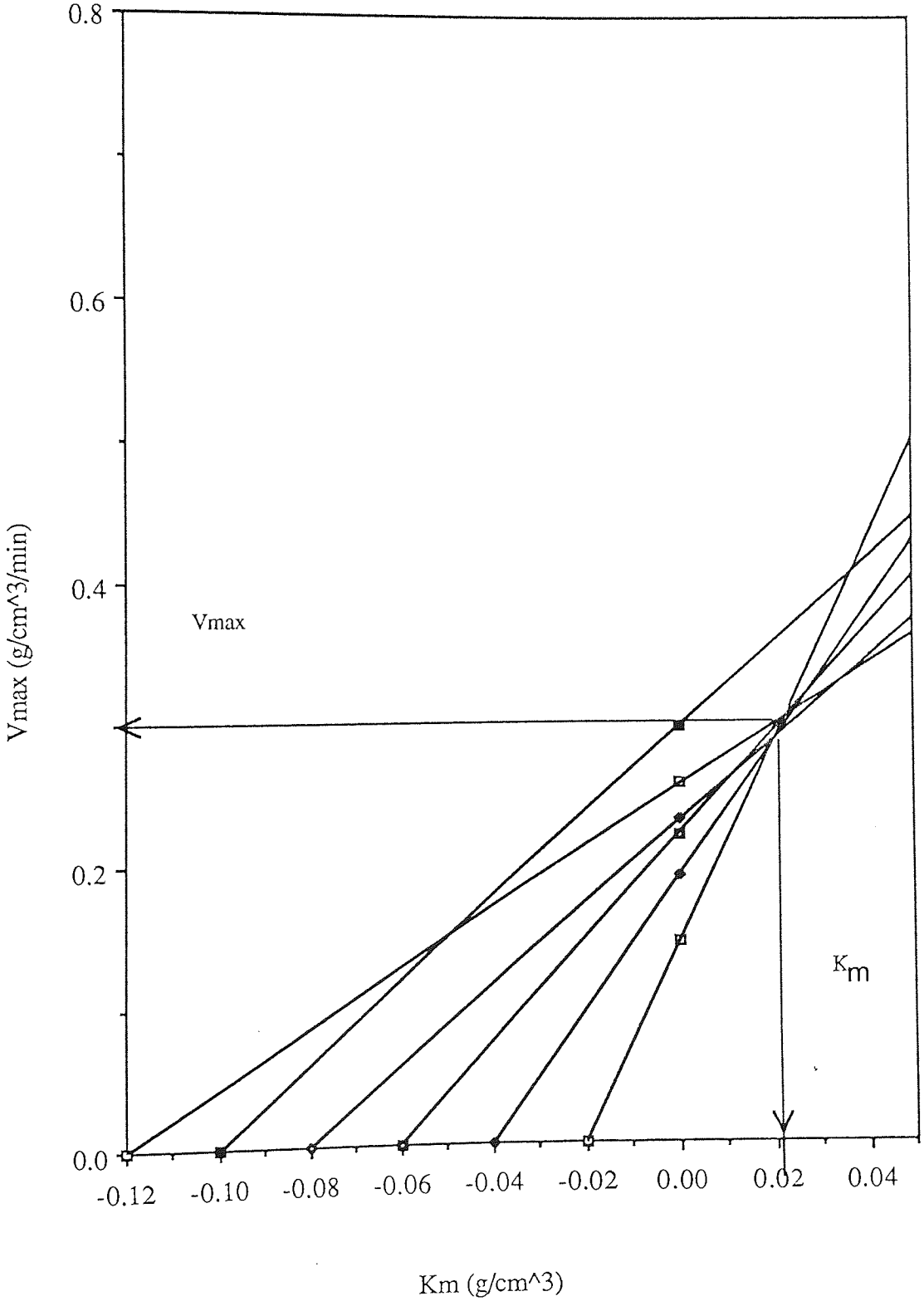
$$V_{max} = \frac{u}{s} K_m + u \qquad 8.2$$

This gives a K_m value of 20g/L (58.48mM) and V_{max} vlaue of 0.28 g/cm^3 min^{-1}.

The substrate inhibition constant (K_{si}) was calculated from Equation 8.1 by differentiating the equation at maximum initial reaction velocity and incorporating the value of sucrose (s_{max}) and the Michaelis constant K_m. A value of 475.3g/L was obtained.

These kinetic constants were used in developing the mathematical model of the SCCR-S1 system (Chapter 11).

Figure 8.2:- Cornish Bowden plot for Invertase Enzyme



Apart from the substrate inhibition effect Bowski et al [146] showed that the effect of water concentration at high sucrose concentrations does affect the kinetics of reaction. The decreasing amount of water at high sucrose concentrations resulted in the sucrose being incompletely hydrated by water. This suggests that sucrose molecules are hydrogen bonded to each other, so as to form clusters that are inaccessible to the active sites of the enzyme. Although this particular subject was not investigated in this work, the slight discrepancy in the k_m values obtained (20g/L) from that of published data [147, 148] might be due to this effect.

Sucrose feed concentrations of over 15% W/V were used in the SCCR-S1 system when acting as a combined bioreactor-separator. At these concentrations it is expected that substrate inhibition will play a significant role on the effect of product yield and the behaviour of the system, by limiting the rate of conversion. However, one of the advantages of a combined reactor-system is the ability to allow conversions above the concentrations which would have been limited by substrate inhibition. This principle was investigated during the course of this work.

8.3 SELECTING THE OPERATING CONDITIONS

In selecting the operating parameters required for reaction and separation to simultaneously take place in the SCCR-S1 system, an understanding of the reaction conditions and separation conditions is essential. The operating conditions required for separation in the SCCR-S1 system have already been discussed in Chapter 7.

In a reaction process, understanding the properties of the biocatalyst and the reaction kinetics of the particular process gives some idea of the reaction's limitation. By working within this limitation, the reaction product yield can be maximised.

The properties of invertase have been described in Chapter 3. The optimum temperature of 55°C and an optimum pH of 5.2 have been quoted [123] for the inversion

reaction. The enzyme is very stable and can therefore be used for the duration of each run (six days) provided it is kept under cold conditions (4°C).

The conditions stated in Chapter 5 were initially selected and used to commission the SCCR-S1 system as a combined reactor-separator. The pH of the deionised water used throughout this work was around 5.2 and therefore there was no need for further adjustments.

8.4 COMMISSIONING RUN FOR THE INVERTED SUCROSE REACTION-SEPARATION STUDIES ON THE SCCR-S1 SYSTEM

The SCCR-S1 was purged out with deionised water after having been used as a separator and recharged with Ca^{2+} ions by flowing through calcium nitrate and then purging out the nitrate with water. The system was checked for leakages, and the flowrates were rechecked for accuracy. Initially the system was designed to allow the enzyme to be introduced continuously into the eluent and purge streams directly at the required strength. The Run (19.5-9-30-30-60-55) was carried out with this arrangement. Initially it was presumed that steady state had not been achieved, and the run was allowed to continue for another five to six cycles. It was observed, however, that there was a continuous increase in the amount of unconsumed sucrose throughout the system. Rechecking all the accessory equipments, showed that the enzyme metering pump was not dosing the enzyme into the eluent stream at the required rate. It was discovered later that due to the back pressure from the system being transmitted through the eluent line, the pumping capacity of the enzyme pump was not able to handle it. The system was then redesigned as shown in Figure 5.7, with enzyme pump dosing the enzyme into a mixing tank at the required strength. Eluent was also added into the mixing tank. The eluent stream (deionised water + enzyme) was then delivered to the pump that fed eluent into the system.

The Run (19.5-9-30-30-60-55) was repeated with the new design and steady state was established after twelve cycles compared to eight cycles in the separation process. This was due to the additional time needed for reaction to take place in a bioreaction-separation process. Table 8.2 shows the product purities of both GRP and FRP streams collected after each cycle. During the first four cycles, the system was still adjusting to the continuous feed of sucrose and enzyme, and only part of the system was being used at this stage.

The experimental conditions and results are shown in Tables 8.3 and 8.4 respectively. Figures 8.3 and 8.4 show on-column profiles (cycles 12 and 14) which are very similar thereby confirming a pseudo-steady state had been reached. Figure 8.5 gives the purge concentration profile.

These results obtained from the commissioning runs looked very promising. A high glucose purity (95%) was achieved in the GRP and the FRP fructose purity was 74% W/V. The low fructose purity was a result of the "cross-over" point being close to the FRP (Column 2) (Figures 8.3 and 8.4). This is due to the fast motion of the stationary phase relative to the mobile phase thereby causing contamination of the FRP stream.

From the on-column concentration profiles (Figures 8.3 and 8.4) the glucose and fructose concentration profiles are very broad compared to the separation profiles of synthetic glucose and fructose (Figure 7.4). An explanation for this broadness is due to the continuous conversion of sucrose throughout the system, and the counter-current movement of the mobile phase relative to the stationary phase.

At the feed point (Column 7), there is a step increase in the concentration of fructose and glucose, with the fructose more prominent. This resulted in the glucose profile

Table 8.2: Product Purities at the end of each Cycle

Cycle No.	GRP % W/V			FRP % W/V		
	S	G	F	S	G	F
5	-	94.3	5.8	-	17.6	82.4
6	-	89.7	10.3	-	19.8	80.2
8	-	88.2	12	-	26	74
10	-	90.1	9.9	-	29.8	70.2
11	-	93.2	6.8	-	22	78
12	-	94.3	5.9	-	20	80
14	-	95	5	-	26	76

S:- Sucrose G:- Glucose F:- Fructose

overlapping the fructose concentration profile near the feed point. A possible cause might be the combination of the counter-current movement of the stationary phase relative to the mobile phase and the background concentration in the feed column due to the presence of a high concentration of sucrose. The sucrose was predominant around the feed column (Columns 6-8). The effect of substrate inhibition on the reaction rate could be significant at this part of the system, and the reaction rate could be limiting.

The purge concentration profile (Figure 8.5) shows a different picture from the on-column concentration profile. The reason being, that sucrose continues to react inside the system after the run has been completed. By the time the purging products were collected outside the system, where all reaction is terminated by the quencher unit (Figure 5.7), all the sucrose had been consumed. As a result the purge profile did not give a representative picture of the system behaviour and so will not be used in the following discussions.

8.5 EFFECT OF SWITCH TIME

To optimise the SCCR-S1 system as a combined reactor-separator, the switch time was varied. The switch time has been found to be the controlling parameter in the operation of SCCR systems [9, 105]. A study of the bioreactor-separator's behaviour and sensitivity to switch time variation was investigated.

8.5.1 Experimental Results and Discussion

From the preliminary studies discussed in Section 8.8, an eluent-to-feed rate ratio of 3.5:1 was used. The experimental conditions and results are shown in Tables 8.5 and 8.6 with the on-column concentration profiles given in Figures 8.6, 8.7, 8.8 and 8.9.

From these profiles, it was observed that the fructose concentration profile gets broader as the switch time increases. The increase in switch time increases the residence

time in the system and also reduces the relative motion of the stationary phase (P) compared to the mobile phase thus allowing more products (glucose and fructose) to be formed with less complexing and more stripping of fructose product with the calcium charge resin along the system.

As the ratio of the mobile phase to the stationary phase flowrate increases (Lm/P) (Table 8.5) the "cross-over" point advances towards the GRP product end (Figures 8.6 - 8.9). This results in better FRP product purities as demonstrated in Section 7.3. It was however, observed that at the post feed section of the system the fructose profile became more prominent as the switch time increased. The two profiles (glucose and fructose) overlap with each other and with increased time the overlap dissappears (Figure 8.9). The possible cause can be found from the work carried out by Andersen et al [58] on the mechanism of invertase action on sucrose. The two step transfer mechanism of invertase resulted in glucose, fructose and some oligosaccharides being formed. The oligosaccharides which are predominantly small chains of fructose due to the affinity of invertase in transferring fructose molecules from the sucrose to carbon receptor, are subsequently hydrolysed to glucose and fructose in different proptions. When a trisaccharide or tetrasaccharide sugar is being formed, the proportion of fructose to glucose can range from 2:1 to 3:1 and with a disaccharide a 1:1 ratio or only fructose could be formed [58]. The combination of this side reaction and the longer time spent by the eluent in the column due to increased switch time might account for the broadness of the fructose profile. Thus an increase in the fructose distribution coefficient.

The analysis of components in each column in the system by HPLC shows that in Columns 7 to 10 an unknown component was formed before the sucrose peak which might be an oligosaccharide. If so this will explain the presence of side reactions taking place in these columns. It was observed that as the switch time increased less amounts of this unknown component (oligosaccharide) were formed with higher amounts of fructose

concentration compared to glucose concentration noted near the feed section of the system. This is an indication that increases in switch time increase the residence time and allows these oligosaccharides enough time to hydrolyse to yield fructose and glucose which are quickly removed by the eluent stream through the system. This brings about the broadening in the fructose profile which in turn overlaps the glucose profile and as a result affects the GRP product purities.

The effect on the GRP product purities is very significant. This was observed when the switch time increased from 31 minutes (Run:- 18.21-9-31.5-31-60-55) to 32 minutes (Run:- 17.82-9-31.5-32-60-55). The "cross-over" point advanced by two columns from Column 3 to Column 5 (Figures 8.7 and 8.8). This brought about a 23% drop in glucose product purity. As a comparison, when the SCCR-S1 was operated as a separator, an increase in switch time by one minute resulted in a 4% drop in purity.

8.5.2 Conclusion

The transfer mechanism of the invertase enzyme [59] which causes a side reaction by forming and hydrolysing oligosaccharides to fructose and glucose, does have a greater effect on the fructose concentration profile than the glucose profile near the feed column. This resulted in a high contamination of the GRP product stream. With the SCCR-S1 system this problem can be overcome by carefully adjusting the switch time. Product purities of over 90% on both GRP and FRP product streams were achieved with the switch time set at 30.5 minutes for a feed concentration of 20% W/V.

8.6 EFFECT OF FEED CONCENTRATION

The feed concentration was varied to study the effect of substrate (sucrose) concentration on the system performance and also to investigate the importance of reaction kinetics of the enzyme during the process.

Tables 8.7 and 8.8 show the experimental conditions and results respectively. Run 34.63-9-31.5-30-60-55 and run 41.8-9-31.5-29.5-60-55 were optimised by adjusting the switch time. A constant enzyme activity (60 U cm^{-3}) was used thereby resulting in a decrease in total enzyme to substrate concentration ratio at increased feed concentrations.

As the feed concentration increases, it was observed that the amount of the unknown oligosaccharide (1-2% concentration relative to fructose concentration), detected during analysis, spread from Column 7 (Feed inlet) to Column 12 (GRP product outlet). The fructose concentration profile became more prominent near the feed column (Columns 6-9) compared to the glucose profile (Figures 8.7, 8.10 to 8.13). This also resulted in the contamination of the GRP product (Table 8.8).

The reduction in the enzyme-to-feed concentration ratio, resulted in low reaction rates and thus high amounts of unreacted sucrose near the feed column. The reaction rate was also limiting due to the presence of oligosaccharides which were subsequently hydrolysed to fructose and glucose in different proportions.

It was also observed that at increased substrate concentration (20-40% W/V) sucrose was detected in the GRP product stream (Figures 8.7, 8.10, to 8.13). Table 8.8 shows that about 4% of the 41.8% W/V sucrose used for Run (41.8-9-31.5-29.5-60-55) did not react.

The kinetics of the invertase enzyme and the increased background concentration of sucrose near the feed point do play a significant role in the yield of the products and the system behaviour. The substrate inhibition effect at high sucrose concentration coupled with a reduced water concentration, resulted in a decrease in reaction rate and less conversion of sucrose. Nelson and Schubert [148] observed that as the concentration of sucrose increases, the water concentration decreases. This they find to be a factor in

determining the magnitude of the velocity of hydrolysis of sucrose by invertase. This coupled with the reduced enzyme-to-substrate ratio explains the increased amount of unreacted sucrose present at high feed concentrations. The high sucrose background concentration near the feed inlet will also have an effect on the distribution coefficient of glucose and fructose, thus affecting their profiles. It should be noted, however, that the relatively small amounts of unreacted sucrose (i.e. < 4%) at these very high feed concentrations illustrate that operating in a combined bioreaction separation mode assists in overcoming the problem of substrate inhibition.

8.7 EFFECT OF ENZYME-TO-SUBSTRATE RATIO

The effect of the enzyme to substrate ratio on product purities and system behaviour was investigated. While all the experimental conditions remained constant the enzyme activity was varied from 43 $U.cm^{-3}$ to 103 $U.cm^{-3}$. This changed the total enzyme to feed concentration ratio from 499 U/g sucrose to 1161 U/g sucrose. The pH in the system was kept around 5.4 and steady state was established well before analysis of the product concentrations were carried out.

The experimental results are shown in Table 8.10 and the on-column concentration profiles in Figures 8.11, 8.14, 8.15 and 8.16.

As the amount of enzyme was increased, the reaction rate increased. This resulted in the formation of lower amounts of oligosaccharides and it therefore caused less broadening of the fructose profile.

At low enzyme activities (run:-30-15-9-31.5-30-43-55), the reaction rate was too low and the reaction time was the limiting factor, thus resulting in the FRP product being contaminated (Figure 8.14). Product purities over 90% were achieved on both the GRP and FRP product streams when the enzyme activity was increased to 60 and 90 $U.cm^{-3}$

(Run:- 34.63-9-31.5-30-60-55 and Run:- 31.72-9-31.5-30-86-85). At enzyme activities over 60 U/cm^3, the reaction time factor could be said not to be limiting.

Enzyme activities higher than 90 U.cm^{-3} resulted in a contaminated FRP product stream (Run:- 30.05-9-31.5-30-103-55). The inversion reaction at this activity tended to be so fast that the reaction rate has a minimal effect and the operation depends on separating power of the equipment. At these activities (>90 U cm^{-3}), all the sucrose reacts in or near the feed column thus more columns are available for separation purposes.

Figure 8.17 shows a plot of product purities in the GRP and FRP stream against enzyme activities. It illustrates that for a set of conditions given in Table 8.9, an optimum reaction rate at enzyme activity of 60 U.cm^{-3} will result in a FRP product purity of around 95%.

8.8 EFFECT OF ELUENT TO FEED RATE RATIO

From previous carbohydrate separation studies carried out on SCCR systems, an eluent to feed rate ratio of 3.1 was found to be the optimum [9]. For the SCCR-S1 system, acting as a combined reactor-separator, an investigation of the best eluent to feed rate ratio was carried out by maintaining the feedrate constant at 9cm^3 min^{-1} and gradually increases the eluent rate. Tables 8.11 and 8.12 show the experimental conditions and results. By increasing the eluent to feed rate ratio, the reaction time in the system was reduced and the movement of the mobile phase relative to the stationary phase was increased. This resulted in lower product formation, due to the shorter reaction time, and from the criteria for separation [9], it led to fast removal of the products formed with the mobile phase. This combined effect caused most of the glucose and some fructose to be removed in the GRP thus increasing the GRP concentration and reducing its purity and as a result led to high FRP purities but at lower concentration.

From the on-column concentration profiles (Figures 8.4, 8.6, 8.18 and 8.19), the glucose concentration profiles became less broad and moved towards the GRP section while the fructose on-column concentration increased. The movement of the glucose profile towards the GRP section reflects the faster movement of the mobile phase as the eluent rate increased.

The broadening of the fructose profile as the eluent rate increases was due to increased 'stripping' or removal of some of the fructose that had been complexed on the resin. This fructose is re-complexed along the system but is effectively shifted by one or more columns. The sucrose concentration was only prominent at the feed point and its concentration dropped to less than 0.5% W/V at the two columns either side of the feed column. No sucrose was detected in any of the product streams showing that 100% conversion was achieved.

From the different eluent to feed rate ratios examined in this work, a 3.5:1 ratio was found to be the optimum and was used throughout the rest of this experimental work. This was because at this eluent to feed rate ratio, high product concentrations were achieved in both the GRP and FRP streams and both the separating and reacting sections (i.e. column 2 to column 12) of the SCCR-S1 system were well utilised.

8.9 EFFECT OF CONSTANT ENZYME TO FEED CONCENTRATION RATIO

At different feed concentrations, the amount of enzyme required to achieve complete conversion was altered thereby keeping the total enzyme to substrate feed concentration ratio approximately constant. Its effect on the system behaviour and product purities was investigated.

Tables 8.13 and 8.14 show the experimental conditions and results respectively. In all runs the switch time was adjusted to give the best product purities in both the GRP and FRP streams. As the feed concentration increased the product purities in the FRP

were reduced. This is reflected in the on-column concentration profiles Figures 8.7, 8.16, 8.20 and 8.21. The fructose profile becomes less broad with increasing feed concentration and only part of the system is fully utilised. In Figure 8.21 eight columns, rather than the full 11 columns in Figure 8.7, are used for the reaction separation.

A possible explanation might be that at high feed concentrations. (40 - 50% W/V), the effect of background concentration is very significant compared to substrate inhibition effect. Hence the distribution coefficients of glucose and fructose are significantly changed. Since no sucrose was present in either the GRP or FRP streams, complete conversion was assumed. The effect of substrate inhibition was less significant at a total enzyme to substrate concentration ratio of around 1000 U/g sucrose.

From the on-column profiles (Figures 8.7, 8.16, 8.20 and 18.21), the glucose profile was not as affected as the fructose profile. A possible explanation can be found in the action of the enzyme invertase on sucrose. The secondary reaction of the enzyme polymerises the fructose to form oligosaccharides and as such has a higher affinity for fructose than glucose. Thus the amount of glucose produced during inversion is approximately constant while the amount of fructose produced may vary depending on the amount of oligosaccharide formed and hydrolysed.

8.10 ENZYME USAGE

The amount of invertase used to carry out the inversion reaction for all runs was calculated relative to the theoretical amount of enzyme required. The calculation was based on the standard definition of the invertase enzyme Unit (U), which is the amount of enzyme required to convert 1 μmol of sucrose in 1 minute at a pH of 5.0 and a temperature of 55°C. The actual rate of enzyme activity entering the system (U/min) was compared to the theoretical enzyme activity required to convert the sucrose fed into the system per minute (g/min).

The relative amounts of enzyme used in the various runs are shown in Tables 8.3, 8.5, 8.7, 8.9, 8.11 and 8.13. Appendix B1 gives an example of the calculation of the relative amount of enzyme. From these results the enzyme used was between 30-40% of the theoretical amount of enzyme required to carry out the same conversion in a conventional fermentor over the same time period.

As an indication of the enzyme used in the continuous bioreaction-separation studies (SCCR-S1), its usage can be compared with the enzyme used in the dextran biosynthesis work by Zafar [25] and Ganetsos [9] on a batch chromatographic bioreactor-separator. In their work the minimum amount possible was about 1.2 times the theoretical amounts of enzyme.

Figure 8.22 shows the enzyme concentration profiles for three different enzyme activites in the SCCR-S1 system during the reaction-separation process.

The enzyme was introduced with the eluent at Column 2 hence the high enzyme activity present in this column. As the enzyme migrates along the system with the eluent, there was a drop in activity due to enzyme deactivation inside the system and the dilution effect of the feed stream. In Column 1, the purge column, no enzyme was detected.

Table 8.15 shows the calculated results of the total enzyme used in the system at steady state over one cycle based on the profiles shown in Figure 8.22. The calculation was based on the total enzyme concentration (U cm^{-3}) present in all the columns in one cycle as compared to the expected theoretical amount of enzyme that would convert the total concentration of substrate (sucrose) present in all the columns.

From the results between 27-38% of the theoretical amount of enzyme was required for the consumption of the feed present which once again showed the efficiency of the SCCR-S1 system.

8.11 PRODUCT CONCENTRATION

To improve the commercial viability of the SCCR-S1 system it is essential to improve the product concentration by reducing the amount of enzyme and deionised water used. To accomplish this task, the combination of a product splitting technique and the recycling of the dilute product fraction was employed. The splitting technique involved the use of a 3 way valve and a timer in each product line (Chapter 5). This allowed the products exiting from each line to be split into two fractions (i.e. the diluted and concentrated fraction).

In the separation of carbohydrates carried out on the SCCR7 by Ganetsos [9], the elution profiles of glucose and fructose were studied. Figures 8.23 and 8.24 show the results over a switch time period. Both profiles are displaced with respect to the ones (Figures 7.1 and 7.2) which were obtained immediately the liquid exited from the respective column exit ports. The relatively long length of the system's product line network between the column exit port and the actual product collection port was the cause of this delay.

From the elution profiles it was apparent that most of the concentrated FRP product can be collected in the initial part of the switch while the concentrated GRP product can be collected in the latter part of the switch. Using a timer on each product line, the concentrated products can be collected by taking into consideration the "hold-up" factor. Ganetsos [9] in his work observed a sudden drop in operating pressure during the switch over, as the last column in the separating section becomes the purge column, resulting in a sudden emmission of the FRP at the beginning of the switch. This phenomena was also observed on the SCCR-S1 system and the technique developed by

Ganetsos was employed (i.e. starting the concentrated FRP fraction collection 1 second before the switch over). The GRP was collected more than halfway into the switch. The diluted fractions were either recycled back into the system or collected for analysis. In this work it was anticipated that recycling the dilute fractions, the enzyme requirement could be significantly reduced.

Two separate runs were carried out to investigate the principle of product splitting and recycling. Tables 8.16 to 8.21 show the run conditions and results. For the first Run (35.9-9-31.5-30.5-90-55), the recycling of the diluted fraction of FRP as purging stream and that of the GRP as eluent stream was started at the beginning of the run. Steady state was achieved after 15 cycles. The concentrated product from each stream was collected separately and the results are shown in Tables 8.17, 8.18 and 8.20. Although there were no substantial changes in product purities, the total product concentrations were increased. The GRP glucose purity increased from 92.3% to 95% while its total product concentration increased from 3.2% W/V to 4.6% W/V. The FRP product concentration increased from 1.82% W/V to 2.88% W/V while the fructose purity remained approximately the same (i.e. 72%) due to contamination by the glucose present in the recycled stream. This recycling technique reduced the enzyme usage down to approximately 16% of the theoretical amount (see Appendix B2). Compared with 30% of the theoretical amount used without recycling. The on-column concentration profile (Figure 8.25) shows a broadening of the sucrose concentration profile which is attributed to the reduced amount of enzyme used during recycling.

In the second Run (34.96-9-31.5-30.5-90-55) the GRP product was split into two fractions and was collected separately instead of being recycled while the FRP diluted fraction was recycled as purge. Tables 8.17, 8.20 and 8.21 show the results of this run. The FRP product concentration increased (1.82% W/V to 3.58% W/V) and the purity also increased from 80.6% to 87%. The concentrated GRP product concentration increased from 3.23% W/V to 5.01% W/V while its purity was 95.5%. The on-column

concentration profile is shown in Figure 8.26. Since there was no actual enzyme recycling in this run, the enzyme loading was maintained constant throughout the run which resulted in shorter sucrose conversion times. This is also apparent from the concentration profile (Figure 8.26).

The recycling of only the FRP dilute fraction gave a better FRP product purity (87%).

Table 8.3: Commissioning Run (19.5-9-30-30-60-55) - Conditions

Experimental Run	Average Flowrates $cm^3\ min^{-1}$			Feed Conc.	Enzyme Activity	Switch Time	Lm/P	pH	Cycle No	Enzyme Usage Actual / Theoretical
	Feed	Enzyme	Purge	% W/V	Eluent U cm^{-3}	(min)				
19.5-9-30-30-60-55	9	30	76	19.5	60	30	0.285	5.4	14	0.368

Table 8.4: Commissioning Run (19.5-9-30-30-60-55) - Results

Experimental Run	Feed Throughput (kg sugar/ m^3 resin/hr	GLUCOSE RICH PRODUCT							FRUCTOSE RICH PRODUCT				TOTAL			MASS BALANCE OUTPUT / INPUT
		Glucose Purity %	% Sucrose in feed Recovered	% Glucose Recovered	Total Product Conc. % W/V	Impurities % S	F	Other	Fructose Purity %	% Sucrose in feed Recovered	% Fructose Recovered	Total Product Conc. % W/V	Impurities % S	G	Other	
19.5-9-30-30-60-55	5.82	95	-	78	11.92	-	5	-	74	-	113	1.865	-	26	-	118

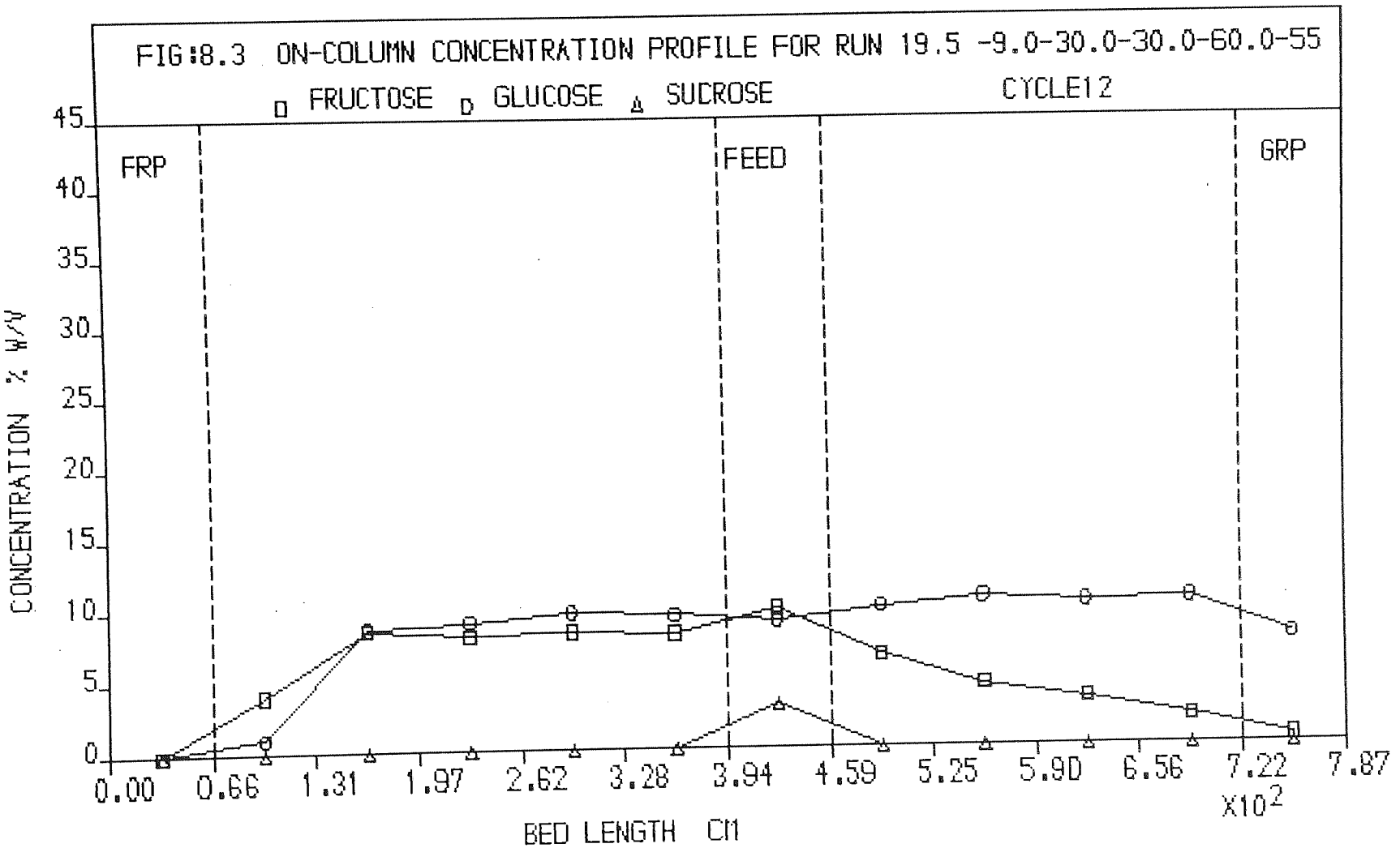


FIG:8.3 ON-COLUMN CONCENTRATION PROFILE FOR RUN 19.5 -9.0-30.0-30.0-60.0-55
□ FRUCTOSE ▫ GLUCOSE △ SUCROSE
CYCLE12
FRP
FEED
GRP
CONCENTRATION % W/W
45
40
35
30
25
20
15
10
5
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10²
BED LENGTH CM

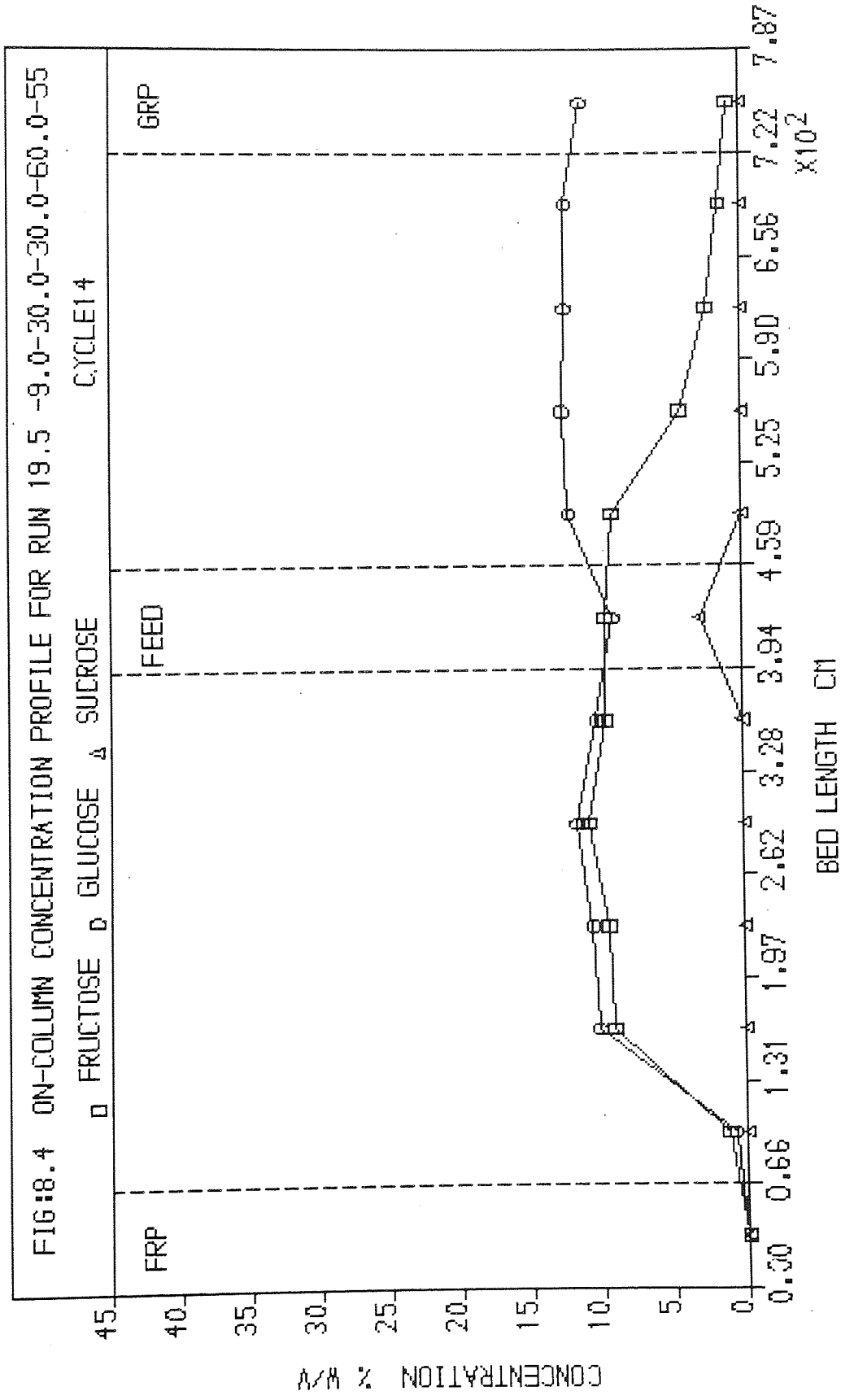


FIG:8.4 ON-COLUMN CONCENTRATION PROFILE FOR RUN 19.5 -9.0-30.0-30.0-60.0-55
CYCLE14
□ FRUCTOSE ○ GLUCOSE △ SUCROSE
FRP
FEED
GRP
CONCENTRATION % W/V
BED LENGTH CM
X10²
0
5
10
15
20
25
30
35
40
45
0.30
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87

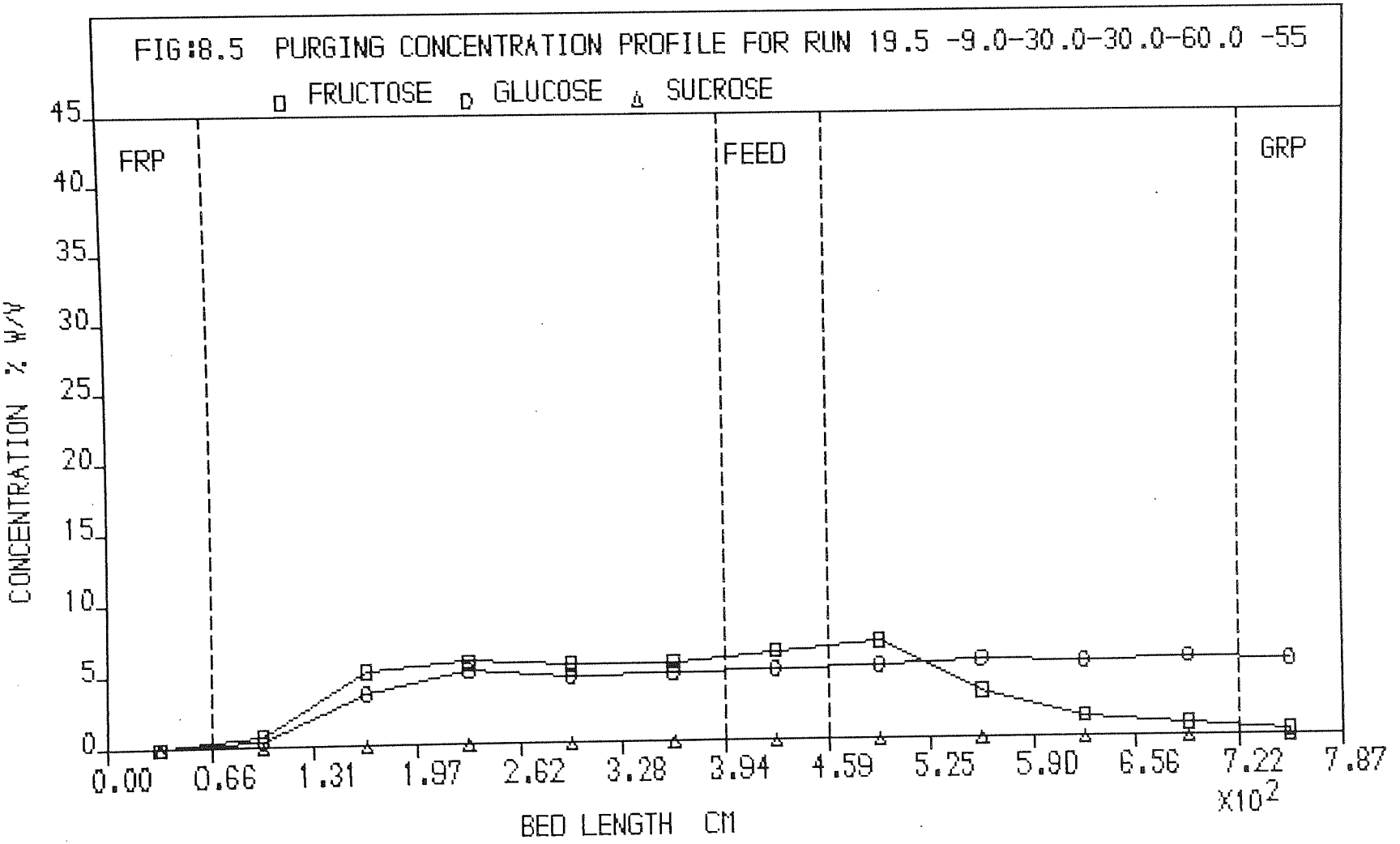


FIG:8.5 PURGING CONCENTRATION PROFILE FOR RUN 19.5 -9.0-30.0-30.0-60.0 -55
FRUCTOSE
GLUCOSE
SUCROSE
FRP
FEED
GRP
CONCENTRATION % W/W
45
40
35
30
25
20
15
10
5
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10^2
BED LENGTH CM

Table 8.5: Effect of Switch Times - Process Condition

Experimental Run	Average Flowrates cm^3 min			Feed Conc	Enzyme Activity in the Eluent Stream	Switch Time	pH	Lm/P	Cycle No.	Relative Enzyme Usage Actual / Theoretical
	Feed	Eluent	Purge	% W/V	U cm^{-3}	(min)				
18.35-9-31.5-30-60-55	9	31.5	76	18.35	60	30	5.6	0.313	12	0.391
20.77-9-31.5-30.5-60-55	9	31.5	76	20.77	60	30.5	5.4	0.324	+8	0.346
18.21-9-31,5-31-60-55	9	31.5	76	18.21	60	31	5.5	0.335	16	0.394
17.82-9-31.5-32-60-55	9	31.5	76	17.81	60	32	5.6	0.357	12	0.403

\+ Indicating the additional number of cycles carried out after making a change in the operating condition without purging the system

Table 8.6: Effect of Switch Time - Results

Experimental Run	Feed Throughput (kg sugar/ m^3 resin/hr	GLUCOSE RICH PRODUCT							FRUCTOSE RICH PRODUCT							TOTAL MASS BALANCE OUTPUT / INPUT
		Glucose Purity %	% Sucrose in feed Recovered	% Glucose Recovered	Total Product Conc. % W/V	Impurities % S	F	Other	Fructose Purity %	% Sucrose in feed Recovered	% Fructose Recovered	Total Product Conc. % W/V	Impurities % S	G	Other	
18.35-9-31.5-30-60-55	5.47	94.4	-	94.8	1.768	~	5.6	-	82	-	102	1.173	~	18	-	100
20.77-9-31.5-30.5-60-55	6.19	95.8	-	107	2.31	~	4.2	-	91.4	-	67	0.93	~	8.6	-	93
18.21-9-31.5-31-60-55	5.42	95.6	-	90	2.10	~	4.4	-	88.8	-	79.4	1.01	~	11.2	-	92
17.82-9-31.5-32-60-55	5.31	73	-	109	3.17	~	27	-	93	-	69	0.816	~	7	-	100

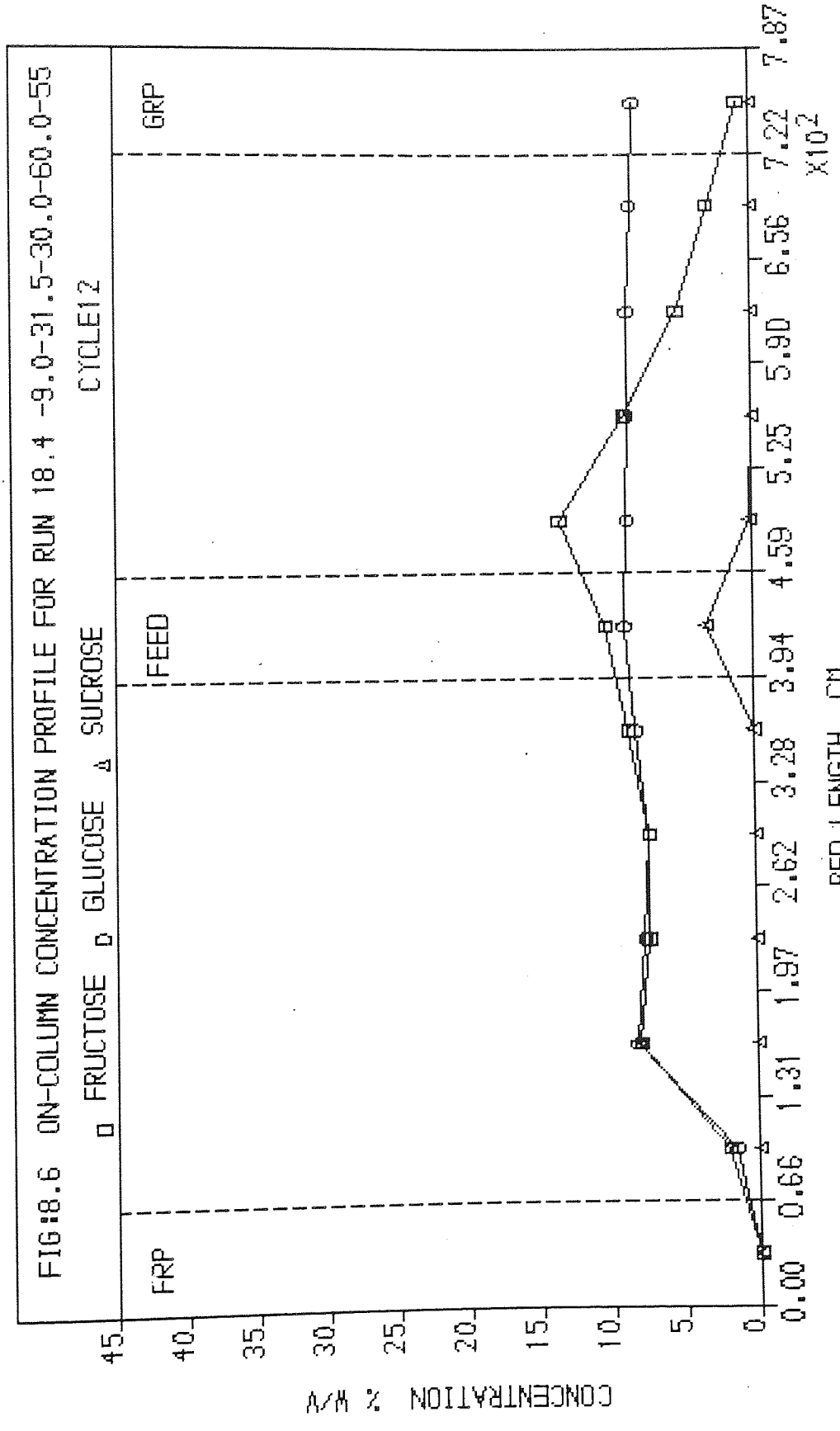


FIG:8.6 ON-COLUMN CONCENTRATION PROFILE FOR RUN 18.4 -9.0-31.5-30.0-60.0-55
CYCLE12
□ FRUCTOSE ○ GLUCOSE △ SUCROSE
FRP
FEED
GRP
CONCENTRATION % W/V
BED LENGTH CM
X10²
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
0
5
10
15
20
25
30
35
40
45

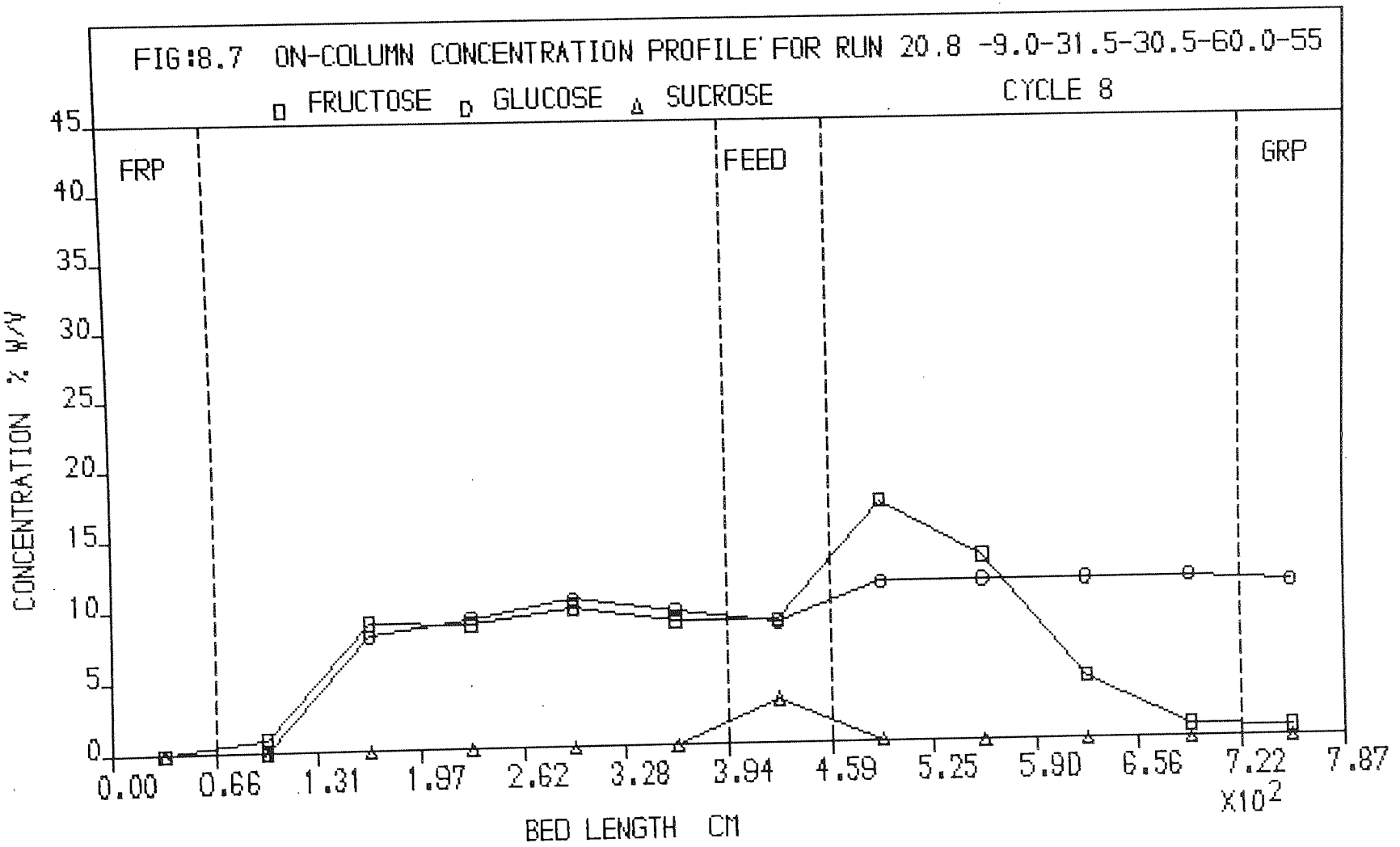


FIG:8.7 ON-COLUMN CONCENTRATION PROFILE FOR RUN 20.8 -9.0-31.5-30.5-60.0-55
FRUCTOSE GLUCOSE SUCROSE
CYCLE 8
FRP
FEED
GRP
CONCENTRATION % W/V
45
40
35
30
25
20
15
10
5
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10²
BED LENGTH CM

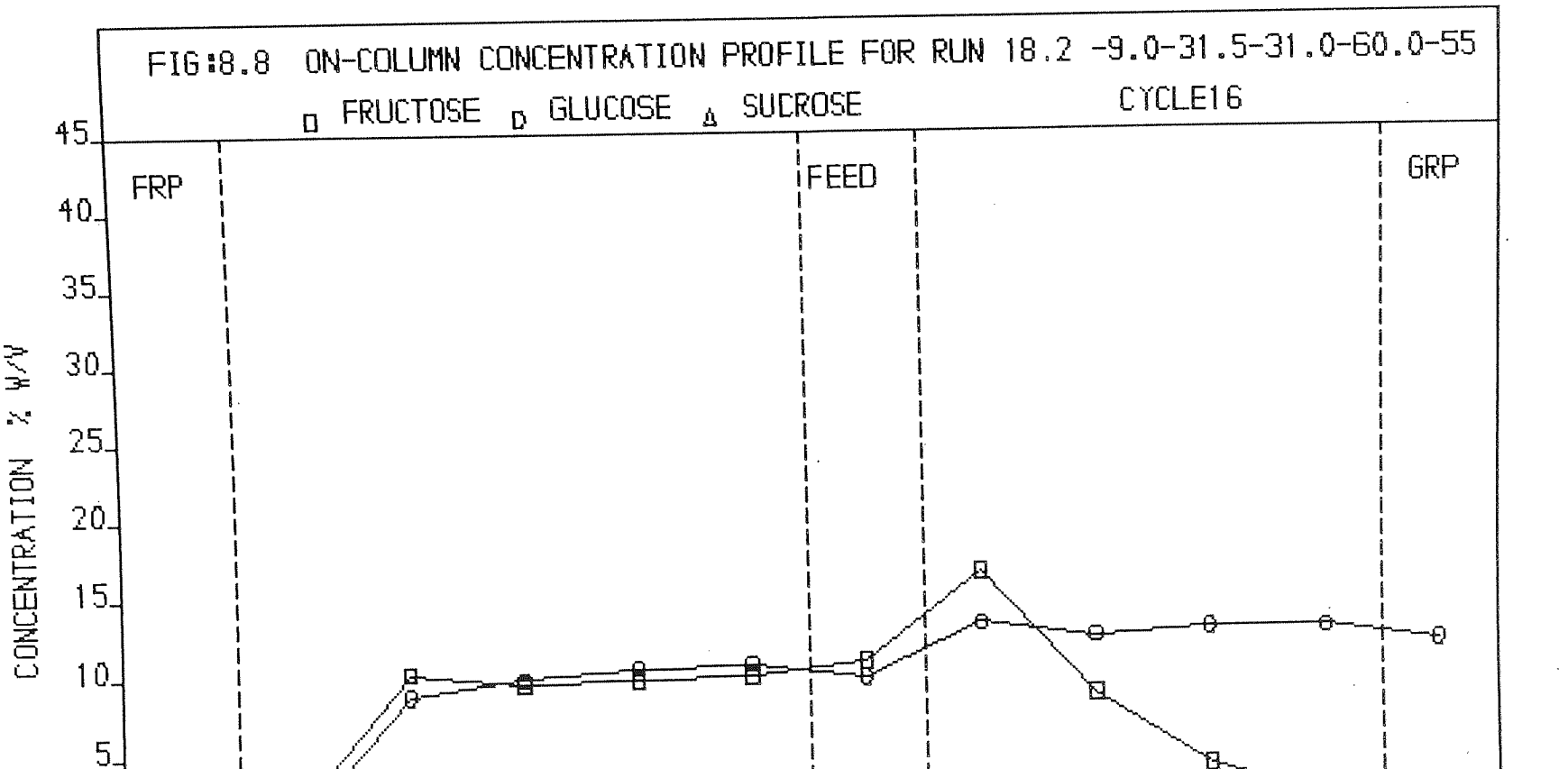


FIG:8.8 ON-COLUMN CONCENTRATION PROFILE FOR RUN 18.2 -9.0-31.5-31.0-60.0-55
FRUCTOSE
GLUCOSE
SUCROSE
CYCLE16
FRP
FEED
GRP
CONCENTRATION % W/W
45
40
35
30
25
20
15
10
5
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10^2
BED LENGTH CM

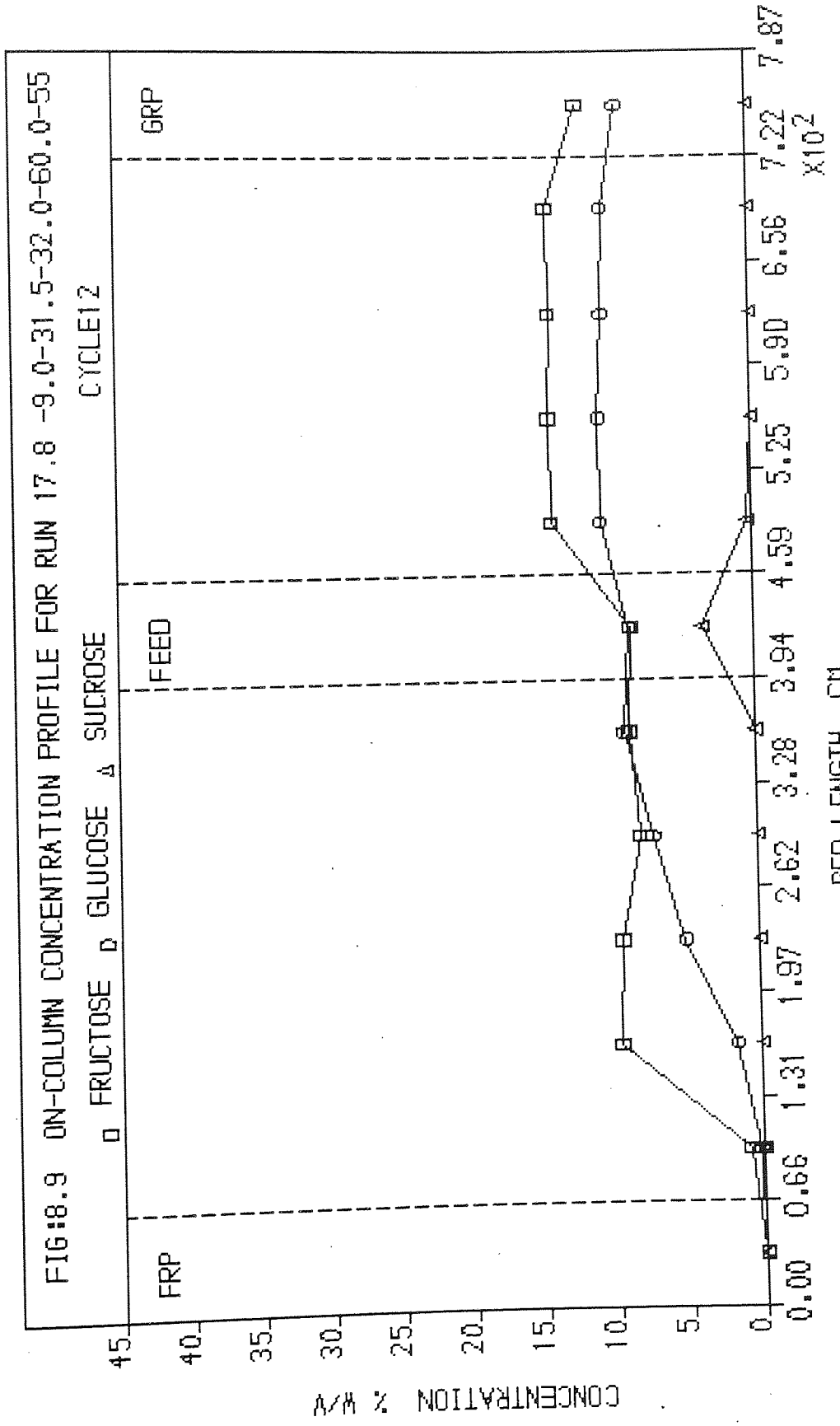


FIG:8.9 ON-COLUMN CONCENTRATION PROFILE FOR RUN 17.8 -9.0-31.5-32.0-60.0-55
CYCLE12
□ FRUCTOSE ○ GLUCOSE △ SUCROSE
FRP
FEED
GRP
CONCENTRATION % W/V
BED LENGTH CM
X10²
0
5
10
15
20
25
30
35
40
45
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87

Table 8.7 : Effect of Feed Concentration - Conditions

Experimental Run	Average Flowrates $cm^3\ min^{-1}$			Feed Conc.	Enzyme Activity	Total Enzyme To Feed Conc. Ratio	Lm/P	pH	Cycle No	Enzyme Usage Actual
	Feed	Enzyme	Eluent Purge	% W/V	U cm^{-3}	U/g				Theoretical
20.77-9-31.5-30.5-60-55	9	33.5	76	20.77	60	1011	0.324	5.4	+8	0.346
33.96-9-31.5-30.5-60-55	9	31.5	76	33.96	60	61.8	0.324	5.8	+9	0.211
*34.63-9-31.5-30-60-55	9	31.5	76	34.63	60	606	0.313	6.0	16	0.217
41.33-9-31.5-30-60-55	9	31.5	76	41.33	60	508	0.313	5.7	9	0.174
*41.8-9-31.5-29.5-60-55	9	31.5	76	41.8	60	502	0.302	5.6	+6	0.172

* Optimised Run

\+ Indicating the additional number of cycles carried out after making a change in the operating conditions without purging the system.

Table 8.8 : Effect of Feed Concentration - Results

Experimental Run	Feed Throughput (kg sugar/ m^3 resin/hr	GLUCOSE RICH PRODUCT							FRUCTOSE RICH PRODUCT							TOTAL MASS BALANCE OUTPUT / INPUT
		Glucose Purity %	% Sucrose in feed Recovered	% Glucose Recovered	Total Product Conc. % W/V	Impurities % S	F	Other	Fructose Purity %	% Sucrose in feed Recovered	% Fructose Recovered	Total Product Conc. % W/V	Impurities % S	G	Other	
20.77-9-31.5-30.5-60-55	6.19	95.8	~	107	2.31	-	4.2		91.4	~	67	0.93	~	8.6		92.5
33.96-9-31.5-30.5-60-55	10.12	89	~	91	4.2	-	11		94.8	~	54	1.18	~	5.2		80
34.63-9-31.5-30-60-55	10.34	92.7	0.65	108	4.8	1.3	6.1		95	~	98.1	2.14	~	5.0		108
41.33-9-31.5-30-60-55	12.33	88.4	0.3	92.6	4.73	0.7	10.8		96.7	~	84	1.81	~	3.3		88
41.8-9-31.5-29.5-60-55	12.44	87.4	3.95	83.5	4.84	8.4	4.2		88	~	92.2	2.65	~	12		100

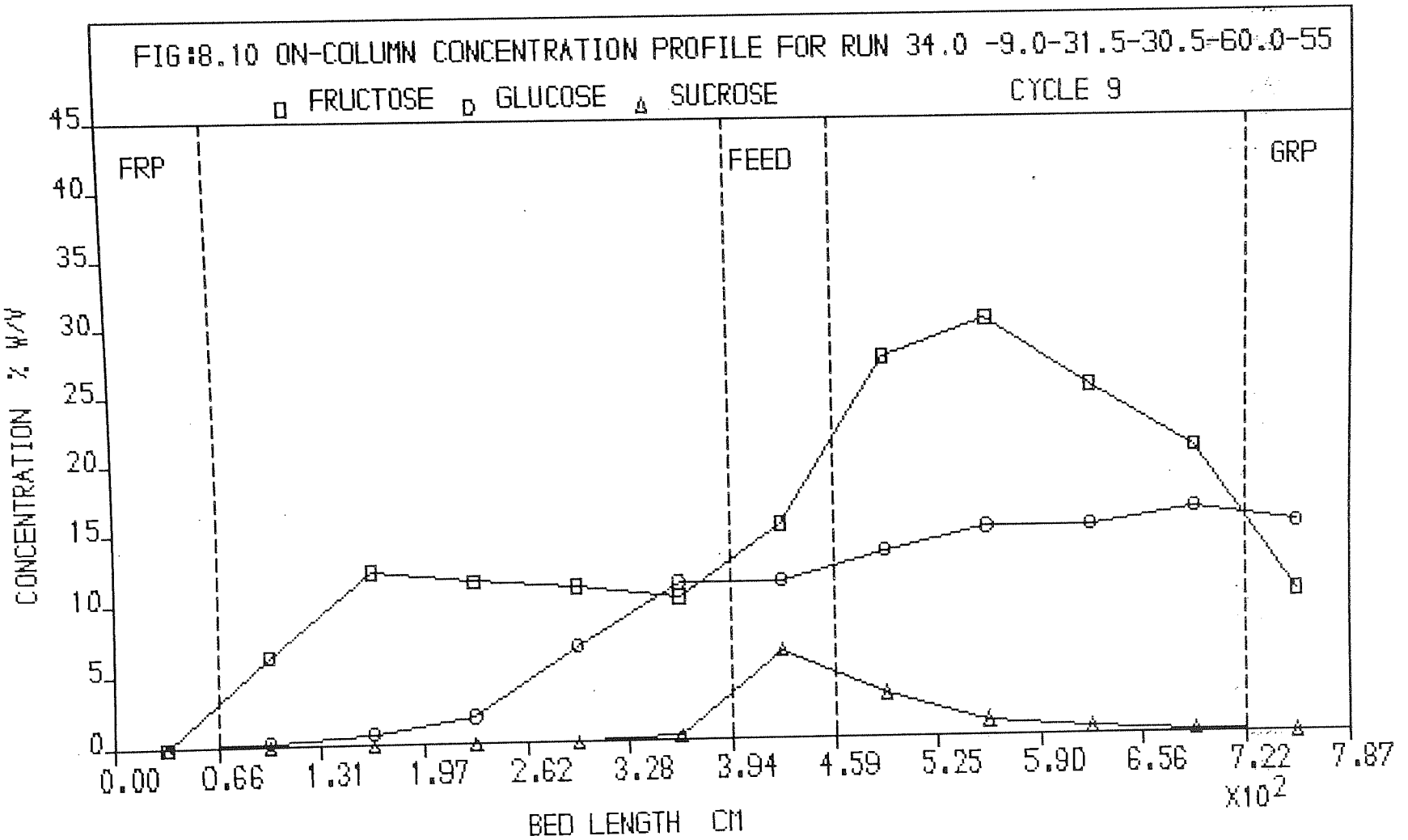


FIG:8.10 ON-COLUMN CONCENTRATION PROFILE FOR RUN 34.0 -9.0-31.5-30.5-60.0-55
FRUCTOSE
GLUCOSE
SUCROSE
CYCLE 9
FRP
FEED
GRP
CONCENTRATION % W/W
45
40
35
30
25
20
15
10
5
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10-2
BED LENGTH CM

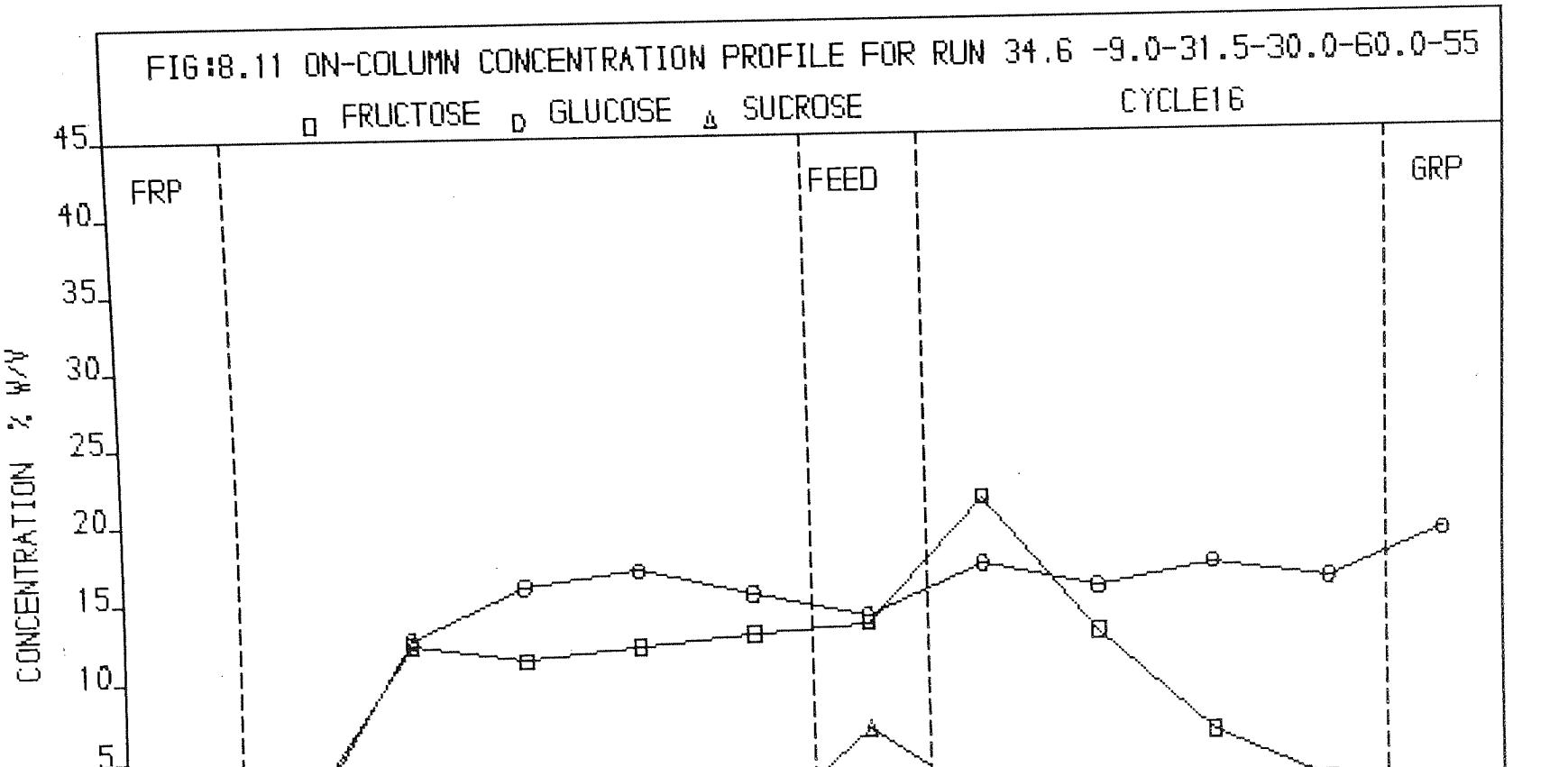


FIG:8.11 ON-COLUMN CONCENTRATION PROFILE FOR RUN 34.6 -9.0-31.5-30.0-60.0-55
FRUCTOSE
GLUCOSE
SUCROSE
CYCLE16
FRP
FEED
GRP
CONCENTRATION % W/W
45
40
35
30
25
20
15
10
5
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10^2
BED LENGTH CM

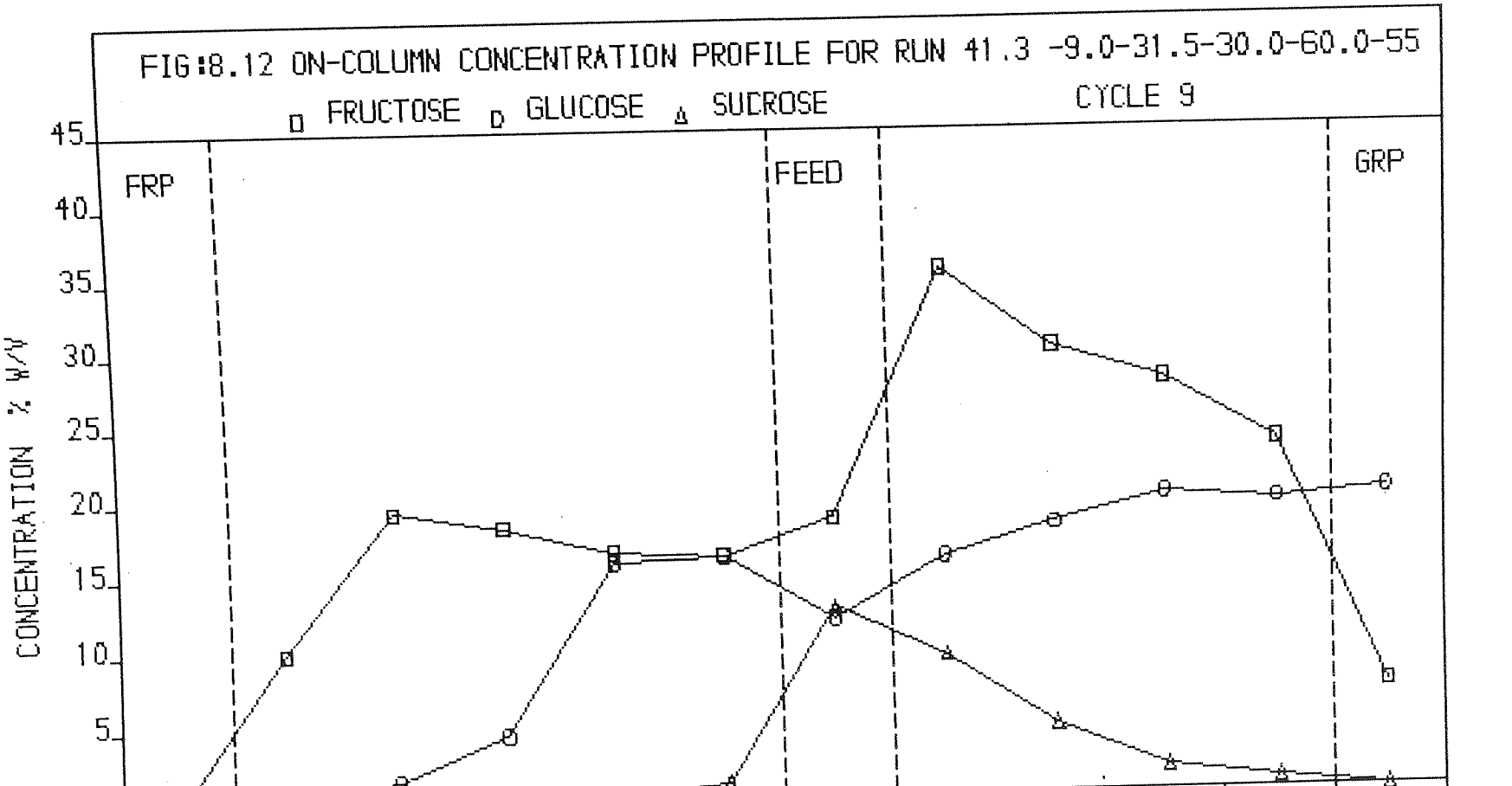


FIG:8.12 ON-COLUMN CONCENTRATION PROFILE FOR RUN 41.3 -9.0-31.5-30.0-60.0-55
FRUCTOSE
GLUCOSE
SUCROSE
CYCLE 9
FRP
FEED
GRP
CONCENTRATION % W/W
45
40
35
30
25
20
15
10
5
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10^2
BED LENGTH CM

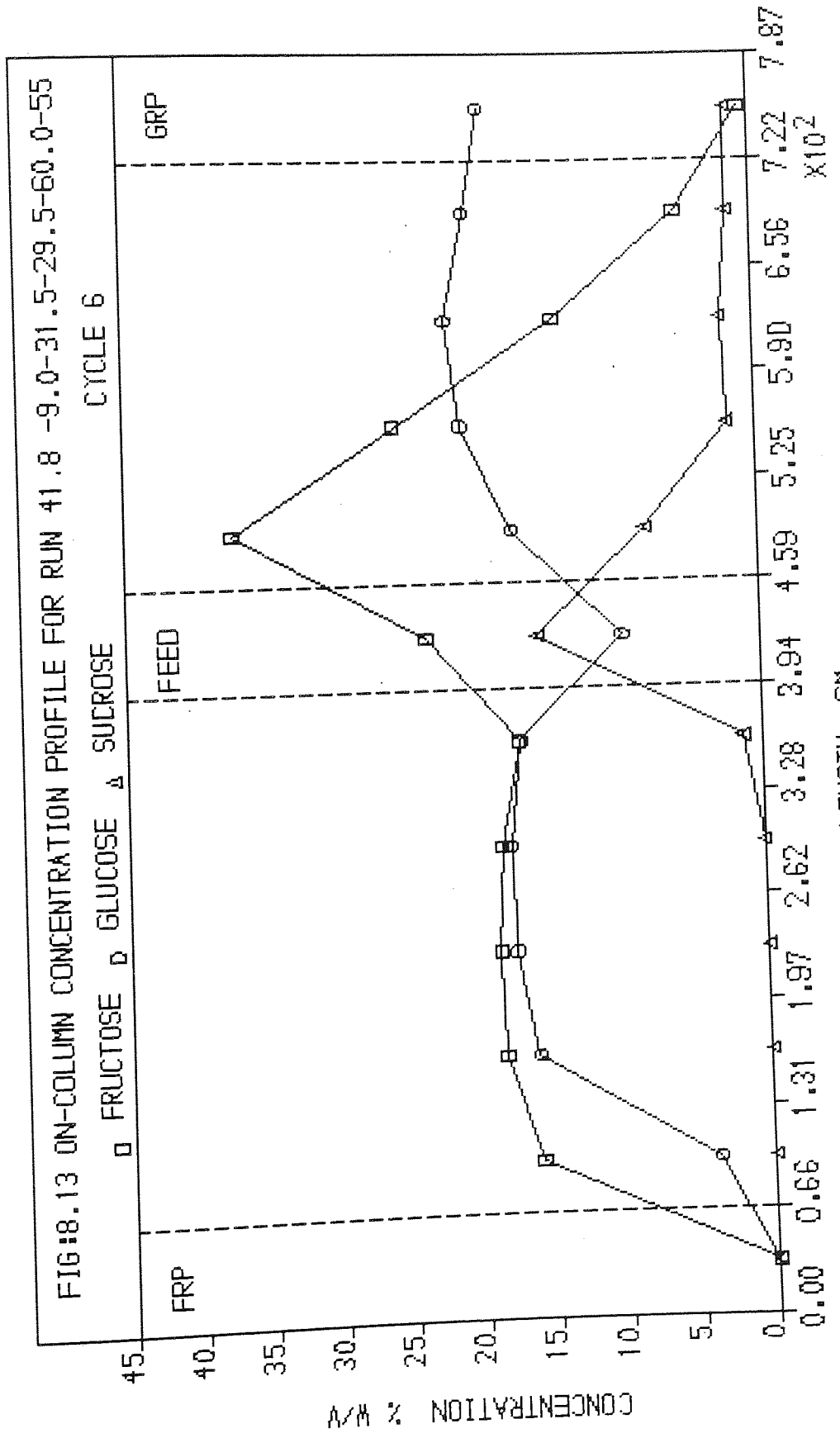


FIG:8.13 ON-COLUMN CONCENTRATION PROFILE FOR RUN 41.8 -9.0-31.5-29.5-60.0-55
CYCLE 6
□ FRUCTOSE ○ GLUCOSE △ SUCROSE
FRP
FEED
GRP
CONCENTRATION % W/V
BED LENGTH CM
X10²
0
5
10
15
20
25
30
35
40
45
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87

Table 8.9 : Effect of Enzyme Activity - Conditions

Experimental Run	Average Flowrates $cm^3\ min^{-1}$			Feed Conc.	Enzyme Activity	Total Enzyme To Feed Conc. Ratio	Lm/P	pH	Cycle No	Enzyme Usage Actual
	Feed	Enzyme	Eluent Purge	% W/V	U cm^{-3}	U/g				Theoretical
30.15-9-31.5-30-43-55	9	31.5	76	30.15	432	499	0.313	5.8	16	0.171
34.63-9-31.5-30-60-55	9	31.5	76	34.63	60	606	0.313	6.0	16	0.207
31.72-9-31.5-30-86-55	9	31.5	76	31.72	86	949	0.313	5.8	12	0.324
31.44-9-31.5-30-103-55	9	31.5	76	31.40	103	1161	0.313	5.9	16	0.41

Table 8.10 : Effect of Enzyme Activity - Results

Experimental Run	Feed Throughput (kg sugar/ m^3 resin/hr	GLUCOSE RICH PRODUCT						FRUCTOSE RICH PRODUCT						TOTAL MASS BALANCE OUTPUT / INPUT
		Glucose Purity %	% Sucrose in feed Recovered	% Glucose Recovered	Total Product Conc. % W/V	Impurities % S	Impurities % F	Fructose Purity %	% Sucrose in feed Recovered	% Fructose Recovered	Total Product Conc. % W/V	Impurities % S	Impurities % G	
30.15-9-31.5-30-43-55	9.01	92.7	-	89	3.3	~	7.3	85	-	106	2.22	~	15	108
34.63-9-31.5-30-60-55	10.34	92.7	-	108	4.8	1.2	6	95	-	98.1	2.14	~	5	108
31.72-9-31.5-30-86-55	9.46	91.8	-	78.5	3.13	~	8.2	90.3	-	80.2	1.71	~	9.7	88
31.4-9-31.5-30-103-55	8.96	93.5	-	79.2	2.85	~	6.5	85		84.5	1.75	~	15	92

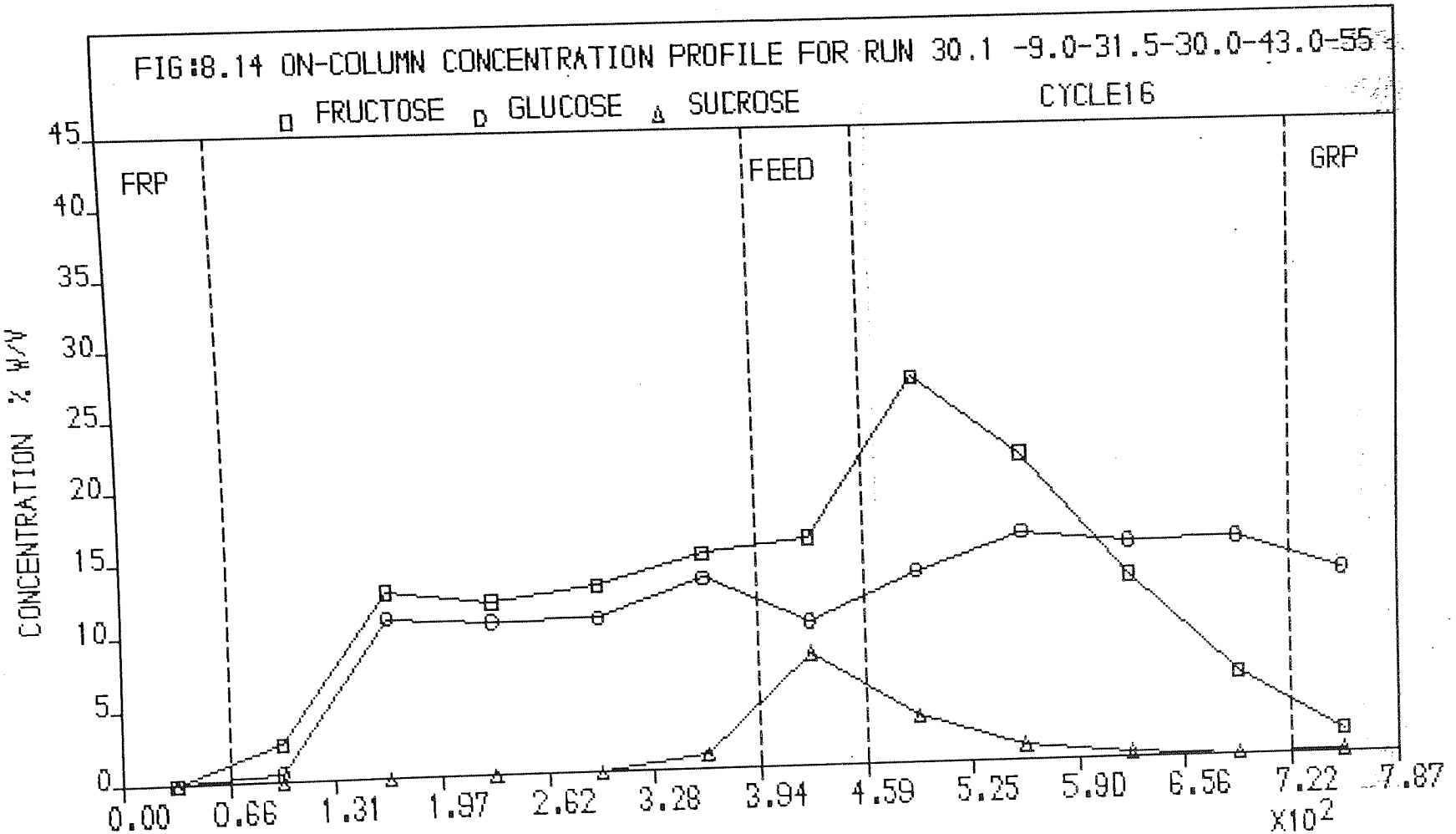


FIG:8.14 ON-COLUMN CONCENTRATION PROFILE FOR RUN 30.1 -9.0-31.5-30.0-43.0-55
FRUCTOSE
GLUCOSE
SUCROSE
CYCLE16
FRP
FEED
GRP
CONCENTRATION % W/V
45
40
35
30
25
20
15
10
5
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10²
BED LENGTH CM

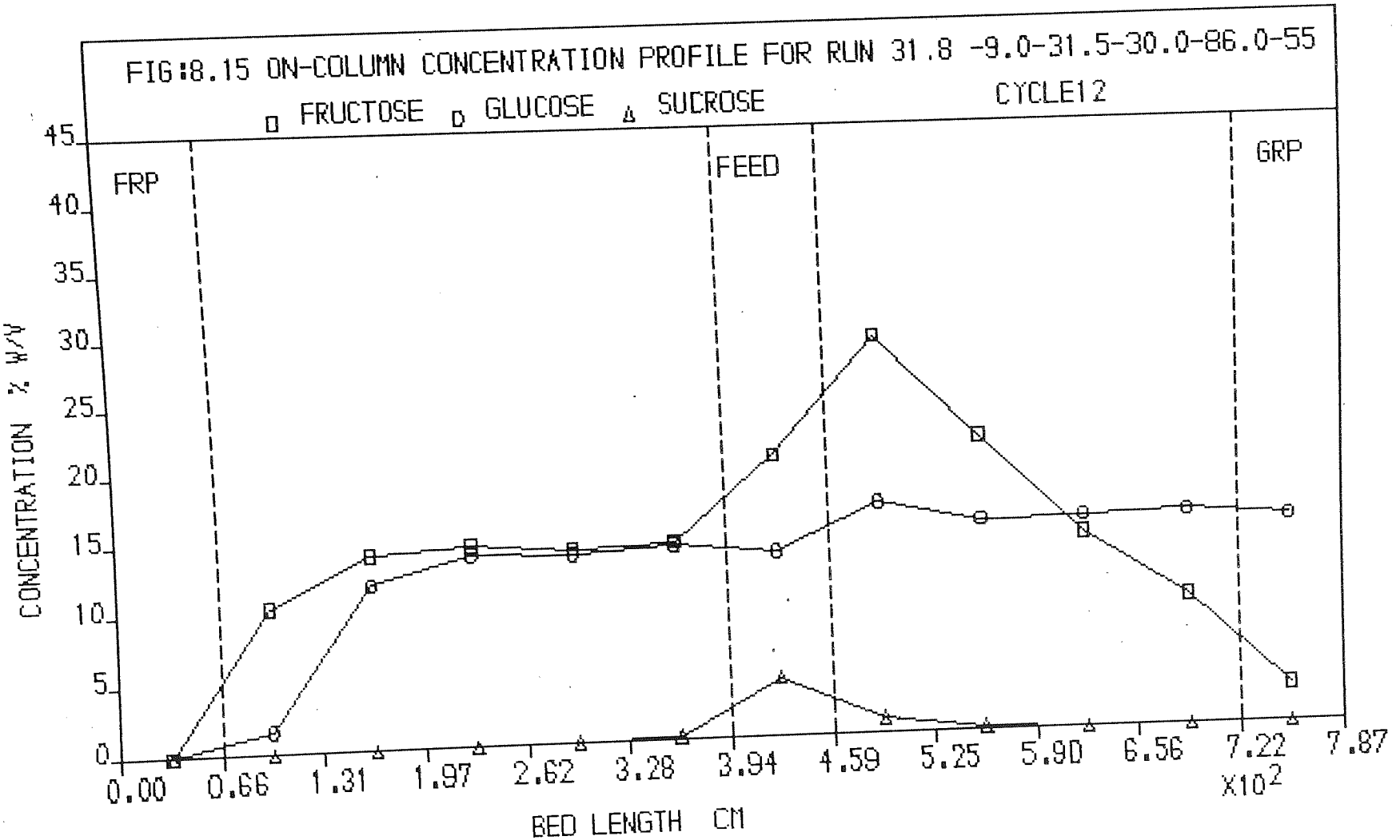


FIG:8.15 ON-COLUMN CONCENTRATION PROFILE FOR RUN 31.8 -9.0-31.5-30.0-86.0-55
FRUCTOSE
GLUCOSE
SUCROSE
CYCLE12
FRP
FEED
GRP
CONCENTRATION % W/V
45
40
35
30
25
20
15
10
5
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10²
BED LENGTH CM

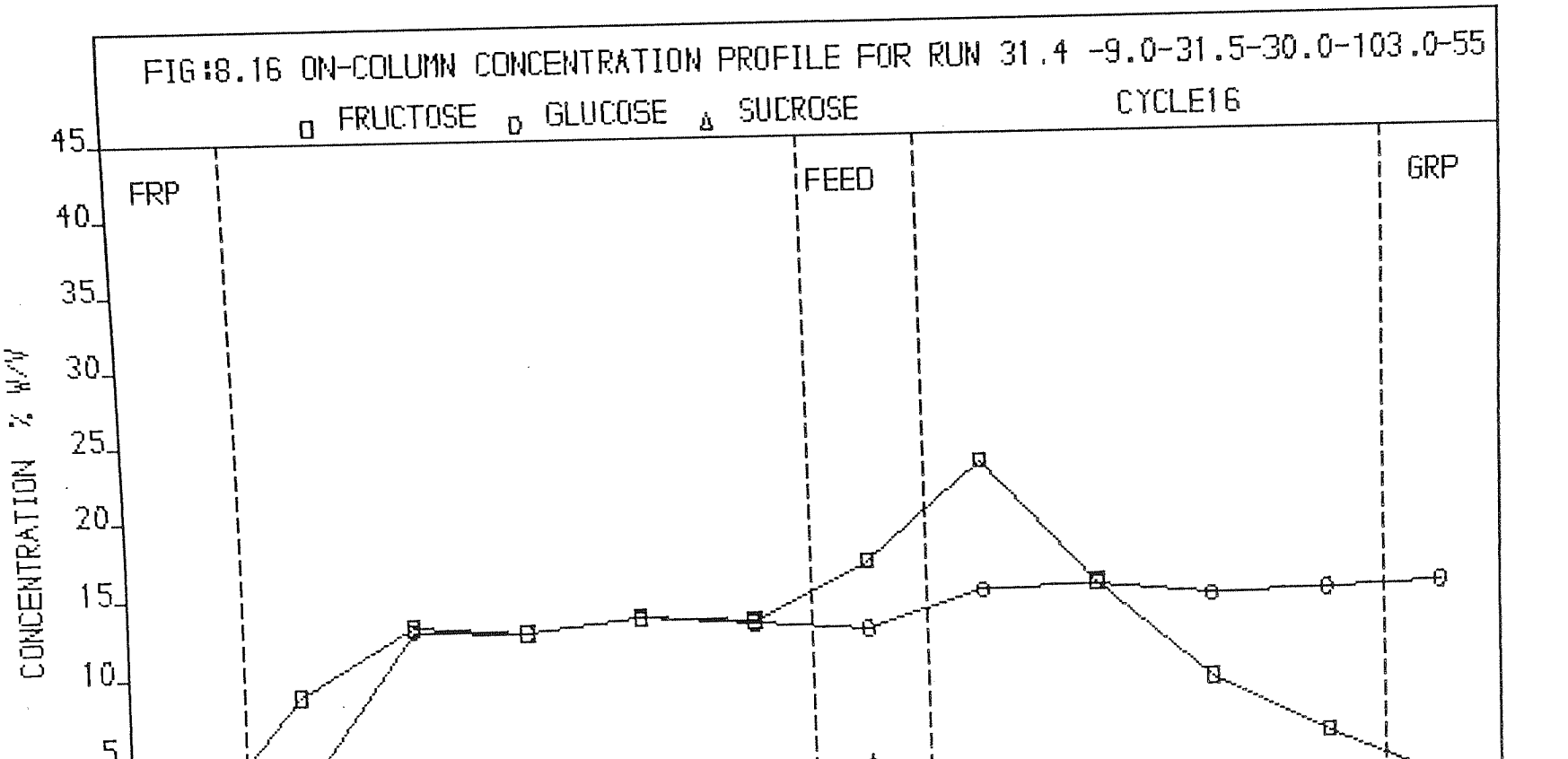


FIG:8.16 ON-COLUMN CONCENTRATION PROFILE FOR RUN 31.4 -9.0-31.5-30.0-103.0-55
FRUCTOSE
GLUCOSE
SUCROSE
CYCLE16
FRP
FEED
GRP
CONCENTRATION % W/W
45
40
35
30
25
20
15
10
5
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10^2
BED LENGTH CM

Figure 8.17:- Determination of the approximate optimum enzyme concentration

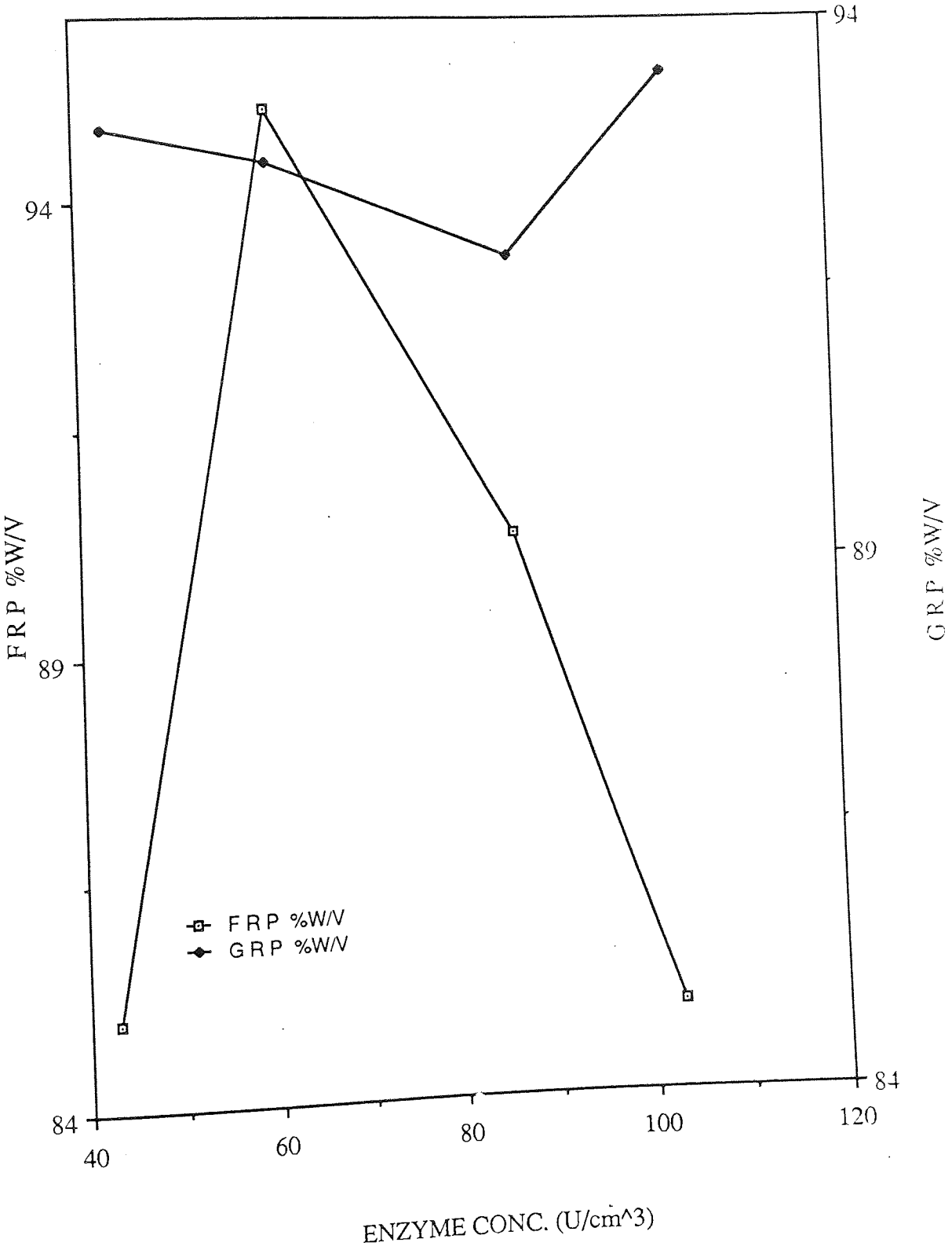


Table 8.11 : Effect of Eluent to Feed Rate Ratio - Conditions

Experimental Run	Average Flowrates $cm^3\ min^{-1}$			Feed Conc.	Enzyme Activity	Eluent To Feed Ratio	Lm/P	pH	Cycle No	Enzyme Usage Actual
	Feed	Enzyme	Eluent Purge	% W/V	U cm^{-3}					Theoretical
19.5-9-30-30-60-55	9	31.5	76	19.5	60	3 1/3 :1	0.285	5.5	14	0.35
19.35-9-31.5-30-60-55	9	31.5	76	18.35	60	3 1/2 :1	0.313	5.6	9	0.391
18.87-9-33-30-60-55	9	33	76	18.87	60	3 2/3 :1	0.34	5.8	9	0.398
20.3-9-36-30-60-55	9	36	76	20.3	60	4 : 1	0.396	5.2	14	0.404

Table 8.12: Effect of Eluent to Feed Rate Ratio - Results

Experimental Run	Feed Throughput (kg sugar/ m^3 resin/hr	GLUCOSE RICH PRODUCT						FRUCTOSE RICH PRODUCT						TOTAL MASS BALANCE OUTPUT / INPUT
		Glucose Purity %	% Sucrose in feed Recovered	% Glucose Recovered	Total Product Conc. % W/V	Impurities % S	Impurities % F	Fructose Purity %	% Sucrose in feed Recovered	% Fructose Recovered	Total Product Conc. % W/V	Impurities % S	Impurities % G	
19.5-9-30-30-60-55	5.72	95	-	78	1.97	-	5	73.7	-	113	1.86	-	26.3	118
18.35-9-31.5-30-60-55	5.47	94.4	-	94.8	1.768	-	5.6	82	-	102	1.17	~	18.0	112
18.87-9-33-30-60-55	5.63	81.8	-	99	2.17	~	18.2	92.6	-	75	0.785	~	7.4	101
20.3-9-36-30-60-55	6.06	64.8	-	105	3.5	-	35.2	93.6	-	41.3	0.56	~	6.4	103

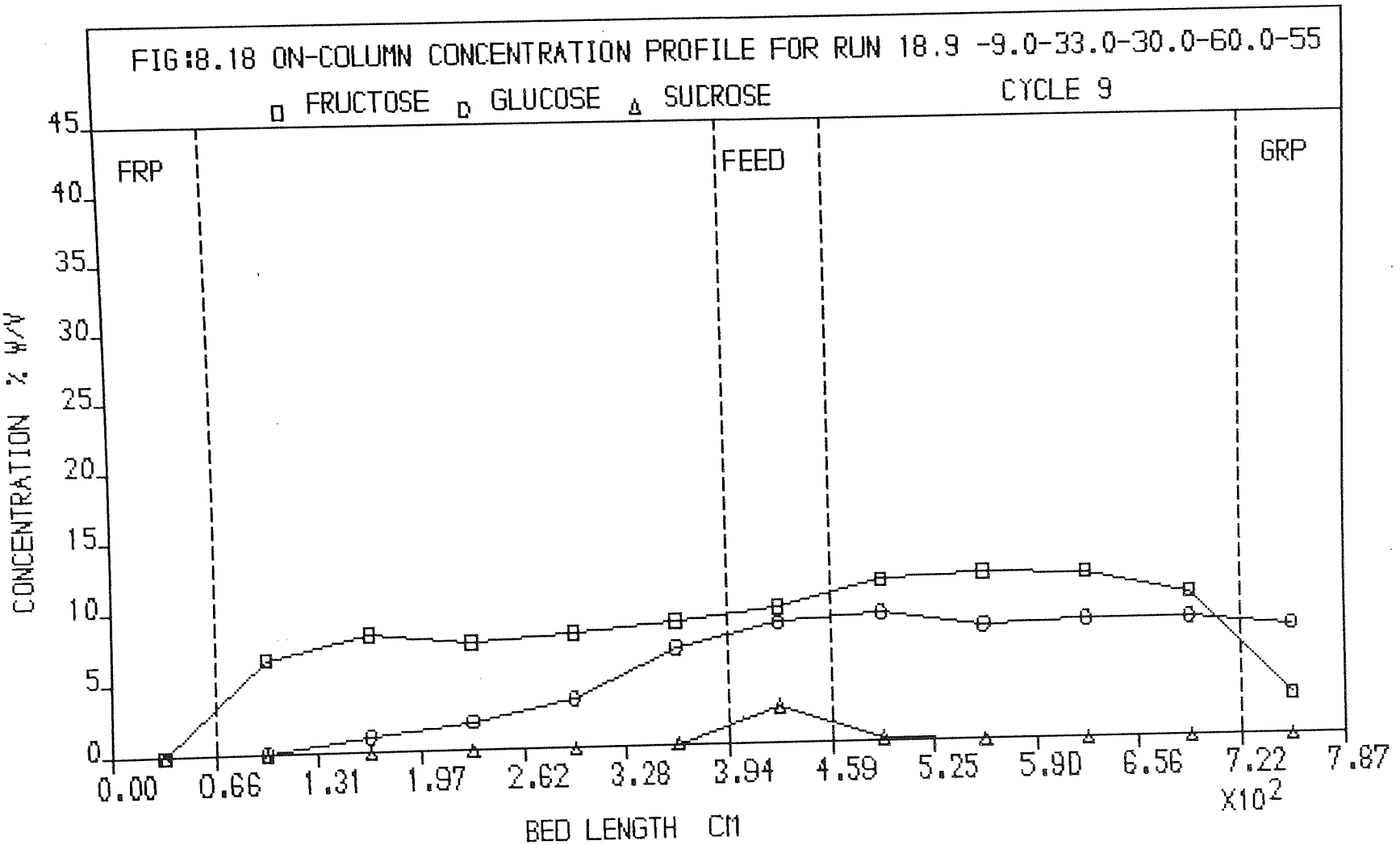


FIG:8.18 ON-COLUMN CONCENTRATION PROFILE FOR RUN 18.9 -9.0-33.0-30.0-60.0-55
FRUCTOSE
GLUCOSE
SUCROSE
CYCLE 9
FRP
FEED
GRP
CONCENTRATION % W/V
45
40
35
30
25
20
15
10
5
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10^2
BED LENGTH CM

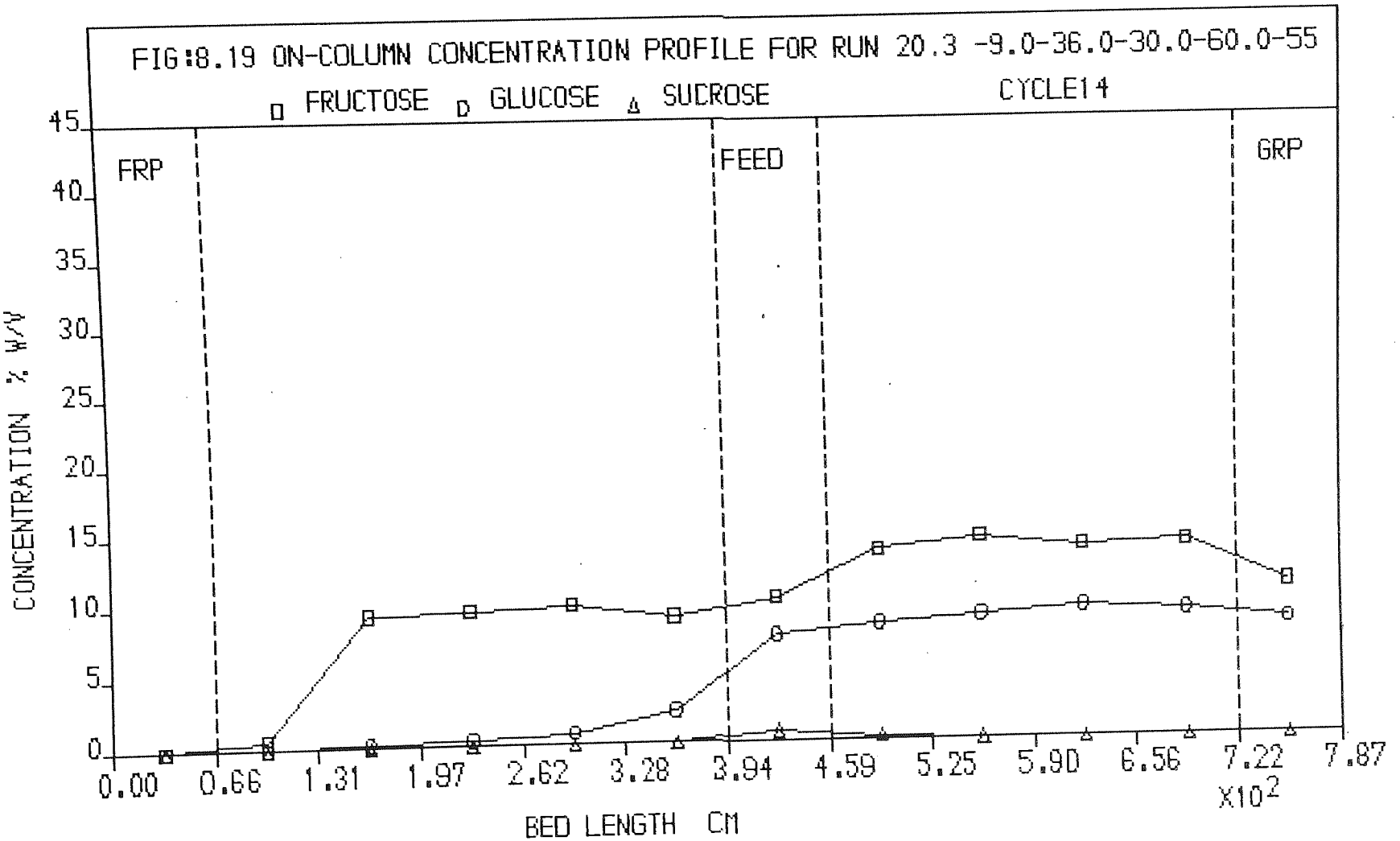


FIG:8.19 ON-COLUMN CONCENTRATION PROFILE FOR RUN 20.3 -9.0-36.0-30.0-60.0-55
□ FRUCTOSE ○ GLUCOSE △ SUCROSE
CYCLE14
FRP
FEED
GRP
CONCENTRATION % W/W
45
40
35
30
25
20
15
10
5
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10²
BED LENGTH CM

Table 8.13 : Effect of Total Constant Enzyme to Feed Concentration Ratio - Conditions

Experimental Run	Average Flowrates $cm^3\ min^{-1}$			Feed Conc.	Enzyme Activity	Enzyme To Feed Conc Ratio	Lm/P	pH	Cycle No	Relative Enzyme Usage Actual / Theoretical
	Feed	Eluent Enzyme	Purge	% W/V	U cm^{-3}	U/g				
20.77-9-31.5-30.5-60-55	9	31.5	76	20.77	60	1011	0.32	5.4	9	0.345
31.4-31.5-30-103-55	9	31.5	76	31.05	103	1148	0.313	5.9	16	0.397
43.32-9-31.5-29.5-122-55	9	31.5	76	43.32	122	985	0.302	5.8	16	0.337
54.88-9-31.5-29.5-155-55	9	31.5	76	54.88	155	1007	0.302	6.0	20	0.338

Table 8.14 : Effect of Total Constant Enzyme to Feed Concentration Ratio - Results

Experimental Run	Feed Throughput (kg sugar/ m^3 resin/hr	GLUCOSE RICH PRODUCT						FRUCTOSE RICH PRODUCT						TOTAL MASS BALANCE OUTPUT / INPUT
		Glucose Purity %	% Sucrose in feed Recovered	% Glucose Recovered	Total Product Conc. % W/V	Impurities % S	Impurities % F	Fructose Purity %	% Sucrose in feed Recovered	% Fructose Recovered	Total Product Conc. % W/V	Impurities % S	Impurities % G	
20.77-9-31.5-30.5-60-55	6.19	95.8	-	107	2.42	~	4.2	91.4	-	67	0.93	~	8.6	92.5
31.4-9-31.5-30-103-55	9.27	93.5	-	79.2	2.85	~	6.5	95	-	84.5	1.75	~	15	92
43.32-9-31.5-29.5-122-55	12.94	95.6	-	67.7	3.54	~	4.4	82.5	-	91.1	2.85	~	17.5	90
54.88-9-31.5-29.5-155-55	16.37	96	-	65	4.34	~	40	76.4	-	85.6	3.69	~	24.6	90

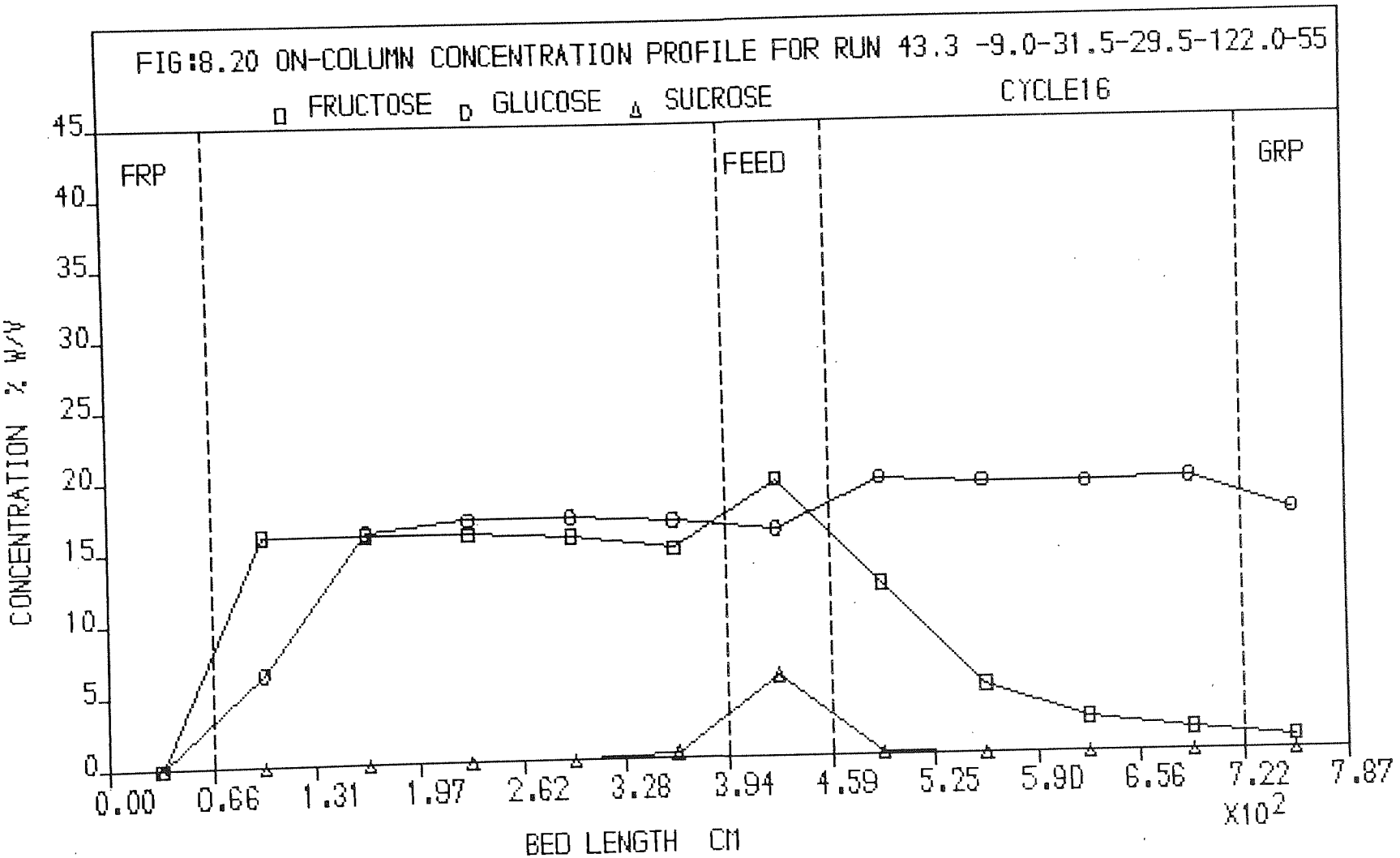


FIG:8.20 ON-COLUMN CONCENTRATION PROFILE FOR RUN 43.3 -9.0-31.5-29.5-122.0-55
FRUCTOSE
GLUCOSE
SUCROSE
CYCLE16
FRP
FEED
GRP
CONCENTRATION % W/V
45
40
35
30
25
20
15
10
5
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10²
BED LENGTH CM

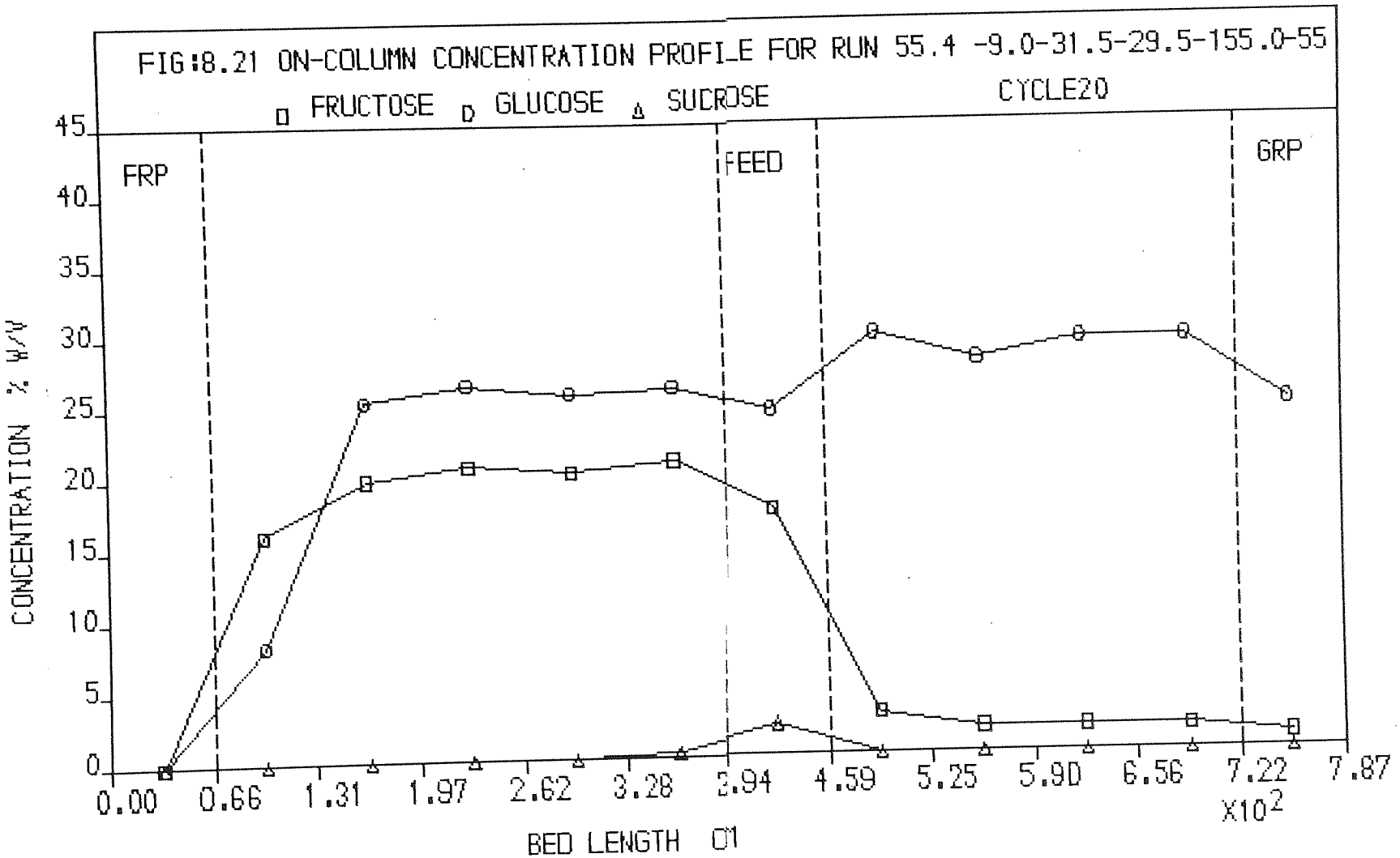


FIG:8.21 ON-COLUMN CONCENTRATION PROFILE FOR RUN 55.4 -9.0-31.5-29.5-155.0-55
□ FRUCTOSE ▷ GLUCOSE △ SUCROSE
CYCLE20
FRP
FEED
GRP
CONCENTRATION % W/V
45
40
35
30
25
20
15
10
5
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10²
BED LENGTH CM

Table 8.15 : Relative Amount of Total Enzyme Used at Different Enzyme Activities

Experimental Runs	Feed Conc % W/V	Enzyme Activity U/ml	Total Weight of Sucrose Consumed/Cycle kg	Theoretical Amount of Enzyme U	Total Enzyme USED/Cycle U	Relative Enzyme Usage ACTUAL / THEORETICAL
31.05-9-31.5-30-110-55	31.05	110	1.004	8156	3112	0.38
31.77-9-31.5-30-90-55	31.77	90	1.025	8328	2794	0.33
43.32-9-31.5-29.5-90-55	43.32	122	1.377	11379	3082	0.27

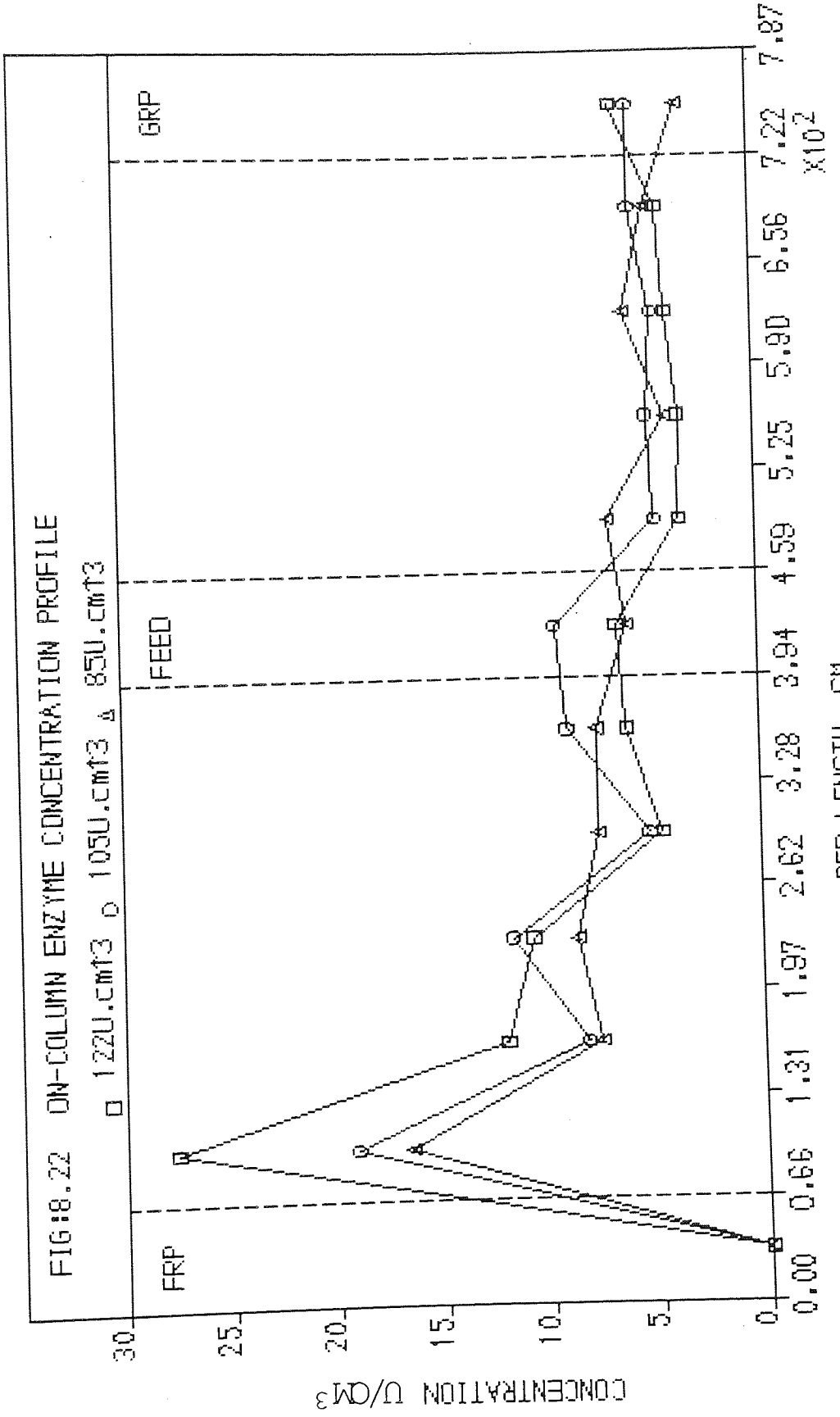


FIG:8.22 ON-COLUMN ENZYME CONCENTRATION PROFILE
□ 122U.cmt3 ○ 105U.cmt3 △ 85U.cmt3
FRP
FEED
GRP
CONCENTRATION U/CM3
BED LENGTH CM
X10^2
0
5
10
15
20
25
30
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87

Figure 8.23:- GRP Elution Profile over a switch
Run:- 60-14.6-40-25-60 (9)

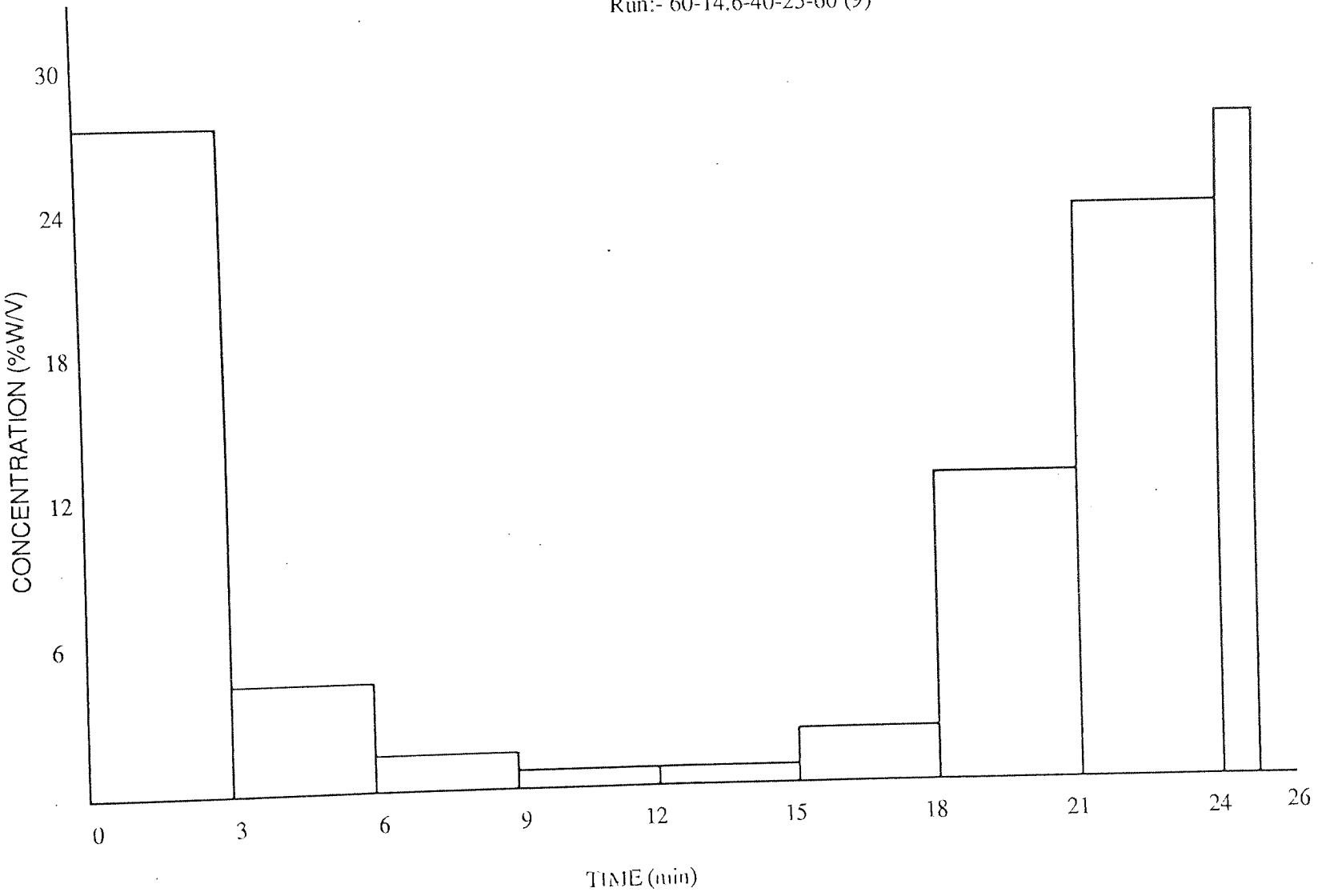


Figure 8.24:- FRP Elution Profile over a switch
Run(60-14.6-40-25-60) (9)

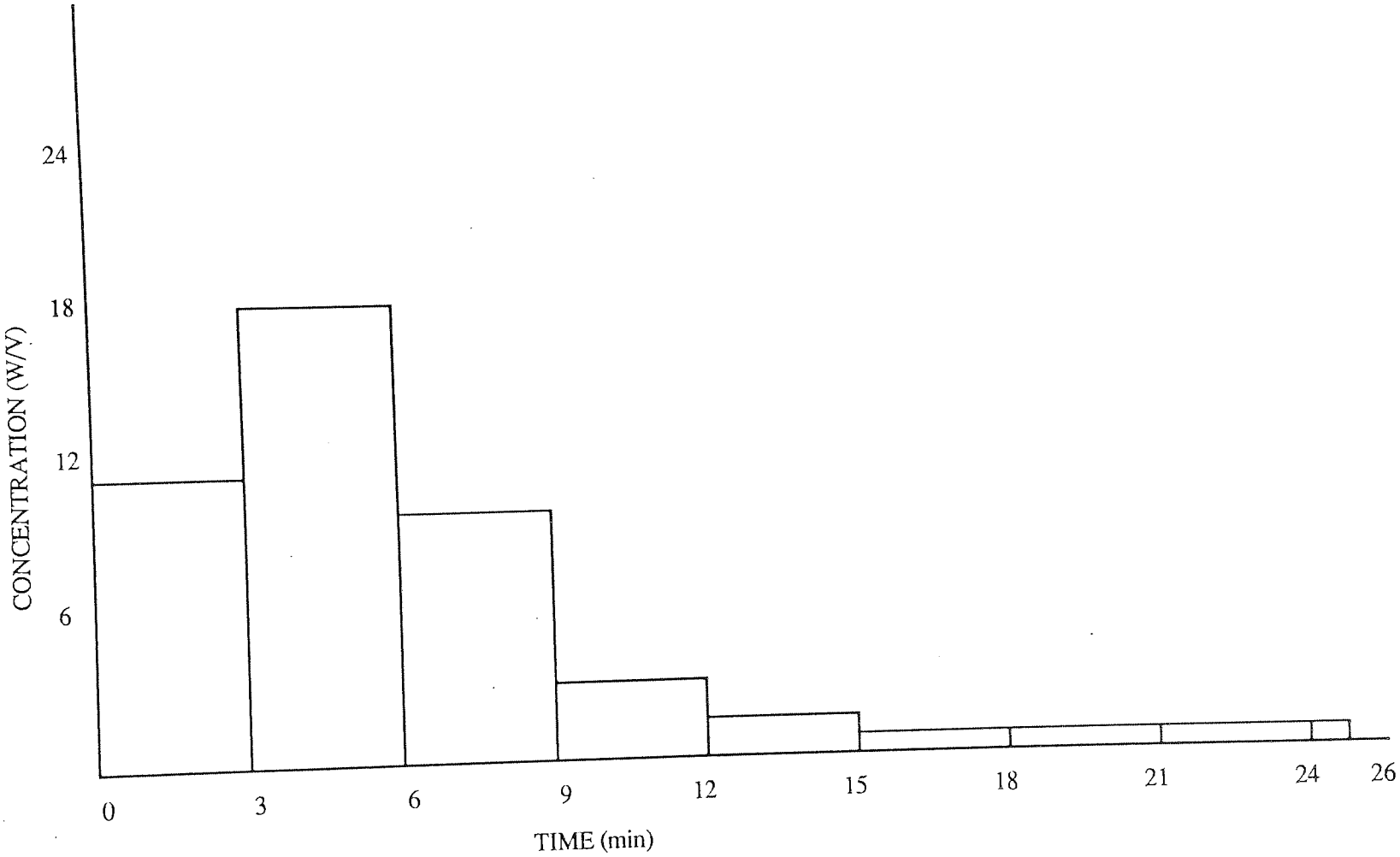


Table 8.16 : Effect of Recycling on Product Concentration - Operating Condition

Experimental Run	Average Flowrates $cm^3\ min^{-1}$			Feed Conc.	Enzyme Activity	Enzyme To Feed Conc Ratio	Lm/P	pH	Cycle No	Relative Enzyme Usage Actual / Theoretical
	Feed	Eluent Enzyme	Purge	% W/V	U cm^{-3}	U/g				
35.9-9-31.5-30.5-90-55	9	31.5	76	35.9	90	877		6.0	16	0.3
34.96-9-31.5-30.5-90-55	9	31.5	76	34.96	90	901		5.8	16	0.308

Table 8.17 : Effect of Recycling on Product Concentration - Results

Experimental Run	Feed Throughput (kg sugar/ m^3 resin/hr	GLUCOSE RICH PRODUCT						FRUCTOSE RICH PRODUCT						TOTAL MASS BALANCE OUTPUT / INPUT
		Glucose Purity %	% Sucrose in feed Recovered	% Glucose Recovered	Total Product Conc. % W/V	Impurities % S	Impurities % F	Fructose Purity %	% Sucrose in feed Recovered	% Fructose Recovered	Total Product Conc. % W/V	Impurities % S	Impurities % G	
35.9-9-31.5-30.5-90-55	10.72	92.3	-	58.6	3.2	~	5	72	-	84.7	1.8	-	28.0	90.6
34.96-9-31.5-30.5-90-55	10.44	95.4	-	76.1	3.23	~	4.6	80.6	-	82.9	1.82	-	19.4	8.6

Table 8.18 : FRP results after recycling the diluted fraction :

Run 35.9-9-31.5-30.5-90-55

COLLECTION PERIOD FROM 1 min to 16 min				
Fructose Purity %	% of Fructose Recovery	Total Product Conc. % W/V	Impurities % S	G
71.34	91.1	2.88	~	28.65

Table 8.19 : GRP results after recycling the diluted fraction :

Run : 35.9-9-31.5-30.5-90-55

COLLECTION PERIOD FROM 16 min to 1 min				
Glucose Purity %	% of Glucose Recovery	Total Product Conc. % W/V	Impurities % S	G
95	60.0	4.6	~	5

Table 8.20 : Results of Run 34.96-9-31.5-30.5-90-55 when Product Splitting was employed on the GRP

0.5 - 16 min Collection					16 to 0.5 min Collection				
Glucose Purity %	% of Glucose Recovered	Total Product Conc. % W/V	Impurities % S	F	Glucose Purity %	% of Glucose Recovered Recovered	Total Product Conc. % W/V	Impurities % S	F
80	16.3	136	~	13.18	95.5	59.8	5.01	~	4.5

Table 8.21 : Results of Run 34.96-9-31.5-30.5-90-55 when recycling was employed in the FRP

COLLECTION PERIOD FROM 0.5 min to 16 min				
Fructose Purity %	% of Fructose Recovery	Total Product Conc. % W/V	Impurities % S	G
87	82.9	3.58	~	13.0

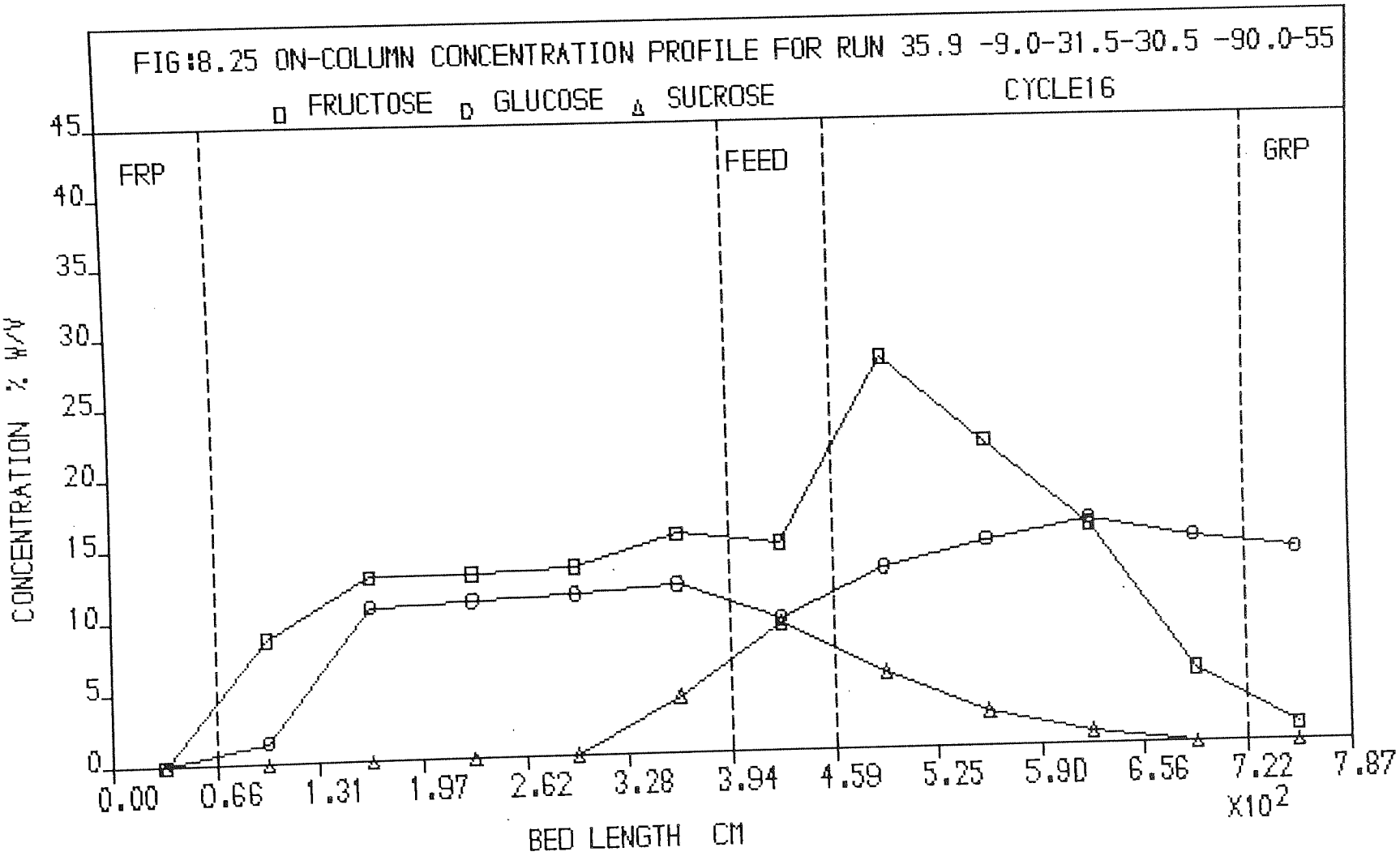


FIG:8.25 ON-COLUMN CONCENTRATION PROFILE FOR RUN 35.9 -9.0-31.5-30.5 -90.0-55
FRUCTOSE
GLUCOSE
SUCROSE
CYCLE16
FRP
FEED
GRP
CONCENTRATION % W/V
45
40
35
30
25
20
15
10
5
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10^2
BED LENGTH CM

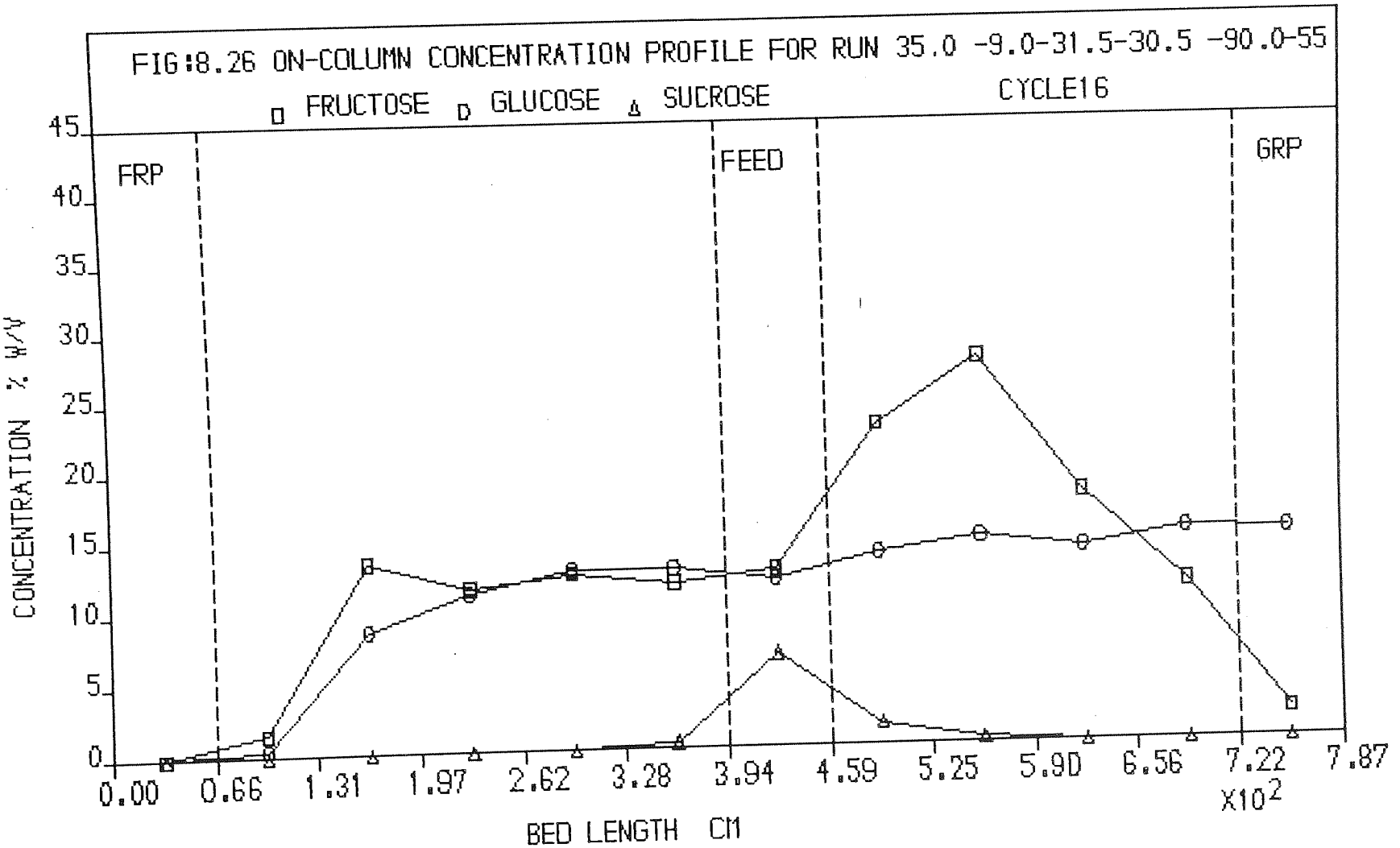


FIG:8.26 ON-COLUMN CONCENTRATION PROFILE FOR RUN 35.0 -9.0-31.5-30.5 -90.0-55
FRUCTOSE
GLUCOSE
SUCROSE
CYCLE16
FRP
FEED
GRP
CONCENTRATION % W/W
45
40
35
30
25
20
15
10
5
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10^2
BED LENGTH CM

CHAPTER 9

PRODUCTION AND PURIFICATION OF DEXTRANSUCRASE

9.0 INTRODUCTION

The invertase enzyme used in the sucrose inversion studies on the SCCR-S1 was commercially available and was purchased in a purified form. Purified dextransucrase enzyme however, was required for the dextran biosynthesis studies on the SCCR-S1 system. As the dextransucrase was not commercially available, it was necessary to consider its production and purification.

Dextransucrase was produced from the bacteria Leuconostoc mesenteriodes in a fed-batch fermenter. The crude enzyme contains a large number of impurities which include dead cells, dextran, fructose, leucrose, mannitol, proteins and other oligosaccharides. These impurities could cause considerable problems during the reaction-separation studies and in the analysis of the products on the HPLC system. The dead cells, dextran and proteins could block the resin and result in high pressure drops. Any fructose and mannitol present could interfere with the fructose formed during the reaction. Similarly, the leucrose, due to a similar retention time as sucrose prevents the unreacted sucrose to be quantified accurately. Also the presence of dextran and proteins in the crude enzyme mixture makes it impossible to investigate the MWD of the dextran produced in the reaction and could foul the HPLC and GPC columns.

All published dextransucrase purification methods [61, 63, 86, 126 - 139] involve a combination of centrifugation, ultrafiltration, precipitation, gel chromatography, ion exchange chromatography and phase partition techniques. The choice of the most suitable purification method was based on the following three criteria:

1. Scale up:- All the methods studied had been carried out on a small scale. Since the quantity of enzyme required for this work was large, it was important to select the best process that could easily be scaled-up, with the least amount of activity loss.

2. Number of stages:- It was desirable to keep the number of stages to a minimum, to reduce the purification time and cost. This will also reduce the loss in enzyme activity.

3. Degree of purification:- There is no need to purify the enzyme more than it is necessary, as the greater the purification, the higher the activity losses.

Figure 9.1 illustrates the different routes of obtaining pure dextransucrase enzyme. The route chosen for this work was the 1a-2-6 route which involves centrifugation and ultrafiltration methods. Using these methods an attempt was made in optimising and scaling up each process.

The purified enzyme produced was then used in the biosynthesis of the dextran reaction.

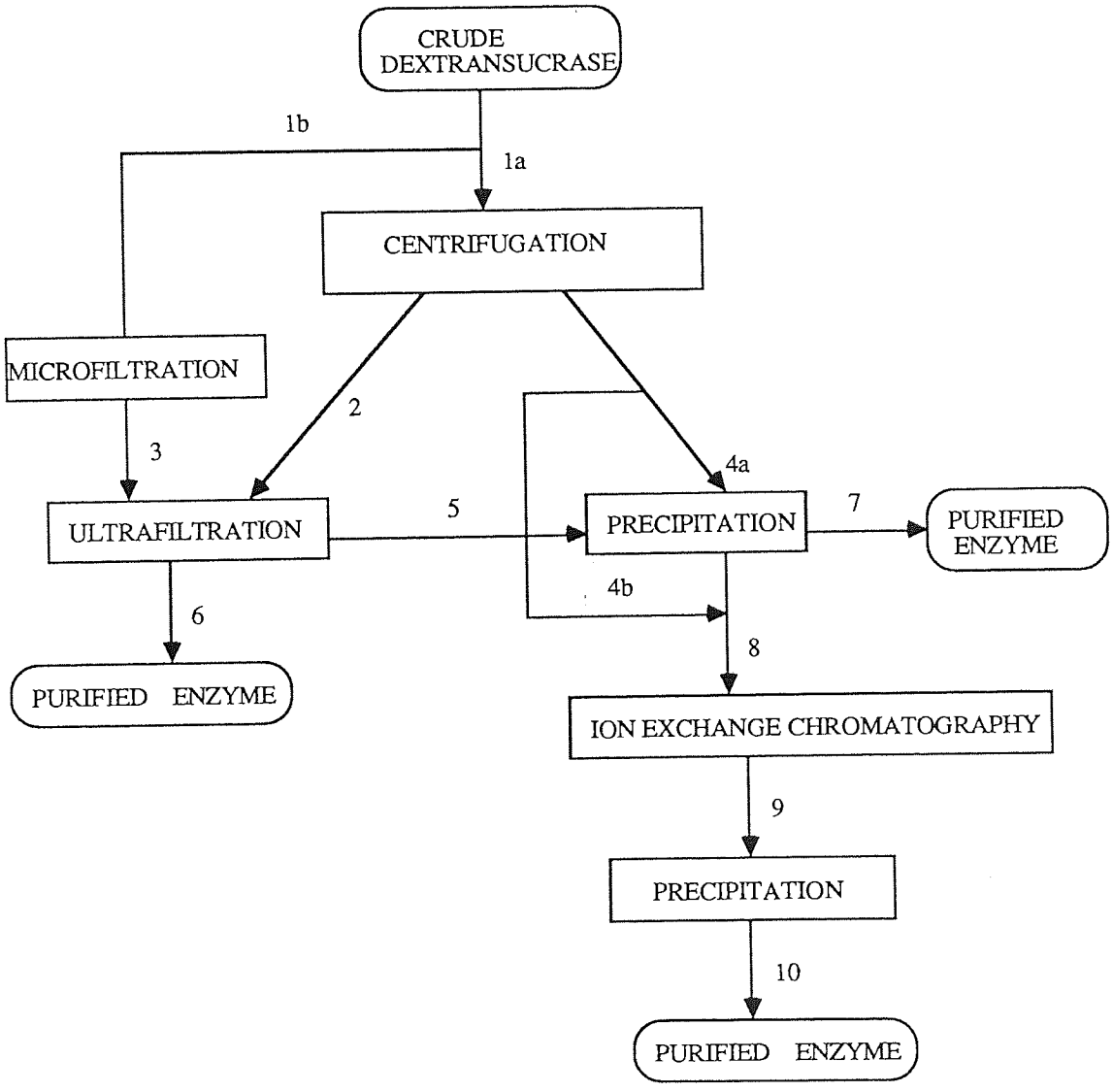
9.2 PRODUCTION OF DEXTRANSUCRASE

Dextransucrase was produced by the culturing of Leuconostoc mesenteroides strain NRRL B512(F) supplied by Fisons Pharmaceuticals plc., Holmes Chapel, Cheshire, England.

The production was carried out on three different scales. In a 16 litre New Brunswick "Microgen" SF116 fermenter, an 80 litre fermenter located at Teeside Polytechnic, Middlesborough and a 1000 litre modified industrial seed fermenter located at Fisons Pharmaceutical plc., Cheshire.

All these fermenters were equipped with temperature and pH controls. The agitation rate on the 16 litre fermenter was automatically controlled. However, the 80 litre fermenter and the 1000 litre fermenter were operated at a fixed agitation rate of 100

Figure 9.1:- Process Routes for Purifying Dextransucrase



rpm and 120 rpm respectively. The fermentation was carried out anaerobically on a fed-batch basis.

This part of the research work was carried out jointly with J. Ajongwen [90] a fellow member of the research team at Aston.

9.2.1 Fermentation Procedure and Operation

The Leuconostoc mesenteroides NRRL B512(F) strain culture was maintained on agar slopes of Lactobacilli MRS (Deman - Regosa - Sharpe) medium supplied by Oxoid Basingstoke.

The inoculation of the fermenters with the culture follow three stages. In the 16 litre fermenter, with a working volume of 6 litres, the culture was transferred aseptically from one of the slopes into 10-15 cm^3 of sterile medium. This was incubated at 23-25°C for 10-15 hours. At the end of this period, or when the medium became turbid (i.e. an optical density (OD_{590}) of 0.1), the contents were transferred into 300-350 cm^3 bottles containing sterile MRS medium. The latter was also incubated for the same period of time at the same temperature. At the end of this second stage the inoculum was ready to be used in the fermenter. For the larger scale fermenters (i.e. 80 litre and 1000 litres with a working volume of 60 and 600 litres respectively), there was a need to have an inoculum to medium ratio identical to that used in the 16 litres fermenter.

The composition of the medium used for all the fermenters was identical to that of Schneider et al. [138] and is shown in Table 9.1. The medium components were dissolved in water and made up to the required volume. The pH was adjusted to 7.0-7.1 and then sterilised at 115°C for 20-25 minutes before it was cooled down to 23°C.

As experiments were conducted on a fed-batch scale, sucrose and NaOH were added continuously.

Table 9.1 : Composition of Fermenter Medium

Sucrose	10.0g/L	
Yeast Extract	40.0g/L	
K_2HPO_4	20.0g/L	
R* Salts	0.5% v/v	
Antifoam	0.1% v/v	
R* Salts	$MgSO_4 \cdot 7H_2O$	4.0g
	NaCL	0.2g
	$FeSO_4 \cdot 7H_2O$	0.2g
	$MnSO_4 \cdot H_2O$	0.2g
	Water	to 100 cm^3

Sucrose addition was coupled to the alkali demand [140]. The composition of the sucrose/alkali mixture used was as shown in Table 9.2.

Table 9.2 : Composition of Sucrose/Alkali Mixture

Sucrose	900g
NaOH	200g
Water	to 1500 cm^3

After inoculation, the pH in the fermenters was allowed to fall from its initial value of pH 7.0-7.1 to pH 6.7 the optimum pH, and then automatically controlled at this value, by the addition of sterile sucrose/alkali mixture. The temperature was controlled at 23°C and the agitation rate set at the required value.

A sample was taken at this point and assayed for the initial cell growth. Further samples were then taken every hour and assayed for cell growth and enzyme activity until the end of fermentation.

Fermentation was terminated when either the enzyme activity had peaked, cell growth terminated or when the rate of addition of sucrose/alkali mxiture had started decreasing.

The enzyme was then harvested and stored in a freezer for purification.

9.2.2 Results and Discussion

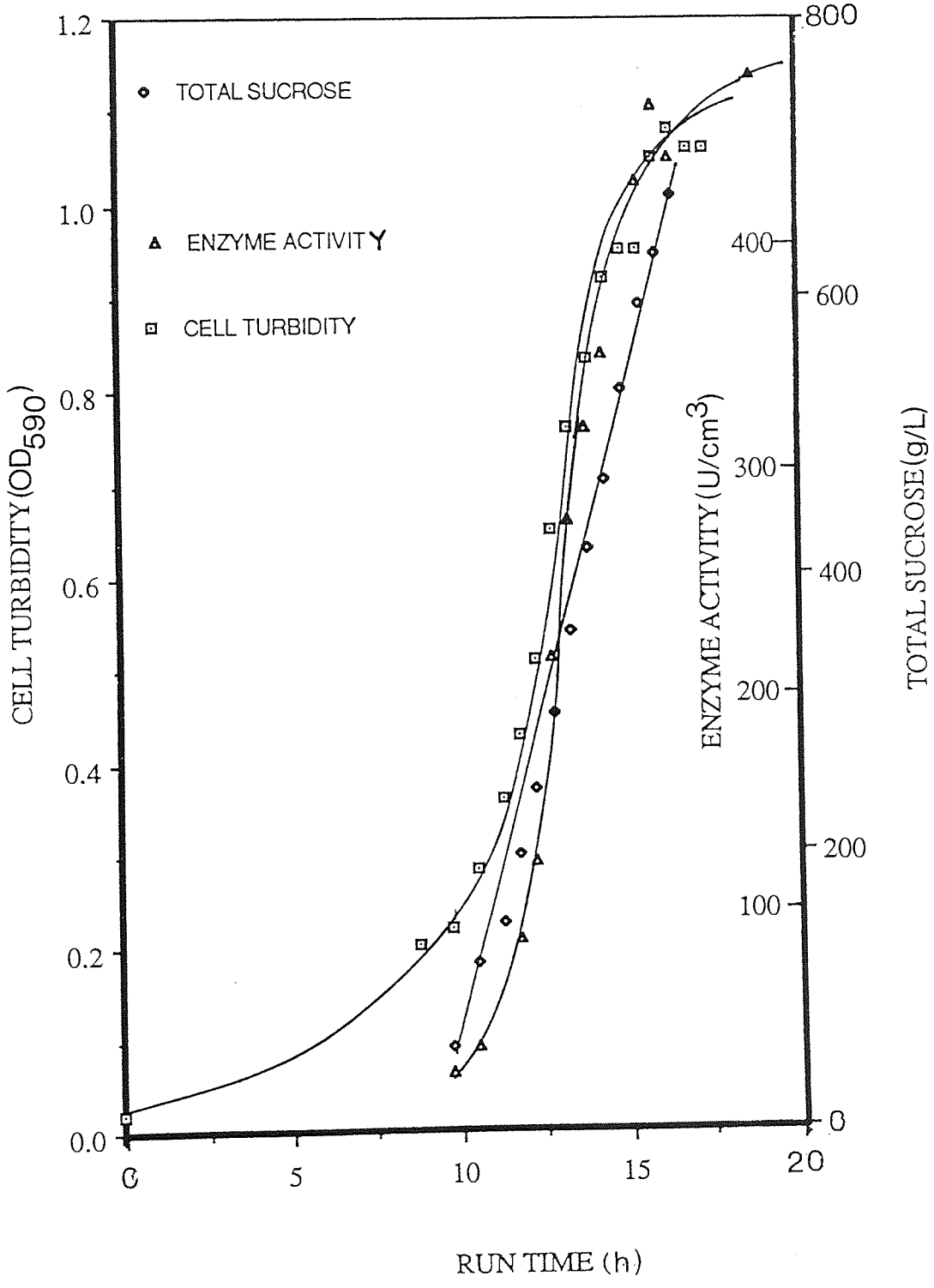
Two runs were carried out on the 16 litres and 80 litres fermenters and one on the 1000 litres fermenter. Table 9.3 shows the set of results obtained and Figure 9.2 is a graphical representation of the fed batch experiment. It shows the cell growth, enzyme activity, total protein present and the total sucrose consumption values against time during the course of the fermentation. The sucrose addition rate was constant during the experimental phase as indicated by the linear relationship of the sucrose consumption.

Table 9.3 : Results of the Anaerobic Fed Batch Fermentation of Dextransucrase

Fermenter Size Litres	Cell Growth OD_{590}	ENZYME Activity DSU/cm^3	ENZYME Total Protein $\mu g/cm^3$	Sucrose Added cm^3	Duration of Fermentation hrs
16	1.08	487	960	684	18
16	1.12	451	800	858	19
80	1.08	300	850	~	26
80	0.85	265	650	~	26
1000	0.95	290	635	~	24

The low yield of enzyme experienced on the 80 litres and 1000 litres fermenters was due to the control of the pH and temperature problem encountered during these runs.

Figure 9.2:- Cell growth, Total sucrose consumed and Enzyme production during Anaerobic Fermentation



The relatively low enzyme activities achieved in the 80 litres fermenter runs were due to problems associated with pH control and due to the long duration of the fermentation [90].

To investigate the scaling up of the fermentation processes and to produce sufficient quantity of enzyme for the biosynthesis and dextran studies, a large scale fermenter (1000 litres) was employed. The pH on this fermenter was controlled by inserting a pH electrode in a recirculating loop that was linked to a controller which activated a dosing pump that delivered the sucrose/alkali mixture to the vessel. Difficulties in maintaining a constant temperature affected the cell growth and the enzyme yield. The cell growth was linear and levelled off at an OD_{590} of 0.67 while the enzyme activity was only 190 DSU cm^{-3}. Adjusting the temperature to 23°C improved the cell growth (OD_{590} = 0.95) and enzyme activity (290 DSU/cm^3). Further information on the production of dextransucrase can be found in J. Ajongwen's thesis [90].

9.3 THE PURIFICATION OF DEXTRANSUCRASE

In the enzyme purification work, any enzyme stability problems need to be identified and overcome especially since the dextransucrase is known to be very unstable [128]. Techniques used in dextransucrase purification include precipitation combined with gel chromatography or ion-exchange chromatography. These techniques involve treating the enzyme with dextranase to eliminate the bound dextran after precipitation before being loaded onto the chromatographic column [86, 139]. The recovery of enzyme after these purification techniques, however, have been found to be quite low (1.9% to 32.8%).

Kaboli [63] used ultrafiltration (Amicon XM - 100A) and gel chromatography without the use of dextranase to obtain a high yield of enzyme (56 percent recovery). Zafar [25] and Ganetsos [9] also carried out the purification of dextransucrase on an ultrafiltration membrane system (Amicon; PM 10, XM-100A, UM-100-and HIP 30-20) and achieved an overall recovery of 60% enzyme activity.

The comparatively low loss in activity, when employing ultrafiltration techniques and the ease of scaling up this process prompted an investigation into the possibility of developing and scaling up this technique.

Ways of removing the solid debris from the fermentation broth were also investigated, with emphasis on the centrifugation process. The potential of a microfiltration process was also considered.

9.3.1 Centrifugation

A Sharples continuous centrifuge (model Lab A) was used in separating the solid debris from the fermentation broth.

The centrifuge was fitted with a variable speed controller to control the centrifugal force. An Ultracentrifuge (MSE 5550) was used as a reference to test the efficiency of the Sharples continuous centrifuge. The ultracentrifuge was a batch centrifuge with a cooling unit incorporated inside it. It had a capacity of 240 cm^3 and a centrifugal force capacity of up to 77,000g (8 x 50 ml angle rotor). For a reference run, the ultracentrifuge was operated at 10,000g for 15 min at 4°C.

The performance of the continuous centrifuge was tested by investigating the effect of feed throughput (Table 9.4) and centrifugal forces (Figure 9.3) on the recovery of enzyme obtained and solid removed.

By increasing the centrifugal forces, the percentage of enzyme recovered decreased, while there was a slight improvement in the amount of solid removed. The loss in activity was the result of the high shear forces on the enzyme molecules. Zafar (25), when working on a CU 5000 batch centrifuge reported a similar effect and found that at 68,000g the enzyme would have lost all its activity.

The effect of feed throughput, at constant centrifugal force (18,000g), shows an enzyme recovery of about 77% with approximately 76% solid removed. The variation of enzyme recovery and solid removal is caused by the variation in operating temperature.

From these results an operating condition of a centrifugal force of 18,000g and a flowrate of 200 cm^3/min with a cooling unit was found to be the optimum.

By installing a cooling unit to the Sharples continuous centrifuge and operating under the above conditions an enzyme recovery of 85% and 80% solid removal was achieved.

Table 9.4 : Effect of Feed Throughput on Enzyme Recovery and Cell Removal]

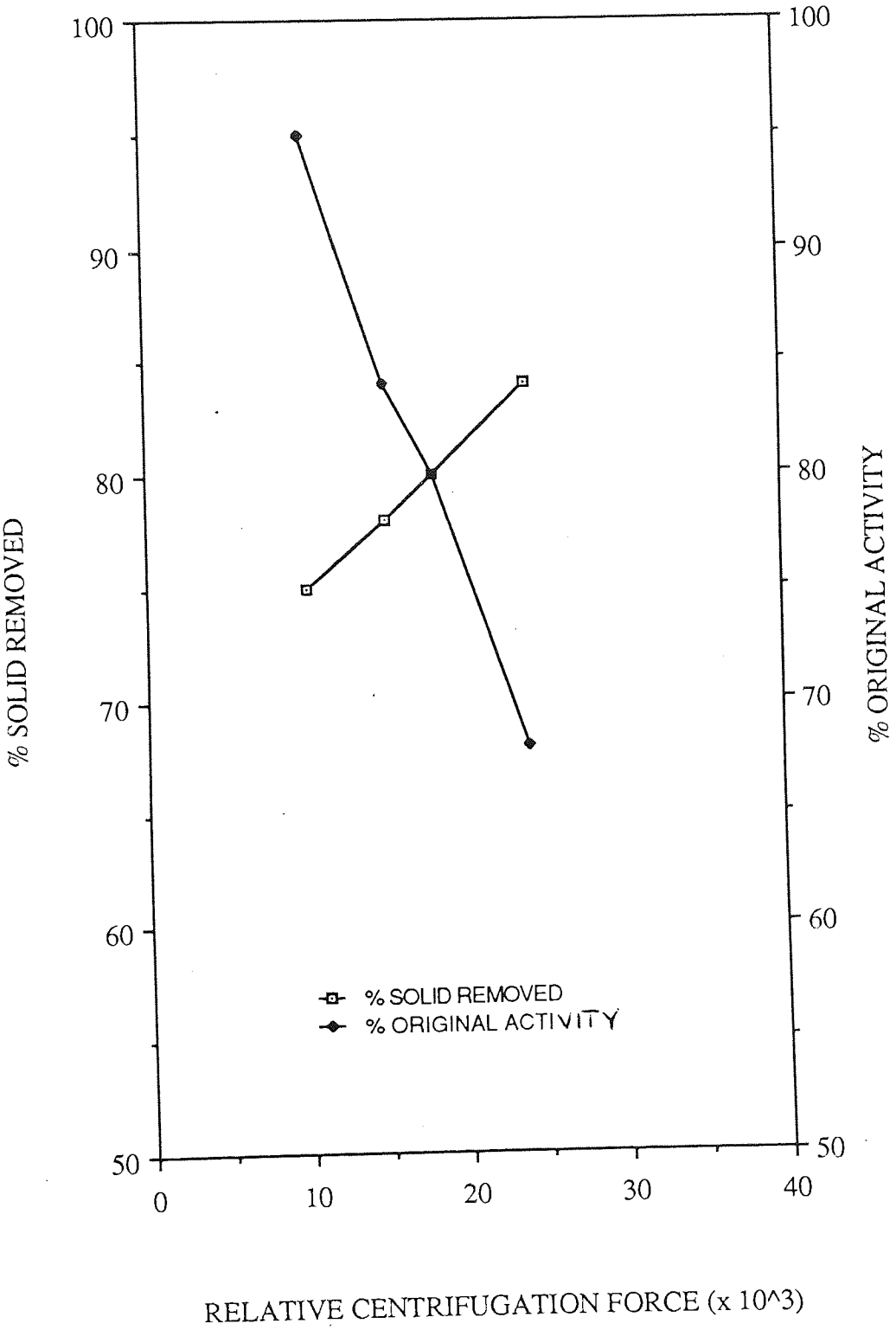
Flowrate cm^3/min	Centrifugal Force g	Temp ℃	% Solid Removal	% Enzyme Recovery
40	18,100	14	76	78
80	18,100	16	87	88
219	18,100	15	76	75
232	18,100	13	77	72
255	18,100	16	81	70

The stability of the enzyme after centrifugation and under a 4°C storage condition indicates that the enzyme can be kept for seven days without significant loss in enzyme activity (< 1% loss).

9.3.2 Microfiltration and Ultrafiltration

Membrane separation techniques were used in investigating the cell and other soluble impurities removal from the fermentation broth. Cross flow microfiltration and

Figure 9.3:- Effect of Centrifugal Force on Enzyme Activity and Solid Removal



ultrafiltration processes were considered. The microfiltration techniques were used to separate the solid debris (dead cells) from the crude dextransucrase and ultrafiltration was used to separate low molecular weight impurities (LMW).

The enzyme activity was measured before and after purification. Impurities in the samples were also determined. The HPLC chromatograph gave an indication of the concentration of solutes presents. From the concentration profile one can calculate the percentage impurities removed from the initial feed and the percentage retained.

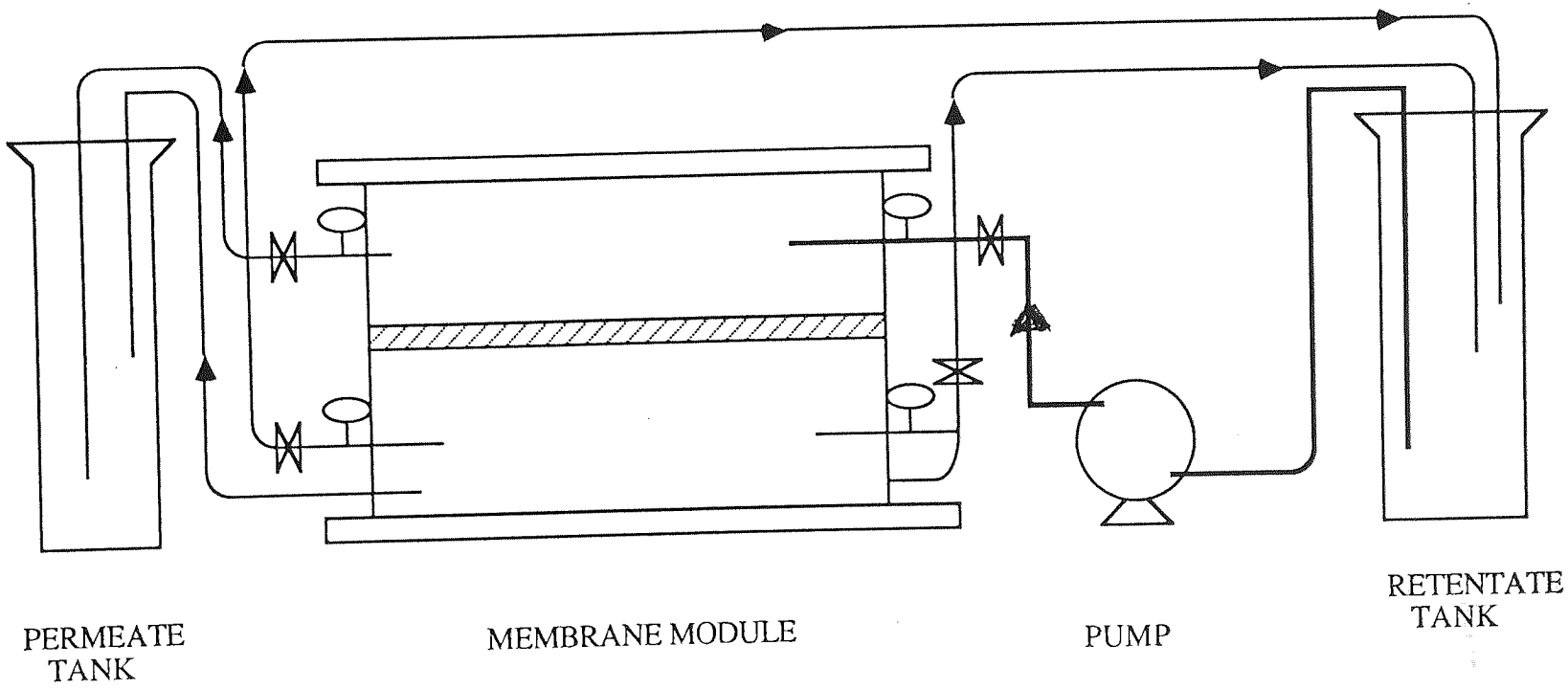
Two membrane systems were investigated to ascertain if it was possible to obtain a high recovery of the enzyme dextransucrase and high percentage removal of low molecular weight impurities: The New Brunswick System and the Millipore System.

9.3.2.1 Equipment Description

The equipment used for this purification work, consisted of storage tanks (measuring cylinders), a pump and the cross flow microfiltration module provided by Millipore plc (XF42000 60 medium PL cell) and New Brunswick (Mega flow membrane TM-100). Figure 9.4 shows a schematic diagram of the apparatus used. Process feed was pumped through the inlet port and flowed under pressure tangentially along two perspex blocks. The filter membrane located between the blocks had its surface continuously washed by the process stream. The turbulent washing action keeps solids in suspension inhibiting macromolecular build-up on the membrane. A fraction of the process stream filters through the membrane to the filter screen. Filtrate flows through the screen into pathways emptying into the filtrate reservoir (permeate solution). The details of operating the systems were as found in the manufacturers manuals [141, 142].

The New Brunswick (TM - 100) system had a crossflow area of 64.5 cm^2 while the Millipore (XF4 2000 PL cell) system was 454.5 cm^2.

Figure 9.4:- Sketch of the Membrane Filtration System.



9.3.2.2 System Behaviour

In a cross flow microfiltration system, there are three important parameters that need to be controlled. The pressure, recirculation rate and permeate flux.

The pressure was measured as the transmembrane presure which can be defined as :

$$Tmp = \frac{Pin + Pout}{2} - Pp$$

where Pin = Inlet pressure

Pout = Recirculation pressure

Pp = Permeate pressure.

To achieve optimum pressure for a particular size of membrane in the system, a pressure excursion was performed. This enabled the working pressure to be estimated.

The recirculation rate chosen should be high enough to reduce fouling and reduce the build up of particulates on the membrane surface, but not excessively high that it will damage the membrane (i.e. 172kN.m^2). To achieve this criterion, a flow excursion was performed. The recommended values quoted by the manufacturers were also taken into consideration.

The response of the permeate flux to changes in the transmembrane pressure gave some idea about the behaviour of the membrane used (e.g. fouling).

9.3.3 The Newbrunswick Megaflow Membrane System

A 10,000 MW and a 100,000 MW membrane were used for the ultrafiltration study and a 0.1µm, 0.2µm and 0.45µm membrane were used for the microfiltration studies.

The pressure excursion graphs for the 10,000 MW and 0.1μm membranes are shown in Figure 9.5. As there was no means of controlling the pressure on the permeate side, the average pressure of the inlet and recirculation presure was used. As shown on the graph, it is very difficult to estimate the optimum pressure. Therefore the manufacturer's recommended value was used (i.e. 138 kN/m^2).

A recirculation rate of 0.9 L min^{-1} was used. The permeate fluxes for the different membrane sizes are generally lower. From Table 9.5, the 0.45 μm membrane, which has a very large pore size, gives a low flux of 19.2 L m^{-2} h^{-1}.

Table 9.5 : Range of Permeate Flux with Different Membrane Sizes

Membrane Sizes (New Brunswick)	Permeate Flux L m^{-2} h^{-1}
0.45μm	19.2
0.2μm	18.73 (7.86)
0.1μm	20.46
10,000 MW cut off	15.8
50,000 MW cut off	14.6
100,000 MW cut off	9.3

All the membrane sizes used had a slight drop in permeate flux ($\simeq$ 20%) from their initial value. A possible explanation is that the film coating on the membrane surface when first purchased was removed during the initial run.

On the microporous membrane (0.1 - 0.45μm), there was an exponential drop in flux with time (Figure 9.6). This characteristic is well known to be associated with microporous membranes (143). It is a measure of the reproducibility of the membrane and it is one of the factors that was considered when using this type of membrane for separation purposes.

Figure 9.5:- Plot of Average Flux vs Average Pressure

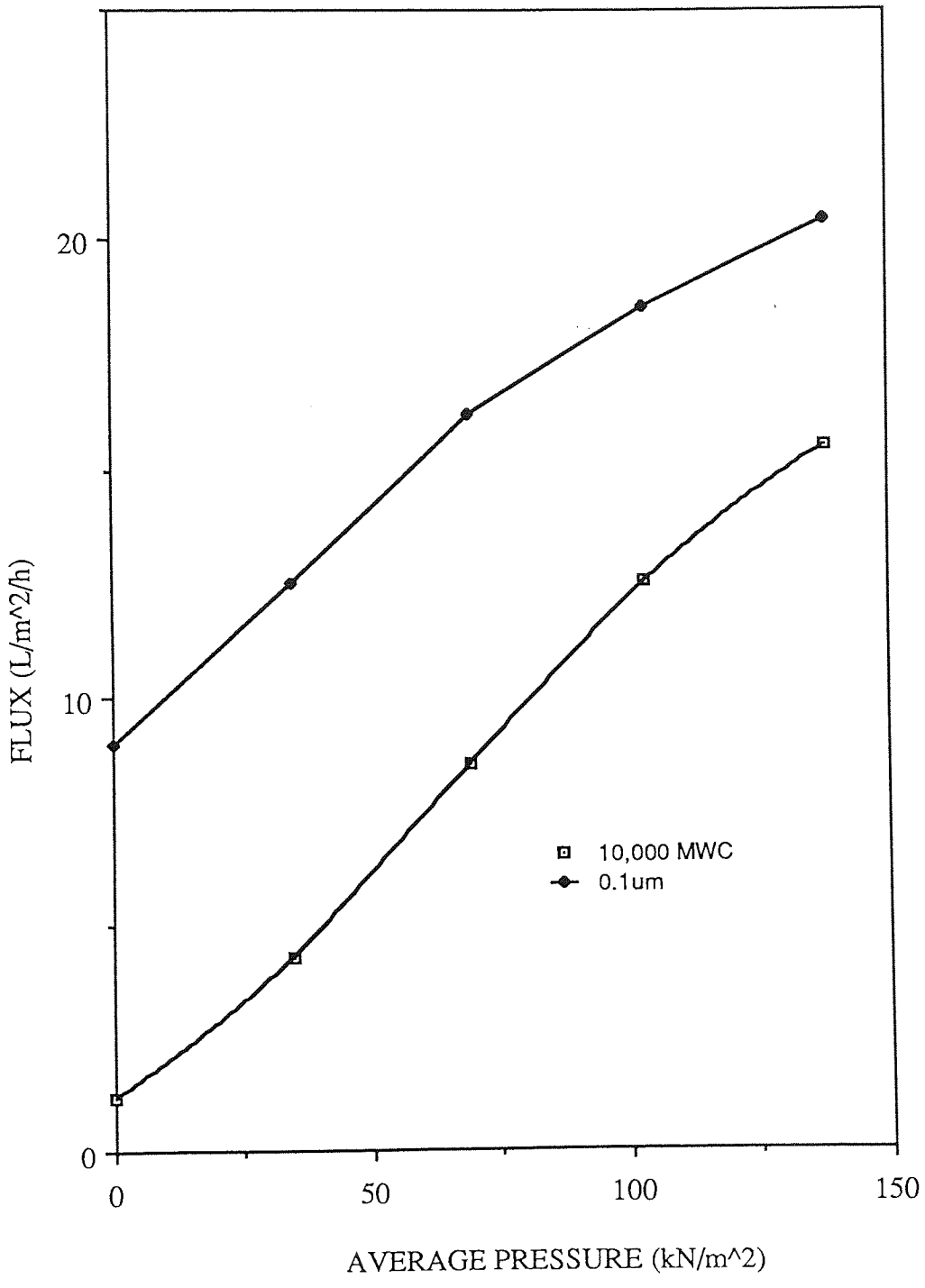
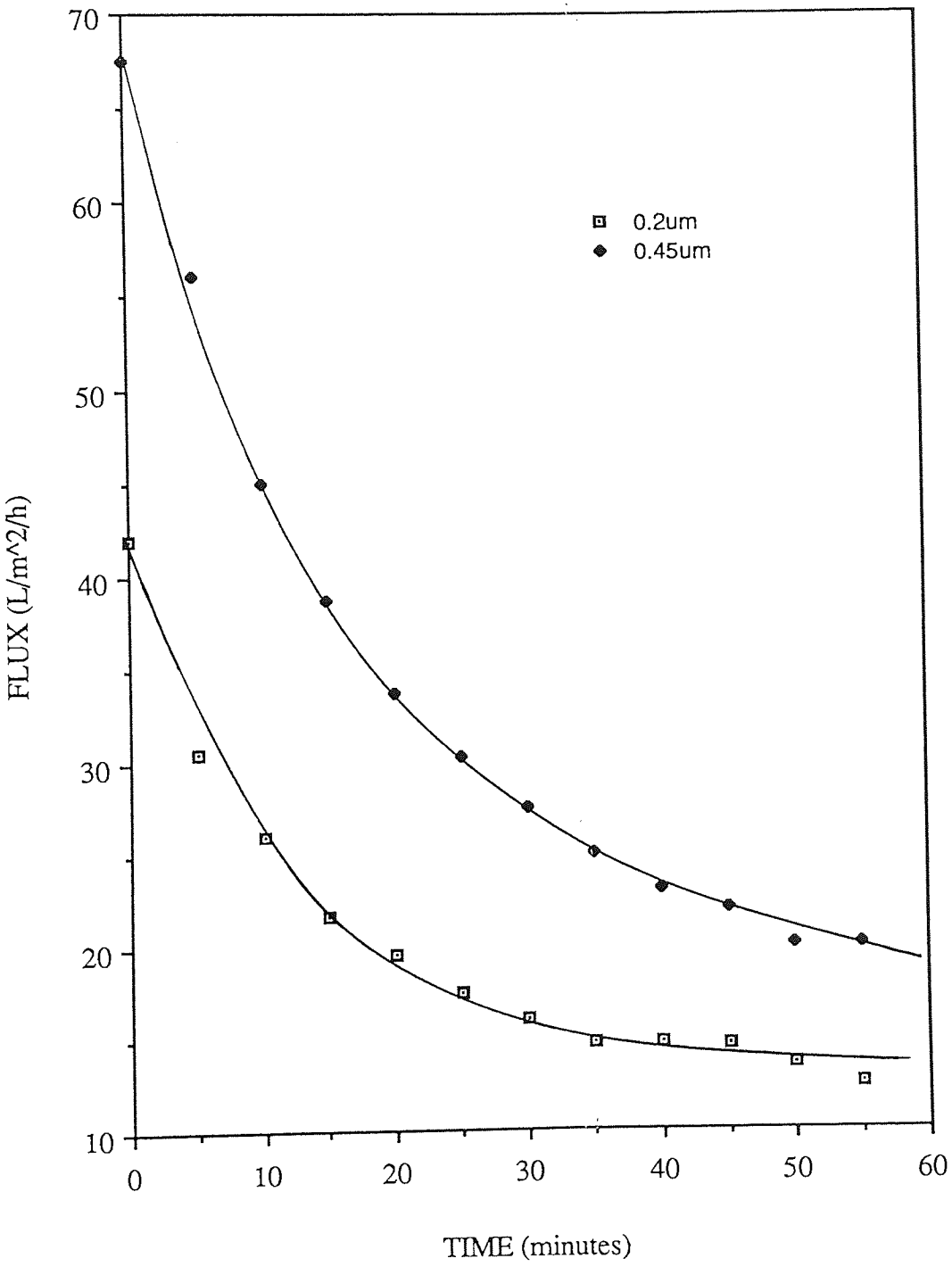


Figure 9.6:- Plot of average flux vs time for Microporus membrane on New Brunswick system



9.3.3.1. Purification Procedure, Results and Discussion

The removal of the soluble impurities in the dextransucrase mixture was carried out by operating in the diafiltration mode. The crude enzyme solution was concentrated down to about half its original volume (500 cm^3) and then diluted back with deionised water. This procedure was carried out a number of times until the enzyme solution was approximately 90 percent pure. The activity of the enzyme was measured at the start and end of each diafiltration. The concentrations of low molecular weight impurities were also monitored after each diafiltration. At the end of each run the membrane was cleaned with 0.1M NaOH to eliminate any fouling and neutralised by acetic acid.

From Table 9.6 a high percentage of the enzyme was retained on the retentate side. This was expected for the ultrafiltration membranes (10,000 MW and 100,000 MW) but not for the microporous membranes (0.1μm - 0.45 μm) since their pore sizes are theoretically large enough for the enzyme molecules (MW ≃ 160,000) to permeate through.

Table 9.6 : Percentage Enzyme Recovery on the New Brunswick (TM -100) System

TYPE AND SIZE OF MEMBRANE (NEW BRUNSWICK)	PERCENTAGE RECOVERY OF ENZYME IN THE RETENTATE DIAFILTRATION SEQUENCE			PERCENTAGE RECOVERY OF ENZYME IN THE PERMEATE DIAFILTRATION SEQUENCE		
	1	2	3	1	2	3
Nylon 0.45μm	51.6	39.5	30.7	16.5	19.7	23.8
Polypropylene 0.2μm	82.6	76	66	2.6	2.9	4.1
Polypropylene 0.1μm	85.6	88	87	6.24	11.9	11.9
Polysulfone 10,000 MW CUT OFF	90.77	77.7	26.4	0.29	0 29	0.29
Polysulfone 100,000 MW CUT OFF	73.9	58.9	-	0.22	0.22	-

Table 9.7 shows the percentage of low molecular weight (LMW) impurities removed after each diafiltration for the different sizes of membranes. After two or three diafiltrations most of the membranes with the exception of the 0.45μm membrane, removed a high proportion of the LMW impurities.

The low enzyme recovery and low percentage removal of solutes on the 0.45μm membrane was possibly caused by the high adsorption of protein molecules onto the membrane surface. This could be caused by the high polarity of the membrane material (Nylon) and the pH of the enzyme (pH 5.2). The pH of the enzyme is very close to its isoelectric point (pI - 4.9) [61] and as a result becomes less soluble, thus allowing adsorption on the membrane material to occur. This becomes pronounced when the polarity of the membrane is very high. The exponential decay of permeate flux which is a characteristic of microporous membranes also contributed to this effect.

The difficulty in relating the recovery of enzyme to membrane sizes is due to the inconsistency of the permeate flux. Table 9.5 shows that two identical membrane sizes (0.2μm) of the same material gave two different flux values under the same conditions.

The low permeate flux of the membranes tested and the inability of the microporous membranes to separate the cells from the crude enzymes efficiently led to the investigation of another membrane system.

Table 9.7 : Solutes Removal on the New Brunswick System

Membrane TYPE AND SIZE	PERCENTAGE SOLUTE REMOVED IN THE PERMEATE			PERCENTAGE SOLUTE REMOVED IN THE RETENTATE		
	NUMBER OF DIAFILTRATION			NUMBER OF DIAFILTRATION		
	1	2	3	1	2	3
Nylon 0.45µm	23.2	34.7	39.7	42	14.3	8.7
Polypropylene 0.2µm	44.3	68.3	80.8	43.2	25.3	2.3
Polypropylene 0.1µm	45.3	74.8	87.2	45.2	27.1	16.2
Polysulfone 10,000 MW CUT OFF	28	76.8	94	36.2	30.5	13.3
Polysulfone 100,000 MW CUT OFF	52.4	80.5	-	49	12.3	

9.3.4 The Millipore XF4 2000 60 Medium PL Cell System

Initial tests carried out on a similar membrane size (0.2µm) used on the New Brunswick TM-100 system showed a much improved performance. A high flux (27 L h^{-1} m^{-2}) was recorded and the microporous membrane did efficiently separate the cells from the crude enzyme. A study of membrane separation was carried out using 0.2µm membrane and a 30,000 MW membrane. The operating conditions used were similar to those used on the New Brunswick System (i.e. constant temperature (4°C) and pH 5.2).

A pressure excursion was carried out for the 30,000MW ultrafiltration membrane to obtain an optimum pressure (Figure 9.7). The working pressure was estimated to be 28 psi (193 kN/m^2). A recirculation rate of 1.4 L h^{-1} was estimated from a flow excursion curve (Figure 9.8) and the permeate flux was found to be constant throughout the whole experiment (35 L h^{-1} m^{-2}).

Figure 9.7:- Pressure Excursion for 30,000 MW
Ultrafiltration Membrane

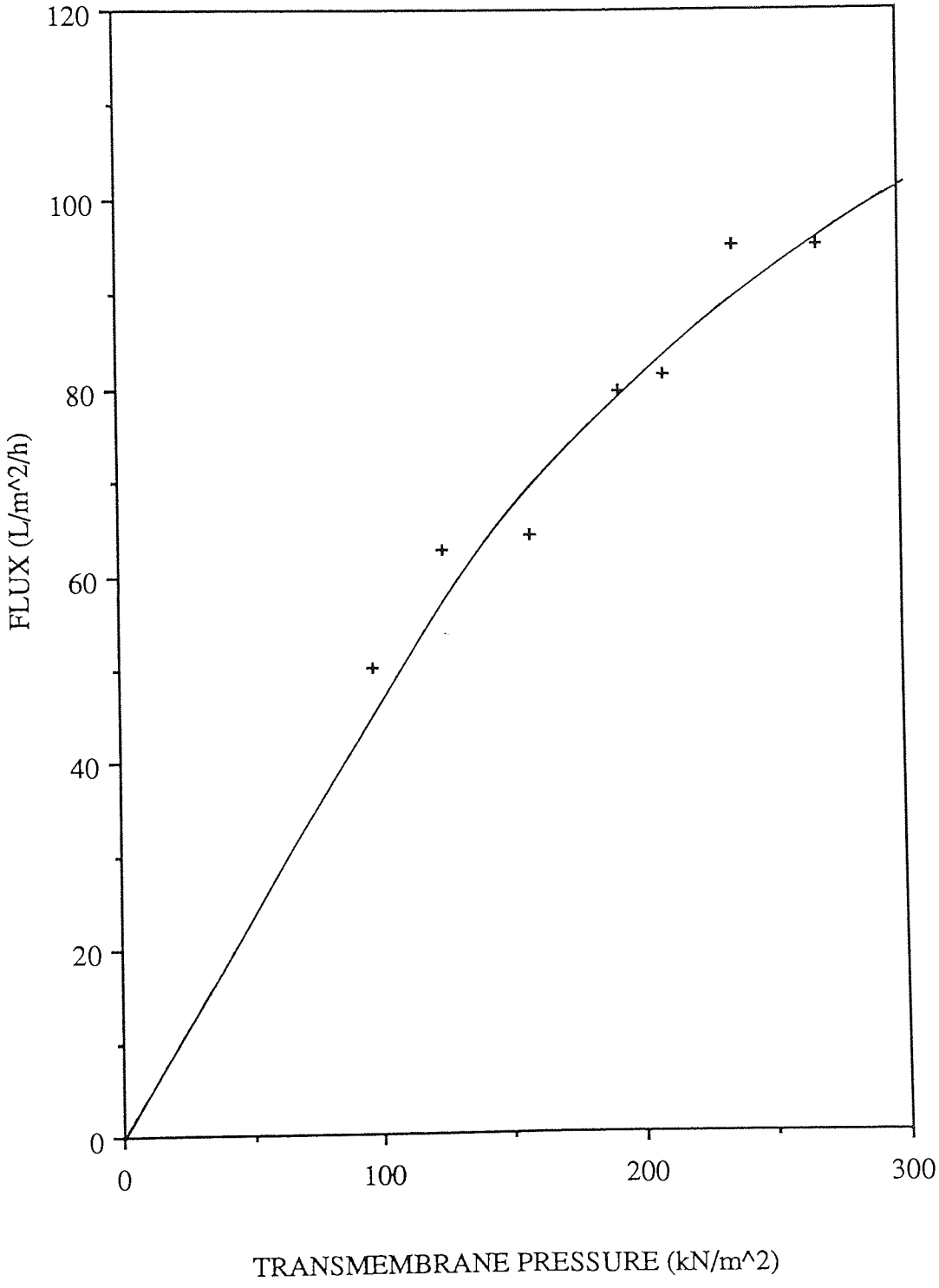
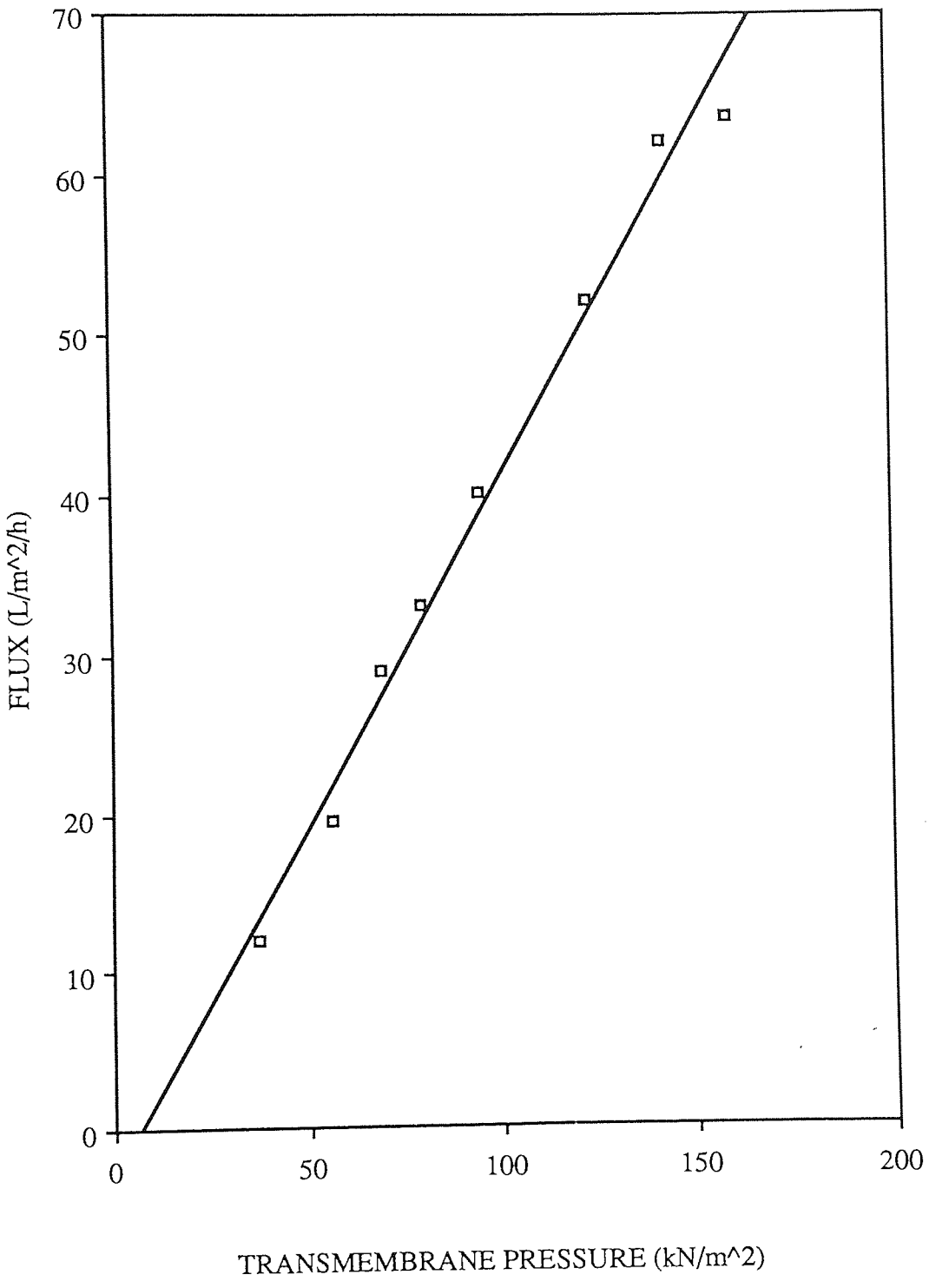


Figure 9.8:- Flow Exursion on the 30,000MW
Ultrafiltration Membrane



For the 0.2μm microporous membrane used in the microfiltration work, the exponential drop of flux with time was investigated under different transmembrane pressures and flowrates (Figure 9.9). It was observed that as the flux reached a steady state, the transmembrane pressure was proportional to the permeate flux at constant recirculation rate. The possibility of employing such a membrane for the separation of the cells from the enzyme molecules led us into investigating and comparing its performance with the established process of centrifugation.

9.3.4.1 Purification Results and Discussion

The purification of dextransucrase was carried out using the diafiltration mode as explained in Section 9.3.3.1. The enzyme was kept at ice temperature and the pH of the solution was constant at 5.2. An increase in temperature was observed which was due to the shearing force the enzyme solution experienced during its passage through the membrane system.

The results obtained using a ultrafiltration membrane (30,000 MW), are given in Tables 9.8 and 9.9. The purification of the enzyme was carried out using a six succesive diafiltrations. The total protein of the enzyme after purification was assayed at 80μg/cm^3. Approximately 99 percent of the low molecular weight solutes were removed and 71 percent enzyme recovery was obtained on the retentate side. A flux of 35 L hr^{-1} m^{-2} was achieved compared to 14 L hr^{-1} m^{-2} recorded for a similar membrane size and type on the New Brunswick system.

Figure 9.9:- Plot of Permeate Flux vs Time for the 0.2µm membrane on the Millipore X-Flow system

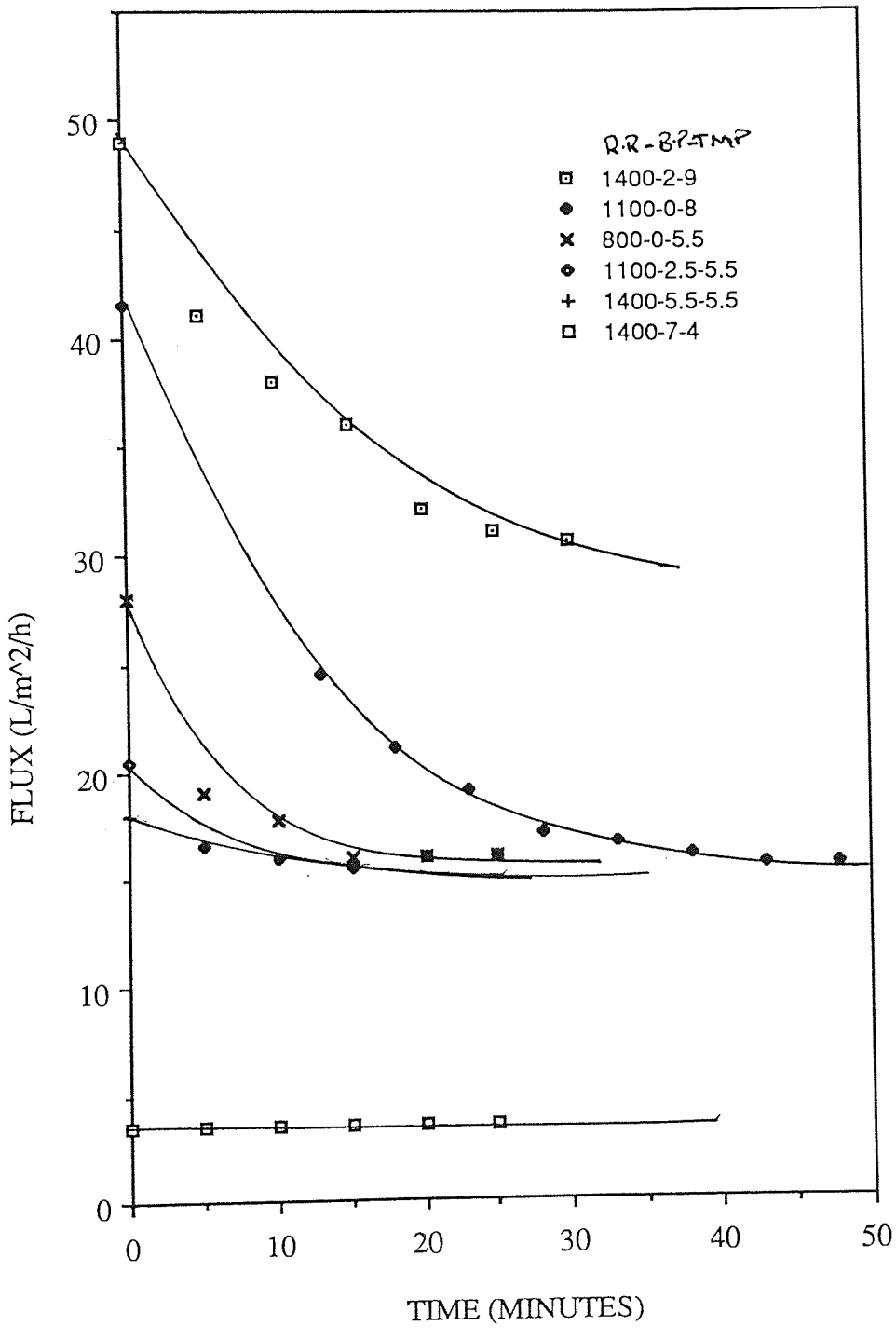


Table 9.8: Recovery of Enzyme Using the 30,000MW Membrane on the Millipore XF4 2000 System.

	Original Solution	Retentate After						Permeate After 6 Diafiltration
		1st Diaf	2nd Diaf	3rd Diaf	4th Diaf	5th Diaf	6th Diaf	
Volume cm^3	1000	1000	1000	1000	1000	1000	1000	3600
Activity DSU/cm^3	340	213	301	268	277	306	241	2.6
Total Activity DSU	340000	213170	301300	268480	276920	305900	241300	9360
% Enzyme Recovery	-	62.7	88.6	79	81	90	71	0.2

Table 9.9 : Solutes Removal on a 30,000MW Membrane on a Millipore XF4 2000 System

	Original Solution	Retentate After						Permeate After 6 Diafiltration
		1st Diaf	2nd Diaf	3rd Diaf	4th Diaf	5th Diaf	6th Diaf	
Volume cm^3	1000	600	600	600	600	600	600	1000
Concentration g/cm^3	0.2	0.155	0.104	0.049	0.02	0.0071	0.0044	0.002
% Removal		46	31.2	14.6	6.1	2.1	1.3	1.06
Overall percentage removal of	-	46	77.8	92.5	98.6	100	102	1.06

Table 9.10 : Enzyme Recovery Using a 0.2µm Membrane on a XF4 2000 Millipore System

	Original Solution	Permeate After Six Diafiltrations	Retentate After Six Diafiltrations
Volume cm^3	1000	360.0	10.0
Activity DSU cm3	312.5	53.02	106.9
Total Activity DSU	312470	190872	106920
Percentage Recovery of Enzyme	-	61	34.2

For the 0.2µm microfiltration membrane, Table 9.10 shows a set of typical purification results. The recovery of enzyme after six diafiltrations was 61 percent on the permeate side and 34 percent on the retentate side.

Although not all the enzyme can be recovered on the permeate side, the high percentage of enzyme achieved and the flexibility of the XF4 2000 system allowed it possible to investigate other factors that could affect the purification of the enzyme.

9.3.4.2. Effect of Transmembrane Pressure

The transmembrane pressure was varied by applying back pressure on the permeate side of the system. From Table 9.11 a decrease in transmembrane pressure resulted in a decrease in enzyme recovery on the permeate side. The highest recovery of enzyme occurred when no back pressure was applied. By applying a back pressure on the permeate side there is a tendency to increase the retention time of solute on the membrane surface which might result in fouling the membrane and thus inhibit the passage of solutes permeating through the membrane.

With the transmembrane pressure kept fairly constant increasing the flux by doubling the crossflow area resulted in a high recovery of enzyme.

Table 9.11 : Effect of Transmembrane Pressure on Enzyme Recovery

Transmembrane Pressure	Membrane Area cm^2	Flux $L\ m^{-2}\ h^{-1}$	% Enzyme Recovered in Permeate	% Enzyme Recovered in Retentate
9.7	464.5	26.3	61	34.2
7.1	464.5	12.9	47	23
4.8	464.5	8.6	32	32
7.3	929	22	84	17
6.2	929	7.6	44	39

9.3.4.3 pH Effect of the Processing Feed on Enzyme Recovery

The pH of the crude enzyme was altered to investigate what effect it would have during purification. Two different batches of feed were used (an anaerobic and an aerobic batches).

The pH was altered for each batch, i.e. pH 6.8 and pH 5.4 and the results are shown in Table 9.12. The pH experiments were carried out with enzyme batches produced either aerobically or anaerobically.

Table 9.12 : Effect of pH and Feed Type on Enzyme Recovery

Enzyme Batch Type	pH	% Enzyme Recovery in Permeate	% Enzyme Recovery in Retentate
Anaerobically Produced	5.3 7.2	61 91	34 23
Aerobically	5.4 6.7	33 59	39 56

An anaerobic batch with pH around 7 gave the highest enzyme recovery on the permeate side. From the above results it seems the type of feed used and its environmental condition is very important. The reason why the anaerobic batch gave a better yield than aerobic batch at same pH might be related to the enzyme history. However this needs to be substantiated.

The solubility of the enzyme at different pH values is one other factor that accounted for the change in enzyme recovery. The isoelectric point (which is the pH at which enzyme and protein molecules are neutrally charged) of dextransucrase was reported to be 4.9 (61). The low recovery of enzyme at pH 5.2 might be due to the charges on the enzyme molecules been repelled by the charges on the membrane surface thus not allowing them to permeate through. Raising the pH above the isoelectric point increased not only the solubility but also the passage of the protein through the membrane.

9.3.5 Microfiltration and Centrifugation

It was decided to compare the microfiltration and centrifugation techniques for the recovery of the enzyme dextransucrase.

The centrifugation was carried out on a MSE 5550 centrifuge operated at 10,000g with a temperature of 4°C. The pH of the feed solution was 6.9 and the temperature was kept below 20°C. An ultrafiltration process was then carried out on the supernatant to remove low molecular weight impurities. An overall enzyme recovery of 60% was achieved after the two processes.

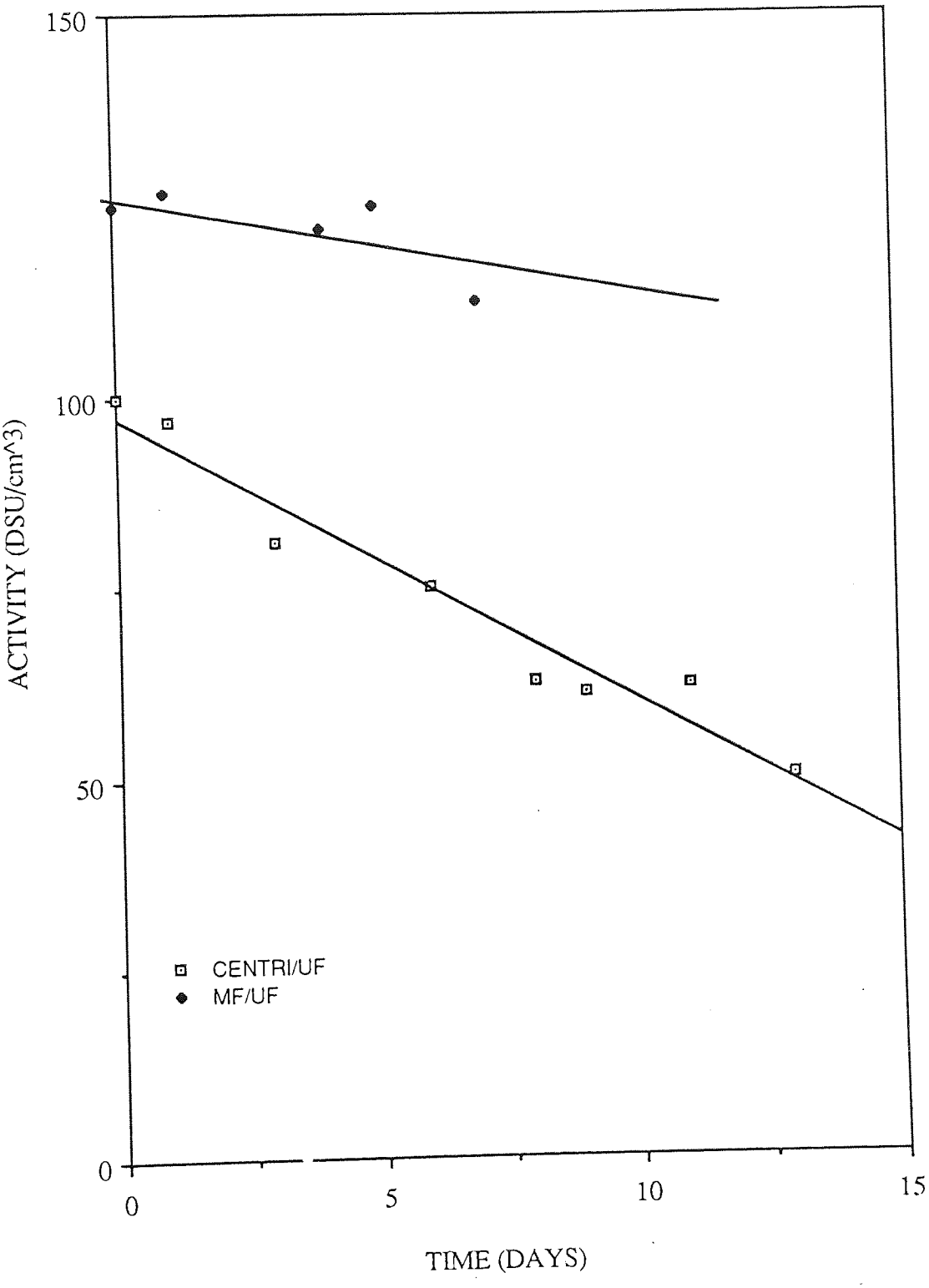
The microfiltration process was also carried out on a similar feed under the same conditions. The operating conditions of the microfiltration runs are reported in Section 9.3.4. An enzyme recovery of 80 percent was achieved on the permeate side. The permeate solution was then passed onto an ultrafiltration process. This led to an overall enzyme yield of 51 percent.

The processing time of the microfiltrtion/ultrafiltration combined operation was longer than the centrifugation/ultrafiltration combination. Due to the overall low enzyme recoveries for both purification combinations the ageing effect of the enzyme was studied to investigate which purification sequence had the lower long term effects on the enzyme. Figure 9.10 shows how the enzyme activity changes with time for each of the purification processes. The instability of the enzyme with time was more pronounced in the centrifuge/ultrafiltration process than the microfiltration/ultrafiltration process. The high shear forces generated during centrifugation have a long time effect on the enzyme molecules.

From these studies, the long processing time, the problems with fouling and the relatively low enzyme recoveries associated with the microfiltration/ultrafiltration process, prompted the use of centrifugation/ultrafiltration process.

The loss of activity experienced with the centrifugation/ultrafiltration process was overcome by storing the enzyme for less than two days before use. This results in only a small drop in enzyme activity i.e. less than 1%.

Figure 9.10:- The Ageing Effect of Dextransucrase after Purification.



9.4 ULTRAFILTRATION SCALE UP

Having established the use of the centrifugation/ultrafiltration purification process, its scaling up had to be considered to enable the production of the relatively high amounts of pure enzyme needed in the dextran production.

The development of the centrifugation process has already been discussed in Section 9.3.1.

From the study carried out on the ultrafiltration process on the Millipore (XF4 2000) system, the size of the system required was estimated, that is, the total membrane area needed and the pumping capacity. The equipment size is a function of flux. To optimise the flux, two factors need to be considered namely, the membrane fouling and the concentration polarisation effects. Membrane fouling is a steady state contamination of the surface of the membrane by feed-stream components and is only reversible by cleaning between runs. It is a function of membrane type and feed stream composition and is only slightly controlled by variation of transmembrane pressure (TMP) and tangential flow.

Concentration polarisation is a dynamic phenomenon caused by a concentration of substrate molecules forming at the membrane surface and creating a secondary membrane like layer. It is controlled by tangential flow across the membrane, which sweeps away the macromolecules layer of solute and re-establishes a homogeneous concentration. It is also increased by increasing the transmembrane pressure and this counteracts the normal hydrostatic flux increase. To optimise these two operational variables, a total recycle mode was used where both retentate and permeate were returned and mixed in the feed tank.

With a working capacity of 1 litre, and a recirculation rate of 81L h^{-1} an optimum transmembrane pressure of 27 psi (186 kN/m^2) and an average flux of 66L m^{-2} hr^{-1} was

achieved. These conditions were found to minimise the fouling and concentration polarisation affects. The pH was maintained around 6.8 to increase the solubility and thus the permeate flux. The processing time after six diafiltration operations was 135min. The concentration factor, defined as:-

$$C_F = \frac{\text{Initial feed stream volume}}{\text{Final feed stream volume}}$$

was 2.5.

The membrane area for a working capacity of 20L and a 1 hour processing time with a transmembrane pressure of 27 psi (186 kN m^{-2}) was calculated as follows:-

$$\text{Membrane area} = \frac{\text{Volume to process}}{\text{Average flux x process time}}$$

given a value of 0.303 m^2.

As the membranes are commercially available in a cassette form with each cassette having a membrane area of 0.4645m^2, a cassette was purchased for the processing of the enzyme purification.

Having established the membrane size and pumping capacity, the large scale ultrafiltration system was constructed. Figure 9.11 depicts the ultrafiltration system used for purifying dextransucrase for the biosynthesis of dextran from sucrose.

It consisted of a cold enzyme storage compartment, the membrane housing module (supplied by Millipore PLC) and a Watson Marlow (701 S/R) pump.

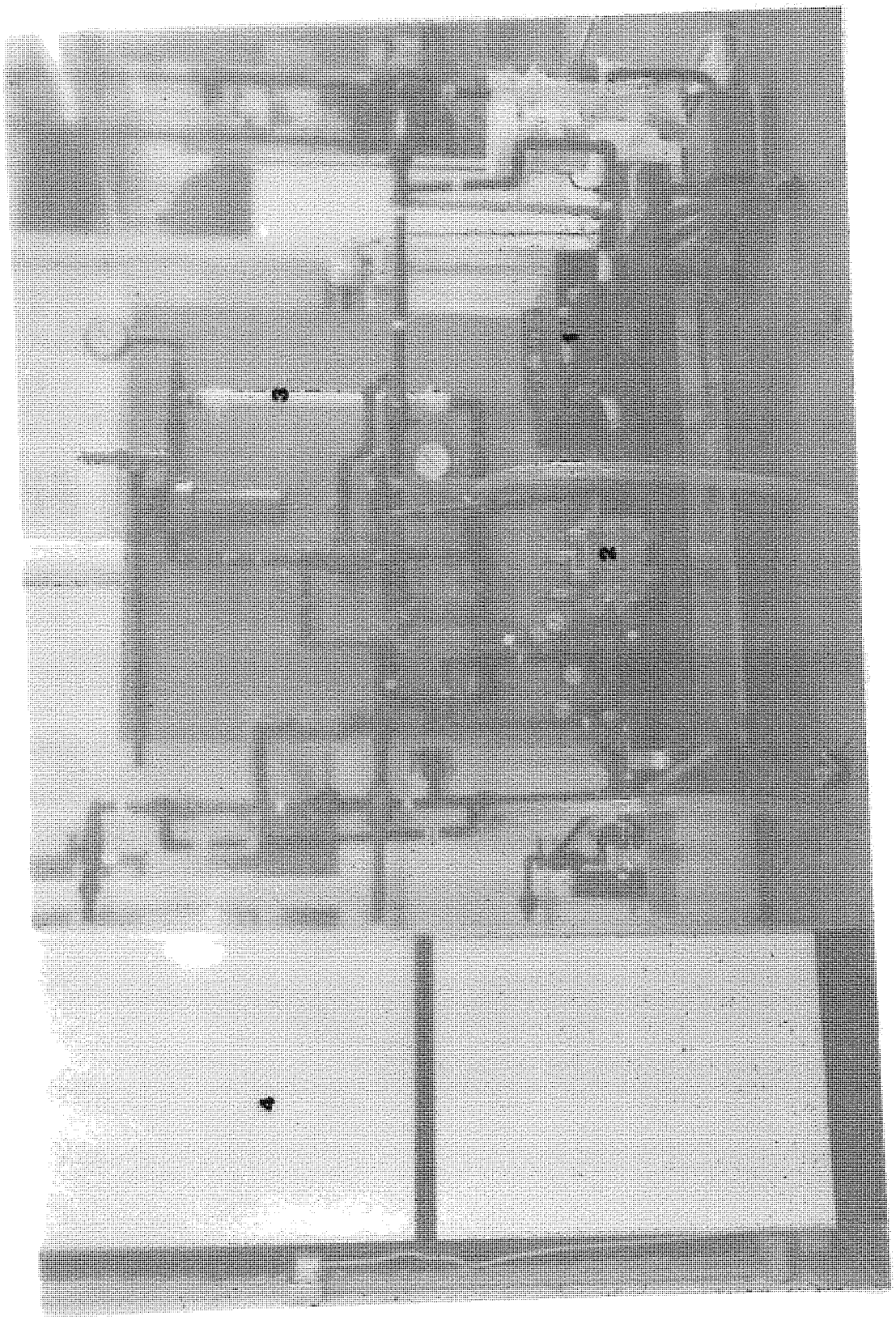
This large scale system was then commissioned and run with 20 litres of supernatant enzyme under the same operating conditions. An enzyme activity recovery of

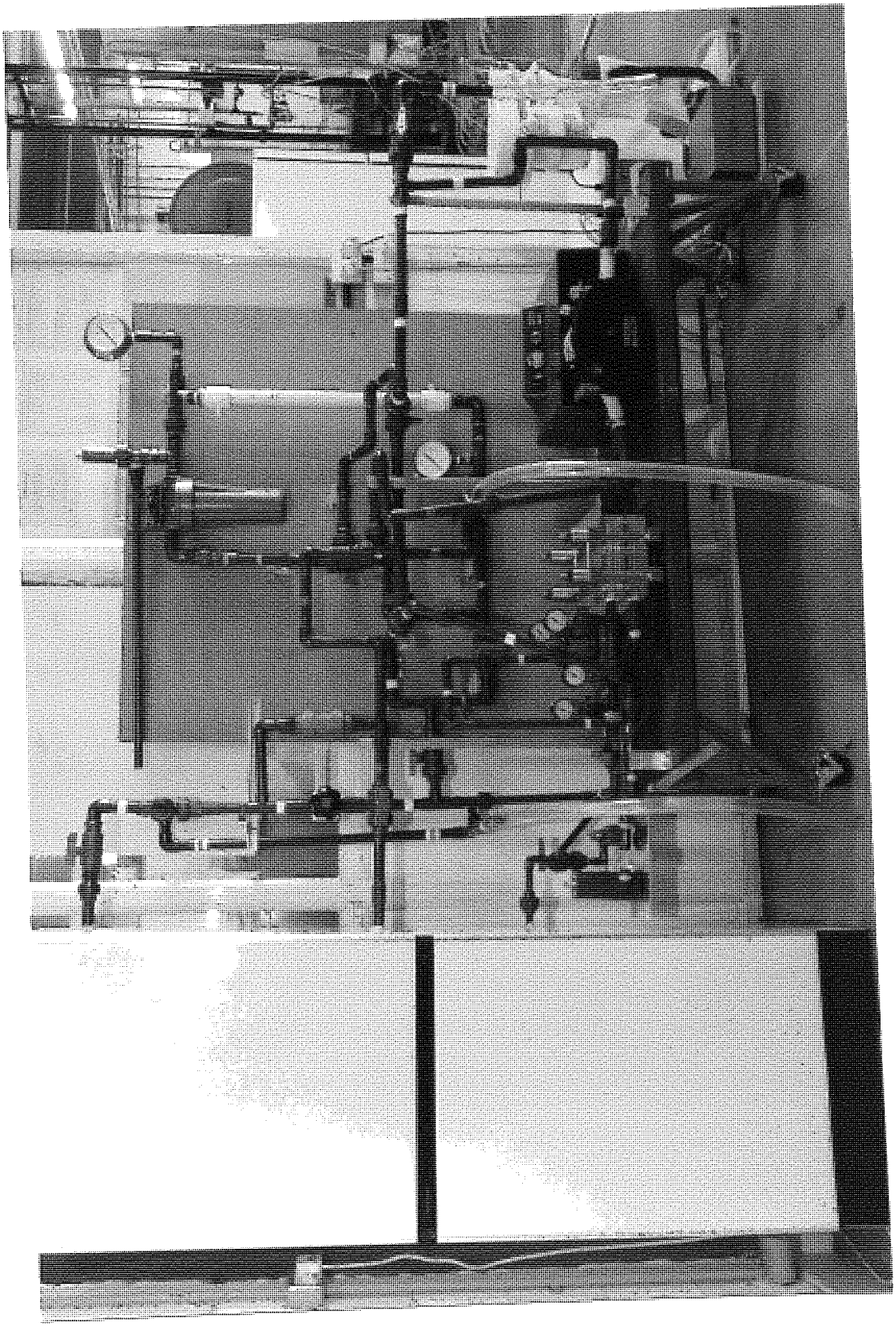
80 percent of the supernatant solution was achieved after six diafiltrations. Over 98 percent of the impurities present were removed.

Figure 9.11 Picture of the ultrafiltration membrane system

Key

1. Pump
2. Flat plate membrane module
3. Hollow fibre membrane module
4. Cold storage compartment





CHAPTER 10

BIOSYNTHESIS OF DEXTRAN ON THE SCCR-S1 SYSTEM

10.0 INTRODUCTION

To consider the versatility of the SCCR-S1 system as a combined reactor-separator, its operation was investigated by studying the biosynthesis of dextran. The bioreaction involved the conversion of sucrose in the presence of the enzyme dextransucrase to dextran and fructose. Fructose is known to act as an acceptor thus terminating the production of high molecular weight dextran [86]. The instantaneous removal of fructose from the reaction mixture by the chromatographic principle is expected to improve the molecular weight of the dextran produced. Work carried out by Zafar [25] and Ganetsos [9] on batch chromatographic reactor separators have shown that the percentage of molecular weight of dextran over 156,980 daltons was 77% at sucrose concentrations of 20% W/V compared with the conventional reactor of 43.7%.

For this part of the research work, the reaction kinetics of dextransucrase enzyme were studied and two commissioning runs were carried out on the SCCR-S1.

10.2 DEXTRANSUCRASE KINETICS

The rate of the dextransucrase action in the biosynthesis of dextran was investigated by measuring the initial reaction rate for a wide range of initial sucrose concentrations (1 - 15% W/V). This study was carried out in a batch mode at constant temperature (25°C) and constant pH (5.2). The enzyme activity (25 DSU/cm^3) was measured by the Hostettler's method (Chapter 6).

Figure 10.1 demonstrates that at low sucrose concentrations (< 7% W/V) the initial reaction velocity increases linearly with sucrose concentration and this corresponds to that of a first order reaction. At higher sucrose concentrations (> 10% W/V), a reduction in reaction rate occurs. It may be noted that the effect of substrate inhibition is minimal compared with the inversion of sucrose reaction using invertase enzyme at these concentrations.

Figure 10.1:- Plot of initial velocity vs sucrose concentration using dextransucrase enzyme

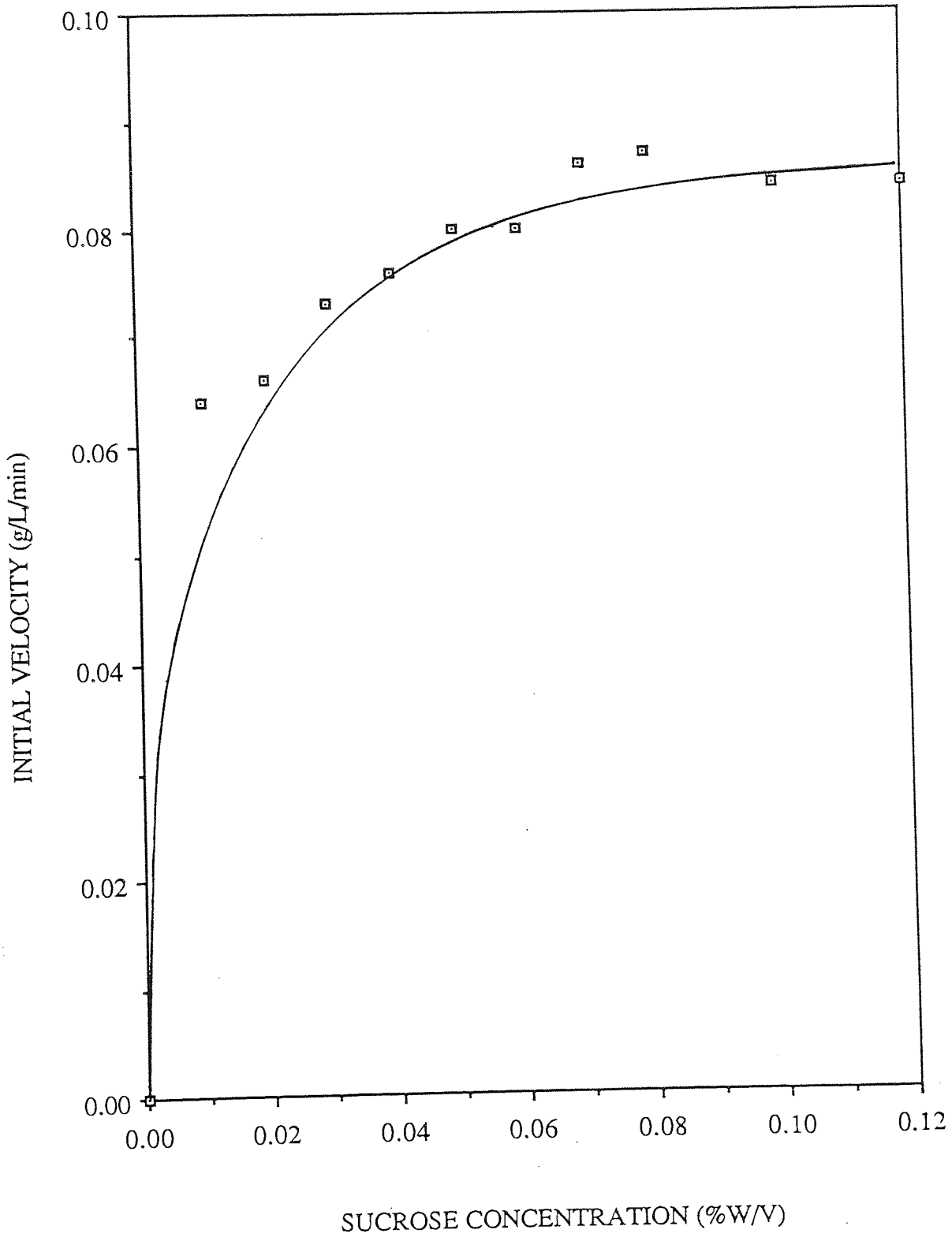
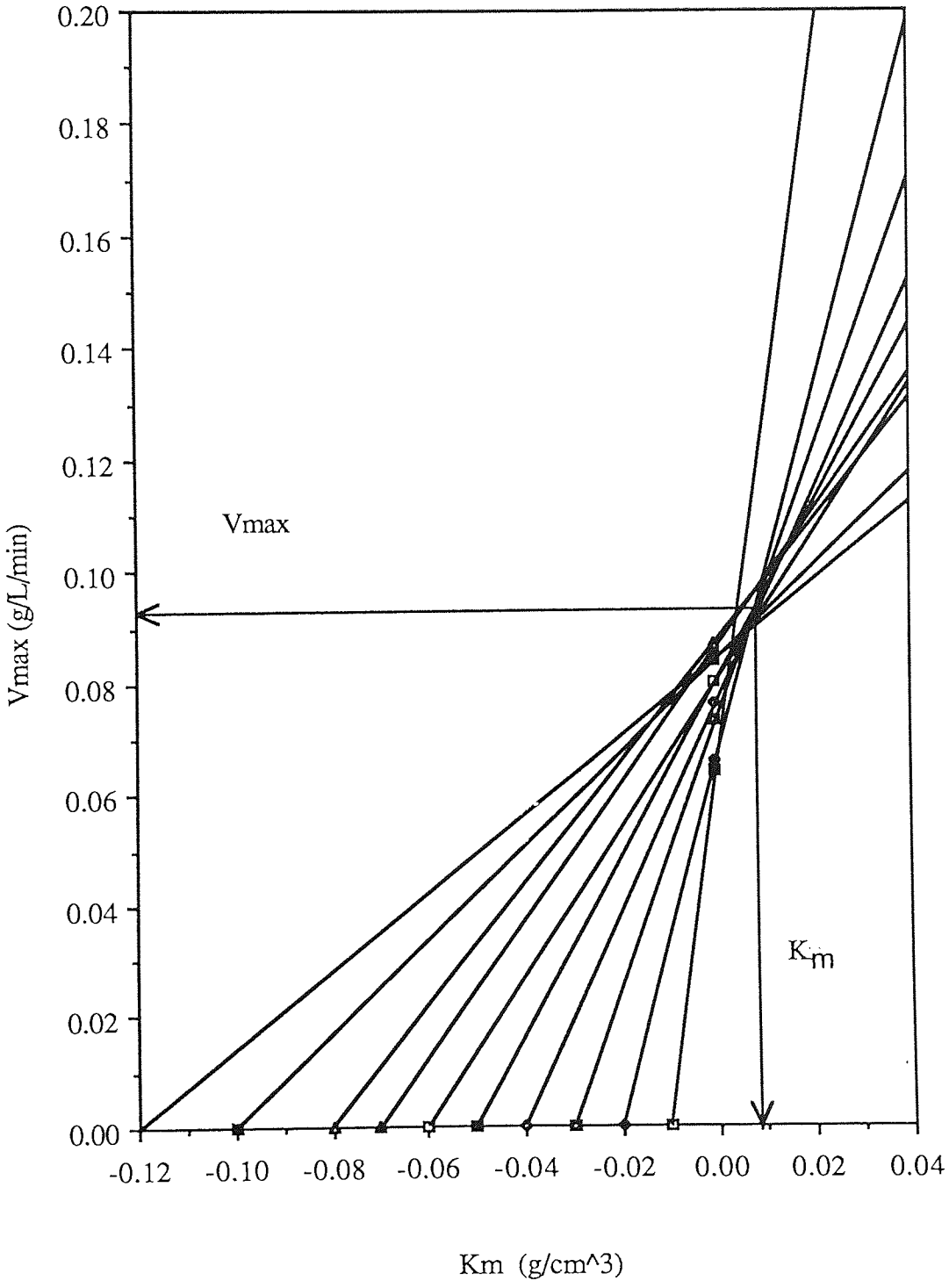


Figure 10.2:- Cornish Bowden Plot for Dextransucrase Enzyme



These results fit well with the Michaelis Menten kinetic model (Equation 3.1). The key parameters Vmax, the maximum initial reaction velocity and Km, the Michaelis constant were calculated using the Cornish Bowden plot [158] (Figure 10.2). This resulted in a K_m value of 6.8g/L and a Vmax value of 0.09 g/L/min. The Km value obtained is in agreement with some of the published work [97, 157].

For sucrose concentrations less than 5% W/V, that is the concentrations used in the commissioning biosynthesis of dextran experiments on the SCCR-S1 system, these kinetic results indicate that the reaction should not be the limiting step in the operation since the reaction velocity at these concentrations will be equivalent to a first order reaction.

10.3 COMMISSIONING AND OPERATING CONDITIONS

The SCCR-S1 system was purged with deionised water and recharged with calcium nitrate solution (10% W/V) before carrying out the biosynthesis reaction separation. This was to ensure the system was free of any unwanted substance that might contaminate the reaction and to replenish any displaced calcium ions on the resin material.

The choice of operating parameters was identical to the inversion reaction-separation parameters (Chapter 8), with the exception of the temperature, which was set at 25°C (i.e. the optimum temperature of reaction for dextransucrase [87]. The pH of 5.2, which was the pH of deionised water used in the SCCR-S1 system, corresponded to the optimum pH for the bioreaction.

10.3.1 Data Analysis

At the end of each run (i.e. after 12 cycles), the bulk product of the dextran rich product (DRP) and fructose rich product (FRP) were analysed. The HPLC analytical

system was used to give the component concentrations and the GPC analytical system to give the molecular weight distribution of dextran produced.

Due to the dilute nature of the product mixtures, the samples were concentrated by evaporation before they were injected into the HPLC system. The dextran product was very difficult to detect on the HPLC system, mainly for the reasons given in Chapter 6. A sample of each bulk product was dialysed and then analysed to give an estimation of the dextran concentration.

The percentage conversion of sucrose in the system was based on the amount of fructose produced or sucrose consumed. The fructose based results were obtained by dividing the actual concentration of the total bulk products (i.e. DRP and FRP products) by the expected concentration at 100% conversion. For example for a 2% W/V sucrose feed, the expected concentration for fructose conversion is 1.052% W/V.

A similar approach was used to work out percentage conversions based on sucrose. However, as noted by Zafar [25] and Ganetsos [9], the conversion calculation based on sucrose were higher than those obtained for fructose. This discrepancy was thought to be due to the following reasons. Small quantities of invertase were present causing some of the sucrose to convert to glucose and fructose. There is also a possibility of the acid hydrolysis of sucrose due to the slightly acidic conditions the SCCR-S1 system was operated at (i.e. pH 5.2). It has also been reported that calcium ions have the tendency of catalysing the isomerisation of glucose to fructose under the right conditions [66]. It is therefore possible that the calcium ion exchange on the chromatographic media caused some partial isomerisation in the system. The detection of glucose during analysis substantiated some of these effects. Another source of discrepancy between the conversion calculations reported above is analytical error. When low molecular weight dextrans are formed their retention times approach that of sucrose.

This is significant at high dextran conversions where an overlap between the two peaks was observed.

The activity of the enzyme was also measured before, during and after each run, using the Hostettler method. This was to check for any activity losses and to adjust the enzyme dosing pump accordingly to maintain a constant enzyme to substrate ratio. The actual amount of enzyme used in the system was compared with the theoretical amount of enzyme needed to convert the same amount of sucrose over the same time period which for this set of runs was 1 minute.

10.4 EXPERIMENTAL RESULTS AND DISCUSSION

Initially, a 20% W/V sucrose feed was used to carry out the biosynthesis for dextran on the SCCR-S1 system. After the first cycle, the pressure inside the system started building up with a pressure reading of approximately 419 kN m^{-2}. By the end of the second cycle the pressure had exceeded the maximum operating pressure set for the system (i.e. 523 kN-m^{-2}). This resulted in an automatic shut down of the equipment.

It was discovered that the build-up of high pressure in the system was caused by the formation of high molecular weight dextran during the biosynthesis reaction. The formation of high molecular weight dextran resulted in a highly viscous and slimy fluid which caused the resin particles to bind together thus forming lumps of solids inside the columns. This greatly affected the chromatographic characteristics of the resin particles and as such the whole system was repacked with fresh resin (KORELA VO7C) and recharacterised using the same method to that described in Chapter 5.

To reduce the problems of high pressure building up lower concentrations of sucrose in the feed were chosen for the following runs (i.e. Run:- 2.99-9-30-30-14-25 and Run 4.23-9-31.5-25-21-25). Up to twelve cycles were achieved for each run. Consecutive on-column concentration profiles indicated that equilibrium had not been

established. This was due to the continuously increasing pressure in the system which had a direct effect on the linear velocity of the mobile phase. Thus the time required to establish equilibrium was prolonged.

The purified enzyme dextransucrase used was found to agglomerate inside the mixing tank thus forming "agglomerates" which tended to block the liquid flow into the system. The use of guard columns on the eluent and purge lines and the use of a magnetic stirrer were found to overcome the above problem.

The results of the two runs are shown in Tables 10.1, 10.2 and 10.3. The high operating pressure in the system was caused by the high viscosity of dextran produced. This had a great effect on the mobile phase relative motion which accounted for the poor product purities achieved (Table 10.2). Figure 10.3 shows the on-column concentration profile for Run (2.99-9-30-30-14-25). It shows a large amount of sucrose present especially at the post feed section of the system, with low concentrations of fructose, dextran and glucose. The high concentration of sucrose present indicates that the total conversion was not obtained during the reaction. This was reflected in the bulk product concentrations in both the dextran rich product and fructose rich product streams (Table 10.2). An overall conversion of 53.4% sucrose was achieved. This low conversion was due to the fact that the actual enzyme used in the dextran synthesis was 2.6% of the theoretical amount compared to the amount of invertase enzyme used in the inversion reaction-separation studies which was 34% of the theoretical amount.

By assuming constant enzyme activity feed into the system the enzyme to substrate concentration ratio was found to be 1569 DSU/g. However, dextransucrase is known to be very unstable at temperatures over 23°C and a pH of 5.2 [86]. The conditions of operation (i.e. 25°C and pH 5.4) indicates a denaturation of dextransucrase which could partially account for the low conversion obtained.

To reduce the operating pressure, it was decided to repack the system with calcium charged resin of higher particle size (i.e. $\simeq$ 450μm). Run 4.23-9-31.5-25-21-25 was then carried out and the results obtained showed that at a slightly higher feed concentration (4.23% W/V), a 44.7% conversion (based on sucrose) and 19.4% conversion (based on fructose) was achieved. The reasons for the differences in the conversion rates stated above have been explained in Section 10.3.1. The relative amounts of enzyme used were similar to the first run with a higher enzyme to feed concentration ratio (1738 DSU/g).

There was not a good separation of products formed for the two runs carried out with the "cross over" point between the fructose and dextran profile being very close to the DRP (Figures 10.3 and 10.4). This is expected since complete conversion was not achieved.

The molecular weight distribution of the dextran produced (Table 10.3) showed that all of the dextran in the DRP was over 156,980 daltons with 99.6% over 4 million dalton (Run:- 2.99-9-30-30-14-25). Of the dextran present in the FRP, only 60% was above 156,980 daltons. This showed that some fractionation also took place.

Although from these sets of experiments more work is required to minimise the problem of high pressure build up in the system and to establish the factors that will affect system performance, the results obtained look very promising.

Table 10.1 Biosynthesis of Dextran Commissioning Runs - Operating Conditions

Experimental Run	Average Flowrates $cm^3\ min^{-1}$			Feed Conc.	Enzyme Activity	Total Enzyme To Feed Conc. Ratio	Pressure Readings $KN\ m^{-2}$			Cycle No	Enzyme Usage Actual
	Feed	Eluent Enzyme	Purge	% W/V	DSU cm^{-3}	U/g	Feed	Eluent	Purge		Actual / Theoretical
2.99-9-30-30-14-25	9	30	75	2.99	14	1561	209	418	161	12	0.026
4.23-9-31.5-25-21-25	9	31.5	75	4.23	21	1738	175	384	105	11	0.029

Table 10.2: Biosynthesis of Dextran Commissioning Runs - Results

Experimental Run	Feed throughput (sugar solid kg h^{-1}	Overall Percentage Conversion Based on S	F	DEXTRAN RICH PRODUCT Dextran Purity %	% Sucrose in Feed recovered	Total Product % W/V	Impurities % S	G	F	FRUCTOSE RICH PRODUCT Fructose Purity %	%Sucrose in Feed recovered	Total Product % W/V	Impurities % D	S	G
2.99-9-30-30-14-25	0.016	53.42	27.5	6.6	43.8	0.4	69.5	16.2	7.62	43.75	2.78	0.08	12.5	31.25	12.5
4.23-9-31.5-25-21-25	0.023	44.7	19.4	10.45	47.2	0.52	85.2	2.4	4.2	35.5	8.05	0.13	28.8	35.75	13.43

Table 10.3 : Molecular Weight Distribution of Dextran Product

Experimental Run	% DEXTRAN IN EACH MOLECULAR WEIGHT RANGE									
	DEXTRAN RICH PRODUCT					FRUCTOSE RICH PRODUCT				
	> 4,131,198	2,131,191 to 4,131,198	1,027,302 to 2,131,191	156,980 to 1,027,302	< 156,980	> 4,131,198	2,131,191 to 4,131,198	1,027,302 to 2,131,191	156,980 to 1,027,302	< 156,980
2.99-9-30-30-14-25	99.58	0.42	-	-	-	16.34	8.92	14.56	21.89	38.29
4.23-9-31.5-25-21-25	66.83	24.3	8.83	1.04	-	6.68	10.55	10.91	15.31	56.55

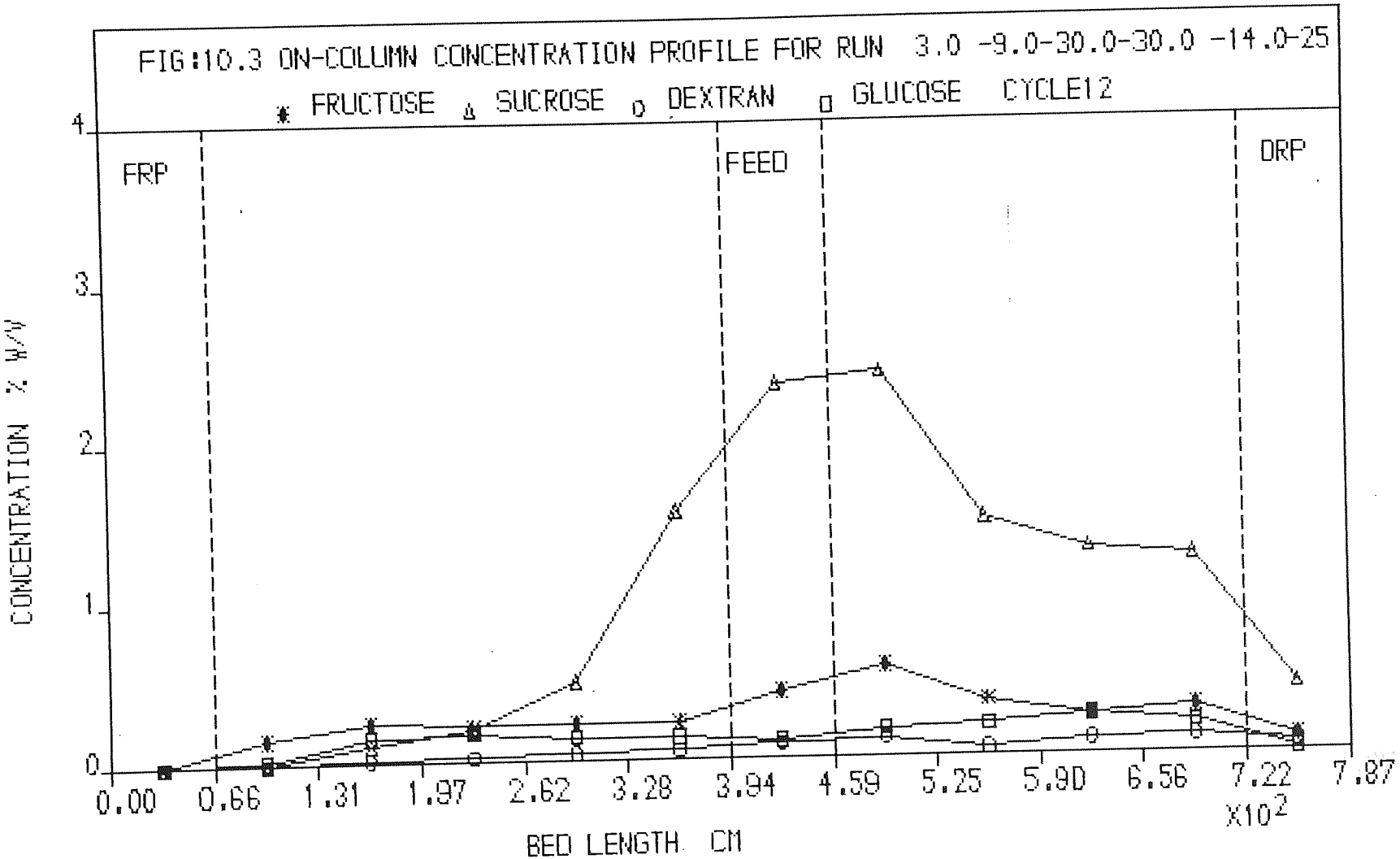


FIG:10.3 ON-COLUMN CONCENTRATION PROFILE FOR RUN 3.0 -9.0-30.0-30.0 -14.0-25
* FRUCTOSE Δ SUCROSE o DEXTRAN □ GLUCOSE CYCLE12
FRP
FEED
DRP
CONCENTRATION % W/V
0
1
2
3
4
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10-2
BED LENGTH CM

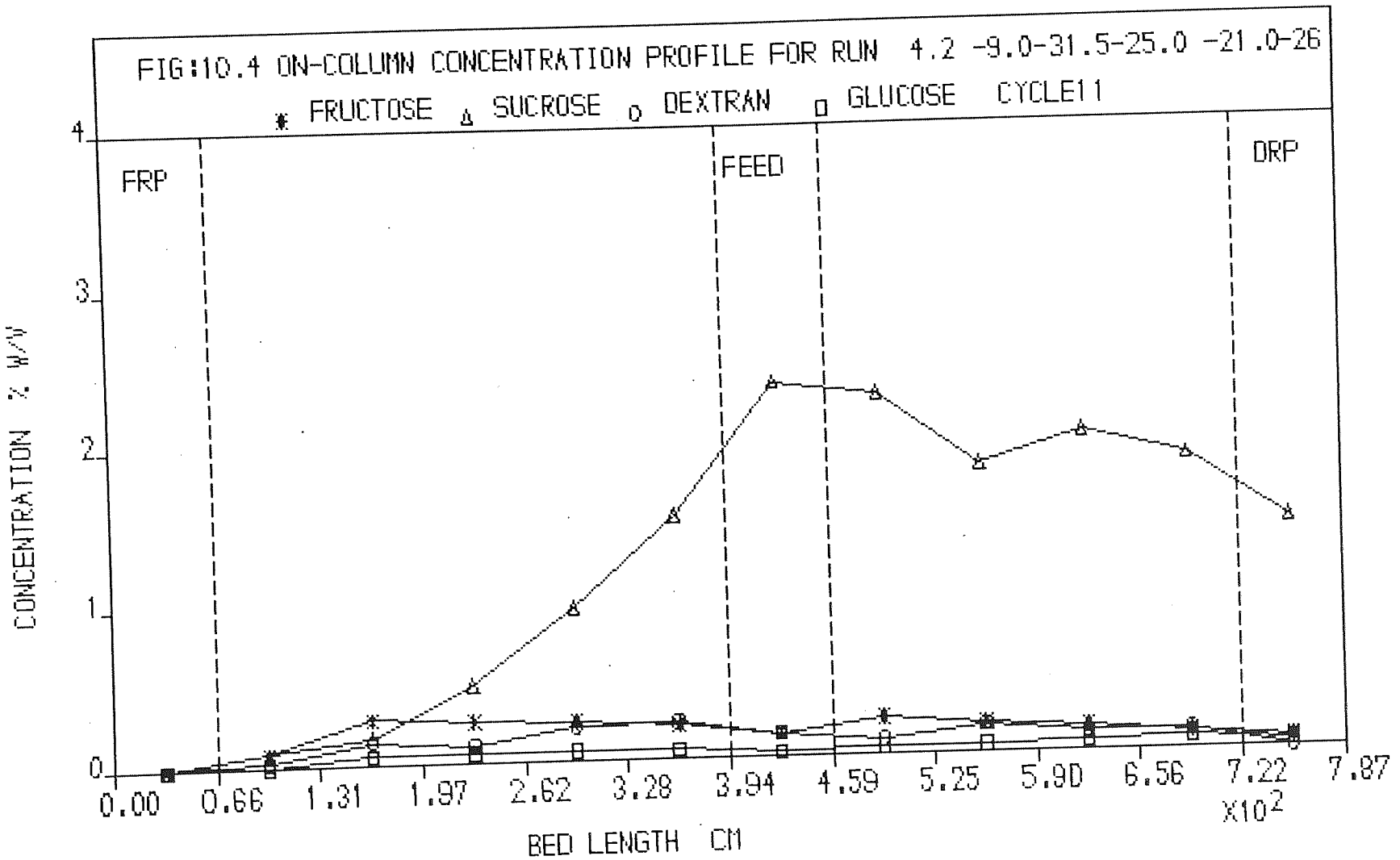


FIG:10.4 ON-COLUMN CONCENTRATION PROFILE FOR RUN 4.2 -9.0-31.5-25.0 -21.0-26
* FRUCTOSE Δ SUCROSE ○ DEXTRAN □ GLUCOSE CYCLE11
FRP
FEED
DRP
CONCENTRATION % W/W
0
1
2
3
4
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10^2
BED LENGTH CM

CHAPTER 11

COMPUTER MODELLING

11.1 INTRODUCTION

A number of mathematical models have been developed to simulate batch chromatographic reactors [4, 29, 149, 153] with the majority of them being based on the chromatographic reactor definition of Langer et al. [4] . A summary of the simulation effects by previous workers is given in Table 11.1. Most of these models refer to chemical reactions which are reversible with the aim of achieving a conversion higher than an equilibrium conversion. Zafar [25] developed a model to simulate a combined chromatographic biochemical reaction-separation on a batch chromatographic column. He studied the biosynthesis of dextran and fructose. His model, which was also based on Langer's work assumed linear adsorption of components and that reaction mainly takes place in the mobile phase. The model uses finite differences analysis to solve the set of partial differential equations derived from mass balances. The simulated results showed reasonably good agreement with the experimental results at low pulse sizes.

Some modelling work on a continuous combined reactor-separator system have also been carried out, Ching et al [155] carried out some work using a fluidised bed reactor-separator studying the isomerisation of glucose to fructose in the presence of immobilised glucose isomerase. Using an axial dispersion model, a set of non-linear ordinary differential equations derived from mass balances were solved numerically using a multiple shooting method. A good fit of the experimental and predicted concentration profile was obtained with high glucose conversion.

Petroulas et al [38] using a chemical reversible reaction A $\rightleftharpoons$ B namely the hydrogenation of mesitylene (MES) to 1, 3, 5-trimethylcyclohexane (TMC) simulated a counter current moving bed chromatographic reactor. The behaviour of an ideal reactor model was examined for different values of feed concentrations and reactor lengths. They predicted that reaction and separation can be achieved simultaneously and that under appropriate operating conditions, a reactor fed at the bottom with the feed mixture is more

favoured by thermodynamic equilibrium and can lead to 100% product purity with overall conversions lower than with a conventional fixed bed reactor.

Other mathematical models carried out on continous chromatographic reactor-separators apply only to separation processes. Hashimoto et al [156] used a simulated moving bed adsorber parked with ion-retardant resin to predict the concentration profiles of a mixture of glucose/NaCL. Two kinds of mathematical models were proposed. The intermittent moving bed model and a continuous moving bed model were used to calculate concentration profiles in the adsorber where the adsorption isotherm of the first component (NaCL) and the second component (glucose) are represented by the Langmuir equation and by a linear equation respectively. The validity of the calculation methods proposed was experimentally confirmed.

However based on the literature, all of the mathematical modelling work carried out on the semi-continuous chromatographic systems (SCCR) were also applied in the separation field. The plate theory concept has been used [9, 10, 105, 154].

For this research work, the aim was to develop a mathematical model and simulate the bioreaction-separation for a SCCR-S1 System. The inversion of sucrose and separation of its products (glucose and fructose) was considered.

Table 11.1 : Modelling of Chromatographic Reactors

Author	Reaction	Adsorption	Axial Dispersion	Input	Experimental Verification
Magee (149)	A⇌B + C	Linear	Negligible	Pulse	No
Chu(151)	A⇌B + C	Langmuir Hinshlewood	Yes	Delayed sinewave	No
Gore (29)	A⇌B +C	Linear	Negligible	Delayed sinewave Gausian	No
Wetherold(150)	A⇌B + C	Freundlish	Negligible	Rectangular Pulse	Yes
Schweich (152)	A⇌B + C	Linear	Yes	Rectangular Pulse	Yes
Zafar (25)	A→ B + C	Linear	Negligible	Pulse	Yes
Petroulas (38)	A⇌B	Langmuir	Negligible	Continuous Feed	Yes
Ching (155)	A⇌B	Linear	Yes	Continuous Feed	Yes

11.2 MATHEMATICAL MODELLING APPROACH

11.2.1 Assumptions

In developing a material balance (or continuity equation) for each of the components in the chromatographic reactor separator over a plate the following assumptions were made:

(1) Constant velocity (i.e. constant fluid density).

(2) Flat concentration and velocity profile.

(3) Adsorption of component is linear and can be defined by Kd = P/C.

(4) Negligible axial dispersion.

(5) Adsorption of a component is independent of its own concentration and is independent of the concentration of other components.

(6) Steady State.

(7) Conversion of sucrose to glucose and fructose according to Michealis-Menten kinetics.

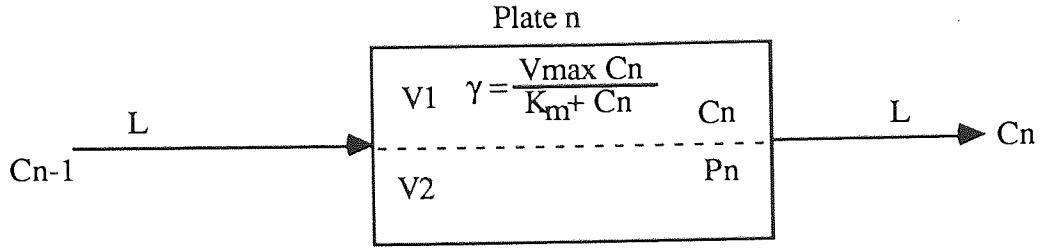
(8) The reaction only takes place in the mobile phase.

(9) Equal molar quantities of fructose and glucose are produced for every molar of sucrose consumed.

(10) The whole separating length of the SCCR-S1 system consists of a number of theoretical plates, each containing a volume of mobile phase and a volume of stationary phase. The mobile phase leaving each plate is at equilibrium with the stationary phase in the plate.

11.2.2 Mass Balance

From the above assumptions a mass balance was carried out for all the components over plate 'n'. The condition around plate 'n' may be represented by:



Consider a mobile phase flowrate L, passing through the plate "n" having an initial solute concentration of Cn-1 and an exit solute concentration of Cn, with the biochemical reaction γ taking place only in the mobile phase, a mass balance for the reactant (sucrose) over the plate "n" gives:

$$V_1 \frac{dCn}{dt} + V_2 \frac{dPn}{dt} = LC_{n-1} - LC_n - \gamma V_1 \qquad 11.1$$

where:

C	=	sucrose concentration in the mobile phase
P	=	sucrose concentration in the stationary phase
V_1, V_2	=	plate volume of mobile and stationary phases respectively
L	=	mobile phase flow rate
γ	=	reaction rate

The reaction rate is assumed to follow the Michaelis-Menten equation and can be expressed as:-

$$\gamma = \frac{Vmax\ Cn}{K_m + Cn} \qquad 11.2$$

where:

Vmax	=	Maximum reaction velocity
K_m	=	Michaelis-Menten Constant.

Equilibrium on the plate is represented by the distribution coefficient Kd, whereby:-

$$Kd_n = \frac{Pn}{Cn} \qquad 11.3$$

Substitution of equation 11.2 and 11.3 into equation 11.1 yields:-

$$(V_1 + V_2\, Kd_s)\, \frac{dCn}{dt} = LC_{n-1} - LC_n - \frac{V_1 \,.\, Vmax\, Cn}{K_m + Cn} \qquad 11.4$$

A similar mass balance for glucose (G) and fructose (F) gives:-

$$(V_1 + V_2\, Kd_G)\, \frac{dGn}{dt} = LG_{n-1} - LG_n + \frac{0.5V_1\, Vmax\, C_n}{K_m + C_n} \qquad 11.5$$

$$(V_1 + V_2\, Kd_F)\, \frac{dFn}{dt} = LF_{n-1} - LF_n + \frac{0.5V_1\, Vmax\, C_n}{K_m + C_n} \qquad 11.6$$

11.2.3 Method of Solution

Equations 11.4, 11.5 and 11.6 are sets of first order non linear differential equations which were solved by using the Runge-Kutta-Merson method. The program was found to consume an excessive amount of computing time (i.e. CPU = 28,800 secs) on the VAX Cluster. Therfore this approach was not followed.

To simplify equations 11.4 to 11.6 the following assumption was made regarding the Michaelis-Menten kinetic equation (11.2). A pseudo-first order reaction for all feed concentrations (i.e. $\gamma = \frac{Vmax}{K_m} Cn$) was assumed. To simplify however, the enzymatic reaction kinetics which follow Michaelis-Menten equation (Equation 3.1), a zero order reaction is commonly used. At high substrate concentrations the inversion reaction kinetics studies (Chapter 8) showed that substrate inhibition does have a considerable effect on the rate of inversion with maximum reaction velocity attained at 9.75% W/V sucrose concentration. Above this concentration there is a sharp drop in the initial reaction velocity (Figure 8.1) compared to the constant or slight fall in the initial reaction

velocity of other enzymatic reactions (Figure 3.2). This indicates that the approximation to a zero order reaction cannot be justified.

Equations 11.4 to 11.6 were therefore modified as follows:-

$$(V_1 + V_2 Kd_s) \frac{dC_n}{dt} = LC_{n-1} - LCn - \frac{V_1 Vmax\ Cn}{Km} \qquad 11.7$$

$$(V_1 + V_2 Kd_G) \frac{dG_n}{dt} = LG_{n-1} - LGn + \frac{0.5\ V_1 Vmax}{Km} Cn \qquad 11.8$$

$$(V_1 + V_2 Kd_F) \frac{dF_n}{dt} = LF_{n-1} - LFn + \frac{0.5\ V_1 Vmax}{Km} Cn \qquad 11.9$$

Equations 11.7 to 11.9 are linear first order differential equations and can be solved analytically using Laplace transforms to give:

$$Cn(t) = \beta C_{n-1} - \beta exp - [(\alpha_s + \gamma_s)\ \Delta t] + Cnoexp - [(\alpha_s + \gamma_s)\ \Delta t] \qquad 11.10$$

$$Gn(t) = G_{n-1} [1 - exp [-\alpha_G \Delta t]] + \gamma_G\ Cn\ exp [-\alpha_G \Delta t] + G_{no}\ exp [-\alpha_F \Delta t] \qquad 11.11$$

$$Fn(t) = F_{n-1} [1 - exp [-\alpha_F \Delta t]] + \gamma_F\ .\ Cn\ exp [-\alpha_F \Delta t] + F_{no}\ exp [-\alpha_F \Delta t] \qquad 11.12$$

where:

$$\beta = \frac{L}{L + \frac{V_1\ .\ Vmax}{Km}}$$

$$\alpha_n = \frac{L}{V_1 + V_2 Kd_i}$$

$$\gamma_n = \frac{V_1 \, Vmax}{K_m (V_1 + V_2 Kd_i)}$$

The zero order reaction ($\gamma = Vmax$) was used to test the assumption of first order reaction for all feed concentrations in inversion reaction separations. The analytical solution for zero order reaction of the respective concentration expressions give:

$$C_{n(t)} = C_{n-1} \left[1-\exp\left[-\alpha_S \Delta t\right]\right] - \emptyset \left[1-\exp[-\alpha_S \Delta t]\right] + C_{no} \exp[-\alpha_S \Delta t] \qquad 11.13$$

$$G_{n(t)} = G_{n-1} \left[1-\exp\left[-\alpha_G \Delta t\right]\right] + 0.5\, \emptyset \left[1-\exp[-\alpha_G \Delta t]\right] + G_{no} \exp[-\alpha_G \Delta t] \qquad 11.14$$

$$F_{n(t)} = F_{n-1} \left[1-\exp\left[-\alpha_F \Delta t\right]\right] + 0.5\, \emptyset \left[1-\exp[-\alpha_F \Delta t]\right] + F_{no} \exp[-\alpha_F \Delta t] \qquad 11.15$$

where:

$$\emptyset = \frac{V_1 \, . \, Vmax}{L}$$

The first term on the right hand side of Equations 11.10 to 11.15 represents the material-transferred from plate (n-1), the second term the reaction taking place in the n^{th} plate and the third term represents the material already present in the n^{th} plate.

When these equations are applied to the post-feed section, they are modified to account for the feed concentration, C_f and feed flowrate F as follows:- the term C_{n-1} is replaced by the ratio:

$$\frac{L C_{n-1} + F C_F}{L + F} \qquad 11.16$$

For the purging column where no reaction is taking place, Equations 11.4 and 11.6 are modified without the reaction term and solved analytically to give:

$$A_n = A_{n-1}\left[1-\exp[-\alpha_n \Delta_E]\right] + A_n o \exp[-\alpha_n \Delta E] \qquad 11.17$$

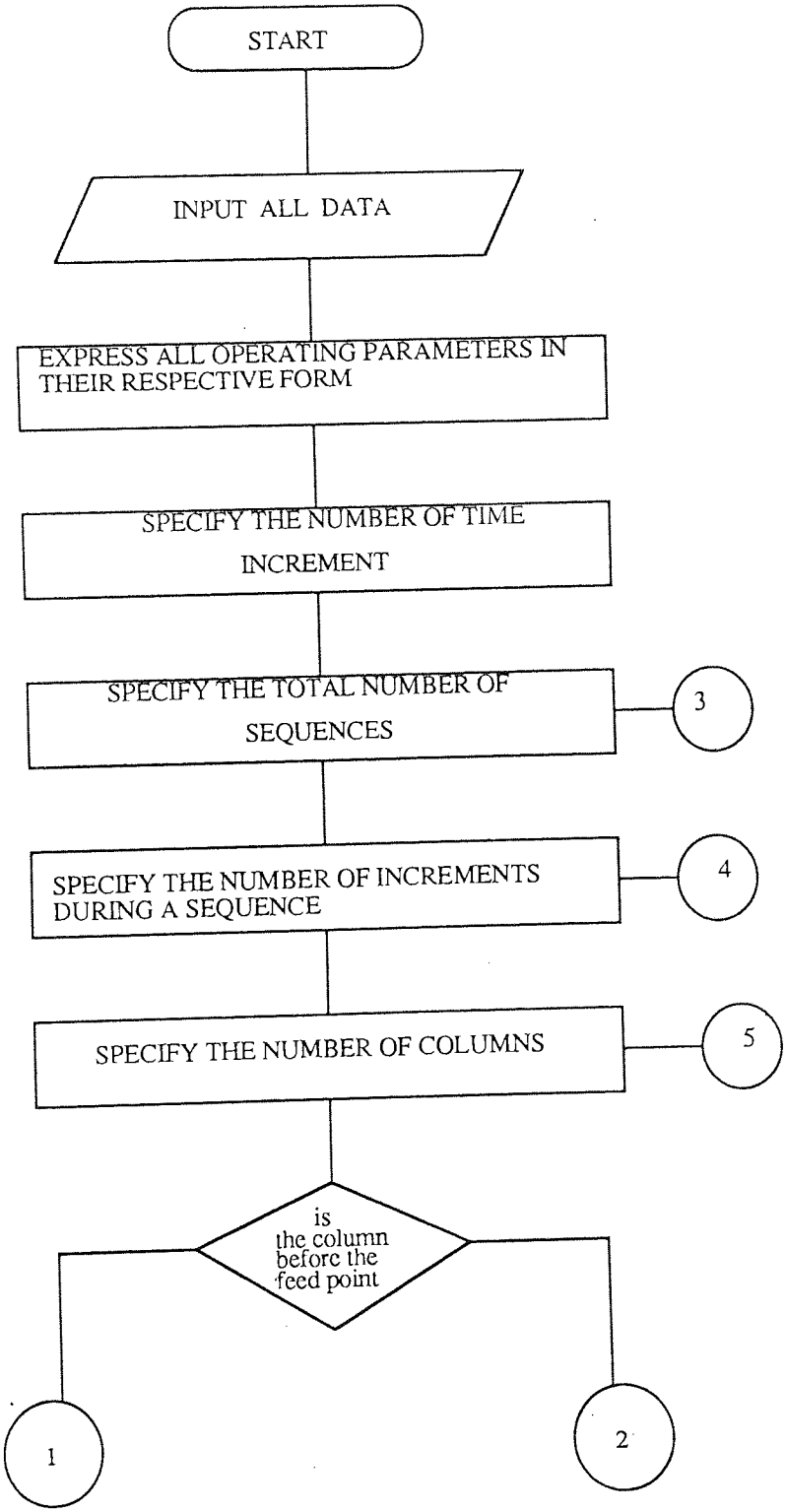
where A_n is the concentration of the components present in the n^{th} plate (i.e. sucrose, glucose and fructose).

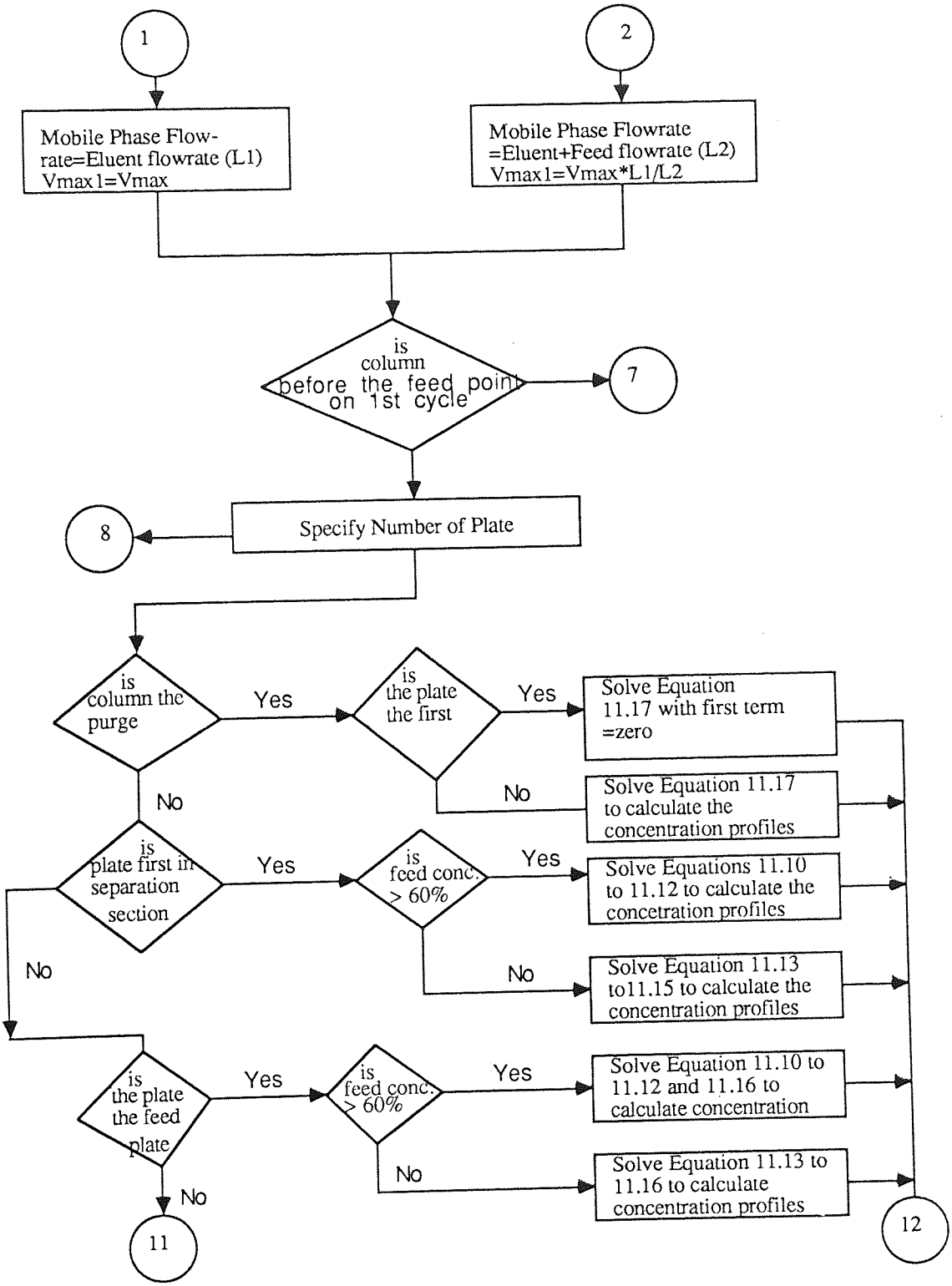
The model predicts the solute concentration in the mobile phase leaving each theoretical plate over a small time increment Δt, and calculations are repeated over the total number of increments, when this predetermined total number of increments, which is equal to the switch period, has been reached, the sequencing counter-current action is simulated by stepping the concentration calculation by one column. The stepping process then continues until a predetermined number of cycles has been completed.

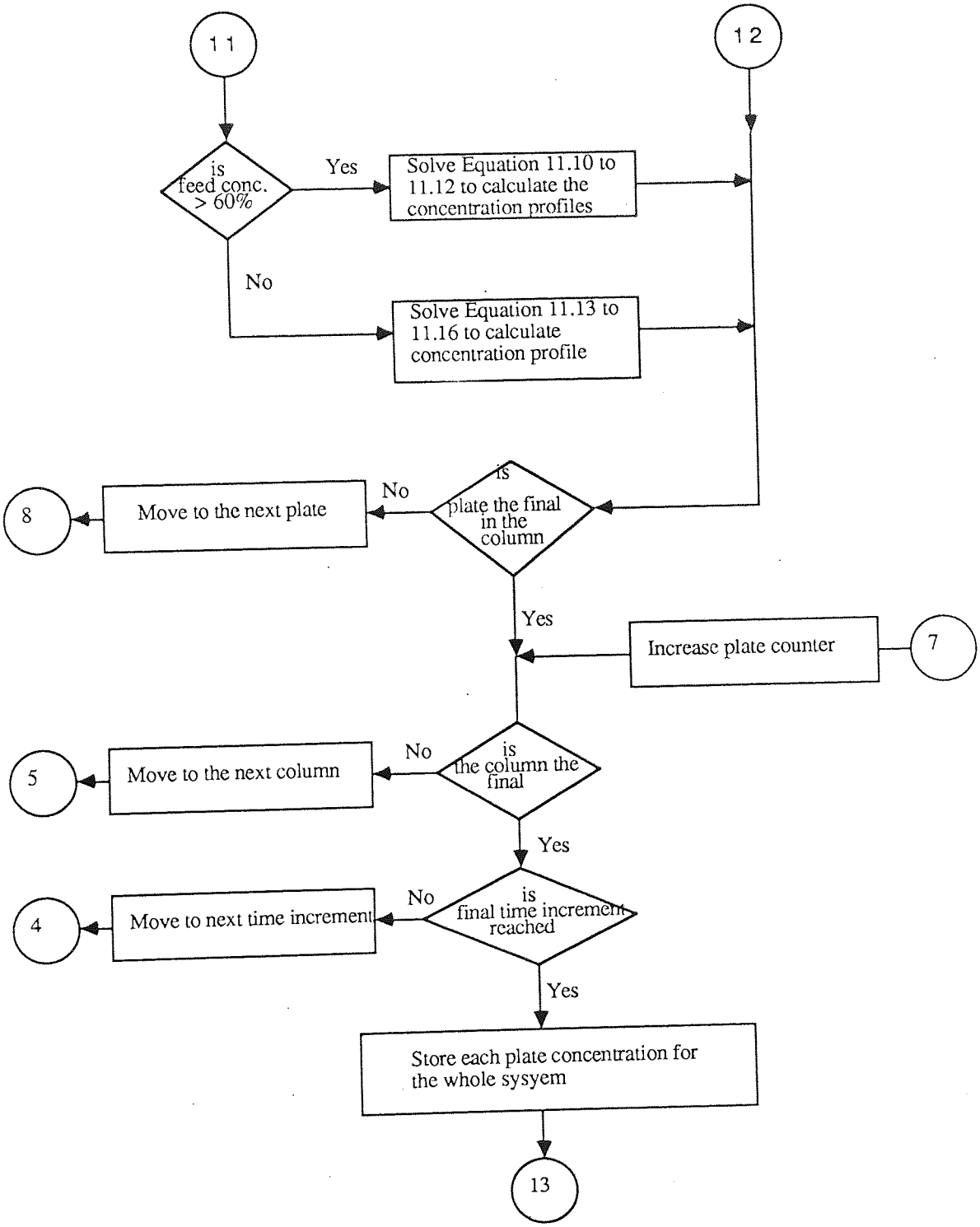
11.3 COMPUTER PROGRAMME

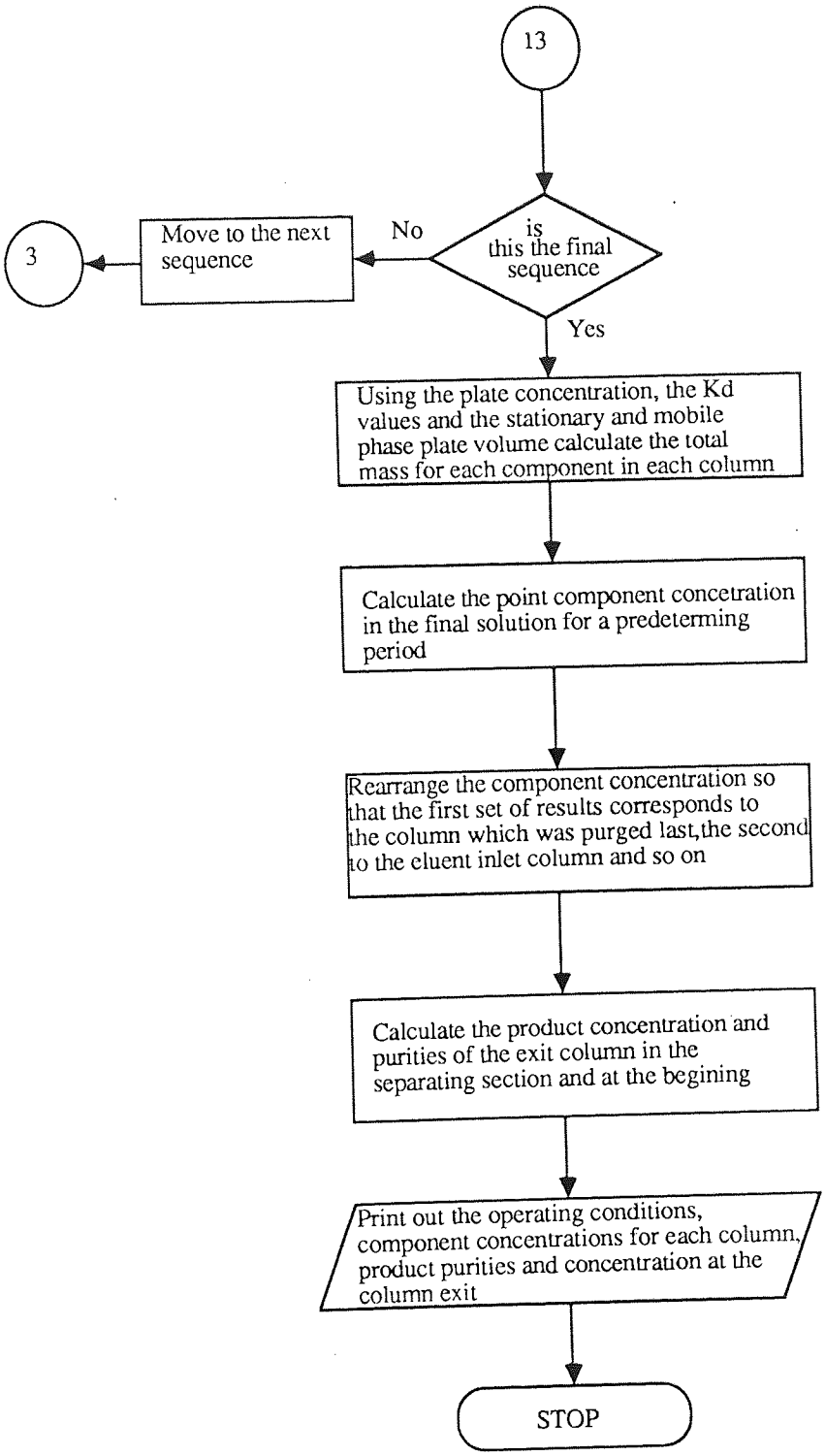
The simulation programme originated from the programme developed by Gould (154) for separation processes on the SCCR4 System. His programme was modified by incorporating the differential Equations 11.10 to 11.17. The rather simpler Gould version was chosen in this work as a basis rather than the more complicated developments by Thawait (105) and Ganetsos (9) since the modelling of continuous bioreaction-separation systems was being carried out for the first time. The computer programme is listed in Appendix C and the flowchart is provided in Figure 11.1.

Figure 11.1:- Computer flow chart for the simulation of the continuous operation of the SCCR-S1 system




1
2
Mobile Phase Flow-rate=Eluent flowrate (L1) Vmax1=Vmax
Mobile Phase Flowrate =Eluent+Feed flowrate (L2) Vmax1=Vmax*L1/L2
is column before the feed point on 1st cycle
7
8
Specify Number of Plate
is column the purge
Yes
is the plate the first
Yes
Solve Equation 11.17 with first term =zero
No
Solve Equation 11.17 to calculate the concentration profiles
No
is plate first in separation section
Yes
is feed conc. > 60%
Yes
Solve Equations 11.10 to 11.12 to calculate the concetration profiles
No
Solve Equation 11.13 to11.15 to calculate the concentration profiles
No
is the plate the feed plate
Yes
is feed conc. > 60%
Yes
Solve Equation 11.10 to 11.12 and 11.16 to calculate concentration
No
Solve Equation 11.13 to 11.16 to calculate concentration profiles
No
11
12


11
12
is feed conc. > 60%
Yes
Solve Equation 11.10 to 11.12 to calculate the concentration profiles
No
Solve Equation 11.13 to 11.16 to calculate concentration profile
8
Move to the next plate
No
is plate the final in the column
Yes
Increase plate counter
7
5
Move to the next column
No
is the column the final
Yes
4
Move to next time increment
No
is final time increment reached
Yes
Store each plate concentration for the whole sysyem
13


13
is this the final sequence
No
Move to the next sequence
3
Yes
Using the plate concentration, the Kd values and the stationary and mobile phase plate volume calculate the total mass for each component in each column
Calculate the point component concetration in the final solution for a predeterming period
Rearrange the component concentration so that the first set of results corresponds to the column which was purged last,the second to the eluent inlet column and so on
Calculate the product concentration and purities of the exit column in the separating section and at the begining
Print out the operating conditions, component concentrations for each column, product purities and concentration at the column exit
STOP

All the system related parameters in the main programme i.e. number of columns, feed inlet location number of theoretical plates, switch time, voidage and stationary and mobile phase plate volume; the operating parameters such as the distribution coefficients, flowrates, feed concentrations kinetic constants (Maximum initial velocity, Vmax, and the Michaelis constant Km) and purging period; and programme related parameters such as number of cycles and time increments were replaced by "dummy" variables which were defined at the beginning of the programme.

The programme was designed to predict the on-column "point" concentration profiles for the inversion reaction. The Michaelis constant, Km, used was a constant value which was calculated from the kinetic studies (Chapter 8). The maximum initial velocity, Vmax, was a variable depending on the enzyme activity used. The programme can be modified to incorporate the biosynthesis of dextran reaction by including the mass balance equations and the kinetic parameters for this reaction.

Different values for the distribution coefficients were used and their choice will be discussed later. In the plate to plate concentration calculations, the apparent number of theoretical plates based on fructose was used. The programme accounted for all the three components present i.e. the retarded fructose product, the less retarded glucose product and the fast moving and reacting sucrose.

The simulation was carried out using a fixed number of time increments (KKINK) per sequence and a fixed switch period. The time increment (DT) was kept constant and was chosen to be 3 seconds. The number of time increments per sequence (KKINK) was obtained using the DT value and the operating switch time for the particular condition.

Finally an estimation of product purity on the GRP stream was carried out to give an indication of the systems performance. The CPU time was 1680 secs.

11.4 EXPERIMENTAL RESULTS AND DISCUSSION

The experimental conditions of Run 20.77-9-31.5-30-60-55 were used to examine the accuracy of the simulation programme. This run was chosen because the product purities were within specification (i.e. 90%) which is the value set out for this research work.

Different types of distribution coefficients (Kdi) were used to test the sensitivity of the SCCR-S1 system. Table 11.2 and Figures 11.2 to 11.4 shows the sensitivity of the SCCR-S1 system to changes in the distribution coefficients of sucrose, glucose and fructose. To compare the experimental results with the simulated results, the infinite dilution distribution coefficient calculated from the column characterisation (see Chapter 5) was first chosen to predict the simulated concentration profiles (i.e. $Kd_F = 0.62$; $Kd_G = 0.27$; $Kd_S = 0.12$). Figure 11.2 shows the disagreement between the experimental and simulated profiles. The simulated results show a high concentration of fructose product across the system with most of the glucose eluted very early thus resulting in a very low concentration of glucose at the post feed section with no glucose present at the prefeed section. With the sucrose concentration profile, the simulated result predicted a broader profile at the post feed section compared with the experimental results which indicated that the sucrose was consumed within three columns around the feed location point. These differences show that the fructose, glucose and sucrose distribution coefficients chosen for the simulation run were not the actual values to give a good representation of the true concentration profile. One important factor for this is that the infinite dilution distribution coefficients used were derived under room temperature (~20°C) conditions and an eluent rate of 25cm^3 min^{-1} while the experiment was carried out at 55°C and an eluent rate of 31.5cm^3/min.

The effect of operating parameters on the distribution coefficients of fructose, glucose and dextran have been carried out by Thawait [105] and Ganetsos [9] and discussed in Chapter 7. Using the predicted distribution coefficient values calculated from the

correlations developed by Ganetsos [9] for fructose and glucose background concentrations, temperature and flowrates to simulate the SCCR-S1 performance (i.e. $Kd_F = 0.58$; $Kd_F = 0.41$; $Kd_S = 0.12$), a slight improvement in the concentration profiles was observed (Figure 11.3). However the lack of sucrose and protein background correlations did not allow comparison between the simulated and experimental profiles to be made.

From the infinite dilution distribution coefficients and the predicted distribution coefficients, it was clear how crucial it is to use the right distribution coefficients in the simulation of the SCCR-S1 operation. With no information of the distribution coefficients in a combined chromatographic reactor-separator system i.e. where there is also an enzyme background effect, it was decided to experiment with some combinations of Kd values to optimise the simulated results. From Table 11.2, the best combination of distribution coefficients were $Kd_F = 0.715$, $Kd_G = 0.41$ and Kd_S 0.3 which gave a predicted GRP product purity of 93%. The predicted profiles shown on Figure 11.4 indicate the closeness in agreement between the simulated concentration profiles and the experimental concentration profiles.

From Figure 11.4 it was also observed that the simulated programme indicated a better separation performance at the pre-feed section of the system than the experimental results indicated. Also the predicted reaction time was longer, therefore there was more column length available for the separation. These differences are attributed to the simplified assumptions made when modelling the SCCR-S1 system. With the first order reaction assumption made in the modelling of the SCCR-S1 system, a better predicted concentration profile (Figure 11.4) was obtained than when a zero order reaction assumption was made (Figure 11.5). The zero order reaction assumption showed the presence of high concentrations of unconsumed sucrose in the GRP product and indicated that conversion was not complete. This also indicated that the effect of substrate inhibition on the reaction rate was quite significant. However, from the experimental result this

effect was negligible (i.e. the operating of the SCCR-S1 as a combined reactor-separator, the substrate inhibition effect was overcome). The first order reaction minimises the significant effect of substrate inhibition by showing a less broader sucrose concentration profile. The effect of substrate inhibition on the performance of SCCR-S1 unit was not taken into account when modelling the system due to the complexity of the kinetic equation (Equation 8.2).

During the experimental runs there was a significant reduction of invertase activity along the system, as shown in Figure 8.22. This, however, was not accounted for during the simulation programme which assumed a constant enzyme activity at the pre-feed and post-feed sections. As mentioned earlier another important component, background concentration which would influence the values of the distribution coefficients is the protein content (invertase) which was not accounted for during the correlation of the predicted Kd values.

For a better agreement between the simulated profile and experimental profile the choice of the right Kd values is critical. All the factors mentioned above need to be taken into consideration and their influence on the contribution of the distribution coefficients must be quantified. To highlight this point, further experimental runs at different feed concentrations (i.e. Run:- 34.6-9-31.5-30-60-55 and Run:- 41.4-9-31.5-29.5-60-55) were simulated. Figures 11.6 and 11.7 illustrate the deviation of the simulated profiles from the experimental profiles. These runs give a better indication of the magnitude of the problem since the distribution coefficients are greatly affected by changes in solute concentrations.

In conclusion, the simulation programme has been shown to predict the trend of the on-column concentration quite well, however, the accuracy of the prediction will only be improved if a more complete set of Kd and operating conditions correlations can be obtained and incorporated into the model.

Table 11.2 : Effect of Distribution Coefficients on the Simulated SCCR-S1 System for Run:- 20.77-9-31.5-30.5-60-55

Distribution Coefficients of Sucrose; Glucose and Fructose Kd_S	Kd_G	Kd_F	GRP Product Purity (% W/V)	Comments
0.12	0.27	0.62	10	Infinite dilution coefficients
0.12	0.41*	0.58*	38.7	Predicted coefficients for Kd_G and Kd_F
0.3	0.41*	0.58*	45.85	Arbitrary Kd_S value
0.12	0.39	0.705	77.4	Arbitrary Kd_G and Kd_F values
0.12	0.39	0.715	81.78	Arbitrary Kd_G and Kd_F values
0.3	0.39	0.715	92.3	Arbitrary Kd Values
0.3	0.41*	0.715	93.6	Arbitrary Kd_S and Kd_F

* Predicted distribution coefficients using the relationships shown in (9) and listed in Chapter 7.

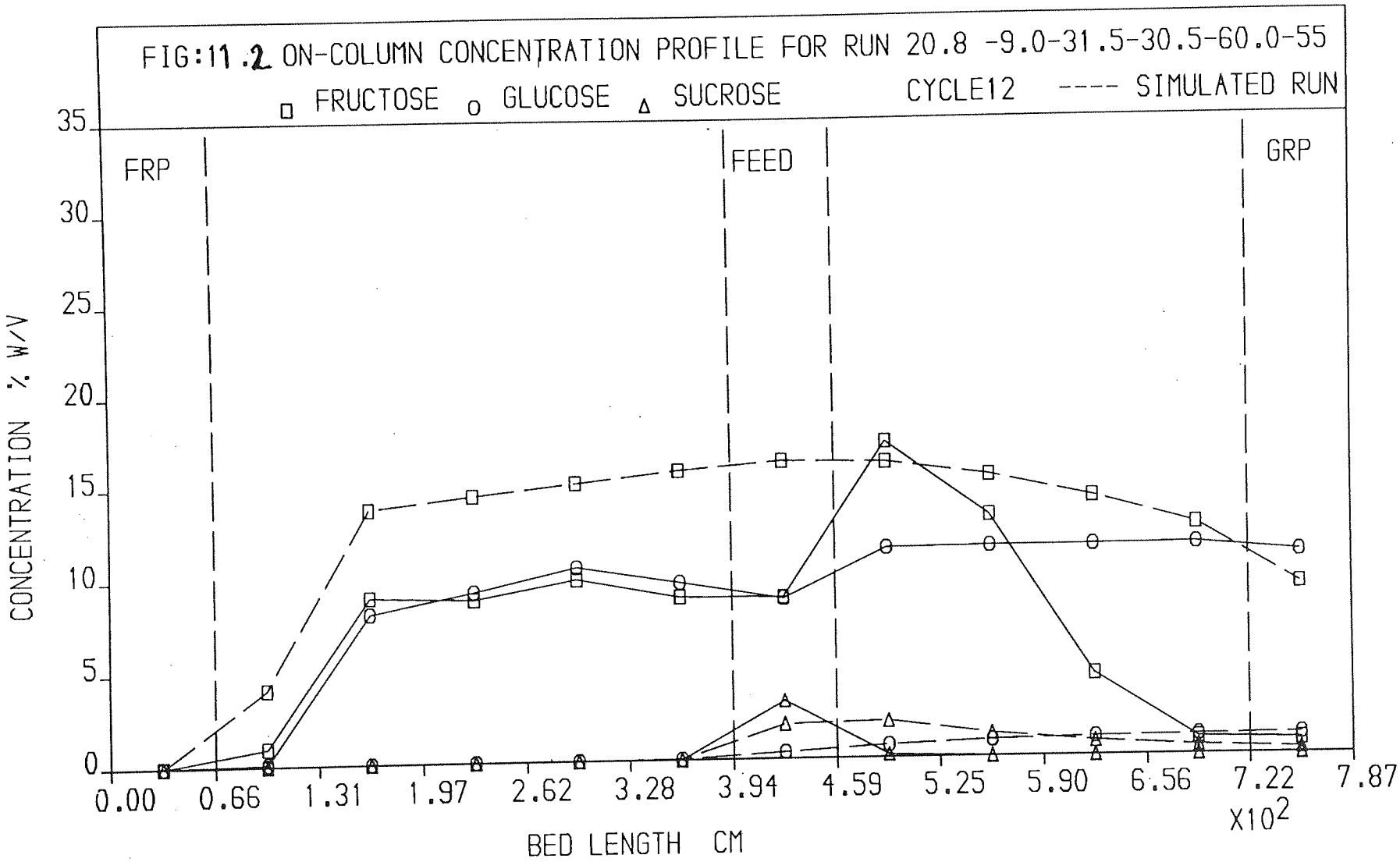


FIG:11.2 ON-COLUMN CONCENTRATION PROFILE FOR RUN 20.8 -9.0-31.5-30.5-60.0-55
□ FRUCTOSE o GLUCOSE Δ SUCROSE CYCLE12 ---- SIMULATED RUN
FRP
FEED
GRP
CONCENTRATION % W/V
35
30
25
20
15
10
5
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10^2
BED LENGTH CM

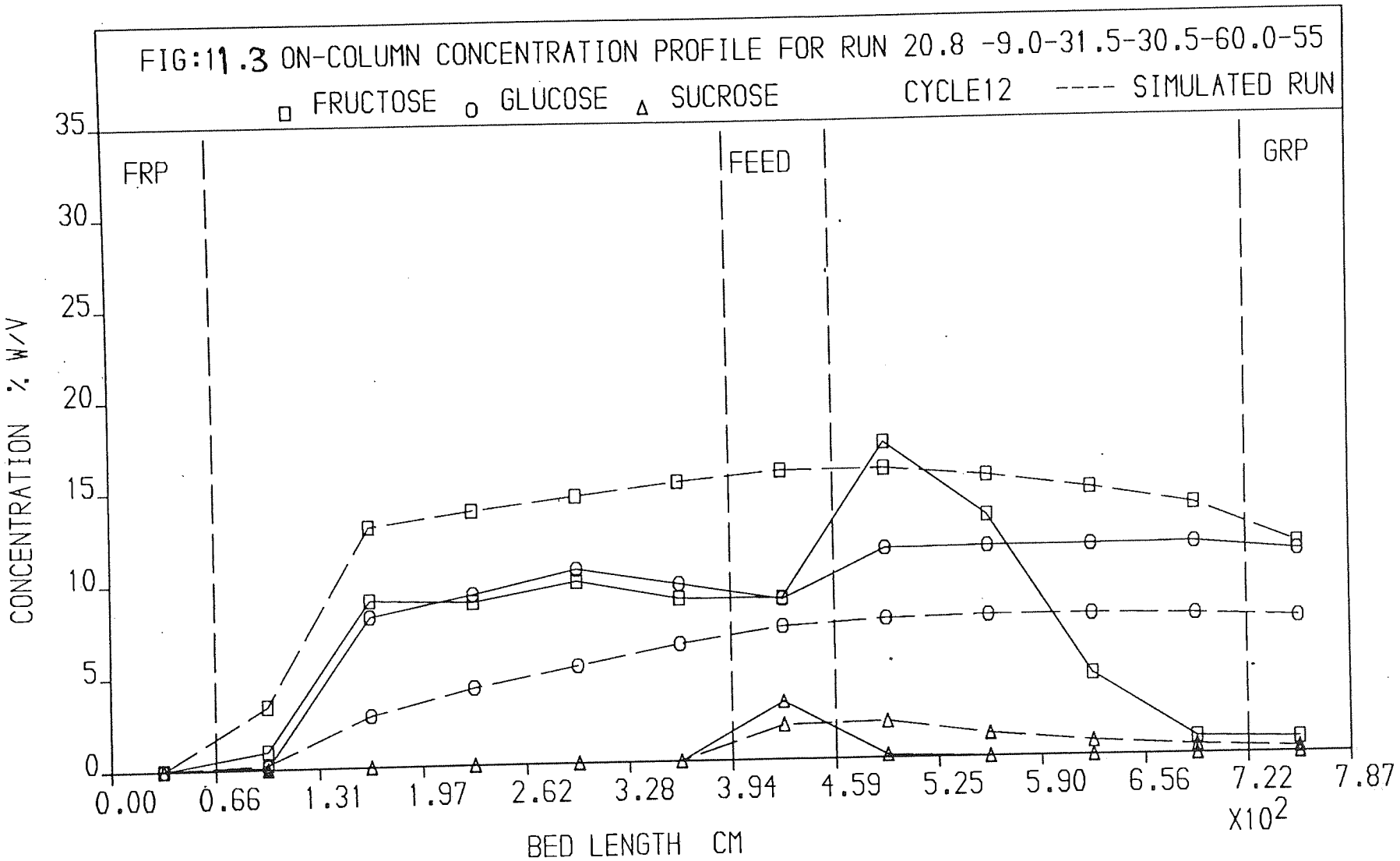


FIG:11.3 ON-COLUMN CONCENTRATION PROFILE FOR RUN 20.8 -9.0-31.5-30.5-60.0-55
□ FRUCTOSE o GLUCOSE △ SUCROSE CYCLE12 ---- SIMULATED RUN
FRP
FEED
GRP
CONCENTRATION % W/V
35
30
25
20
15
10
5
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10²
BED LENGTH CM

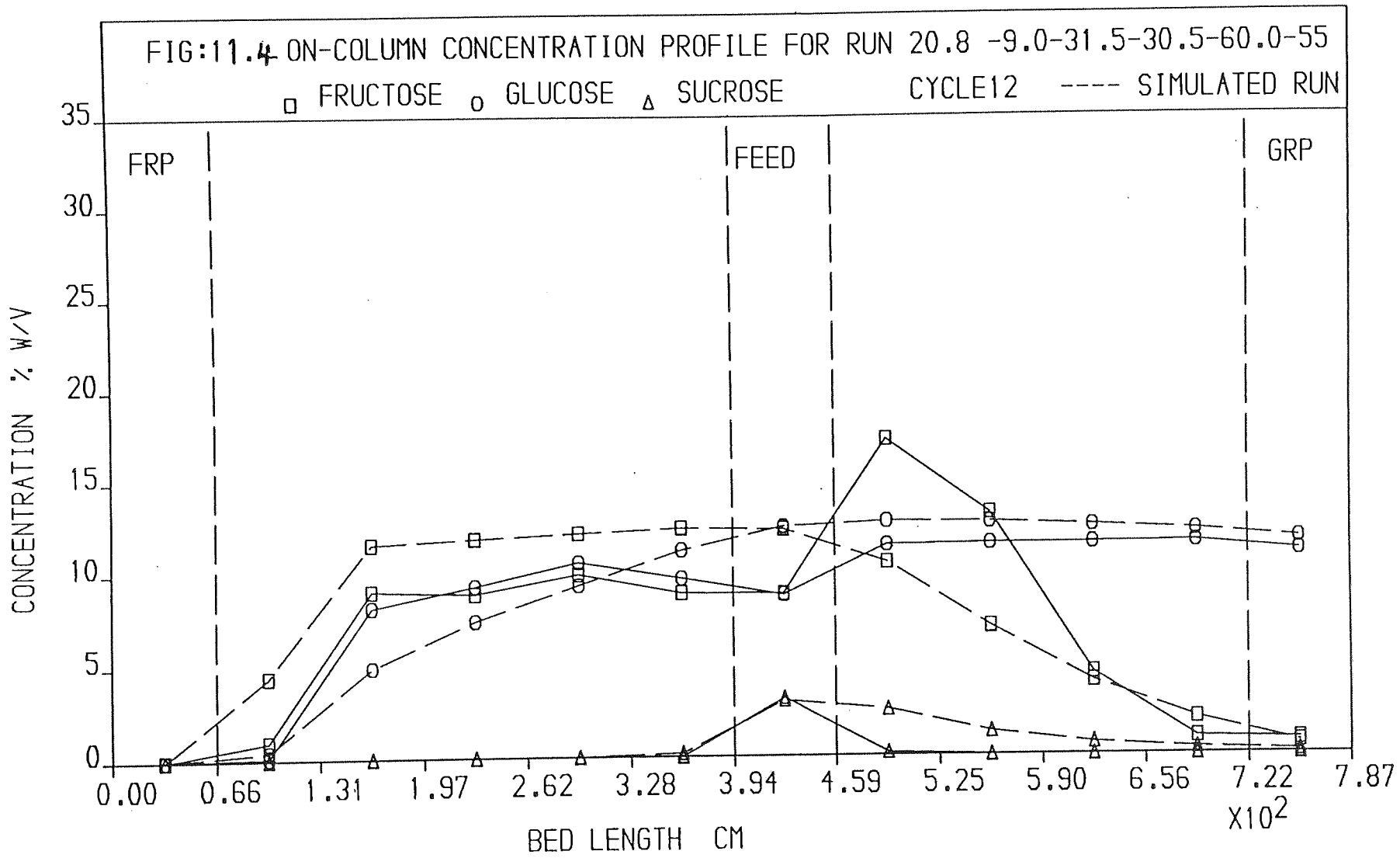


FIG:11.4 ON-COLUMN CONCENTRATION PROFILE FOR RUN 20.8 -9.0-31.5-30.5-60.0-55
□ FRUCTOSE o GLUCOSE Δ SUCROSE CYCLE12 ---- SIMULATED RUN
FRP
FEED
GRP
CONCENTRATION % W/V
35
30
25
20
15
10
5
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10²
BED LENGTH CM

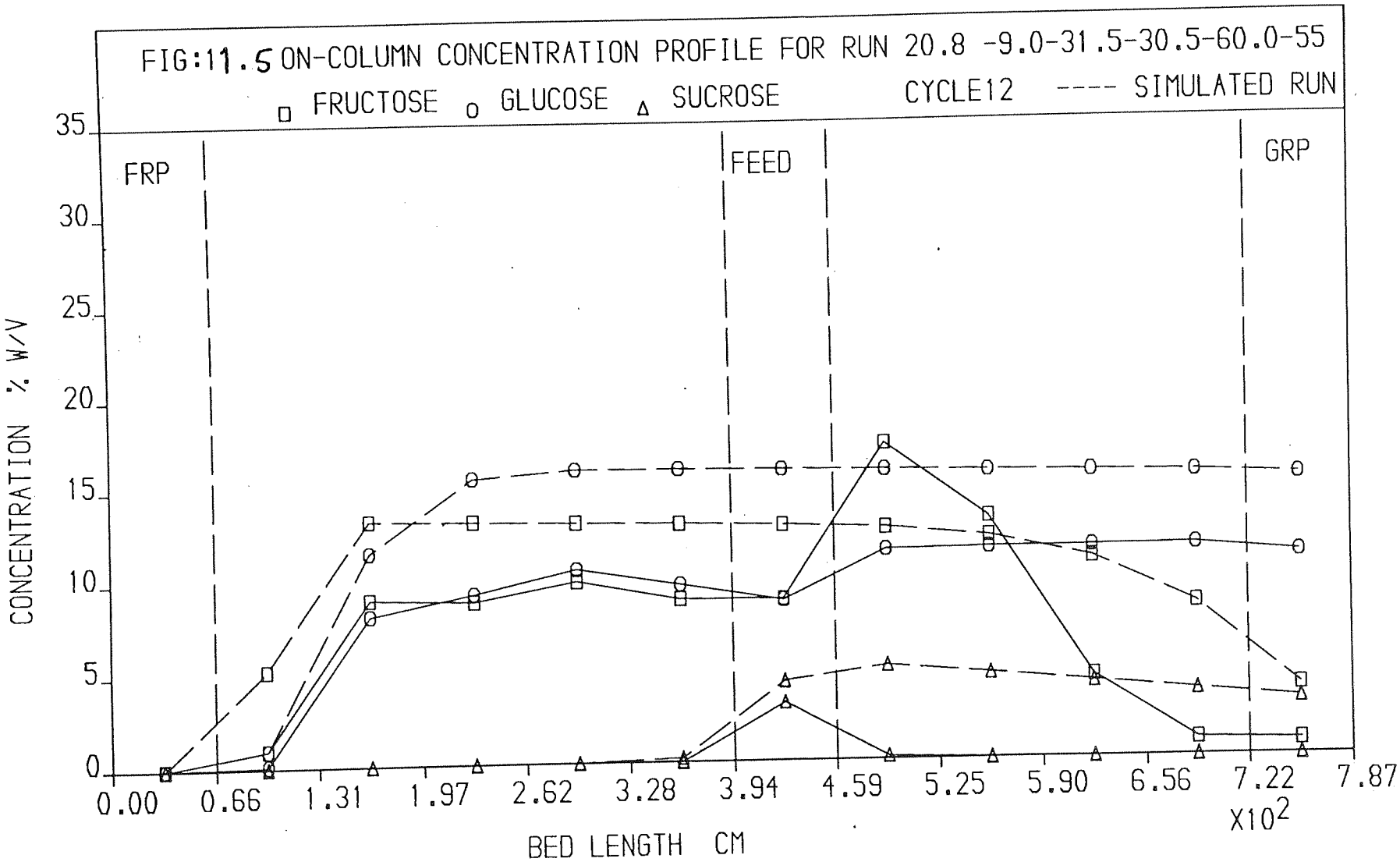


FIG:11.5 ON-COLUMN CONCENTRATION PROFILE FOR RUN 20.8 -9.0-31.5-30.5-60.0-55
□ FRUCTOSE o GLUCOSE Δ SUCROSE CYCLE12 ---- SIMULATED RUN
FRP
FEED
GRP
CONCENTRATION % W/V
35
30
25
20
15
10
5
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10²
BED LENGTH CM

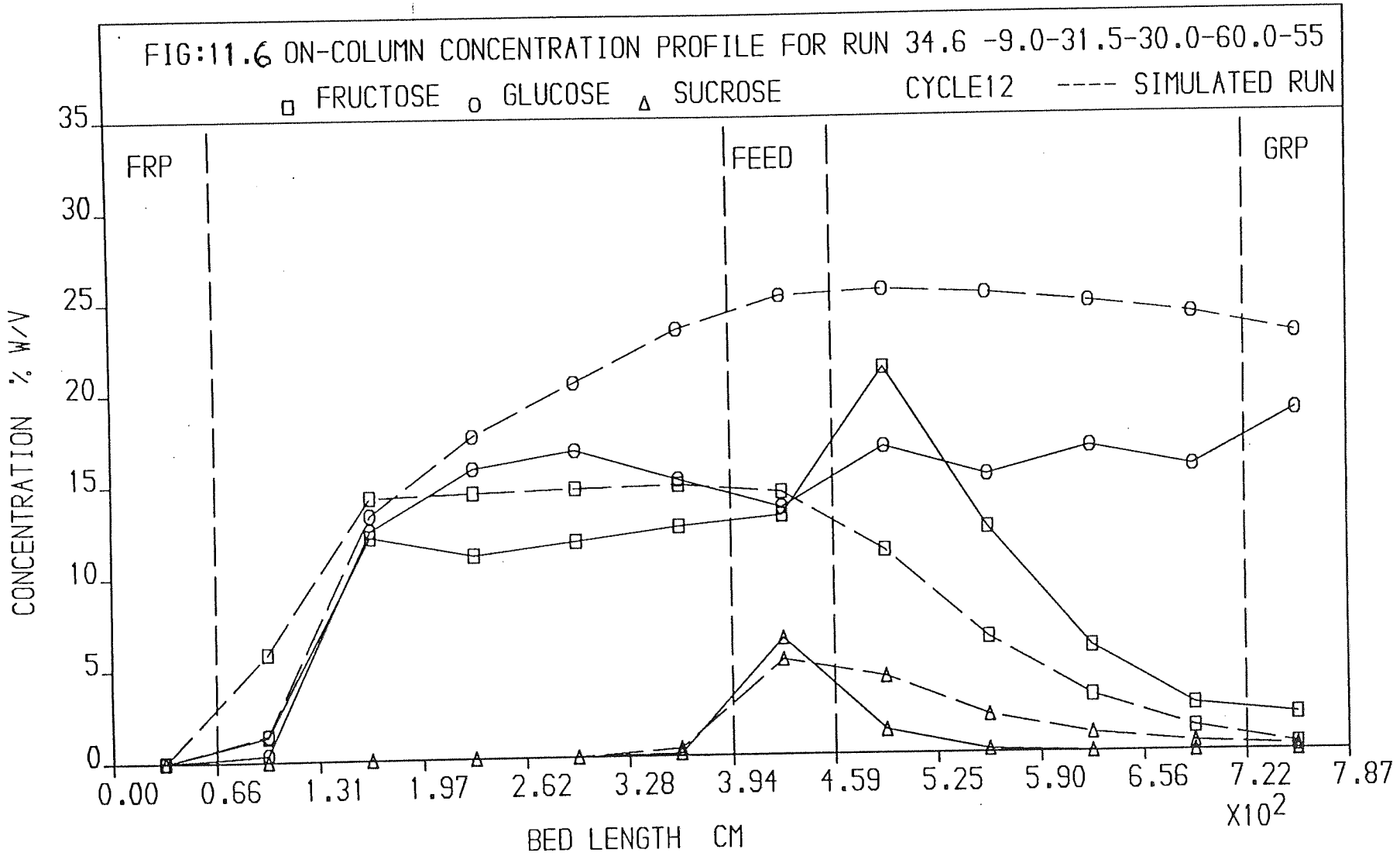


FIG:11.6 ON-COLUMN CONCENTRATION PROFILE FOR RUN 34.6 -9.0-31.5-30.0-60.0-55
□ FRUCTOSE o GLUCOSE Δ SUCROSE CYCLE12 ---- SIMULATED RUN
FRP
FEED
GRP
CONCENTRATION % W/V
35
30
25
20
15
10
5
0
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87
X10²
BED LENGTH CM

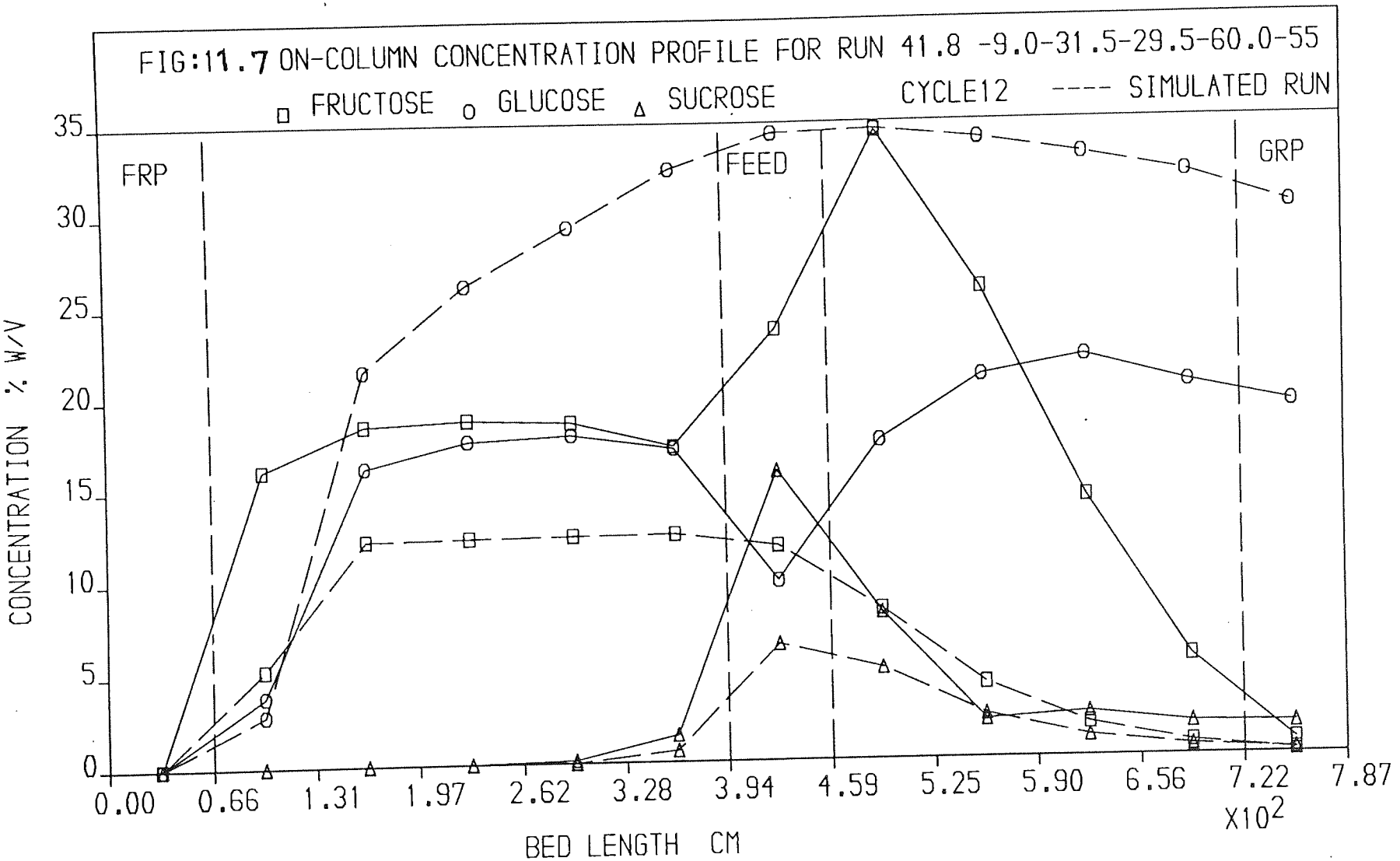


FIG:11.7 ON-COLUMN CONCENTRATION PROFILE FOR RUN 41.8 -9.0-31.5-29.5-60.0-55
FRUCTOSE
GLUCOSE
SUCROSE
CYCLE12
---- SIMULATED RUN
FRP
FEED
GRP
CONCENTRATION % W/V
BED LENGTH CM
X10^2
0
5
10
15
20
25
30
35
0.00
0.66
1.31
1.97
2.62
3.28
3.94
4.59
5.25
5.90
6.56
7.22
7.87

CHAPTER 12

CONCLUSIONS AND RECOMMENDATIONS

12.0 CONCLUSIONS AND RECOMMENDATIONS

12.1 CONCLUSIONS

The aim of this work was to investigate for the first time the use of a semi-continuous chromatographic refiner (SCCR-S1) as a combined biochemical reactor and separator and to optimise its operation by identifying the effects of various operating parameters. Two bioreactions were considered, namely, the enzymatic inversion of sucrose in the presence of invertase enzyme and the biosynthesis of dextran using the enzyme dextransucrase. The overall findings and conclusions can be summarised as follows:-

12.1.1. Reaction-Separation Work on the SCCR-S1 Using the Enzymatic Inversion of Sucrose by Invertase

1. The reaction-separation results on the inversion of sucrose have shown for the first time that continuous bioreaction-separation using SCCR type equipments is possible. Complete conversions and product purities of 90% and above have been achieved. The problems related to substrate inhibition at high feed concentrations in the case of sucrose inversion, were successfully overcome.

2. The switch time was the controlling parameter during the operation. Changes in the switch time affected the residence time and concentration profiles of the reaction products (glucose and fructose) and the feed (sucrose). An increase in switch time increases the residence time and broadens the on-column concentration profiles. The broadening of the fructose concentration profile was more pronounced than the glucose concentration profile due to the secondary reactions (i.e. formation of oligosaccharides and subsequent hydrolysis), taking place at the post-feed section. This affected the product purities of the glucose rich product (GRP) stream significantly. Operating at an optimum value of 30.5 minutes switch time for 20% W/V sucrose concentration and an enzyme activity

of 60U/cm^3 gave 90% product purities in both the FRP and GRP product streams. Complete conversion of the sucrose was also achieved.

3. Kinetic studies of the invertase reaction have shown that substrate inhibition limits the inversion rates especially at concentrations over 10% W/V. However, when operating on the SCCR-S1 system at increased feed concentrations and at constant invertase enzyme activities, this effect was minimised. Only at very high sucrose concentrations (ca. 50% W/V), the extent of conversion was reduced slightly (i.e. approximately 96 - 100% conversion was achieved). In fact the limiting factor of the SCCR-S1 system's separation performance, at high feed concentration, was the background sucrose concentration which has a great effect on the distribution coefficients of the solutes present in the system and thus the product purities.

4. By operating at a constant feed concentration and increasing the amount of invertase activity (i.e. from 43U/cm^3 to 155U/cm^3), the inversion rate was found not to be the limiting step in the operation of the SCCR-S1 system. The operation of the system therefore depended on the separating power of the equipment. An enzyme to substrate ratio above 1000U/g resulted in complete conversion. However, when operating at high sucrose concentrations the background concentration effects on the distribution coefficients (Kds) resulted in reduced purity at the FRP.

5. An eluent to feed rate ratio of 3.5:1, was found to be the optimum with respect to efficient utilisation of the separating and reacting sections of the SCCR-S1 system. Higher eluent to feed rate ratios (i.e. 4:1) resulted in very dilute product concentrations. Operating at lower eluent to feed rate ratios (i.e. 3:1) the product concentration increased, but the separation efficiency and thus the product purities were affected.

6. The amount of enzyme used for the conversion of sucrose in the SCCR-S1 system over a 1 minute period was 35% of the theoretical amount that will convert the same amount of sucrose in a conventional fermenter. Enzyme denaturisation has been found to take place along the system length and some dilution due to the introduction of the feed stream does take place. This indicates that even lower amounts of enzyme were used.

7. To improve the product concentrations and reduce the enzyme usage further the product splitting technique was employed for both product streams. The diluted fraction of the GRP stream was recycled as eluent and the dilute fraction of the FRP stream as purge. This resulted in higher glucose product purities in the GRP stream, i.e. from 92.3 to 95% and GRP product concentration increased from 3.2% W/V to 4.6% W/V. The FRP product concentration also increased from 1.82% W/V to 2.8% W/V with the fructose product purity remaining approximately constant. When the dilute product recycling was employed the enzyme usage was reduced to 16% of the theoretical amount needed to convert the same amount of sucrose in a conventional fermeter over the same time period of 1 minute. This enzyme usage was actually 52.8% lower than the amount used when operating without recycling.

8. By recycling only the dilute fraction of the FRP stream as purge, and splitting the GRP stream into two fractions (diluted and concentrated), the FRP product concentration increased from 1.82% W/V to 3.6% W/V and the fructose purity increased from 80.6% to 87%. The concentration fraction of the GRP increased by approximately 58% and the glucose product purity was 95.5% W/V.

9. The computer simulation for the SCCR-S1 as a combined reactor-separator has been carried out successfully. The "plate" model approach was employed. First

order reaction and constant invertase enzyme activity were assumed throughout the simulation. The point on-column concentration profiles of sucrose, glucose and fructose were predicted from estimated distribution coefficients of the solutes. The CPU time to run the whole simulation programme was approximately 1680 seconds. The best combination of distribution coefficients of sucrose, glucose and fructose that gave a good agreement between the simulated profiles and experimental value for run condition 20.77-9-31.5-30.5-60-55 were 0.3, 0.41 and 0.715 respectively. It was found however that the availability of distribution coefficient correlations which take into account the effect of solute background concentration, protein background concentration, temperature and eluent rates are absolutely necessary since the accuracy of the simulation programme depends heavily on the availability of accurate distribution coefficient prediction data.

12.1.2 Reaction Separation Work on the SCCR-S1 System Using the Biosynthesis of Dextran by Dextransucrase Enzyme

In the biosynthesis of dextran reaction-separation work, the preliminary results looked very promising. In these preliminary studies the limiting factor in operating the SCCR-S1 system was the high viscosity of the dextran produced.

10. Operating at a feed concentration of 3% W/V and an enzyme activity of 14 DSU/cm^3, 53% sucrose conversion based on sucrose was achieved. The FRP fructose purity obtained was 43.7%. It was difficult to analyse the concentration of dextran molecules due to problems associated with the HPLC analytical system, namely the adsorption of dextran on the chromatographic media.

11. During the reaction some glucose was produced probably for the following reasons: Inversion due to invertase impurities present in the enzyme solution,

the calcium ion effect which causes partial isomerisation of glucose in the system and acid hydrolysis due to the acidic operating conditions (pH 5.2).

12. Using the GPC analytical system, 100% of the dextran produced in the dextran rich product (DRP) stream was found to have a molecular weight of over 156,980 with 99.6% above 4 million daltons. In the FRP product stream, 60% of the dextran molecular weight was above 156,980 daltons, with 16% above 4 million.

12.1.3 Purification Studies of Dextransucrase Enzyme

13. Dextransucrase enzyme was produced anaerobically by the strain bacteria Leuconustoc Mesenteriodes NRRL B512(F) in a large scale (1000 litre) fed batch seed fermenter. The activity of the enzyme produced under optimum conditions (i.e. pH 6.7 and 23°C) was 290 DSU/cm^3.

14. In the purification of dextransucrase, the solid debris removal was achieved by using a continuous centrifuge. The results showed that by operating at high centrifugation forces and high throughput, the enzyme dextransucrase denaturates very rapidly. 60% of the initial activity was lost when operating at a centrifugal force of 36000g and a throughput of 520cm^3/min. The percentage of solids removed was 58%. Operating at a centrifugal force of 18,000g and a throughput of 200cm^3/min gave an enzyme activity recovery of 85% and 80% solid removal.

15 The removal of all other soluble impurities was carried out by using membrane separation. Two commercial systems were used, the New Brunswick TM-100 system and the Millipore XF4-200 system. With the New Brunswick system, membranes with different types and sizes were tested. The 10,000MW cut off and 100,000MW cut-off polysulfone membranes were used in the

ultrafiltration process for removing low molecular weight impurities. Enzyme recoveries of between 80-90% were achieved with over 95% low molecular weight impurities being removed. The study of the possibility of cell removal by microfiltration as an alternative to centrifugation was also carried out. Microporous membranes of 0.1μm, 0.2μm and 0.45μm sizes were used. The recovery of dextransucrase on these membranes however, was very low (i.e. down to 40%).

With the Millipore membrane system, better purification with both the microporous membrane (0.2μm) and ultrafiltration membranes (30,000MW cut-off) were achieved. Using the 30,000MW ultrafiltration membrane an average of 90% enzyme recovery was achieved with 100% removal of low molecular weight impurities. With the microporous membrane, about 60% of the dextransucrase was recovered.

16. The loss of enzyme activity during the membrane separation process was low compared to the centrifugation process with an average loss of 2% of the original activity. However, the processing time was long (2 hours compared to a centrifugation process of 20 min).

17. To study the best combined purification techniques required to give a desired pure enzyme solution, the centrifugation/ultrafiltration techniques and the microfiltration/ultrafiltration techniques were considered. The centrifugation/ ultrafiltration combined technique was found to give a better overall enzyme recovery (i.e. 60%) and shorter processing time.

The ultrafiltration membrane system was scaled up and a dextransucrase recovery of 80% and 98% removal of low molecular weight impurities was obtained.

12.1.4 Separation Work on the SCCR-S1 System

18. There was a difference in separation power between the SCCR-S1 system and a similar system (SCCR7) used in previous research work [9] for the separation of a glucose/fructose synthetic feed. The product purities achieved were between 95-97% on both the FRP and GRP product streams on the SCCR-S1 system compared to a 99.9% purity reported for the SCCR7 system.

19. The partial isomerisation of glucose which was initiated by the calcium charge ions on the chromatographic media under the right conditions (i.e. pH $\leq$ 6 and 25°C) [145] was found to prevent the SCCR-S1 system from achieving 99.9% pure products. Other possible factors included the differences in infinite dilution distribution coefficients.

20. With a dextran/sucrose/fructose synthetic feed mixture, a fructose product purity of 97% was obtained for the FRP product stream and a dextran rich product (DRP) stream was obtained containing equal amounts of dextran and sucrose.

21. Operating at the same feed concentration (18.6% W/V) and under similar operating conditions and using the inverted sucrose feed and glucose/fructose synthetic feed mixtures, the differences in concentration profiles (i.e. the shift of the cross-over point towards the FRP end) were attributed to the different proportions of fructose to glucose in the feed and on the amount of β-D-fructopyranose (the sweetest fructose form) present in the feedstock [99].

12.2 RECOMMENDATIONS

The potential of the SCCR-S1 system as a combined reactor-separator opens new horizons in the application of such systems. From the work carried out in this research study, the following recommendations have been derived:

1. Carry out more reaction-separation experiments with the inversion of sucrose and investigate the effect of operating temperature, feed throughput and pH to provide a better understanding of the kinetics in the SCCR-S1 system.

2. With the biosynthesis of dextran, the use of larger particle size resin is required to reduce the high pressure caused by the highly viscous dextran produced.

3. Carry out experiments using strong acceptors to tailor the molecular weights of dextran produced to commercial specifications. This might also solve the problem of high pressure build up in the system.

4. Carry out a study of the rate of denaturation of invertase and dextransucrase enzyme in the SCCR-S1 system.

5. More experimental work is required to investigate the effect of temperature, eluent rate, carbohydrates (sucrose, glucose, fructose and dextran) background concentration and protein background concentration on the distribution coefficients.

6. The computer simulation programme needs to be modified to include the substrate inhibition effect of the Michaelis-Menten kinetics, the rate of enzyme denaturation inside the SCCR-S1 system and the effect of the operating parameters on the distribution coefficient mentioned above. The dextransucrase bioreaction needs to be mathematically modelled and simulated.

7. It is recommended that the SCCR-S1 system be used to study other types of biochemical or chemical reactions in which the reactions are limited due to equilibrium and product or substrate inhibition.

8. Other purification techniques such as two phase partition techniques and affinity chromatography need to be investigated on a large scale, to find the best purification method that will give pure dextransucrase without any great loss in activity. This will minimise any side reactions taking place and will enable an estimate of the degree of immobilisation taking place.

9. Finally a feasibility study and an economic evaluation needs to be carried out to estimate the commercial viability of the SCCR-S1 as a reactor-separator.

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NOMENCLATURE

b	Calibration constant
C_F	Concentration factor
C_m'	Resistant to mass transfer in mobile phase
C_{no}	Initial concentration of sucrose in plate (g/cm^3)
C_S'	Resistant to mass transfer in stationary phase
d	dilution factor
D	Polydispersity ratio
DRP	Dextran rich product
DSU	One dextransucrase unit
e	Natural logarithm
F_{no}	Initial concentration of fructose in plate (g/cm^3)
FRP	Fructose rich product
G_{no}	Initial concentration of glucose in plate (g/cm^3)
GRP	Glucose rich product
h	Peak height (cm)
H	Plate height (cm)
HETP	Height equivalent to theoretical plate (cm)
Ki	Capacity factor
Kdi	distribution coefficient of component i
$K^{\infty}di$	Infinite dilution distribution coefficient of component i
$K^{G}di$	Glucose background distribution coefficient of component i
^{25}Kdi	distribution coefficient of component i at 25°C
K_m	Michaelis Menton Constant (g/cm^3)
K_{si}	Substrate inhibition constant (g/cm^3)
L_e	Mobile phase flowrate ($cm^3.min^{-1}$)
L1	Eluent flowrate ($cm^3.min^{-1}$)
L3	Purging flowrate ($cm^3.min^{-1}$)

M	Molecular weight
Mn	Average molecular number
M_W	Average molecular weight
N	Number of theoretical plates
N^*	Apparent number of theoretical plates
ODi	Optical density of inculated sample
ODs	Optical density of 2g/L fructose standard
ODu	Optical density of unincubated sample
P	Stationary phase effective flowrate ($cm^3.min^{-1}$)
P_{in}	Inlet pressure ($kN.m^{-2}$)
P_{out}	Outlet pressure ($kN.m^{-2}$)
P_p	Permeate pressure ($kN.m^{-2}$)
R_S	Resolution of chromatographic column
S	Switch time (min)
[S]	Substrate concentration ($g.cm^{-3}$)
t	time (min)
t_{Ri}	Retention time of component i(min)
TMP	Transmembrane pressure ($kN.m^{-2}$)
U	One enzyme unit
u	Initial reaction velocity ($g/cm^3/min$)
V	Mobile phase velocity ($cm.sec^{-1}$)
V_i	Retention volume of component i (cm^3)
V_{max}	Maximum initial velocity ($g/cm^3/min$)
Vo	Total void volume in a column (cm^3)
V_S	Volume of stationary phase (cm^3)
V_T	Total empty column volume (cm^3)
V_1	Plate volume of mobile phase (cm^3)
V_2	Plate volume of stationary phase (cm^3)
W_i	Peak width at the base of elution curve of component i (cm^3)

X_i	Component concentration in stationary phase ($g.cm^3$)
Y_i	Component concentration in mobile phase ($g.cm^{-3}$)
γ	Reaction rate ($g.cm^{-3}.min$)
δ_1	Fraction changes due to effect of concentration on distribution coefficient
δ_2	Fractional changes due to effect of system characterisation on distribution coefficient

SUPERSCRIPT

D	Dextran
F	Fructose
G	Glucose

SUBSCRIPT

f	fructose
g	glucose
i	any solute component
s	sucrose

APPENDIX A

Computer program used to calculate the molecular weight distribution

```
5 REM PROGRAM USED TO CALCULATE THE MOL.
10 REM WEIGHT DISTRIBUTION OF A DEXTRAN
12 REM SAMPLE
15 REM          BY A. AKINTOYE 1987
17 REM
20 DIM A(400),Z(5),B(170),VIN(170),S(170),S5(170),
MAX(2),AMIN(170)
40 X=0.0
60 PRINT 'RUN A VO-VT ... YES OR NO'
80 INPUT ANS$
100 IF ANS$='YES' THEN X=1 : GOTO 320
120 PRINT 'THEN INPUT TO AND TT'
140 INPUT MAX(1),MAX(2)
150 X=2.0
160 PRINT 'INPUT THE FLOW RATE'
180 INPUT RATE
200 PRINT 'TYPE IN BATCH NUMBER'
220 INPUT KR$
240 PRINT 'TYPE IN DATE'
260 INPUT KP$
280 PRINT 'READY TO START ... TYPE GO'
300 INPUT NUM$
320 I=1: T1=TI: Y=1
340 GOSUB 1570
360 E=TI-T1
380 IF E<120 THEN 360
400 IF Y=5 THEN 460
420 Y=Y+1: T1=TI
440 GOTO 340
460 T1=TI
480 A(I)=(Z(1)+Z(2)+Z(3)+Z(4)+Z(5))/5
500 PRINT 'READING AT';I/6; 'MINS =';A(I)/4
520 IF I >= 282 THEN 580
540 Y=1.0: I=I+1
560 GOTO 340
580 IF X=1.0 THEN GOSUB 1380
```

```
600 IF X=0.0 THEN 150
610 GOSUB 1860
620 GOSUB 2140 : GOSUB 2520
640 OPEN 1,4
660 OPEN 2,4,1
680 OPEN 3,4,2
700 F$='999 99999999.99 99999999999999. 9999.99
99999999.9999 999999.99'
720 PRINT#3, F$
740 PRINT#1, CHR$(1) 'BATCH NUMBER : '; KR$
760 PRINT#1 : PRINT#1
780 PRINT#1, CHR$(1) 'DATE OF ANALYSIS: '; KP$
800 PRINT#1 : PRINT#1
820 PRINT#1, 'FLOWRATE ='; RATE; 'ML/MIN'
840 PRINT#1,' VO='; MAX(1)
860 PRINT#1, ' VT='; MAX(2)
880 PRINT#1, ' VS='; BEGIN
900 PRINT#1, ' VF='; FINISH
920 PRINT#1
940 PRINT#1, 'WEIGHT AVERAGE MOL WT =' AARW
960 PRINT#1, 'NUMBER AVERAGE MOL WT =' AVMN
1000 PRINT#1, ' MW/MN RATIO =' SPR
1020 PRINT#1 ; PRINT#1 : PRINT#1
1040 HD$= 'POINT '+' '+'KD VALUE '+' '
1060 GH$= 'MOL WEIGHT '+' '
1080 FH$= 'HEIGHT '+' '+'WT FRAC '+' CUMM%'
1100 EH$= HD$+GH$+FH$
1120 PRINT#1, EH$
1140 FOR I=NMAX TO 1 STEP -1
1160 PRINT#2, I, VIN(I), AMIN(I), B(I), S(I), S5(I)
1180 NEXT I
1200 PRINT#1 : PRINT#1 : PRINT#1
1220 PRINT#1, 'ASTON GPC CONSTANTS'
1240 PRINT#1, ' B1= ';B1
1260 PRINT#1, ' B2= ';B2
1280 PRINT#1, ' B3= ';B3
1300 PRINT#1, ' B4= ';B4
1320 PRINT#1, ' B5= ';B5
1340 CLOSE 1 ; CLOSE 2 ; CLOSE 3
1345 GOSUB 3060
1355 GOTO 200
1360 END
1380 REM TO FIND TO AND TT
```

```
1400 Z=1.0
1420 FOR I = 126 TO 282
1440 IF A(I)<200 THEN 1500
1460 IF A(I)>=A(I-1) THEN MAX(Z)=I/6 ; GOTO 1500
1480 Z=2.0
1500 NEXT I
1510 PRINT ' '
1520 PRINT ' TO =' ;MAX(1); 'TT= ' ;MAX(2)
1530 PRINT ' '
1540 X=0.0
1560 RETURN
1570 REM HEIGHTS FROM REWFRACTOMETER
1580 OPEN 1, 9, 15
1600 GET#1, J$, K$
1620 IF K$=' ' THEN K=-224 ; GOTO 1660
1640 K=ASC(K$)-224
1660 IF K<0 THEN D= (K+32)*-1
1680 IF K>=0 THEN D=K
1700 D=D*256
1720 IF J$=' ' THEN J=0 ; GOTO 1760
1740 J=ASC(J$)
1760 IF K<0 THEN J=J* -1
1780 Z(Y)=J+D
1800 PRINT Z(Y), Z(Y)/4
1820 CLOSE 1
1840 RETURN
1850 REM CALC START AND FINISH TIMES
1860 SUM=0.0 : NMAX=1.0 : SAP=0.0
1870 FOR I = 100 TO 270
1880 IF A(I) > SAP THEN SAP=A(I) : PEAK=1
1890 IF (I>102) AND (I=< 120) THEN SUM=SUM+A(I)/4
1900 NEXT I
1910 MEAN= SUM/18
1920 FOR I=120 TO PEAK
1930 IF A(I)/4-MEAN>2 THEN S=I ; GOTO 1950
1940 NEXT I
1950 FOR I=PEAK+1 TO 282
1955 IF A(I)<0.0 THEN A(I)=0.0 : T=I : R=0.0 : GOTO 1985
1960 IF (A(I)-A(I-1))/4<0.6 THEN R=A(I+1)/4 : T=I+1:
GOTO 1985
1980 NEXT I
1985 B(1)=0.0
```

```
1990 FOR I=S TO T
2000 NMAX=NMAX+1
2020 IF PEAK>=I THEN B(NMAX)=A(I)/4=MEAN ; GOTO 2050
2040 B(NMAX)=A(I)/4-R
2050 NEXT I
2060 BEGIN=(S-1)/6 ; FINISH=(T)/6
2120 RETURN
2130 REM CALC OF ELUTION VOLUMES
2140 VIE=RATE*BEGIN
2160 VFE=RATE*FINISH
2180 VI=(VIE-(RATE*MAX(1)))/(RATE*(MAX(2)-MAX(1)))
2200 VF=(VFE-(RATE*MAX(1)))/(RATE*(MAX(2)-MAX(1)))
2220 VH=(VF-VI)/(NMAX-1)
2240 RETURN
2520 REM CALC OF MWD
2540 B1=-12.859 : B2=9.005 : B3=-1.986 : B4=15.516 : B5=-15044.768
2560 S1=0.0 : S2=0.0 : S3=0.0
2580 FOR I=1 TO NMAX
2600 VIN(I)=VI+VH*(I-1)
2620 IF VIN(I)>1.0 THEN 2680
2640 IF VIN(I)<0.0 THEN 2720
2660 AMIN(I)=B5+EXP(B4+B1*VIN(I)+B2*(VIN(I)^2)+
B3*(VIN(I)^3)) : GOTO 2740
2680 AMIN(I)=B5+EXP(B4+B1+B2+B3)
2700 GOTO 2740
2720 AMIN(I)=B5+EXP(B4)
2740 S1=S1+B(I) : S2=S2+(AMIN(I)*B(I))
2760 S3=S3+(B(I)/AMIN(I)) : NEXT I
2780 AAAW=S2/S1
2800 AVMN=S1/S3
2820 SPR=AAAW/AVMN
2840 PRINT 'WEIGHT AVERAGE MOL WT=';AAAW
2860 PRINT 'NUMBER AVERAGE MOL WT=';AVMN
2880 PRINT '                    MW/MN RATIO";SPR
2900 S4=0.0
2920 FOR I=1 TO NMAX : S4=S4+B(I) : NEXT I
2940 FOR I=1 TO NMAX : S(I)=B(I)*100/S4 : NEXT I
2960 S5(NMAX+1)=0.0
2980 FOR I=NMAX TO 1 STEP -1
3000 S5(I)=S5(I+1)+S(I)
3020 NEXT I
3040 RETURN
```

```
3060 PRINT 'IS THE DATA OK ....IF SO TYPE YES'
3080 PRINT 'ELSE PRESS ANY LETTER'
3100 INPUT ROP$
3120 IF ROP$= 'YES' THEN 3640
3140 FOR I=126 TO 282
3160 OPEN 1, 4, 1
3180 OPEN 2, 4, 2
3200 F$= '9999.99        S99999.99       S9999.99'
3220 PRINT#2, F$
3240 PRINT#1, I/4, A(I), A(I)/4
3260 CLOSE 1 ; CLOSE 2
3280 NEXT I
3300 PRINT 'DO YOU WISH TO RUN THE '
3320 PRINT 'DISTRIBUTION YOURSELF IF SO '
3340 PRINT INPUT ANY LETTER , ELSE TYPE '
3360 PRINT 'NO'
3380 INPUT POP$
3400 IF POP$= 'NO' THEN GOTO 3640
3420 PRINT ' TYPE IN THE START AND FINISH TIMES '
3440 INPUT AB , BC
3460 NMAX=0.0 : SAP=0.0
3500 FOR I=100 TO 270
3520 IF S(I)>SAP THEN SAP=A(I) : PEAK=I
3530 NEXT I
3535 IF A(BC)<0.0 THE A(BC)=0.0
3540 FOR I=AB TO BC
3550 NMAX=NMAX+1
3560 IF PEAK>=I THEN B(NMAX)=(A(I)-A(AB)))/4 : GOTO 3600
3580 B(NMAX)=(A(BC))/4
3600 NEXT I
3620 BEGIN=AB/6 : FINISH=BC/6
3630 GOTO 620
3640 RETURN
```

APPENDIX B

B1 : ENZYME USAGE CALCULATION FOR A STANDARD RUN

INVERTASE ENZYME UNIT DEFINITION (U)

1 Unit of Enzyme is the amount of enzyme that will convert 1 μmol of sucrose in 1 minute at pH 5.0 and a temperature of 55°C.

I.e. 1 Unit ——> 3.44×10^{-4} g sucrose in 1 minute

2907 Unit ——> 1 gram sucrose in 1 minute

EXPERIMENTAL CONDITION :- Run (20.77-9-31.5-30.5-60-55)

Enzyme activity used = $60U/cm^3$

Volumetric Rate of Enzyme fed into the system = $31.5cm^3/min$

Total enzyme activity used = $60 \times 31.5 = 1890$ U/min

Concentration of sucrose fed in system = 20.77% W/V

Volumetric rate of sucrose fed into the system = $9\ cm^3/min$

Mass rate of sucrose fed into the system = $0.2077 \times 9 = 1.8693$g/min

$\therefore$ 1890 U of enzyme converts 1.8693g/min in 1 minute.

Theoretically the amount of enzyme required to convert 1.8693g/min

$= 2907 \times 1.8693 = 5434.0$

$$\therefore \quad \text{ENZYME USAGE} = \frac{\text{ACTUAL ENZYME USED}}{\text{THEORETICAL ENZYME USED}}$$

$$= \frac{1890}{54340} = 0.348$$

B2 ENZYME USAGE CALCULATION FOR A RECYCLING RUN

ASSUMPTIONS

(i) Enzyme from the enzyme storage tank was injected into the system over half of the cycle and the residual enzyme in the recycled GRP for the other half of the cycle.

(ii) Due to the deactivation of enzyme along the system the amount of enzyme recycled on the GRP stream was equivalent to the amount exiting from column 13; i.e. U/cm^3 for run:

EXPERIMENTAL CONDITION:- RUN (35.9-9-31.5-30.5-90-55)

Amount of pure enzyme used = $90U/cm^3$

Amount of enzyme in the recycled stream $\simeq 4U/cm^3$

Eluent rate fed into the system = $31.5cm^3/min$

Recycle rate fed into the system = $40.5cm^3/min$

Switch time = 30.5min

Total amount of enzyme fed in one cycle

$$= (90 \times 31.5 \times \frac{30.5}{2} \times 12) + 4 * 40.5 \times \frac{30.5}{2} \times 12)$$

$$518805 + 29646$$

$$= 548451 \text{ Unit}$$

Concentration of sucrose fed into the system = 35.9% W/V

Volumetric rate of feed = $9cm^3/min$

Mass Rate of feed = $0.359 \times 9 = 3.231g/min$

Mass Rate fed in one cycle = $3.231 \times 30.5 \times 12 = 1182.54g$

Theoretically, 2907 Unit convert 1 gram in 1 minute

$\therefore$ Amount of enzyme to convert 1182.54g

$= 1182.54 \times 2907$

$= 3437661$ Units

$$\text{ENZYME USAGE} = \frac{\text{ACTUAL ENZYME}}{\text{THEORETICAL ENZYME}} = \frac{548451}{3437661}$$

$$= 0.16$$

For a standard run with an enzyme activity of $90U/cm^3$ fed continuously into the system in one cycle, the total enzyme =

$$90 \times 31.5 \times 30.5 \times 12 = 1037610 \text{ Unit}$$

$$\text{ENZYME USAGE} = \frac{1037610}{3437661} = 0.302$$

APPENDIX C

```
      DIMENSION S(500),F(500),G(500),AS(500),AF(500),AG(500),SMASS(500)
     1SCUM(500),FMASS(500),FCUM(500),GMASS(500),GCUM(500),SCONC(500),
     2FCONC(500),GCONC(500),VS1(20),VF1(20),VG1(20)
      REAL KD1,KD2,KD3,KM,VMAX,VMAX1,KC2,KSC
      INTEGER KNL(20),M,NTCLS
C     INPUT ALL OPERATING PARAMETERS

      CFLOW=0.525
      FFLOW=0.15
      SFLOW=1.266
      DT=3
      TEMP=55
       SFEED=0.414
      ACTI=60.0
      ACT=ACTI*(3.44E-4)/60
      KSC=0.4575
      KD1=0.30
      KD2=0.715
       KD3=0.41
      KC2=0.01356
      NFEED=7
       KM=0.02
       VMAX=KC2*ACT
      NNBED=33
      NTCLS=12
      KCYC=12
      KTOTAL=NTCLS*KCYC
      SWT=1770
      CFLOWM=CFLOW*60.0
      SFLOWM=SFLOW*60.0
      FFLOWM=FFLOW*60.0
      TFEED=SFEED
      CFEED=TFEED*100.0
      V1=702.0/NNBED
      V2=803.0/NNBED
      SWP=SWT/60.0
C     PRINT OUT ALL OPERATING PARAMETERS

      WRITE (15,5)
      WRITE (15,6)
      WRITE (15,7)
      WRITE (15,8)
      WRITE (15,51) CFEED,CFLOWM,FFLOWM,SFLOWM,SWP,NNBED,TEMP,ACTI
      WRITE (15,9)
      WRITE (15,12) KCYC
      WRITE (15,10)
      WRITE (15,34)
    5 FORMAT(1H1,///,6X,'FEED',1X,'ELUENT',1X,'FEED',3X,'PURGE',2X,
     1'SWITCH',1X,'NO',6X,'TEMP',5X,'ENZYME')
    6 FORMAT(6X,'CONC',1X,'FLOW',3X,'FLOW',3X,'FLOW',3X,'PERIOD',1X,
     1'OF',15X,'ACTIVITY')
    7 FORMAT(8X,'%',2X,'RATE',3X,'RATE',3X,'RATE',3X,'MINS',3X,
     1'PLATES')
    8 FORMAT(11X,'ML/MIN',1X,'ML/MIN',1X,'ML/MIN',8X,'/COL',
```

```
  113X,'U/ML',//)
  9 FORMAT(//,10X,'AVERAGE  CONCENTRATION OF SUGARS ON EACH',
   111X,'COLUMN')
 12 FORMAT(10X,'AFTER',I3,'CYCLES')
 10 FORMAT(//,9X,'COL NO',2X,'AV SUCROSE CONCN',2X,'AV',
   11X,'FRUCTOSE CONCN',2X,'AV GLUCOSE CONCN'/)
 34 FORMAT(23X,'%W/V' ,15X,'%W/V' ,15X,'%W/V'/)
 51 FORMAT(6X,F4.1,1X,F5.1,2X,F4.1,3X,F5.1,2X,F4.1,3X,I2,4X,F6.2,
   16X,F6.2)
    DO 99 I=1,500
    S(I)=0.0
    F(I)=0.0
    G(I)=0.0
    AS(I)=0.0
    AF(I)=0.0
    AG(I)=0.0
 99 CONTINUE
    NNTOT=NNBED*NTCLS
    NNNINE=NNBED*(NTCLS-1)+1
    NNFEED=(NFEED-1)*NNBED+1
    DO 100 K=1,KTOTAL
    IF(K.EQ.1) ISTKK=0
    ISTKK=SWT*(K-1)+1
    LSTKK=SWT*K
    DO 200 KK=ISTKK,LSTKK,DT
    DO 300 N=1,NTCLS
    IF(N.LE.(NFEED-1))CFLOWC=CFLOW
    VMAX1=VMAX*(CFLOW/CFLOWC)
    IF(N.GE.NFEED)CFLOWC=CFLOW+FFLOW
    VMAX1=VMAX*(CFLOW/CFLOWC)
    IF(N.LE.(NFEED-K))GO TO 300
    NNFST=NNBED*(N-1)+1
    NNLST=NNBED*N
    DO 400 NN=NNFST,NNLST
    IF(N.EQ.1)GO TO 90
    IF((N.EQ.2).AND.(NN.EQ.NNFST))GO TO 40
    IF(NN.EQ.NNFEED) GO TO 50
    GO TO 70
 40 S(NN-1)=S(NNBED)
    F(NN-1)=F(NNBED)
    G(NN-1)=G(NNBED)
    GO TO 75
 70 IF(S(NN-1).LT.0.1E-10)S(NN-1)=0.0
    IF(F(NN-1).LT.0.1E-10)F(NN-1)=0.0
    IF(G(NN-1).LT.0.1E-10)G(NN-1)=0.0
 75 RR=CFLOWC/(V1+V2*KD1)
    SS=CFLOWC/(V1+V2*KD2)
     RA1=V1*VMAX1/(V1+V2*KD1)
     RA2=(0.5*V1*VMAX1)/(V1+V2*KD2)
     RA3=(0.5*V1*VMAX1)/(V1+V2*KD3)
     FRE=CFLOWC/(V1+V2*KD3)
    IF(SFEED.LE.0.60) THEN
    RR1=EXP(-(RR+(RA1/KM))*DT)
    SS1=EXP(-SS*DT)
    TT1=EXP(-FRE*DT)
    S(NN)=(RR/(RR+RA1/KM))*S(NN-1)*(1-RR1)+S(NN)*RR1
    F(NN)=(F(NN-1)*(1-SS1))+(S(NN)*(RA2/KM)+F(NN))*SS1
    G(NN)=(G(NN-1)*(1-TT1))+(S(NN)*(RA3/KM)+G(NN))*TT1
    ELSE
    RR1=EXP(-RR*DT)
    SS1=EXP(-SS*DT)
    TT1=EXP(-FRE*DT)
    S(NN)=(S(NN-1)-RA1/RR)*(1-RR1)+S(NN)*RR1
    F(NN)=(F(NN-1)+RA2/SS)*(1-SS1)+F(NN)*SS1
    G(NN)=(G(NN-1)+RA3/FRE)*(1-TT1)+G(NN)*TT1
    END IF
```

```
      GO TO 150
   50 IF(S(NN-1).LT.0.1E-10)S(NN-1)=0.0
      IF(F(NN-1).LT.0.1E-10)F(NN-1)=0.0
      IF(G(NN-1).LT.0.1E-10)G(NN-1)=0.0
       RR=CFLOWC/(V1+V2*KD1)
       SS=CFLOWC/(V1+V2*KD2)
       RA1=V1*VMAX1/(V1+V2*KD1)
       RA2=(0.5*V1*VMAX1)/(V1+V2*KD2)
       RA3=(0.5*V1*VMAX1)/(V1+V2*KD3)
       FRE=CFLOWC/(V1+V2*KD3)
       IF(SFEED.LE.0.60) THEN
      RR1=EXP(-(RR+(RA1/KM))*DT)
      SS1=EXP(-SS*DT)
      TT1=EXP(-FRE*DT)
      S(NN)=(RR/(RR+(RA1/KM)))*((CFLOW*S(NN-1)+FFLOW*SFEED)/CFLOWC)
     1*(1-RR1)+S(NN)*RR1
      F(NN)=F(NN-1)*(1-SS1)+(S(NN)*(RA2/KM)+F(NN))*SS1
       G(NN)=G(NN-1)*(1-TT1)+(S(NN)*(RA3/KM)+G(NN))*TT1
      ELSE
      RR1=EXP(-RR*DT)
      SS1=EXP(-SS*DT)
      TT1=EXP(-FRE*DT)
      S(NN)=(((CFLOW*S(NN-1)+FFLOW*SFEED)/CFLOWC)-RA1/RR)*(1-RR1)+
     1S(NN)*RR1
      F(NN)=(F(NN-1)+RA2/SS)*(1-SS1)+F(NN)*SS1
      G(NN)=(G(NN-1)+RA3/FRE)*(1-TT1)+G(NN)*TT1
      END IF
      GO TO 150
   90 IF(NN.EQ.NNFST)GO TO 95
      IF(S(NN-1).LT.0.1E-10)S(NN-1)=0.0
      IF(F(NN-1).LT.0.1E-10)F(NN-1)=0.0
      IF(G(NN-1).LT.0.1E-10)G(NN-1)=0.0
      GO TO 97
   95 S(NN)=0.0
      F(NN)=0.0
      G(NN)=0.0
      GO TO 400
   97 RR=SFLOW/(V1+V2*KD1)
      SS=SFLOW/(V1+V2*KD2)
      FRE=SFLOW/(V1+V2*KD3)
      RR1=EXP(-RR*DT)
      SS1=EXP(-SS*DT)
      TT1=EXP(-FRE*DT)
      S(NN)=S(NN-1)*(1-RR1)+S(NN)*RR1
      F(NN)=F(NN-1)*(1-SS1)+F(NN)*SS1
      G(NN)=G(NN-1)*(1-TT1)+G(NN)*TT1
  150 IF(K.EQ.KTOTAL.AND.KK.EQ.(LSTKK*SWT))GO TO 160
      GO TO 400
  400 CONTINUE
  300 CONTINUE
  200 CONTINUE
  160 DO 500 NN=1,NNBED
      AS(NN)=S(NN)
      AF(NN)=F(NN)
      AG(NN)=G(NN)
  500 CONTINUE
      DO 600 NN=1,NNTOT
      IF(NN.GE.NNNINE)GO TO 2010
      NNADJ=NN+NNBED
      S(NN)=S(NNADJ)
      F(NN)=F(NNADJ)
      G(NN)=G(NNADJ)
      GO TO 600
 2010 NNADJ=NN+1-NNNINE
      S(NN)=AS(NNADJ)
      F(NN)=AF(NNADJ)
```

```
      G(NN)=AG(NNADJ)
  600 CONTINUE
  100 CONTINUE
      SCUM(1)=0.0
      FCUM(1)=0.0
      GCUM(1)=0.0
      DO 11 I=1,NNTOT
      SMASS(I)=S(I)*V1+S(I)*KD1*V2
      FMASS(I)=F(I)*V1+F(I)*KD2*V2
      GMASS(I)=G(I)*V1+G(I)*KD3*V2
   11 CONTINUE
      L=NNBED
   27 DO 14 I=1,NNTOT+1
      IF(I.EQ.(L+1))SCUM(I)=SMASS(I)
      IF(I.EQ.(L+1))FCUM(I)=FMASS(I)
      IF(I.EQ.(L+1))GCUM(I)=GMASS(I)
   14 CONTINUE
      L=L+NNBED
      IF(L.EQ.(NNTOT+NNBED))GO TO 15
      GO TO 27
   15 L=NNBED
      I=2
      Z=0
      SCUM(1)=0.0
      FCUM(1)=0.0
      GCUM(1)=0.0
   13 DO 16 M=I,L
      SCUM(M)=SMASS(M)+SCUM(M-1)
      FCUM(M)=FMASS(M)+FCUM(M-1)
      GCUM(M)=GMASS(M)+GCUM(M-1)
      SCONC(M)=SCUM(M)/2700.0
      FCONC(M)=FCUM(M)/2700.0
      GCONC(M)=GCUM(M)/2700.0
      KOLNO=M/NNBED
      IF(M.EQ.L) GO TO 18
      GO TO 16
   18 Z=Z+1
      KNL(Z)=KOLNO
      VS1(Z)=SCONC(M)*100
      VF1(Z)=FCONC(M)*100
      VG1(Z)=GCONC(M)*100
   16 CONTINUE
      I=L+2
      L=L+NNBED
      IF(L.EQ.(NNTOT+NNBED)) GO TO 355
      GO TO 13
  355 WRITE(15,4)KNL(1),VS1(NTCLS),VF1(NTCLS),VG1(NTCLS)
      FINLST=NTCLS-1
      DO 886 Z=1,FINLST
       TST=Z+1
       WRITE(15,4)KNL(TST),VS1(Z),VF1(Z),VG1(Z)
  886 CONTINUE
    4 FORMAT(11X,I2,9X,F5.2 ,15X,F5.2 ,14X,F5.2)
      GO TO 17
   17 P=SWT
       FRU=VF1(1)/100
       GLU=VG1(1)/100
       SCU=VS1(1)/100
       FRPC=FRU*2700./(P*SFLOW)
       GLPC=GLU*2700./(P*SFLOW)
       SLCP=SCU*2700./(P*SFLOW)
       SU=VS1(11)/100
       GU=VG1(11)/100
       FU=VF1(11)/100
       PUF=(FRPC/(FRPC+GLCP+SLPS))*100
       PUG=(GU/(GU+FU+SU))*100
```

```
      WRITE(15,80)
   80 FORMAT(//24X,'FRU PRO',22X,'GLU PRO',/,13X,'FCONC',3X,'GCONC',
     1 3X,'PURITY',8X,'FCONC',3X,'G/SCONC',6X,'PURITY',/,60X,'GLU',
     2 6X,'SUC')
      WRITE(15,66) FRPC,GLPC,PUF,FU,GU,PUG,PUS
   66 FORMAT(13X,F5.2,2X,F5.2,2X,F6.2,10X,F5.2,2X,F5.2,5X,F5.2,2X,F5.2
      STOP
       END
```

FEED CONC %	ELUENT FEED FLOW RATE ML/MIN	FEED FLOW RATE ML/MIN	PURGE FLOW RATE ML/MIN	SWITCH PERIOD MINS	NO OF PLATES /COL	TEMP	ENZYME ACTIVITY U/ML
34.0	31.3	9.0	76.0	30.0	33	55.00	60.00

AVERAGE CONCENTRATION OF SUGARS ON EACH COLUMN
AFTER 12CYCLES

COL NO	AV SUCROSE CONCN %W/V	AV FRUCTOSE CONCN %W/V	AV GLUCOSE CONCN %W/V
1	0.00	0.00	0.00
2	0.00	5.73	1.41
3	0.00	14.26	13.28
4	0.00	14.46	17.55
5	0.00	14.64	20.47
6	0.40	14.79	23.35
7	5.14	14.35	25.18
8	4.16	11.07	25.49
9	2.05	6.28	25.22
10	1.00	3.07	24.67
11	0.49	1.30	23.99
12	0.24	0.36	22.86

FRU PRO			GLU PRO			
FCONC	GCONC	PURITY	FCONC	G/SCONC	PURITY GLU	PURITY SUC
0.07	0.02	80.34	0.00	0.23	97.47	1.01

LIST OF SYMBOLS USED FOR THE SIMULATION OF THE CONTINUOUS OPERATION OF THE SCCR-S1 SYSTEM

Ai	Array Containing concentrations of component i
ACTI	Enzyme activity ($U.cm^{-3}$)
C_{FLOW}	Mobile phase flow rate ($cm^3.S^{-1}$)
D_T	Time increment
F	Array containing the fructose concentration in each plate ($g.cm^{-3}$)
FFLOW	Feed flow rate
G	Array containing the glucose concentration in each plate ($g.cm^3$)
iCONC	Average concentration of components in each column ($g.cm^{-3}$)
iCUM	Cumulative mass of component i (g)
iMass	Arrays containing the mass of component i for each plate (g)
ISTKK	First time increment in a sequence
KC2	Reaction rate constant
KCYC	Total number of cycles
KD1	Distribution coefficient of sucrose
KD2	Distribution coefficient of fructose
KD3	Distribution coefficient of glucose
KKINK	Number of time increments in a sequence
KM	Michaelis Menton constant
KSC	Substrate inhibition constant
KTOTAL	Total number of sequence
LSTKK	Last time increment in a sequence
N	Column number
NFEED	Number of feed column
NN	Plate counter
NNBED	Number of "apparent" plates in each column (based on fructose)
NNFEED	Number of feed plate

NNFST	First plate in a column
NNLST	Last plate in a column
NNNINE	Last plate in the previous column
NNTOT	Total number of "apparent" plates on the system
NTCLS	Total number of columns
S	Array containing the sucrose concentration in each plate ($g.cm^{-3}$)
SFEED	Concentraiton of sucrose in feed ($g.cm^{-3}$)
SFLOW	Purge flowrate ($cm^3\ S^{-1}$)
SWP	Switch time (min)
TEMP	Operating temperature (°C)
V1	Mobile phase plate volume (cm^3)
V2	Stationary phase plate volume (cm^3)
Vmax	Maximum initial velocity

The subscript i represent sucrose, glucose and fructose.