BIOREACTION AND SEPARATION IN BATCH CHROMATOGRAPHIC COLUMNS

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Doctor of Philosophy

THE UNIVERSITY OF ASTON

September 1994

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SUMMARY

The objective of this work has been to study the behaviour and performance of a batch chromatographic column under simultaneous bioreaction and separation conditions for several carbohydrate feedstocks. Four bioreactions were chosen, namely the hydrolysis of sucrose to glucose and fructose using the enzyme invertase, the hydrolysis of inulin to fructose and glucose using inulinase, the hydrolysis of lactose to glucose and galactose using lactase and the isomerization of glucose to fructose using glucose isomerase. The chromatographic columns employed were jacketed glass columns ranging from 1 m to 2 m long and the internal diameter ranging from 0.97 cm to 1.97 cm. The stationary phase used was a cation exchange resin (PUROLITE PCR-833) in the Ca^{2+} form for the hydrolysis and the Mg^{2+} form for the isomerization reactions.

The mobile phase used was a diluted enzyme solution which was continuously pumped through the chromatographic bed. The substrate was injected at the top of the bed as a pulse. The effect of the parameters pulse size, the amount of substrate solution introduced into the system corresponding to a percentage of the total empty column volume (% TECV), pulse concentration, eluent flowrate and the enzyme activity of the eluent were investigated.

For the system sucrose-invertase complete conversions of substrate were achieved for pulse sizes and pulse concentration of up to 20 % TECV and 60 % w/v, respectively. Products with purity above 90 % were obtained. The enzyme consumption was 45 % of the amount theoretically required to produce the same amount of product as in a conventional batch reactor. A value of 27 kg sucrose/m^3 resin/h for the throughput of the system was achieved. The systematic investigation of the factors affecting the performance of the batch chromatographic bioreactor-separator was carried out by employing a factorial experimental procedure. The main factors affecting the performance of the system were the flowrate and enzyme activity.

For the system inulin-inulinase total conversions were also obtained for pulses sizes of up to 20 % TECV and a pulse concentration of 10 % w/v. Fructose rich fractions with 100 % purity and representing up to 99.4 % of the total fructose generated were obtained with an enzyme consumption of 32 % of the amount theoretically required to produce the same amount of product in a conventional batch reactor.

The hydrolysis of lactose by lactase was studied in the glass columns and also in an SCCR-S unit adapted for batch operation, in co-operation with Dr. Shieh, a fellow researcher in the Chemical Engineering and Applied Chemistry Department at Aston University. By operating at up to 30 % w/v lactose feed concentrations complete conversions were obtained and the purities of the products generated were above 90%. An enzyme consumption of 48 % of the amount theoretically required to produce the same amount of product in a conventional batch reactor was achieved.

On working with the system glucose-glucose isomerase, which is a reversible reaction, the separation obtained with the stationary phase conditioned in the magnesium form was very poor although the conversion obtained was compatible with those for conventional batch reactors. By working with a mixed pulse of enzyme and substrate, up to 82.5 % of the fructose generated with a purity of 100 % was obtained.

The mathematical modelling and computer simulation of the batch chromatographic bioreaction-separation has been performed on a personal computer. A finite difference method was used to solve the partial differential equations and the simulation results showed good agreement with the experimental results.

KEY WORDS: bioreactor-separator, chromatographic reactor, invertase, lactase, inulinase.

Dedicated to the memory of my mother and to my patient family who followed me to this country and to the other part of the family who stayed in Brazil.

ACKNOWLEDGEMENTS

The author wishes to thank the following:

Professor B Tighe, Dr. E L Smith and the Department of Chemical Engineering and Applied Chemistry for making available the facilities for this research.

Professor P E Barker, who supervised the work, for his advice, guidance, constructive criticism and cooperation in revising the text of this thesis.

Dr. E L Smith who co-supervised the work for the fruitful exchange of ideas, advice and his helpful support in revising this thesis.

Dr. M T Shieh for his cooperation on the batch mode operation of the SCCR-S unit.

All the staff in the Department, specially Mr. M Lea, Mr D Bleby, Mrs. L Wright, Mrs. R Wright, Mr. M Santoro, Mr. D Walton, Dr. M Perry, Mr S Ludlow

Dr. J Ajongwen of Merck Sharp and Dohme for his friendly support and inestimable help during my first steps in 1991.

Dr. S Setford and my fellow research students in the Separation and Purification Group for their practical help and useful discussions.

Professor Claudio Costa Neto from Universidade Federal do Rio de Janeiro, Brazil, who first showed me the door to the chromatographic world back in 1964 and for his support in obtaining the sponsorship.

Professors Regina Sandra Nascimento and Adelaide Antunes from Universidade Federal do Rio de Janeiro for their most appreciated friendship, help and support during my stay in England.

To Universidade Federal do Rio de Janeiro who allowed me to take a leave of absence.

Finally I would to thank the Coordenação do Programa RHAE - Recursos Humanos em Áreas Estratégicas and Conselho Nacional de Desenvolvimento Científico e Tecnológico for the sponsorship.

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CHAPTER ONE

INTRODUCTION

1.1 Simultaneous Bioreaction and Separation in Batch Chromatographic Columns

In the last 90 years, chromatography has evolved from a laboratory analytical tool to the industrial scale. The wide range of applications include the isolation of small amounts of very expensive active substances in the pharmaceutical sector to millions of tons per year of high fructose syrups used in the food and drink sector. With this latter application, columns ranging from 2 to 5 m diameter and from 10 to 15 m long are being used. They perform the chromatographic enrichment of glucose and fructose mixtures obtained by the isomerization of glucose syrups to produce products with fructose purities ranging from 55 % to pure fructose.

Starting in the late 1950's chromatographic columns became of great interest for the performance of simultaneous chemical reaction and separation of the products formed (1). Its main feature was the enhancement of reaction rates and yields. By separating the products from the reaction zone, as soon as they are formed, the displacement of the equilibrium towards the product side is achieved.

The feasibility of using of the chromatographic column to perform simultaneous bioreaction and separation was established by Barker and Zafar in 1986 (2,3). This work was linked with Fisons Pharmaceuticals who were interested in the production of dextran, a polyglucose, with molecular weights of up to $2x10^6$ daltons. They studied the biosynthesis and separation of dextran working with a chromatographic column containing a cationic exchange resin in the Ca^{2+} form. A dilute solution of dextransucrase continuously flowed through the chromatographic bed as an eluent.

By performing the bioreaction-separation in a chromatographic column, Barker and Zafar were able to produce dextran with improved yields of high molecular weight dextran as compared to that produced in a conventional batch reactor. The yield was improved because some of the fructose molecules were removed from the reaction zone, as they were retained by the Ca^{2+} resin, and therefore prevented from acting as acceptors. Using this approach the production of a high purity fructose fraction with a high market value was also achieved. In the conventional dextran production process the fructose is carried all the way through the downstream processing steps. It becomes contamined by metallic ions and other impurities thereby presenting severe disposal problems.

The batch bioreaction separations work by Barker and Zafar (2,3) has been successfully extended to other bioreaction studies in annular and in semi-continuous chromatographic separators (4,5,6).

The chromatographic chemical reactor, as a multifunctional reactor, enhancing reaction rates and yields is not a new concept in chemical engineering (7). Some industrial applications have been mentioned in the area of trans-esterifications and ethanol production (8,9). In esterifications and trans-esterifications, the products are separated in order to enable an equilibrium reaction to move to completion. In traditional fermentations using the micro-organism *Saccharomyces cerevisiæ*, the accumulation of ethanol reduces the specific cell growth and consequently the ethanol production. The continuous removal of the ethanol produced during the fermentation by distillation minimises the product inhibition effect (9).

Product inhibition is very common in bioprocesses. This type of inhibition was found in the lactose hydrolysis investigated by Griethuysen-Dilber *et al.* in a pilot continuous biochemical reactor using immobilized microbial cells (10). Other approaches can be used to eliminate the substrate and product inhibition effects. Setford (11,12) using a rate-zonal centrifugation technique successfully carried out simultaneous bioreaction and separation. Using this technique he was able to produce dextran with a molecular weight distribution comparable to that obtained from a conventional batch reactor. This was due to the removal of acceptor fructose molecules from the sedimenting reaction zone by the action of the centrifugal forces.

Bioreaction and the downstream processing steps are the most important components of bioprocess activities (13). Due to the complex and sensitive nature of the bioprocess streams, these steps are regarded as technically challenging and incur high costs. The components of the process streams often include biopolymers, microbial cells and salts. They usually occur in dilute solutions due to inherent product and substrate inhibition effects. Downstream processing alone accounts for 50-80 % of the total production costs of a biotechnology operation (14). The demand to improve performance in bioprocess activities with consequent reduction of costs within the industry is increasing.

The integration of the bioreaction and product recovery in a single step is one of the approaches being used to improve the performance of biotechnology processes, as in the case of ethanol production. The main feature of this approach is that the reaction and separation take place simultaneously in the same equipment unit especially for processes where product or substrate inhibition is present. In this context, the chromatographic column employed as a bioreactor-separator is one form of meeting these objectives and worthy of further studies.

1.2 The Research Objectives

Because of the nature of the dextran obtained in the previous batch chromatographic bioreactor-separator studies (2), it was difficult to model with confidence the system and to fully elucidate the behaviour of the system properly. The objectives of this research work therefore were to select systems with different challenges so that the behaviour and performance of batch chromatographic bioreaction separations could be placed on a firmer basis. The four systems listed below have been chosen for study on 2 m long and 1 and 2 cm ID jacketed glass columns packed with cationic exchange resin.

a - Hydrolysis of sucrose by invertase

This system was chosen because it is a well known enzymatic reaction and regarded as a relatively simple process. The substrate and enzyme were relatively pure and inexpensive to purchase. The invertase used in this research was obtained from *S. cerevisæ*.

b - Hydrolysis of inulin, a polyfructan, by inulinase

This system was selected because it represents an alternative to the traditional process for the production of high fructose syrups using inulin from plants of the *Compositæ* family. The inulinase used in this work was obtained from *Aspergillus niger*.

c - Hydrolysis of lactose by lactase

The lactose-lactase system was chosen as an example of a typical bioreactor-separator process. It can be considered as an alternative to the traditional batch process for galactose production for two reasons. Firstly, the main feature of the chromatographic process can be practised: galactose which is a product of the bioreaction and strongly inhibits the enzyme lactase can be removed from the reaction zone and, consequently, the reaction rate and yield enhanced. Secondly, the process offers the alternative of the recovery of both reaction products. In the traditional galactose production, glucose is removed by further fermentation with yeasts, and therefore is not recovered. The lactase used in this work was obtained from *A. oryzæ*.

d - Isomerization of glucose to fructose by glucose isomerase

Since all the reactions (a-c) are unidirectional a reversible bioreaction namely the isomerization of glucose by glucose isomerase was also chosen for study. This is an important isomerization reaction largely employed in the manufacture of fructose syrups. The enzyme used in this research was obtained from *Actinoplanes missouriensis*.

The basic philosophy of conducting the bioreactions to their completion was adopted for the experimental work, although this did not prove to be possible with system (d).

1.3 Thesis Outline

This thesis has the following organisation. Chapter 2 presents a survey of the literature on chromatography, chromatographic reactors, enzymes and carbohydrates. The survey on chromatography covers a very brief history since its "discovery" by Tswet, chromatographic theory, techniques and the most important industrial applications of chromatography. Chromatographic reactors covers the principles of liquid chemical reaction-separations, bioreaction-separation and modelling. The enzyme section focuses on kinetics and the main characteristics of the species used in this research. Carbohydrates covers the main aspects of the sugars related to this thesis.

Chapter 3 describes the materials and methods employed in this project. It covers the chemical products used, equipments, experimental and analytical procedures developed for the quantitative analysis of carbohydrates, enzyme assays and particle size measurement applied to the ion exchange resins. The methods employed for the determination of the maximum reaction rate and the Michaelis constants for the enzyme kinetics are also discussed.

Chapter 4 covers the chromatographic physical separation of the products generated in the bioreactions and the kinetics of the bioreaction systems used in this research. The preparation of the stationary phase including size grading and resin conditioning is described. The data obtained for the characterisation of the chromatographic columns employed, namely distribution coefficients, resolution of products, separation factors, are presented and discussed. The effect of background concentrations, temperature and flowrate on the distribution coefficients are also introduced here. The results from the determination of the maximum reaction rate and the Michaelis constants for the enzyme kinetics make up the final section.

Chapter 5 describes the preliminary experiments related to the system sucrose-invertase and the systematic investigation employing a factorial experimental procedure. The effects of pulse size, pulse concentration, enzyme activity and flowrate were explored. Two levels for each factor were studied giving a total of 16 sets of experimental conditions. The main factors affecting the performance of the system are discussed.

Chapter 6 covers the system inulin-inulinase. Two sets of experiments were conducted. In the first set, the eluent employed was a diluted enzyme solution and the substrate injected as a pulse. In the second, the substrate and the enzyme were mixed and injected as a pulse.

Chapter 7 describes the batch bioreaction-separation experiments for the system lactose-lactase. Two sets of experiment were conducted. In the first one, the column employed was

the jacketed glass column 2 m long x 2 cm ID. The second set was performed in the modified SCCR-S1 equipment in co-operation with Dr. Shieh, a fellow researcher in the Department.

Chapter 8 describes the system glucose-glucose isomerase. The experiments were carried out in a 2 m x 1 cm ID column using cation exchange resin in the Mg^{2+} form as stationary phase. Both techniques previously employed for the system inulin-inulinase were again used for the present system. The results, nevertheless, indicated that this system needs a much fuller investigation than was possible here.

Chapter 9 describes the mathematical modelling and computer simulation of the batch chromatographic bioreactor-separator for the systems investigated. The set of differential equations developed based on the continuity equation was solved by a finite difference method and implemented on a personal computer using a FORTRAN algorithm.

Finally, chapter 10 presents the main conclusions of the research and also lists suggestions for further investigations.

CHAPTER TWO

CHROMATOGRAPHY, CHROMATOGRAPHIC REACTORS, ENZYMES AND CARBOHYDRATES

2.1 Chromatography

2.1.1 A Brief History

The term "chromatography" was coined by Michail Semenovich Tswett in his paper "On a New Category of Adsorption Phenomena and Their Application to Biochemical Analysis" presented at a meeting of the Biological Section of The Warsaw Society of Natural Sciences on March 21st 1903 (15). This novel method was a further development of his M.S. thesis work on the studies of chlorophyll in 1901 (16).

In the early 1940s Martin and Synge developed 'partition chromatography' stating that "the mobile phase need not to be a liquid but a vapour" (17) and it was followed by a publication of Consden *et al.* (18) presenting 'paper chromatography', another variant of liquid chromatography. During the 1950s partition gas-liquid chromatography was introduced by James and Martin (19). By the middle of the 1960s the knowledge accumulated in the previous two decades in general chromatographic theory, the appearance of new types of stationary phases, the development of mechanical devices that could overcome the high pressures developed in the systems by the use of very small particles as stationary phases and the modern small-volume detectors, to mention just a few points, initiated the modernisation of liquid chromatography. The development of the experimental work presented in this thesis will be based on this technique.

2.1.2 Chromatographic Techniques

The unit operation of chromatography can be defined as a separation method based on the differential migration velocity of solutes, due to their different adsorption and desorption characteristics. The solutes percolate through a column, which is essentially a system of two phases, the mobile phase - the eluent and the stationary phase - the support. Depending on the nature of the phases, if gaseous, liquid or solid, four chromatographic systems can be devised: liquid-solid chromatography, meaning liquid mobile phase and a solid stationary phase, gas-solid, with a gaseous mobile phase and a solid stationary phase, liquid-liquid chromatography, with a liquid mobile phase and a liquid stationary phase and

gas-liquid, with a gaseous mobile phase and a liquid stationary phase. A detailed description of these techniques can be found elsewhere (20,21).

The increasing use of supercritical fluids or SF's (high density gases above their critical temperature) being used as mobile phases recently introduced the classification SFC, Supercritical Fluid Chromatography (21, 22)

The core of a chromatographic system is the stationary phase where the solutes will be more or less retained depending on the nature of their retention mechanism. There are four types of retention mechanisms: adsorption, if the retardation is caused by adsorption on granular solids; partition, if the retardation is caused by a liquid impregnated in a porous material; ion-exchange (or simply ion chromatography), whenever ionic groups from the ion exchange materials used as stationary phase exchange positions with ions in the mobile phase, and size exclusion, where the solutes, usually macromolecules, are excluded from the stationary phase rather than being differently attracted.

Chromatographic separations are accomplished by the differential migration of solutes along the column. The following methods can be used: frontal analysis or adsorptive filtration (20), elution development, gradient elution where the composition of the eluent is varied continuously and displacement chromatography which is characterised by the fact that the eluent is more strongly retained by the stationary phase than the solutes and displaces completely the solute from the stationary phase. Liquid chromatography is characterised by elution development. In this technique the relative distribution of the solutes between the mobile and the stationary phases occurs and, as they migrate at different rates, the separation occurs. Typical elution chromatograms are thus obtained in which, ideally, each peak is separated from the following one by a band of pure eluent.

The adsorption isotherm may be either linear or non-linear. A linear isotherm implies that the amount of non sorbed solute is proportional to the amount sorbed. Linearity holds only for very dilute solutions in adsorption chromatography which is known, in this case, as linear chromatography (21). At higher concentrations a noticeable departure from linearity occurs and peak position depends on sample size. This condition is called overloading and results in non-linear chromatography, a characteristic of preparative chromatography, where the Langmuir isotherms apply.

Furthermore, chromatographic separations can be operated in three different ways, all aiming to increase the throughputs in preparative and production scale: batch, including repetitive feed injection technique, semi-continuous cross-current and counter-current and

continuous annular chromatography. Some considerations about these types will be made in the sections related to chromatographic bioreactor-separators.

2.1.3 Chromatographic Theory

2.1.3.1 Principles of Chromatography

Separation in chromatography is achieved through the differential migration of solutes which results from their relative distribution between the mobile phase and the stationary phase, expressed by the distribution coefficient, K_{di} , defined as:

$$K_{di} = \frac{q_i}{C_i} \qquad (2.1)$$

where, q_i is the concentration of component 'i' in the stationary phase and C_i the concentration of component 'i' in the mobile phase.

The chromatographic process is a resultant of other inseparable processes such as convective transport, diffusion and sorption equilibration. Nevertheless, it is the difference in the distribution coefficients of solutes that determines the differential migration rates resulting in a final separation. To those factors should be added the geometrical irregularities of the chromatographic bed and the hydrodynamic properties of the fluids.

2.1.3.2 Chromatographic Concepts and Definitions

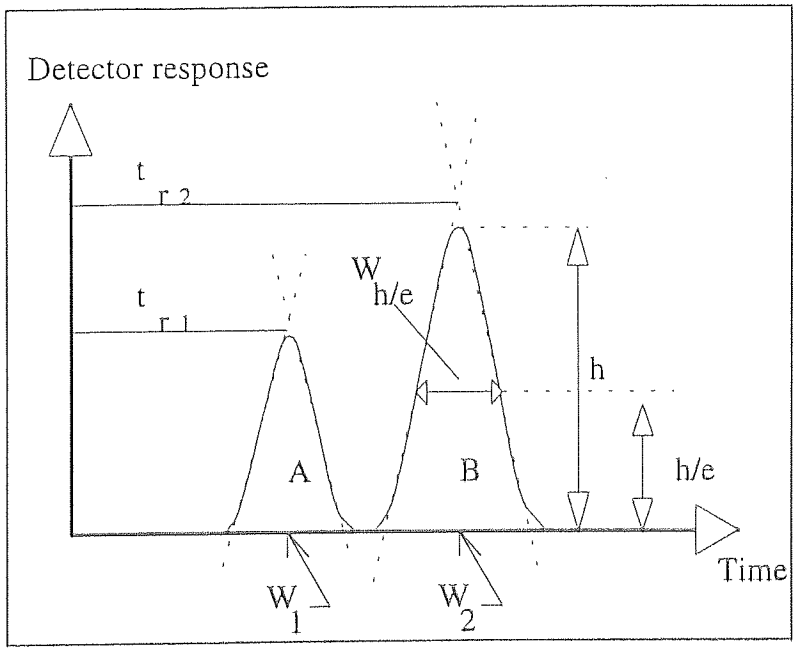
If the concentration of the product stream is measured and plotted with time, the resulting profile or elution curve is called a chromatogram. A typical chromatogram is shown in Figure 2.1. The retention time t_{ri} (t_{r2} and t_{r1} for the components 2 and 1, respectively, in Figure 2.1) is the average time the component 'i' has taken to migrate along the chromatographic bed and is measured at its maximum concentration value.

The retention volume, V_i , is the volume of mobile phase required for the complete elution of a component 'i'. The relation between retention volume and the distribution coefficient, K_{di}, for a component 'i' is given by:

$$V_i = V_0 + K_{di} V_s \qquad (2.2)$$

where, V_0 is the total void volume in the column occupied by the mobile phase and V_s the volume of the stationary phase.

Figure 2.1 - Typical chromatogram showing the elution profile for two solutes



Equation 2.2 is regarded as the Retention Equation. V_0, for practical means, is usually defined as the elution volume of the eluent molecules and measured for an unretained substance. Therefore,

$$V_s = V_T - V_0 \qquad (2.3)$$

where, V_T is the total volume of the column.

Combining equations 2.2 and 2.3 we get:

$$K_{di} = \frac{V_i - V_0}{V_T - V_0} \qquad (2.4)$$

The separation factor α is a measure of the separation potential of the column related to two components A and B , according to the following relation:

$$\alpha = \frac{K^{\infty}_{dA}}{K^{\infty}_{dB}} \qquad (2.5)$$

were, K^{∞}_{dA} is the distribution coefficient at infinite dilution for solute A and K^{∞}_{dB} the distribution coefficient at infinite dilution for solute B

The infinite distribution coefficients may be calculated from equation 2.4.

The degree of separation R_S of two substances in chromatography is called resolution and is defined by the following relation:

$$R_S = \frac{2\,(t_{r2} - t_{r1})}{(W_1 + W_2)} \qquad (2.6)$$

where, t_{r2} and t_{r1} are the retention times for the components 2 and 1 and W_1 and W_2 their peak width measured at the base of the elution curve all referred to the same units, either cm or min. The notation $R_{1\text{-}2}$ will also be used in the text of this thesis.

2.1.3.3 Theory of Band Broadening

When a sample band is pulsed into a chromatographic column it is broadened or spread by diffusion processes associated with flow velocity and rates of various equilibration processes. There are two important theoretical approaches to describe the phenomena which have been extensively treated by Giddings (23): the theoretical plate concept and the rate theory.

The theoretical plate concept was first applied by Martin and Synge in 1941 (17) in analogy with the concepts developed for distillation. They considered a column consisting of a 'number of theoretical plates' within each of which perfect equilibrium between the two phases occurred. Glueckauf (24) using a similar approach improved the model of Martin and Synge. On reducing the plate volume to an infinitesimal value he converted the discontinuous model into a continuous one. The retention time t_{ri} was related to the variance of the "apparent" number of plates, N*, for a chromatographic column through the following correlation:

$$N^* = 8\left(\frac{t_{ri}}{W_{h/e}}\right)^2 \qquad (2.8)$$

where, $W_{h/e}$ is the peak or band width measured at a height equal the relation h/e, h being the height of the peak and 'e' the base of the natural logarithm (see Figure 2.1)

The model failed to take into account the molecular distribution, sorption phenomena and flow patterns causing zone spreading although it is used to measure column efficiency.

The role of the dispersion process in the chromatographic theory was first recognised and treated by Lapidus and Amundsen in 1952 (25). Van Deemter *et al.* (26) developed the model further and introduced the contribution of the so-called eddy diffusion to the band broadening and the longitudinal diffusion effects as well. The model is referred to as the

rate theory model and the equation 2.9, known as Van Deempter equation, expressed for H (plate height) has general validity for chromatography.

$$H = A + \frac{B}{u} + C_M . u + C_S . u \qquad (2.9)$$

where, A is the eddy diffusion term, B the longitudinal diffusion term, C_M the resistance to mass transfer in the mobile phase, C_S the resistance to mass transfer in the stationary phase and u the mobile phase linear velocity

2.1.4 Continuous Chromatography

Chromatography has been used as a very powerful technique for the purification of small amounts of expensive active substances in the pharmaceutical sector and for the production of commodities for example the high fructose corn syrups (see section 2.4.2.3). Batch operation is still used in both cases. Production scale based on batch operation uses automated repetitive operation of large diameter columns. Simulated moving bed chromatography which allows the continuous introduction of a feed and continuous removal of product fractions is an alternative approach which can give increased throughputs.

Continuous chromatographic processes can be classified in two ways: the cross-current and the countercurrent systems. In the cross-current systems the chromatographic bed moves perpendicularly to the direction of fluid motion within the bed. In this case, the chromatographic process that takes place in the continuous-cross current chromatograph is a steady state separation occurring in both axial and circumferencial directions of the bed (27). This is the principle used by the continuous annular chromatographs first described by Martin in 1949 (28).

The counter-current systems uses two approaches to obtain the relative movement of the mobile and the stationary phases: the moving-bed and the moving-column systems. In the moving-bed continuous systems the stationary phase flows under gravity countercurrent to a stream of mobile phase flowing upward (29). The moving-column continuous systems are characterised by a circular array of interconnected columns, which rotate as a whole past fixed inlet and outlet ports (30). Both counter-current systems face difficulties in achieving a reliable seal between the static ports and the moving columns.

Simulated moving-bed or moving port systems circumvented these mechanical difficulties. In these systems a number of static columns are interlinked and the counter-current movement is effectively achieved by sequentially moving the inlet and the outlet ports

associated with each column, in the direction of the mobile phase. There are two main approaches: the Sorbex process developed by Universal Oil Products (UOP) (31) and the semi-continuous chromatographic refiners (SCCR's) developed at Aston University (32).

2.1.5 Some Industrial Applications of Chromatography

Elf-Aquitaine (France), operating now as Separex, and Societe de Recherches Techniques et Industrielles have also been very active in batch gas chromatography. Their column diameter ranged from 1 cm to 50 cm for the separation of flavours and fragrances from terpene feedstocks and normal paraffins from light naphta feedstocks (33).

Separex is also specialised in batch high performance liquid preparative chromatography (HPPLC). This system is characterised by columns packed with extremely fine particles, ranging from 10 μm to 30 μm. They usually operate in overload conditions and develop pressures up to 100 bar. The main use of these systems are the pharmaceutical, fine chemicals, flavours and fragrances and biotechnology industries (34).

Batch chromatographic equipment for high performance high pressure liquid chromatography is being commercialised by many other companies. Prochrom S. A., Merck GmbH, Amicon Inc., Pharmacia Biotech AB and Fisons Instruments UK are some companies that are very active in marketing their equipment (35-39).

Finnish Sugar Co. Ltd (Finland) specialised in the desugarization of beet molasses, recovering sucrose through the ion-exclusion principle, and in the production of high purity fructose and HFCS (high fructose corn syrups) using cation exchange resins in the calcium form. Their first unit was based on a 2.7 m diameter x 6 m high column. Their technology is now based on modular units consisting of seven 3.6 m diameter x 12 m high columns processing 60,000 tons/yr. of molasses (40).

Xyrofin (Finland) is also operating a plant producing 13,000 tons/year of crystalline fructose from corn syrup based on Finnish technology, in the USA (41).

Munir Zucker AG (Germany) reported a process for the treatment of molasses, employing three 1m diameter x 6 m high columns packed with 4 % Lewatit TSW 40 cation exchange resin in the calcium form (42).

Staley (USA) are probably the largest producers of HFCS and their level of production reaches some millions tons per year outputted by eight production centres all over the world. They also participate in join ventures in other countries (43).

IMASA-Industrias de Maiz S.A. (Argentina) has installed a plant in Argentine based on Staley's technology, with a capacity of 10,000 tons/yr. of HFCS. Another plant is planned to be installed in Uruguay, under the management of IMASA (44).

STARCOSA GmbH (Germany) and IWT-Illinois Water Treatment have licensed plants and equipment for HFCS production and the latter one also licenses technologies for molasses desugarization based on the Finnish Sugar Co. process (45,46).

GETEC, a Brazilian manufacturer of glucose, sorbitol and mannitol, have recently upgraded their technology. They adopted the chromatographic separation of glucose from fructose, obtained from acid hydrolysis of cane sugar, to be used as raw material in their plant in substitution of inverted sugar and tapioca starch. The equipment was supplied by IWT and is basically one chromatographic column which is operated continuously (47).

The simulated moving bed principle has been developed by many companies and the most active in licensing the technology is Universal Oil Products (UOP). It was reported that more than 40 plants were in operation world-wide with a total capacity of 3.5 million tons/year in 1989 (48). Advanced Separation Technologies Inc. developed and are currently operating an industrial unit consisting of 30 columns for the purification of fermentation products (49). According to this reference there are many plants in operation based on this technology.

2.2 Chromatographic Reactors

2.2.1 Introduction

Chromatographic columns have been used as chromatographic reactors since the late 1950's (1) mostly applied to gas phase reactions. The first patents on the subject were awarded to Magee (50) and Dinwiddie and Morgan (51), both in 1961. From the 1960's to the 1970's the literature covering the subject showed that the chromatographic column was a very powerful tool for measurements of kinetic rates and the determination of mechanisms of some catalysed organic reactions (52-63).

The development of the first mathematical models was due to Magee (52) for the reaction $2\,HD \leftrightarrow H_2 + D_2$ using a simplified differential model and Roginskii *et al.* for the reaction of the type $A \leftrightarrow B$, showing the main peak tailing or with a slow ascending front depending on the distributions coefficients, K_{di}'s, for the products (54, 55).

Early applications involving gas chromatographic systems led to several discussions of the advantages of chromatographic reactors relative to conventional static and flow reactors (55,56,60,64). Some studies also showed the improvement of the equilibrium conversions for some industrial chemical reactions such as the dehydrogenation of cyclohexane into benzene, butylenes into butadiene and the Haber process in the synthesis of ammonia (61,65).

Starting from the late 1960's, liquid chromatography emerged as one of the most powerful techniques available for the separation, identification and production of chemicals because of its versatility and freedom from the volatility requirement of gas chromatography. The merits of speed, efficiency, accuracy and ease of use have permitted modern liquid chromatography to be applied to compounds that range from simple molecules to complex macromolecules.

While the possibility of carrying out reactions to advantage in gas chromatographic columns has been realised in many instances, reaction kinetic studies and applications in liquid chromatography columns are less developed. Due to the increasing use of large scale preparative liquid chromatographic equipment by the chemical industry in general (66) and the recent appreciation of the features of the liquid chromatographic reactor, more opportunities for broader applications of modern liquid chromatographic systems could emerge (67).

Chromatographic reactor-separator devices can be defined as the systems where chemical species are totally or partially converted into products which are simultaneously separated by the principles that govern chromatographic separations. The chemical reaction can take place either in the mobile phase or in the stationary phase or both (57). The attractive features of using chromatographic columns as chemical reactors include the following:

- the presence of concerted reaction and separation processes throughout the column
- the possibility of exceeding equilibrium conversions for reversible reactions as well as selective production of reaction intermediates with high purity in series reactions
- the ease of manipulation and control of chromatographic apparatus and associated detectors
- the relative ease in measuring the rate of reaction specially in catalytic reactions.

Although chromatographic systems have been relatively exploited but restricted to the study of chemical reactions, their use in the biochemical field is still emerging. There are

only two groups which have employed chromatographic columns as bioreactor-separators. The first, Hashimoto *et al.* (68,69) from Kyoto University, worked with a mixed system of chromatographic columns and columns containing immobilized glucose-glucose isomerase enzyme for the production of high purity fructose. The second group, Barker *et al.* (5,6,70,71) from Aston University, has studied important bioreactions using batch, semi-continuous and continuous annular systems employing flowing enzyme systems.

2.2.2 Principles of Chromatographic Reaction-Separation

According to Matsen *et al.* (61), based on a single pulse technique, the chromatographic reactor could be advantageous when the reaction involved is a reversible one, the equilibrium constant for the reaction is small and the reaction rates are high enough. In this case the product separation is the limiting step rather than the reaction rate. Gore (64) developed the model further adopting repetitive pulses and observed better conversions although with higher consumption of catalyst. Antonucci *et al.* (63) have also observed the equilibrium of the dehydrogenation of ethane over cadmium-exchanged zeolite 4A, performed in a chromatographic column, being displaced towards the product side.

The chromatographic reaction-separation principle is based on the assumption that the rate of a chemical process is much lower than that of the mass transfer between the phases. The following cases applied to liquid reactions illustrate the principle.

Case 1: Reactions of the type A → B + C

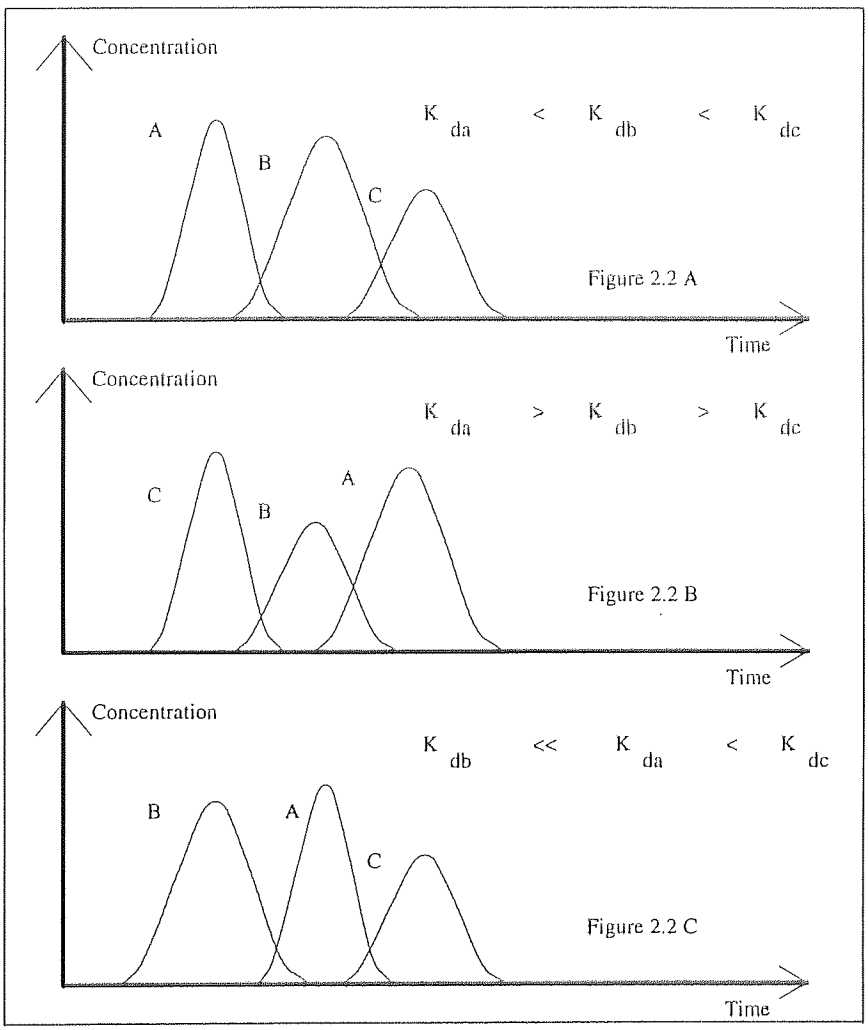
If $K_{dA} < K_{dB} < K_{dC}$, the products B and C are formed continuously and appear as tails at the rear of the unreacted moving band of component A (Fig. 2.2 A). Also the overlapping between the products B and C regenerates some A, thus restricting the extent of the conversion. Similarly, when $K_{dA} > K_{dB} > K_{dC}$, the inadequate separation between B and A presents similar problems and indicates that high conversion require very long columns (Fig. 2.2 B). The ideal situation, however, is when $K_{dB} < K_{dA} < K_{dC}$ or $K_{dC} < K_{dA} < K_{dB}$, enabling high conversions and high product purity (Fig. 2.2 C).

This is the case for hydrolysis reactions catalysed by enzymes, for instance, which will be discussed in detail in section 2.2.3 of this chapter.

Case 2: Reactions of the type A + B ↔C + D (or A (+ B) ↔ C + D)

This case comprises the esterification of alcohols by carboxylic acids and trans-esterification reactions, both general equilibrium limited reactions. Reactions of such type are only possible when $K_{dA} = K_{dB} \neq K_{dC} \neq K_{dD}$. If this holds, then A and B are eluted together and the alternative permutations are similar to that of the previous reaction.

Figure 2.2 - Concentration profiles for partial conversions obtained under pulse chromatographic conditions



The earliest reported work on liquid chromatographic reactors by Wetherold *et al.* (72) falls within this case. They performed an experimental and computational study of the liquid phase hydrolysis of methyl formate to methanol and formic acid occurring under chromatographic conditions. The system used comprised a chromatographic column containing activated charcoal as stationary phase.

Another example is the esterification reaction of acetic acid and ethanol to ethyl acetate studied by Sardin and Villermaux (73). The reaction was performed in a chromatographic column 1.5 m long by 7 mm ID containing a mixture of activated alumina and cation exchange resin in the acidic form as stationary phase to catalyse the reaction and separate the products. The product, ethyl acetate, was not adsorbed and was obtained pure in the eluent, separated from the reactants. The eluent used was a mixture of heptane and dioxane. The conversion of about 90 % was obtained for both pulsed the regime and single injection as compared to 67 % equilibrium conversion using a conventional continuous reactor.

A further example is the trans-esterification reaction of menthol with ethyl acetate to menthyl acetate and ethanol studied by Sardin and Villermaux (74). The reaction was carried out in a chromatographic column containing cation exchange resin in the acidic form as stationary phase.

More recently, Cho *et al.* (75) proved it was possible to perform a liquid chromatographic chemical reaction and separation using a continuous rotating annular chromatograph. They carried out the hydrolysis of methyl formate to methanol and formic acid. The chromatograph was made of two concentric cylinders, the outer being 20 cm outside diameter, which formed an annulus 30.5 cm long x 0.6 cm wide. Activated charcoal was used as stationary phase and a complete conversion was achieved compared to 75 % equilibrium conversion with a non separating reactor. Very poor separation of the products formed was obtained due to the formic acid peak which wrapped completely around the cylinder. The methanol peak was reasonable well-formed. The mathematical model developed for the reactor performance was based on an ideal chemical reactor with a Freundlich adsorption isotherm.

Case 3: Reactions of the type $nA \rightarrow B_n + nC$

This case represents the chemical or biochemical synthesis of macromolecules B and especially when one of the products acts as an acceptor C terminating the polymer chain growth. In this example, the removal of C from the reaction mixture improves the yield of the long chain polymer B. An example of this type of reaction is the biosynthesis of dextran by the enzyme dextransucrase using sucrose as substrate (2,6) which will be discussed in section 2.2.3.

Case 4: Reactions of the type A ↔ B + C

According to Coca et al (76) in the case of reversible reactions of the type A ↔ B + C it is possible to enhance conversions through product separation so that reverse reactions are minimised. This increases product yield and permits an approach to conversions considerably beyond equilibrium values according to the observations of Basset and Habgood (58) in the isomerization of cyclopropane to propylene.

Case 5: Isomerization reactions of the type A ↔ B

One of the features of chromatographic reactors is the displacement of the reaction towards the products side. According to Sardin *et al.* (77) this is not possible because whatever product B is formed in the column and whatever the separation between A and B, the backward reaction destroys B unless it is catalysed by A. The mathematical model for this type of reaction was developed by Villermaux (78). The chromatograms were predicted using Laplace transforms. It was shown that the conversion in a pulsed regime was identical to the conversion at steady state. According to the authors, to improve the conversion of B, a heating zone moving at the same velocity as the pulse of A should be used. In this pulse, the temperature would be higher than elsewhere. Away from the pulse, from which B is extracted, the lower temperature can stop the reverse reaction and this would lead to conversions higher than the steady-state conversion.

Carta et al. (79) worked on the chromatography of reversibly reacting mixtures of glucose and fructose and showed the mutarotation effects in sugar separations for temperatures ranging from 0.1 °C to 60 °C. A mathematical model based on a differential equation was developed to describe the chromatographic behaviour of the two linear adsorbed components. At lower temperatures two distinct peaks of the tautomeric forms were obtained. The results obtained for the reversibly reacting mixtures of glucose and fructose confirmed the predictions from the model developed by Villermaux (78).

2.2.2.1 Conclusions

The actual use of liquid chromatographic reactors on a production scale has been addressed only recently and is still in a early stage of development. The examples given are related to situations where enhanced product yield for reversible reactions was a major concern. The potential for reduction of downstream purification costs where the liquid chromatographic reactor is applied is another positive aspect where economics are important. With these

features this type of reactor becomes a prominent candidate for consideration when significant production of costly chemicals involves either reversible or consecutive reactions (78).

2.2.3 Bioreaction-separations in chromatographic columns

2.2.3.1 Introduction

The possibility of performing simultaneous bioreaction and separation in batch chromatographic columns using a diluted solution of enzyme as eluent, which ensures intimate contact between substrate and enzyme, was established by Barker and Zafar in 1986 (2,3). They studied the biosynthesis of dextran using the enzyme dextransucrase and sucrose as substrate. The technique of using a diluted enzyme solution as eluent is very appropriate when the enzyme and substrate have different distribution coefficients values. They chose the flowing enzyme method with this system because dextransucrase does not readly immobilise.

Two other possibilities of performing batch chromatographic bioreaction-separations in chromatographic columns can be adopted. The first one uses the technique of pulsing a mixture of the enzyme and substrate into the chromatographic column, which has been demonstrated in this thesis (see chapters 6 and 8). This method is appropriate for bioreaction-separations when the enzyme and substrate have similar distribution coefficients values. In this case both enzyme and substrate percolate together along the column allowing the reaction to take place. The advantage of this method is the fairly low consumption of enzyme compared to other methods, although the reactions performed showed that 50 % of the substrate was left unreacted.

In the second one, the enzyme is immobilized on a support and alternate columns are packed with the immobilized enzyme and the stationary phase (adsorbent). This technique was used by Hashimoto *et al.* (68,69,80) for the production of high fructose syrups using glucose as substrate.

In the following sections the utilisation of three types of chromatographic reactor systems, namely batch, semi-continuous and continuous annular, will be reviewed.

2.2.3.2 Batch Chromatographic Bioreactor-Separators

Zafar and Barker (2,3) carried out the biosynthesis of dextran using two batch chromatographic bioreactor-separators with sizes of 150 cm x 0.97 cm I.D. and 175 cm x 1.94 cm I.D. They reported that the use of these systems not only allowed biochemical reaction and simultaneous product separation, but improved the dextran yield due to the continuous removal of the acceptor fructose from the reaction mixture. They observed that working at 15 % w/v sucrose concentrations, 79 % of the dextran produced had a molecular weight over 157,000 daltons while by conventional fermentation only 40 % of the dextran produced was over 157,000 daltons.

This work is the only work related to batch chromatographic bioreaction-separation which is reported in the literature and the potential of this technique has still to be fully realised. The research group under Prof. Barker has extended the technique to annular chromatography and semi-continuous chromatographic systems. A new line of investigation is being implemented using stationary phases with a much smaller particle size which demand special columns to maintain the internal pressure developed (81).

2.2.3.3 Continuous Chromatographic Bioreactor-Separators

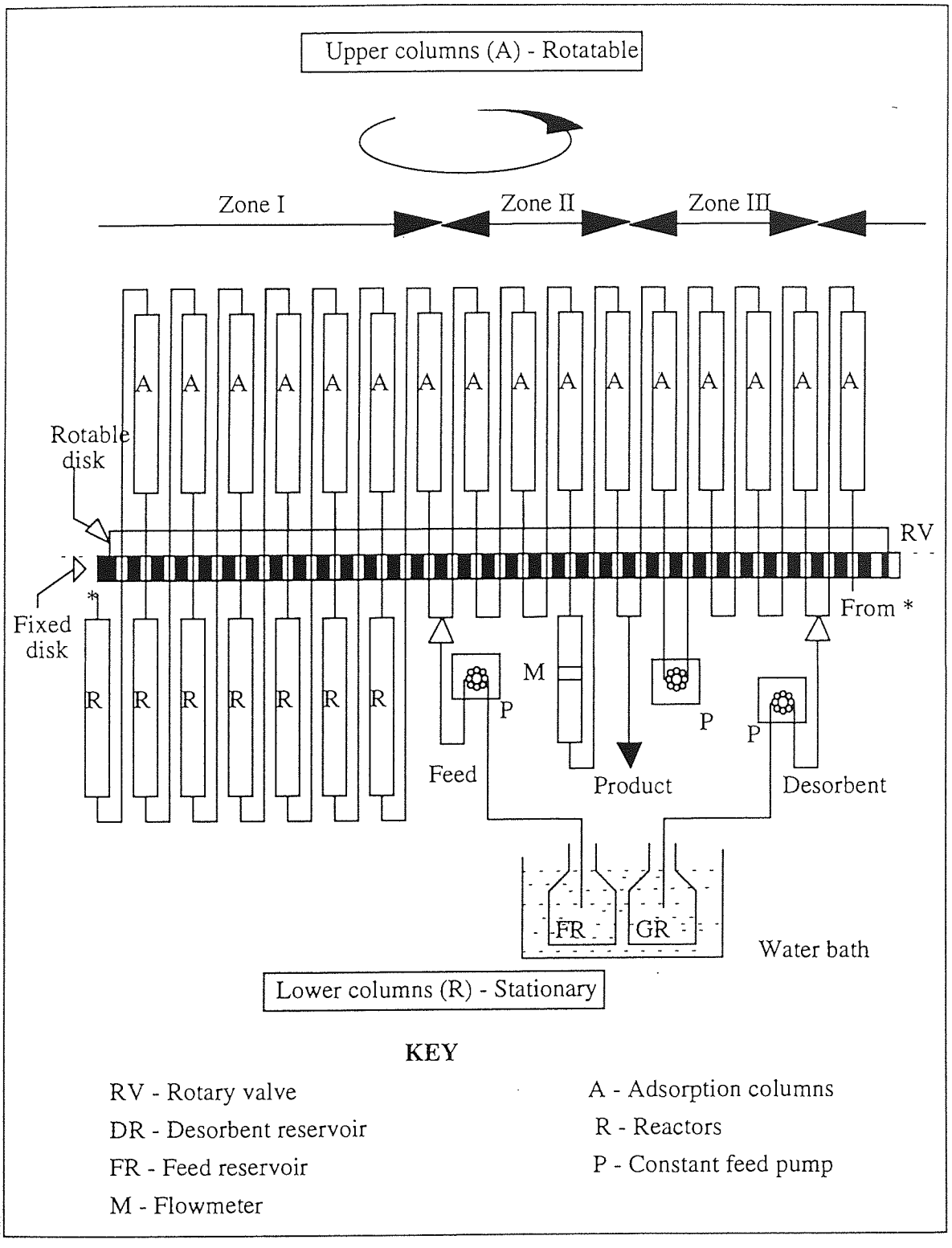
2.2.3.3.1 The Hashimoto *et al.* Simulated Moving Bed Systems

The isomerization of glucose to fructose, using a mixed system of columns packed with immobilized glucose isomerase enzyme alternating with columns packed with the adsorbent used for the separation of the sugars, was studied by Hashimoto *et al.* (69).

The isomerization of glucose to fructose was carried out in a simulated moving bed countercurrent chromatographic reactor. The schematic diagram of the equipment is shown in Figure 2.3. This same equipment has been used for the continuous separation of a glucose-fructose mixture (68).

The experimental apparatus consisted of 16 jacketed chromatographic (or adsorption) columns and 7 jacketed reactors. Each chromatographic column had a dimension of 1.38 cm in diameter and 10.2 cm in length. Each reactor was 1.38 cm in diameter and 18.0 or 10.2 cm long. The chromatographic columns were packed with the Ca^{2+} form of Y zeolite supplied by Tohso (Japan) and the reactors packed with immobilized glucose isomerase from Nagase Sangyo (Japan). The chromatographic columns and the reactors were connected to form a closed loop.

Figure 2.3 - Schematic diagram of the continuous moving bed experimental apparatus used by Hashimoto *et al.*



The system was divided into three zones. The reactors and adsorption columns were alternately arranged in zone I. Zones II and III consisted only of adsorption columns.

The countercurrent movement between the liquid stream and the adsorbent was simulated using a rotary valve, which consisted of two stainless steel disks as shown in Figure 2.3. Each flow of liquid through holes in the valve was isolated by using an "O"-ring to prevent escape of liquid from the boundary between the disks.

The lower disk was stationary whereas the upper disk could be rotated. The adsorption columns were connected to the upper rotary disk of the valve. The upper disk was rotated counter-clockwise 22.5° (= 360° / 16) at regular intervals (2 to 5 min). On the other hand, the positions of feed, desorbent and product streams were fixed.

An equimolecular mixture of glucose and fructose introduced as a feed stream flows into the adsorption column located in zone I. Fructose in the mixture is preferentially adsorbed, together with a small quantity of glucose. The glucose content of the solution leaving the adsorption column becomes higher than the equilibrium level and is introduced into the next reactor, where the glucose is converted to fructose almost to the equilibrium level.

By introducing the mixture through the adsorption columns and reactors alternately in zone I, most of the glucose mixture is converted to fructose, which is completely adsorbed on the adsorbent. Thus the liquid emerging from zone I contains very little of either glucose or fructose, and can be reused as part of the desorbent. The fructose adsorbed in zone I was transferred to zones II and III by the movement of the adsorption columns. The fructose adsorbed was desorbed in zone III as the final product.

Their system was capable of producing syrups with fructose content of 55 % using less desorbent than in the equivalent fixed bed batch process.

2.2.3.3.2 The Barker *et al.* Moving Port Systems

The developments of Barker *et al.* at Aston University represent a significant contribution to the behaviour and performance of simultaneous bioreaction and separation in the continuous chromatography field. Their systems were originally developed for the physical separation of carbohydrate feedstocks (82-93) and the fractionation of dextran (94,95).

The modifications introduced to one of these systems, the SCCR-S, enabled studies on the hydrolysis of sucrose by invertase (4,71), the biosynthesis of dextran by dextransucrase using sucrose as substrate, the hydrolysis of lactose by lactase and the hydrolysis of modified starch by maltogenase for the production of maltose and dextrins (6).

The SCCR-S1 used by Akintoye (4) and Shieh (6) consisted of 12 stainless steel columns 75 cm long x 5.4 cm ID, connected at the top and the bottom to form a closed loop. The columns were packed with cation exchange resin in the Ca^{2+} form. Six pneumatic poppet valves were connected to each column namely the feed, eluent and purge valves, the glucose rich (GRP) and the fructose rich (FRP) product valves and the transfer valve to the next column.

The operation principle of the SCCR-S is illustrated in Figure 2.4. The two diagrams correspond to two consecutive switches. In switch (A) the feed and the eluent are fed to columns 7 and 2, respectively, and flow in a clockwise direction. The less strongly adsorbed component glucose being eluted by the eluent towards the glucose product outlet at column 11. Meanwhile, column 12 is isolated from the loop by closing the transfer valves on either side of the column. A purge stream of deionised water is fed to the column to desorb the adsorbed fructose and exits from the column as the fructose rich product.

After a set time interval known as the switch time, all the ports functions are advanced by one column in the direction of the mobile phase as shown in switch (B). This advancement of port function results in a simulated movement of the stationary phase in an opposite direction to the mobile phase flow. Column 1 is then purged and, at the same time, the feed and the eluent enter column 8 and column 3. The glucose rich products exits from column 12 and the operation is repeated at the next switching. A cycle of 12 sequences are completed when each column has been used as the feed and purge location. To achieve the separation of the reaction products, the rate of port advancement should be greater than the more retained product migration velocity and lower than the less retained and the unreacted substrate migration velocity.

Akintoye (4) working with the SCCR-S demonstrated that, using the cation exchange resin KORELA VO7C (Finnish Sugar, Finland) in the Ca^{2+} form and working with sucrose solutions up to 50 % w/v, complete hydrolysis of sucrose could be obtained. He found that the consumption of the enzyme invertase was only 34 % of the theoretical amount of enzyme required by a conventional batch process. Product purities of over 90 % were obtained. Product concentrations and product throughputs of over 5 % w/v and 16 kg sugar/m^3 resin/h, respectively, were achieved. Additionally, the studies performed on the SCCR-S showed that substrate inhibition was minimised with this type of processing, contrary to what is generally observed when working with conventional reactors.

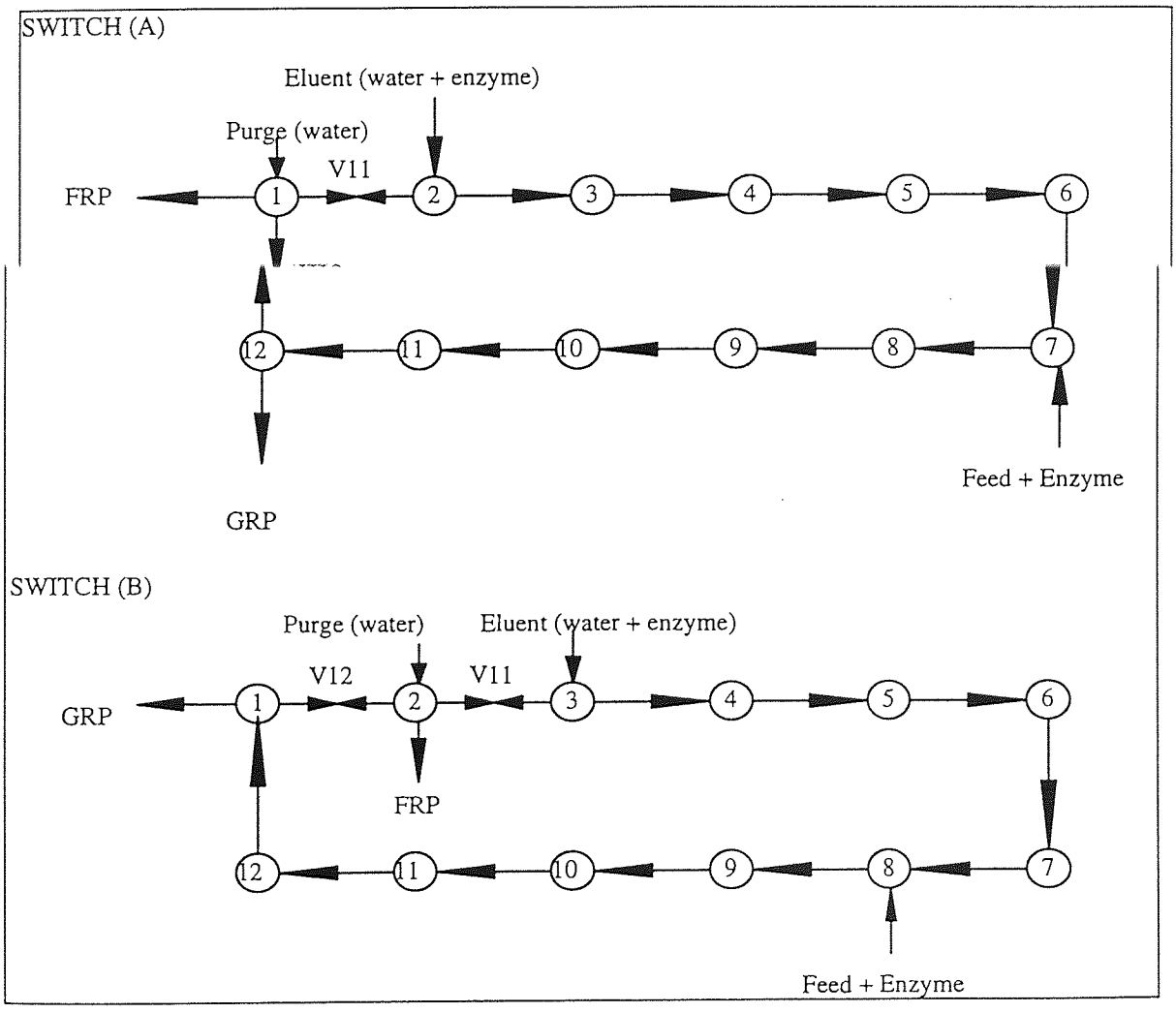
The second bioreaction carried out in the SCCR-S was performed by Shieh (6). The system was packed with DOWEX 50W-X4 (150 μm mean particle size) cationic resin in the Ca^{2+} form. Shieh worked with the system sucrose-dextransucrase for the biosynthesis of dextran.

The operation was similar to that performed by Akintoye. Dextran was the fastest eluted compound, due to its size exclusion, while fructose was retarded by the resin. At 2-5 % w/v sucrose concentrations, 100 % sucrose conversion was achieved. Over 60 % of the dextran produced had molecular weights greater than 2,000,000 daltons.

The hydrolysis of modified starch by maltogenase for the production of maltose was the third system investigated by Barker *et al.* Shieh, using feed throughputs of up to 113 g/h, obtained conversions of up to 60 % and maltose purities up to 95.8 % (6).

Shieh also investigated the performance of the SCCR-S1 equipment working with the system lactose-lactase (6). He obtained products with purities above 90 % using only 50-60 % of the theoretical amount of enzyme required by a conventional batch process.

Figure 2.4 - Schematic representation of the SCCR-S1 operating principle

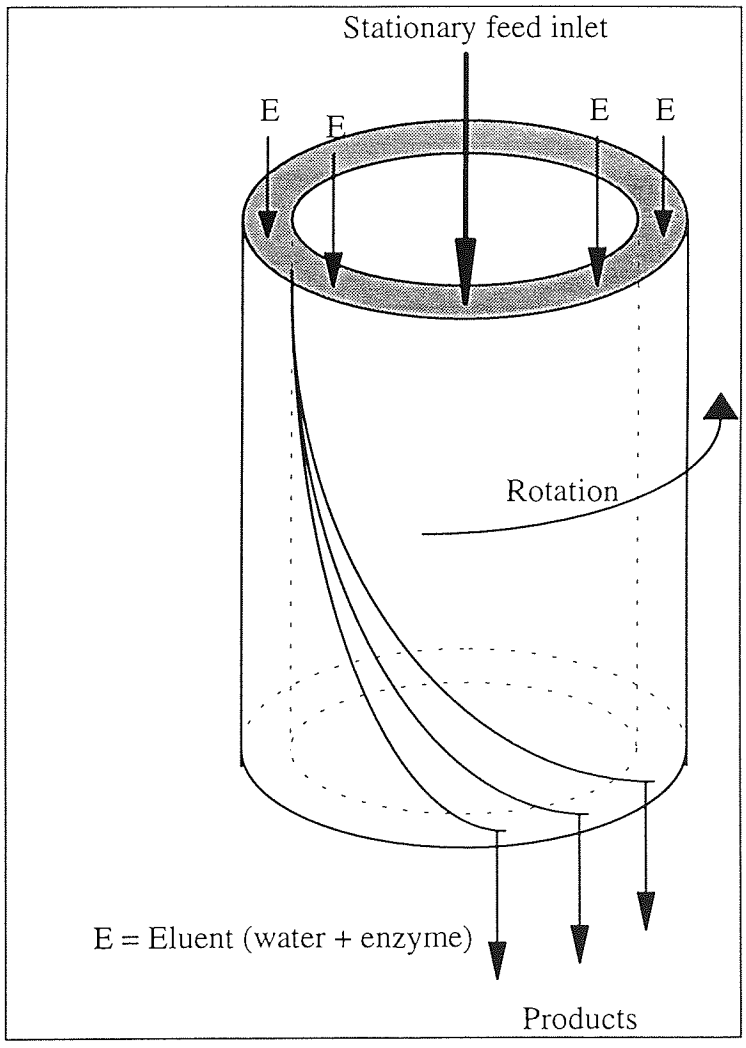


2.2.3.3.3 Continuous Rotating Annular Bioreactor -Separator

Based on published information, only Barker and co-workers have explored the use of the continuous rotating annular chromatograph (CRAC) in the biochemical field (96,97). Two bioreaction-separations were conducted by Sarmidi (5) on this equipment namely the inversion of sucrose by the enzyme invertase and the saccharification of modified starch to maltose by the enzyme maltogenase.

The CRAC consisted of two concentric cylinders which formed an annulus 140 cm long, 29.7 cm external diameter and 1.2 cm wide giving an annular volume of 14.5 dm^3. The stationary phase used was the cation exchange resin DOWEX-X4 in the Ca^{2+} form with a mean particle size of 150 μm. The cylinders were mounted on a turntable which was slowly rotated by a variable speed motor. The CRAC was enclosed in a temperature controlled cabinet. The principle of the operation is illustrated in Figure 2.5.

Figure 2.5 - Operating principle of the continuous rotating annular bioreactor-separator



The operation started by switching on the motor drive and adjusting the speed controller to the required speed. The eluent solution (E - Figure 2.5) was continuously pumped through the inlet distributer block located in the top flange of the CRAC. The feed was continuously introduced through a nozzle adapted in the inlet distributor block (stationary feed inlet - Figure 2.5). The samples were collected from one hundred evenly spaced exit holes located at the lower plate fitted at the bottom of the CRAC.

The results obtained by Sarmidi (5) for the bioreaction-separation of sucrose to glucose and fructose showed that a feed throughput up to 17.2 kg of sucrose/m^3 resin/h could be achieved. Products with concentrations of up to 5 % w/v were obtained using 78 -120 % of the theoretical amount of enzyme required by a conventional batch process.

On working with the saccharification of modified starch for the production of maltose using the enzyme maltogenase, Sarmidi (5) obtained starch conversions of up to 82 % with 80 % maltose recovery.

2.2.4 Models Developed for the Liquid Chromatographic-Separator Systems

2.2.4.1 Batch Chromatographic Reaction-Separation Models

According to Jeng and Langer (67) two approaches have been used for the mathematical analysis of the batch chromatographic reactor-separator: the mixing cells model and the differential model.

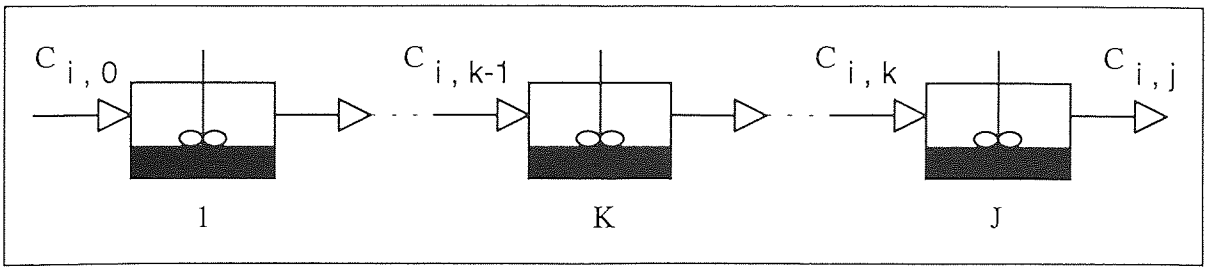
The mixing cell model was first reported by Villermaux in 1972 (98) and later reviewed by Schweich and Villermaux (99). This approach has also been adapted by Cretier *et al.* (100) for the modelling of physical chromatographic separations where no chemical reaction is involved. The model is similar to the one that Martin and Synge developed in 1941 (17). The column is regarded as being divided into J cells of identical volume V/J as shown in Figure 2.6.

The mass balance on component A_i in cell number k is expressed by the following relation:

$$Q C_{i,k-1} \pm r\frac{V}{J} = Q C_{i,k} + (1 + \alpha_i)\frac{V}{J}\frac{d C_{i,k}}{d t} \qquad (2.10)$$

where, Q is the volumetric flowrate, α_i the capacity factor of the column, r the reaction rate, V the volume of the mobile phase or the stationary phase and C_i the concentration of the substance i.

Figure 2.6 - The Martin and Synge or the mixing cells model



To solve the model a set of ordinary differential equations were developed instead of a set of partial differential equations as with the continuous model.

The use of the differential model is regarded as a more general approach for modelling the dynamics of liquid batch chromatographic reactors (67). A material balance for a substrate eluting through a differential section Δz of an ideal chromatographic column, at position z and time t, as shown in Figure 9.1 can be expressed as:

$$\frac{\partial c_i}{\partial t} + \left(\frac{1-\varepsilon}{\varepsilon}\right)\frac{\partial q_i}{\partial t} + + u_0 \frac{\partial c_i}{\partial z} = D_{i\,ax} \frac{\partial^2 c_i}{\partial z^2} - r \qquad (2.11)$$

$$(1-\varepsilon)\frac{\partial q_i}{\partial t} = k_0\, a\, F_{s/m}\ (q_i, c_i) \qquad (2.12)$$

where c_i is the concentration of the reactant in the mobile phase, q_i the concentration of the reactant in the stationary phase, ε the voidage of the chromatographic bed, u_0 the interstitial velocity of the eluent, r the reaction rate, $D_{i\,ax}$ the axial dispersion coefficient, k_0 the mass transfer coefficient, $F_{s/m}$ the relationship between concentration of solute in the mobile phase and the stationary phase, t the time and z the space along the bed.

Jeng and Langer (101) solved the equation analytically using Laplace transforms and a statistical moments method. Chu and Langer (59) included the axial dispersion in their model and used the same method of solution. The set of equations 2.11 and 2.12 can only be solved numerically if the process develops under non-linear adsorption isotherms, characteristic of overloaded chromatography, and higher order reaction rates (102-105).

The differential model was adopted by Barker and Zafar (2,3) for the simulation performed in their work. The reaction was assumed to occur only in the mobile phase and that the kinetics obeyed the Michaelis-Menten model. Some other simplifying assumptions were made. The adsorption relationship was assumed to be linear and the process taken to be under conditions of negligible axial dispersion. Equation 2.11 was reduced to equation

2.13, expressed for sucrose concentration C_s, and a finite difference method was adopted for the solution.

$$(1+\frac{1-\varepsilon}{\varepsilon} K_{dS}) \frac{\partial C_S}{\partial t} + u_0 \frac{\partial C_S}{\partial z} = \frac{C_S + V_{max}}{C_S + K_M} \qquad (2.13)$$

2.2.4.2 Continuous Chromatographic Reactor-Separator Models

2.2.4.2.1 The Hashimoto *et al.* Model for the Simulated Moving Bed Systems

Hashimoto *et al.* proposed an intermittent moving bed model for their simulated moving bed system. The model is similar to that proposed for analysis of a simulated moving-bed adsorber performing only chromatographic separations (68). The bioreaction-separation model includes the reaction term represented by the Michaelis-Menten model.

The development of the intermittent model was based on the assumption that the system included no real movement of the adsorption particles. Each adsorption column was considered to be a fixed bed except at the moment of rotating the columns. In the adsorption column, the mass balance equations for the concentration of component *k* (glucose or fructose) in the mobile phase C_k and the component k equilibrated to C_k, C_k*, are given by equations 2.14 and 2.15.

$$\varepsilon_b \frac{\partial C_k}{\partial t} = -v_n \frac{\partial C_k}{\partial z} - K_f a_v (C_k - C_k^*) \qquad (2.14)$$

$$(1-\varepsilon_b) m_k \frac{\partial C_k^*}{\partial t} = K_f a_v (C_k - C_k^*) \qquad (2.15)$$

where K_f . a_v is the overall volumetric mass transfer coefficient, m_k the distribution coefficient for component k, v_n the superficial velocity and ε the bed voidage of the adsorption column.

2.2.4.2.2 The Model for Chromatographic Bioreaction-Separations in Countercurrent Simulated Moving Port Systems

The mathematical model developed for the simulation of bioreactions performed in the simulated moving port systems by Barker *et al.* was based on the mixing cell model as shown in Figure 2.7 (4,71). In the model pseudo-first order reaction kinetics for sucrose inversion were assumed.

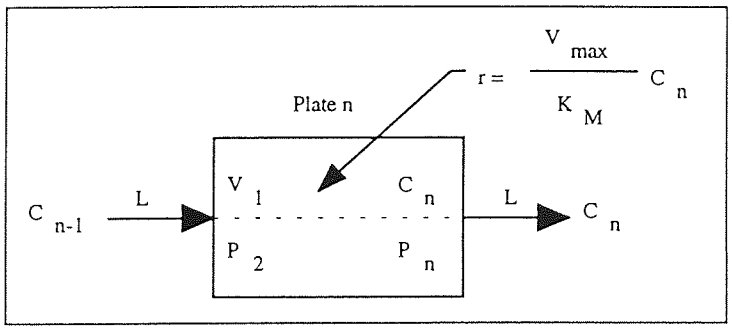
A mass balance for the reactant over plate n gives:

$$V_n \frac{dc_n}{dt} + V_n \frac{dP_n}{dt} = Lc_{n-1} - rV_1 \qquad (2.16)$$

where, V_1 is the volume of the mobile phase, V_2 the volume of the stationary phase, P_n the concentration of solute in the stationary phase, c_n the concentration of solute in the mobile phase and r the reaction rate expressed as $V_{max}/K_M.C_n$.

Similar equations for glucose and fructose were obtained. Under linear equilibrium distribution conditions the linear differential equations were solved analytically using the Laplace transformation technique. Good agreement between experimental and theoretical predictions was obtained.

Figure 2.7 - The mixing cell model



2.2.4.2.3 The Model for Continuous Rotating Annular Chromatographic Bioreaction-Separations

The CRAC was modelled and simulated by Sarmidi (5) for the inversion of sucrose to glucose and fructose by the enzyme invertase. The model was based on the continuity equations for the flowing mobile phase in a conventional chromatographic column and then applied to an annular chromatographic column by translating the time dimension t to angular position θ. The following relationship was used: $t = \theta / \omega$, where θ is the angular co-ordinate and ω the rotation rate. Equation 2.17 shown below was derived from a steady state mass balance for sucrose. It was solved by a finite difference method. Similar equations were obtained for glucose and fructose. The Michaelis-Menten model with substrate inhibition for the sucrose inversion was assumed.

$$\omega \frac{\partial c_s}{\partial \theta} + \omega \left(\frac{1-\varepsilon}{\varepsilon} \right) \frac{\partial q_s}{\partial \theta} + u_0 \frac{\partial c_s}{\partial z} + \frac{c_s V_{max}}{c_s + K_M + \frac{c_s^2}{K_i}} = D_{iax} \frac{\partial^2 c_s}{\partial z^2} \qquad (2.17)$$

where, c_s is the concentration of sucrose, z the axial distance, $D_{i\,ax}$ the axial diffusivity, L the bed length, θ the angular co-ordinate, u_0 the mobile phase interstitial velocity, ε the voidage of the bed, ω the rotation rate, V_{max} the maximum reaction rate, K_M the Michaelis constant and K_i the inhibition constant.

The product profiles generated by the simulation showed good agreement with the experimental profiles.

2.3 Enzymes

2.3.1 Introduction

Enzymes are the catalysts of biological processes. They are produced by living cells and have the capacity of increasing the rate of chemical reactions, generally first or second order reactions, without the formation of by-products. The protein content of the enzymes consists of L-aminoacids linked together in a defined sequence termed the prime structure and coiled in a complex fashion with an active site, where it binds to the reactant, in this case known as a substrate, catalysing the reaction.

They are highly dependent on the conditions of the reaction environment, concerning pH, temperature and substrate concentration and also highly specific in the type of reaction they catalyse. The activity of an enzyme or its capacity to transform a raw material into a product is defined by the International Unit U, defined by The Enzyme Commission of the International Union of Biochemistry in 1961, meaning the conversion of one µmol of substrate in one minute, under optimised standard conditions of temperature and pH.

For catalysis, the enzymes usually require the presence of a non proteic substance such as some metal ions (Ca^{2+}, Fe^{2+}, Mn^{2+} or Zn^{2+}), called cofactors, or a complex organic molecule, known as a coenzyme. Depending on the type of the reaction they catalyse, the enzymes are classified into six different groups: oxidoredutases, transferases, hydrolases, lyases, isomerases and ligases.

2.3.3 Enzyme Kinetics

Theories to explain the kinetics of reactions catalysed by enzymes have emerged since the late 1800's. A wide range of mechanistic models have been developed for investigating the structure and function of enzymes. The most commonly used is the Michaelis and Menten model represented by equation 2.18 below.

$$v = \frac{[S] + V_{max}}{[S] + K_M} \tag{2.18}$$

where [S] is the substrate concentration, V_{max} the maximum reaction velocity and K_M the Michaelis constant. K_M is a characteristic of each enzyme preparation and represents the substrate concentration at half the maximum reaction rate.

For the systems where there is enzyme inhibition equation 2.18 is transformed into equation 2.19 below (106).

$$v = \frac{[S] + V_{max}}{[S] + K_M + \frac{[S]^2}{K_i}} \tag{2.19}$$

where K_i represents the inhibition constant.

According to Ghose (107) the constant K_i can be calculated by differentiating equation 2.19 for the maximum initial reaction rate. After some manipulation equation 2.19 becomes:

$$K_i = \frac{[S_{max}]}{\sqrt{K_M}} \tag{2.20}$$

The irreversible one-substrate model used to derive the Michaelis-Menten equation is unsatisfactory for the treatment of reversible reactions as pointed out by Wharton and Eisenthal (108). This is explained by the reversible nature presented, in theory, by all chemical reactions and to the product-substrate complex formation caused by structure similarities between substrate and product.

The modified model proposed to represent reversible reactions is generally expressed by Equation 2.21 below.

$$\nu = \frac{\frac{V^{S}_{max}[S]}{K^{S}_{M}} - \frac{V^{P}_{max}[P]}{K^{P}_{M}}}{1 + \frac{[S]}{K^{S}_{M}} + \frac{[P]}{K^{P}_{M}}} \qquad (2.21)$$

where V^{S}_{max} is the maximum reaction rate for product formation (forward reaction), V^{P}_{max} the maximum reaction rate for the substrate formation (reverse or backward reaction), K^{S}_{M} the Michaelis constant for the forward reaction, K^{P}_{M} the Michaelis constant for the backward reaction, [S] the substrate concentration and [P] the product concentration.

At equilibrium the rates of the forward and the backward reactions are equal and the net rate ν is zero. Equation 2.21 is then expressed as:

$$\frac{[P]_{eq}}{[S]_{eq}} = \frac{V^{S}_{max} K^{P}_{M}}{V^{P}_{max} K^{S}_{M}} = K_{eq} \qquad (2.22)$$

Equation 2.22 is known as the Haldane relationship, where the subscript "eq" refers to the equilibrium condition.

The values of V_{max} and K_M are generally obtained either by graphical methods such as the Lineweaver-Burk plot and the Edie-Hofstee plot or by computational procedures (109,110). In the Lineweaver-Burk plot the Michaelis-Menten model is expressed according to equation 2.23.

$$\frac{1}{\nu} = \frac{K_M}{V_{max}[S]} + \frac{1}{V_{max}} \qquad (2.23)$$

Equation 2.23 is linear and therefore by plotting 1/V against 1/S, the values of V_{max} and K_M can be obtained. The Edie-Hofstee plot uses a modified form of the Lineweaver-Burk equation 2.23. By multiplying both sides of equation 2.22 by $V_{max} \cdot \nu$ another linear relationship, equation 2.24 expressed in ν, is obtained.

$$\nu = V_{max} - \frac{K_M \nu}{[S]} \qquad (2.24)$$

By plotting ν against $\nu/[S]$ the values of V_{max} and K_M can also be obtained.

Details of the methods used in the experimental work and comments about the margin of error for each one are presented in section 3.7.3.

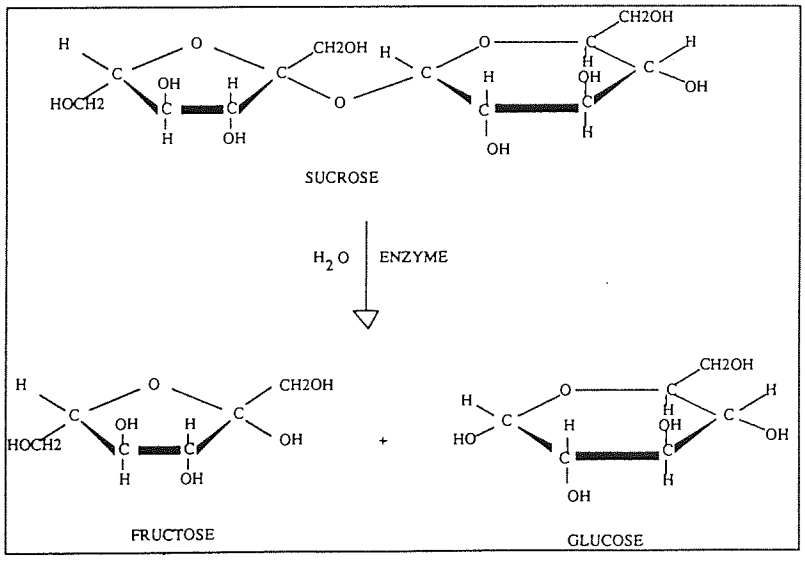
2.3.4 Enzymes Used in the Experimental Work of this Thesis

2.3.4.1 Invertase

Invertase is an hydrolase. This enzyme is also called sucrase, carbohydrase or β-fructofuranosidase (E.C. 3.2.1.2.6) and is derived from *S. cerevisiæ*. It catalyses the hydrolysis of the β-D-fructofuranosyl linkage in sucrose, raffinose, gentibiose, methyl and β-fructofuranoside. The invertase used in this project was obtained from a strain of *S. cerevisiæ*. It operates at an optimum pH of 4.5-5.5 and optimum temperature of 48-60 °C. The hydrolysis of sucrose by invertase produces an equimolar mixture of glucose and fructose, according the reaction presented in Figure 2.8.

Oligosaccharides are generally formed by the transferase action of the invertase during the hydrolytic process (111-114) and are partially or totally hydrolysed thereafter. These sugars introduce a significant error in polarimetric measurements used in determining initial reaction rates, and, therefore, they interfere by producing an erroneously low reaction rate. The subject was studied by Bowski *et al.* (115) and they found that the experimental results did not fit the kinetics model at sucrose concentrations greater than 9.75 w/v.

Figure 2.8 - Reaction of the hydrolysis of sucrose



Apart from the substrate inhibition Bowski *et al.* showed that the effect of water concentration at high sucrose concentrations also affects the reaction kinetics. The decreasing amount of water at high sucrose concentrations resulted in 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 site of the enzyme. Consequently, they have suggested a modified model taking into account a combined effect of water concentration and substrate inhibition.

2.3.4.2 Inulinase

Inulinase is an hemicellulase (116). It is also a β-fructofuranosidase (E.C. 3.2.1.7). Several enzymes capable of hydrolysing inulin have been described in the literature as originating either from plant material such as inulin-containing roots and tubers or from certain microorganisms. The microbial enzymes described originate mainly from yeasts, *K. fragilis* (117) and *Candida* species, but also fungal inulinases from *Aspergillus*, *Penicillum* and *Fusarium* species are mentioned (118). The microbial enzymes are reported to operate at an optimum pH of 3.5-5.5 and optimum temperature of 45-55 °C and lose activity rapidly at 60 °C.

Enzymes from strains of *A. niger* and *A. ficuum* have been described in the literature (118,119) as more appropriate for industrial applications as they are more stable at higher temperatures which helps to prevent infection of the reaction medium. These enzymes operate at an optimum pH of 4.7 and optimum temperature of 55-65 °C. The inulinase used in this project was obtained from a strain of *A. niger.* Industrial fungal inulinase preparations actually are a mixture of endo- and hexo inulinases in proportion 1/1 and they also possess measurable invertase activity.

Inulinase acts on the inulin molecule liberating the fructose units and also one unit of glucose per molecule, according to the reaction presented in Figure 2.9.

2.3.4.3 Lactase

Lactase is a β-galactosidase used for the hydrolysis of lactose in dairy products. The hydrolysis produces an equimolecular mixture of glucose and galactose. This enzyme is produced by a number of different microorganisms namely the bacterium *Escherichia coli*, the yeast *S. lactis*, the mold *A. niger* and the fungal *A. oryzæ*. The lactase used in this project was of *A. oryzæ* origin. This enzyme operates at an optimum pH of 4.5-5.5 and optimum temperature of 55-65 °C. The hydrolysis of lactose by lactase produces an equimolar mixture of glucose and galactose, according to the reaction presented in Figure 2.10.

Figure 2.9 - Reaction of the hydrolysis of inulin

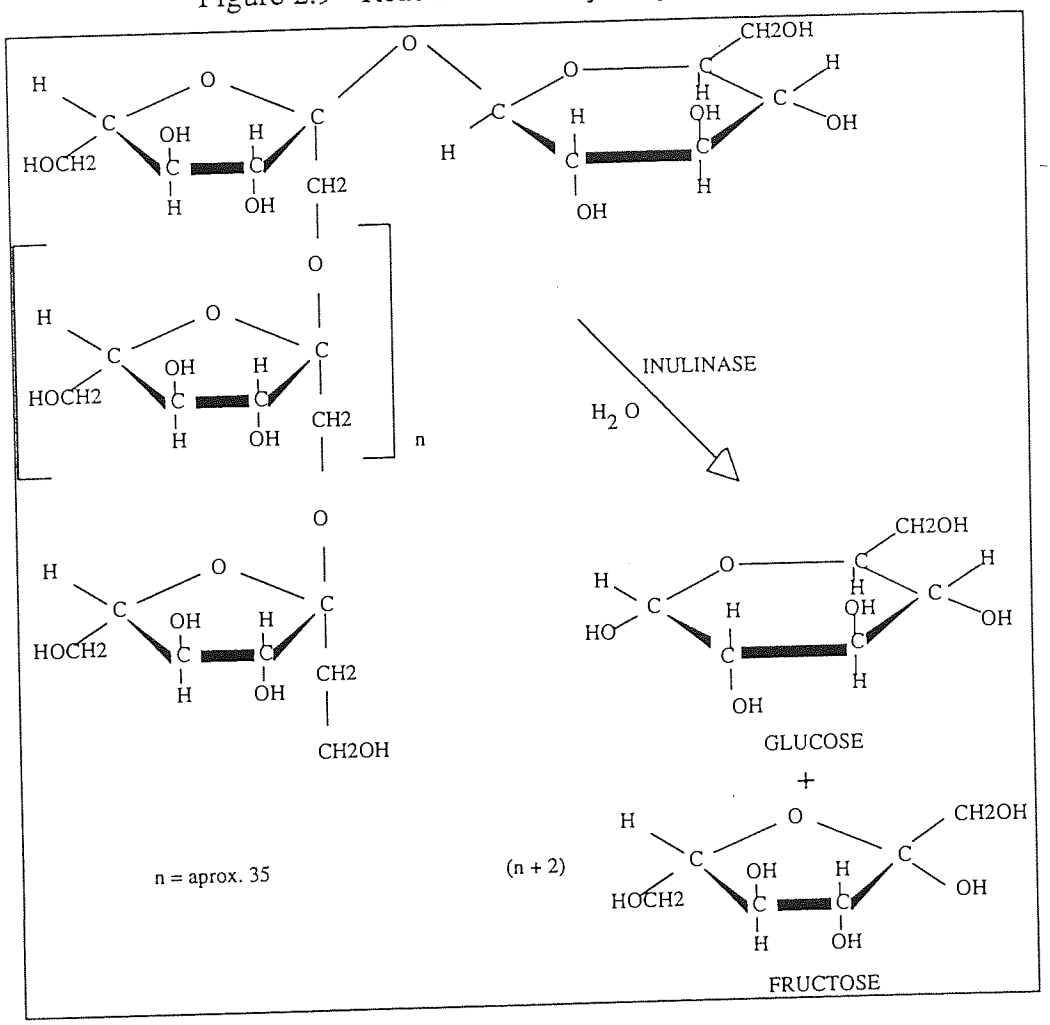
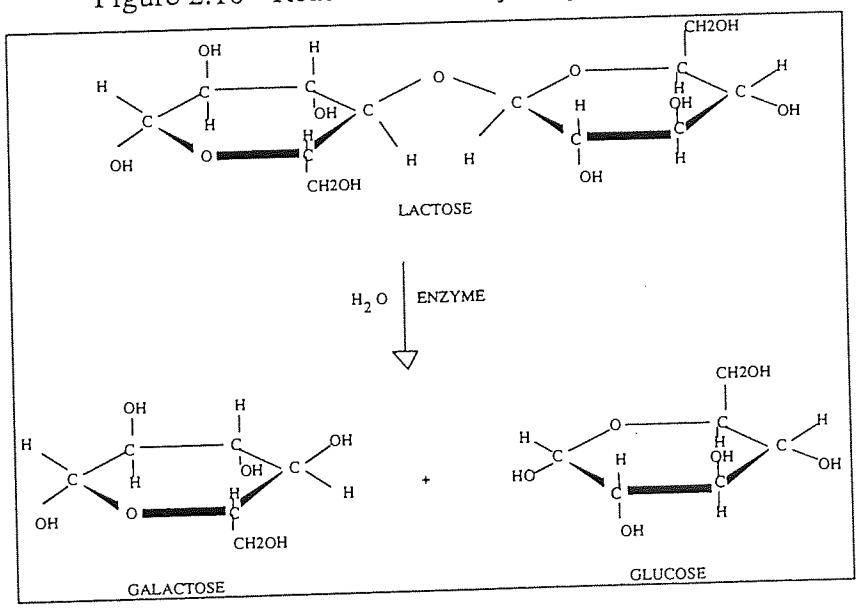


Figure 2.10 - Reaction of the hydrolysis of lactose

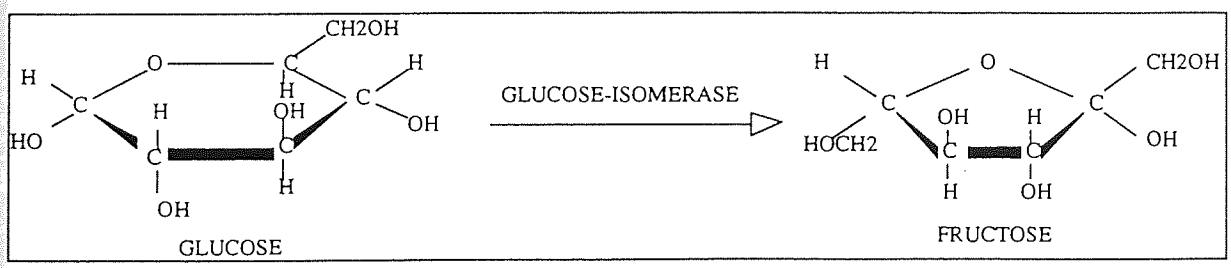


Under closer inspection, the enzymatic hydrolysis of lactose is a complex process involving a multitude of sequential reactions with sugars other than the monosaccharides glucose and galactose being formed. It was also observed by Friend and Sahani (120) that the action of the lactase produced by *A. oryzæ* was inhibited by the galactose produced during the hydrolysis. Many models have been presented in the literature to explain the mechanism of the hydrolysis and the competitive and the anti-competitive inhibition characteristics of the α-galactose, the competitive characteristics of the β-galactose released during the enzymatic reaction and its mutarotation yielding a mixture of both anomeric pyranoses (120). The models proposed by Prenosil *et al.* (121) considered at least the production of trisaccharides which seem to be the most important oligosaccharides formed during the hydrolysis.

2.3.4.4 Glucose Isomerase

Glucose isomerase, also known as xylose isomerase, was raised to prominence in the sucrose shortage of the early 1970's. It can be produced as an intracellular enzyme by several microorganisms. *Pseudomonas hydrophila*, *Streptomyces phaechromogenes*, *S. olivochromogenes*, *Bacillus coagulans*, *A. missouriensis* and *Lactobacillus* species are the main sources of glucose isomerase (122). This enzyme generally requires metal activators such as Mg^{2+}, Co^{2+} and Fe^{3+}. Ca^{2+} acts as inhibitor, by displacing Mg^{2+} from the enzyme molecule. The commercially important glucose isomerases show superior affinity to xylose and are classified as xylose isomerase, E.C. 5.3.1.5. The glucose isomerase used in this project was of *A. missouriensis* origin. This enzyme operates at an optimum pH of 7.0-7.8 and optimum temperature of 53-70 °C, according to the reaction presented in Figure 2.11.

Figure 2.11 - Reaction of the isomerisation of glucose



The enzyme glucose isomerase was originally developed to be used in conventional batch reactors for the production of rich fructose syrups, as substitute for cane sugar. Due to the increased demand for these syrups, the enzyme has been subject to much investigation in the last three decades. Microorganisms have been genetically engineered aiming for the production of an enzyme that could work in an immobilized form and suitable for large

scale use in continuous reactors. The enzymes found commercially are either supplied in the immobilised form on inert supports, or already prepared for immobilisation on various supports, including anion exchange resins, as used in regenerable isomerization units (122).

2.4 Carbohydrates

2.4.1 Introduction

Carbohydrates are the most abundant class of organic compounds found in living matter. The term carbohydrate originated from the belief that they were hydrates of carbon since elemental analyses of common carbohydrates such as lactose, sucrose, starch and cellulose led to the empirical formula $C_x(H_2O)_y$ (123). They are classified into three basic categories: monosaccharides, oligosaccharides and polysaccharides. The oligosaccharides are generally two to ten monosaccharides linked together by the formation of glycosidic bonds. These are divided into disaccharides (e.g. sucrose, lactose and maltose), tri-saccharides (e.g. rafinose), depending on the number of carbohydrate units they contain. Polysaccharides contain more than ten carbohydrate units (or monomeric units): starch, cellulose, dextran and glycogen are the most important ones.

A brief description of the importance, the industrial production and the interrelation of the carbohydrates used in this project are presented in the following sections.

2.4.2 The Carbohydrates Used in this Project

2.4.2.1 Sucrose

Sucrose is the most important industrial sugar, followed by D-glucose, lactose, maltose and fructose (123). It is obtained from sugar cane and from beet, either by squeezing the sugar cane or by countercurrent extraction of the beet slices (cossetes) with water. In both cases, the juice obtained from the sugar extraction step is filtered, chemically treated, decolorized, crystallised, centrifuged and dried.

Sucrose can be inverted (hydrolysed) by acid or enzymatic means for the production of invert syrups, which contain 10-90 % of invert sugar, a mixture of glucose and fructose. Since the presence of invert sugar minimises the danger of crystallisation, these liquid sugars can be shipped at higher densities than sucrose. Invert liquid sugars are used in

carbonated beverages, glacé fruits, canning, bread baking and as an humectant in the tobacco sector.

2.4.2.2 Glucose

Glucose is a monosaccharide and it is also called dextrose, as it is dextrorotatory, with a specific rotation of $(\alpha)^{20}_D$ = 52.7° in water. It is used as a raw material in the pharmaceutical sector mainly in the manufacture of intravenous solutions. In the chemical sector it is used as raw material in the manufacture of ascorbic acid (vitamin C).

Glucose is obtained by the hydrolysis of starch performed in two steps. Liquefaction, carried out by bacterial α-amylase, followed by saccharification with glucoamylase and pullanases to increase the yield in DE (Dextrose Equivalent). The crude solution obtained is then filtered, treated with charcoal, deionised, crystallised, centrifuged and dried to produce either a monohydrate or anhydrous glucose.

2.4.2.3 Fructose and High Fructose Syrups

Fructose is a monosaccharide found in many fruits, hence one of its name is 'fruit-sugar'. It is also called levulose, as it is lævorotatory, with a specific rotation of $(\alpha)^{20}_D$ = -132.8° in water. The main use of fructose is as a substitute to sucrose in beverages as well as in various diabetic food products and low calorie products namely jams, jellies, cakes and conserves. In the pharmaceutical sector it is used as a raw material for the manufacture of intravenous solutions as an alternative to glucose for patients with low tolerance to intravenous glucose.

Fructose was originally obtained by treatment of invert sugar with CaO, followed by the separation and hydrolysis of the precipitate, colour treatment, crystallisation with organic solvents, centrifugation and drying to produce the pure product. With the development of large scale chromatography, fructose is now obtained in pure liquid form, without chemical treatment. The invert sugar is chromatographed and a fructose fraction obtained, which may be 100 % pure, is then crystallised, centrifuged and dried to produce the fructose crystals.

The principal attraction of fructose nowadays is its sweetness, 1.2 to 1.8 times that of sucrose on a weight basis, the precise factor depending on the circumstances of measurement (122). In practice, fructose is very difficult to crystallise from aqueous solution, and organic solvents are used. This increases the costs of production very

considerably so that crystalline fructose is not competitive with sucrose on a cost basis. However, fructose in solution, as a syrup containing glucose, is very competitive with sucrose when the latter is used in solution.

Fructose in solution is obtained either from pure glucose solutions produced from starch hydrolisates or from hydrols. Hydrols are the mother liquors obtained as sub products in the crystallisation step of glucose manufacture. As fructose in solution is obtained from corn starch, they are generally known as high fructose corn syrups.

Glucose solutions obtained by the hydrolysis of starch may contain up to 93 % of the sugar on a dry basis while hydrols have a much lower glucose content and therefore a much higher oligosaccharides content. The isomerisation is carried out at 40-45 % w/w of substrate concentration in continuous reactors packed with glucose isomerase immobilized on inert supports. Glucose isomerase catalyses the production of an equilibrium mixture of glucose and fructose, which contains 55-65 % fructose, the precise level varying slightly with the temperature of the reaction. In practice, the time taken to reach equilibrium is too long for commercial use so all manufacturers aim to produce a syrup containing 42 % fructose. If glucose with 93 % purity is used, the product leaving the isomerization reactors has the following composition: 42 % fructose, 51 % glucose and 7 % oligosaccharides. This product is 'iso sweet' (122) with sucrose syrups of the same solids content.

The upper limit for the fructose content for syrups produced industrially by the continuous isomerisation of glucose by immobilised glucose isomerase is 42 %. This syrup is insufficiently sweet for acidic soft drinks. To reach the same sweetness level of sucrose, fructose syrups containing 55 % fructose are required. This fructose content has been achieved by the chromatographic separation of fructose from the other components of the syrup. This produces a stream containing 90 % fructose and 10 % glucose and a separate stream containing 87 % glucose, 5 % fructose and 8 % oligosaccharides. The fructose-rich stream is blended with the normal stream to give the required 55 % fructose content. These syrups are known as 'ultra-high fructose syrups'.

2.4.2.4 Lactose and Galactose

Lactose is a disaccharide and is present in the milk of all mammals at 2-8 % concentration (123). It is produced from whey that has been treated with acid and heat to remove the proteins. The filtrate is decolorized with carbon and concentrated to produce crystalline lactose. The commercial product is the monohydrate of the α-form.

Galactose is a frequent constituent of oligosaccharides, notably lactose, melibiose and raffinose. Galactose is produced by acid or enzymatic treatment of lactose. The glucose is removed by fermentation with yeasts and the remaining liquid purified by filtration and decolorization, concentrated, crystallised, centrifuged and dried to produce galactose. Two crystalline isomers of galactose are known. The stable α-form obtained under most conditions and the β-isomer prepared by crystallisation from cold alcoholic solution.

2.4.2.5 Inulin

Inulin is a β-2,1-linked fructose polymer terminated by a sucrose unit residue as shown in Figure 2.13. It is also classified as a polyfructan. Its molecular weight is approximately 6,000 with a specific rotation of $(\alpha)^{20}_{D} = -43^{o}$. Inulin occurs as an energy reserve in various plants, particularly those of the *Compositæ* family namely chicory, Jerusalem artichoke and dahlia. It was isolated first from elecampane (*inula helenium*) (122). Pure inulin is almost insoluble in cold water, but can be dissolved in hot water (124). In plant material, part of the inulin is present as fructose polymers with lower chain length which are more soluble in water. These lower chain length polymers have a glucose content between 5-15 % compared with aprox. 3 % glucose content in pure inulin (125).

Inulin can be extracted from sliced plant material at elevated temperatures. The raw juice can be purified by liming followed by carbonation as in sugar beet processing, decolorized by carbon, crystallised, centrifuged and dried to produce the pure material (126). The use of inulin has already been considered as a potential source of fructose (117). The enzyme inulinase may be employed as a step in this process, but its cost makes it unlikely to be chosen in favour of acid hydrolysis (122).

CHAPTER THREE

EXPERIMENTAL PROGRAMME, MATERIALS, EQUIPMENTS AND METHODS

3.1 The Experimental Programme

The experimental programme comprised two lines of study:

1. The physical separation of carbohydrates that were being used as substrates and the products formed in the bioreaction selected in this research programme.

The purpose of doing the physical separation studies was to establish the degree of resolution obtainable without the hindrance of a bioreaction taking place in the column. The following mixtures were subjected to physical separations:

- sucrose-glucose-fructose
- glucose-fructose
- lactose-glucose-galactose

2. Chromatographic bioreaction-separations

- hydrolysis (inversion) of sucrose to glucose and fructose using the enzyme invertase
- hydrolysis of inulin to fructose and glucose using the enzyme inulinase
- hydrolysis of lactose to glucose and galactose using the enzyme lactase
- isomerization of glucose to fructose using the enzyme glucose-isomerase

3.2 Materials

A list of materials used in the experimental work is presented in Table 3.1. If a product has a commercial name it is shown between brackets and the lot or batch identification numbers are only presented for the enzymes. In case of products from different origins the specification will only be mentioned in the text to identify the material used.

Table 3.1 - Materials

Product	Specification	Supplier	City/ Country
Acetic acid	General purpose reagent	BDH Laboratory Supplies	Poole/UK
Acetonitrile	General purpose reagent	BDH Laboratory Supplies	Poole/UK
Buffer solution pH 7.0	General purpose reagent	Fisons Scientific Equipment	Loughborough/ UK
Calcium acetate	Analytical grade	Fisons Scientific Equipment	Loughborough/ UK
Calcium nitrate tetra-hydrate	Technical grade	Berk Ltd. UK	Dorset/UK
Dextran Blue	General purpose reagent	Sigma Chemicals Co.	St. Louis/USA
Dextran T-2000	Analar	Pharmacia LKB-Biotechnology	Uppsala/Sweden
3,5 Dinitro salicylic acid	General purpose reagent	BDH Laboratory Supplies	Poole/UK
β-D (-) Fructose	Analytical grade	Sigma Chemicals Co.	St. Louis/USA
β-D (-) Fructose	FF	Roquette Freres	Lestrem/France
D (+) Galactose	Analytical grade	Fisons Scientific Equipment	Loughborough/ UK
D-Glucose anhydrous	Analar	BDH Laboratory Supplies	Poole/UK
Glucose-isomerase (G-ZYME)	Food grade Batch G993 Activity: 1800-2200 U/g	Rhone Poulenc Chemicals Ltd.	Watford/UK
Glucose-isomerase (Maxazyme GI)	Food grade Batch 2394/SPE 0316 Activity: 900 U/g	International Bio-Synthetics BV	Rijwijk/The Netherlands
Hydrochloric acid	General purpose reagent	BDH Laboratory Supplies	Poole/UK

Table 3.1 - Materials (cont.)

Product	Specification	Supplier	City/ Country
Inulin (Chicory root origin)	General purpose reagent	Sigma Chemicals Ltd.	Dorset/UK
Inulinase (NOVOZYM 230)	Food grade Lot In 223; Activity: 2,000-2,300 U/cm^3	Novo Nordisk	Bagsvaerd/ Denmark
Ion exchange resin (cationic type) 8% cross linked (PCR-833Na)	General purpose reagent capacity: 1.6 meq/cm^3, sodium form	Purolite International UK Ltd.	Pontyclum/ Wales, UK
Invertase (Bioinvert NL)	Food grade Lot 07 ; Activity: 11,600 U/cm^3	Biocon Biochemicals Ltd	Cork/ Ireland
Lactase (Biolactase)	Food grade Lot: mnl 331 Activity: 22,500 U/g	Biocon Biochemicals Ltd	Cork/ Ireland
Lactose	Analar	BDH Laboratory Supplies	Poole/UK
Lactose monohydrate (Pharmatose)	USP-NF/EP/JP	The Melkin Industrie Vegel V	The Netherlands
Magnesium sulphate heptahydrate	Analar	BDH Laboratory Supplies	Poole/UK
Nitric acid	General purpose reagent	BDH Laboratory Supplies	Poole/UK
Potassium dihydrogen orthophosphate	Laboratory reagent Analytical grade	Fisons Scientific Equipment	Loughborough/ UK
Potassium monohydrogen orthophosphate	Laboratory reagent Analytical grade	Fisons Scientific Equipment	Loughborough/ UK
Potassium sodium tartrate	Analytical grade	May & Baker (M&B)	Dagenham/UK

Table 3.1 - Materials (cont.)

Product	Specification	Supplier	City/ Country
Sodium acetate	Laboratory reagent Analytical grade	Fisons Scientific Equipment	Loughborough/ UK
Sodium azide	Laboratory reagent Analytical grade	Fisons Scientific Equipment	Loughborough/ UK
Sodium chloride	Analar	BDH Laboratory Supplies	Poole/UK
Sodium hydroxide	Laboratory reagent Analytical grade	Fisons Scientific Equipment	Loughborough/ UK
Sucrose	BP	British Sugar	Kidderminster/ UK
Tris (hydroxy-methyl) methylamine	Laboratory reagent Analytical grade	Fisons Scientific Equipment	Loughborough/ UK

3.3 The Batch Chromatographic Bioreactor-Separator (BCBS)

3.3.1 General Description

The batch chromatographic bioreactor-separator used in this study was specially built for the development of the proposed experimental work. It was based on the work reported by Zafar and Barker at Aston (2,3). Construction details regarding the injection port and the column outlet were based on Snyder and Kirkland (127) and on the papers of Wilhem and Riba (128). The photograph and the schematic diagram of the BCBS are shown in Plate 3.1 and Figure 3.1. It basically consists of a column, or columns in series, feed and eluent pumps, a heating unit, detection and product collection units as described in sections 3.3.1 to 3.3.9. An alphanumeric code has been used to identify the equipments. For instance, **P**-1 means pump number **1**.

Polypropylene and polyethylene tubing as short as possible to minimise hold up and polypropylene fittings were used for the necessary connections. Special care was taken to avoid extra column effects such as band broadening due to unnecessary dilution in the feed port and at the column outlet.

3.3.2 Columns Employed

The chromatographic columns employed in the experimental work are listed in Table 3.2. They varied according to the reaction system under evaluation or the nature of the tests being made, such as enzyme adsorption, determination of distribution coefficients, effect of temperature, flowrates and background concentrations on the distribution coefficients.

Table 3.2 - Columns employed in the experimental work

Length (cm)	I. D. (cm)	Material	Uses
30	0.78	stainless steel	pre-tests and determination of the effect of temperature and flowrates on the distribution coefficients
100	0.90	PYREX glass (jacketed)	pre-tests and determination of the effect of background concentrations on the distribution coefficients
200	1.96	PYREX glass (jacketed)	bioreaction-separations (Batch Chromatographic Bioreactor-separators BCBS-I and BCBS-II)
200	1.0	PYREX glass (jacketed)	bioreaction-separations (Batch Chromatographic Bioreactor-separator BCBS-III)

The 30 cm stainless steel column was manufactured by Bio-Rad UK Ltd., Watford, and the 100 cm glass column was supplied by BDH Laboratory Supplies, Poole, UK. The 200 cm glass columns were manufactured by J. B. Cutforth Ltd., Birmingham, UK.

Plate 3.1- Photograph of the Batch Chromatographic Bioreactor-separator (BCBS) and Elutriation System

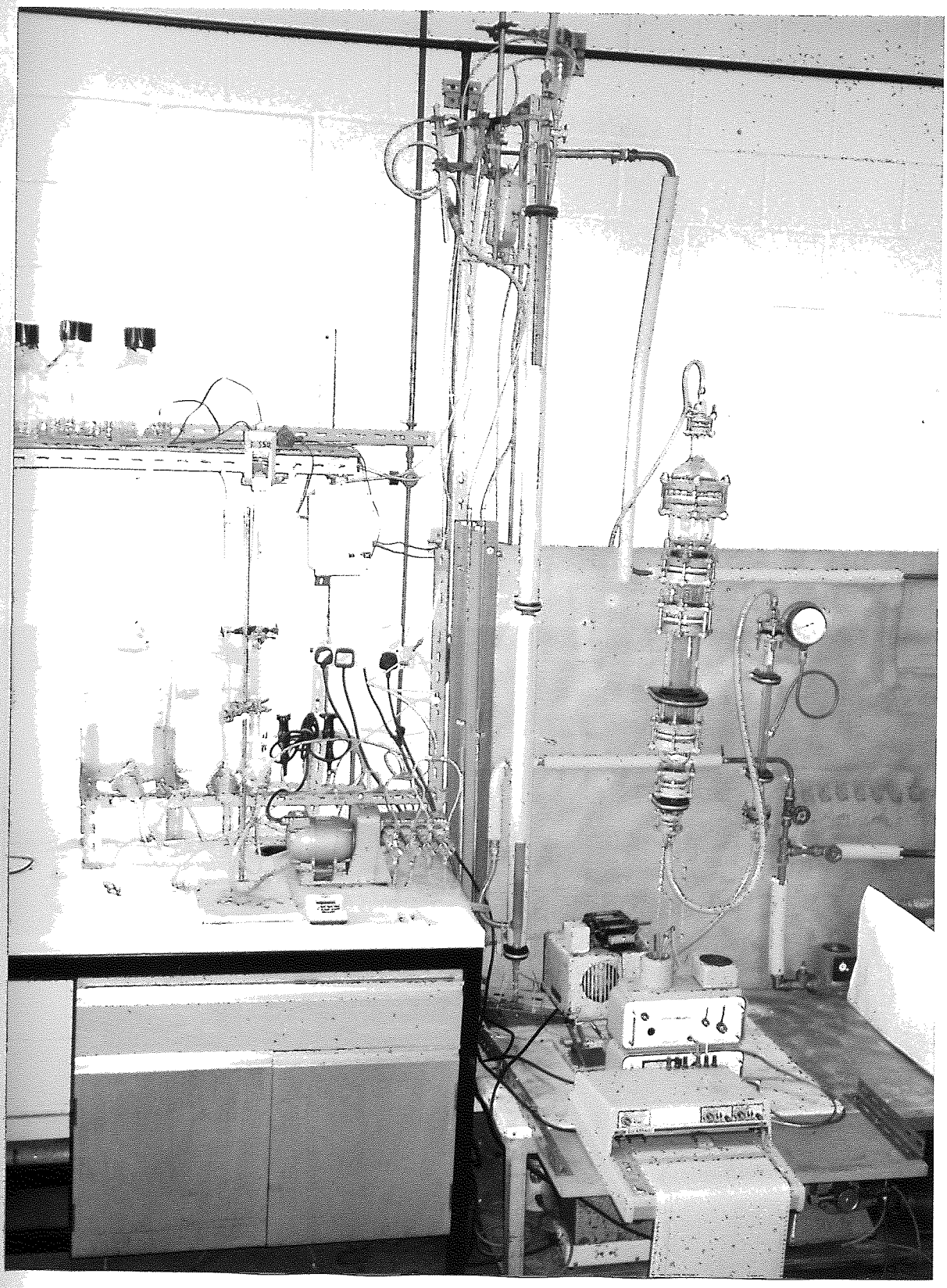
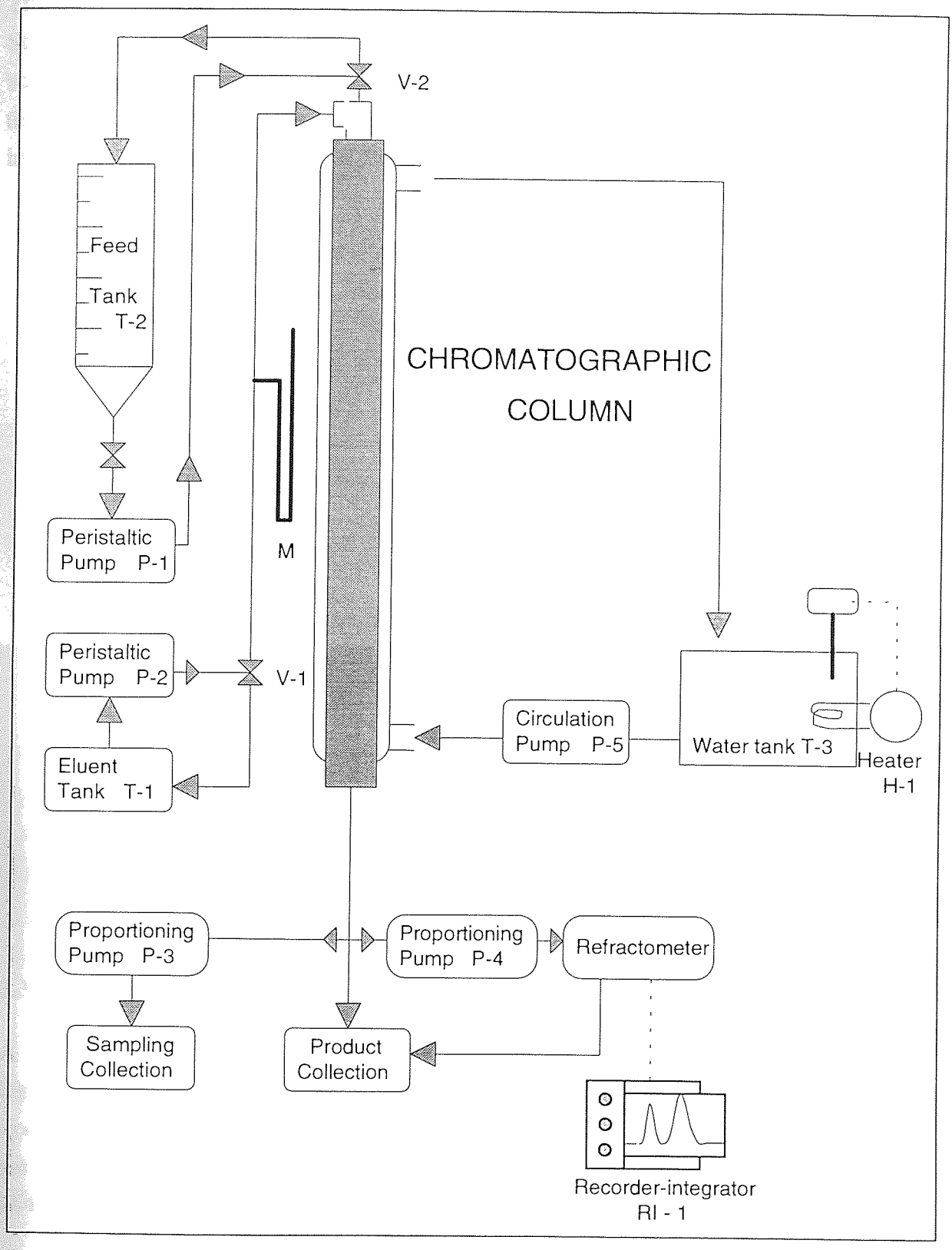


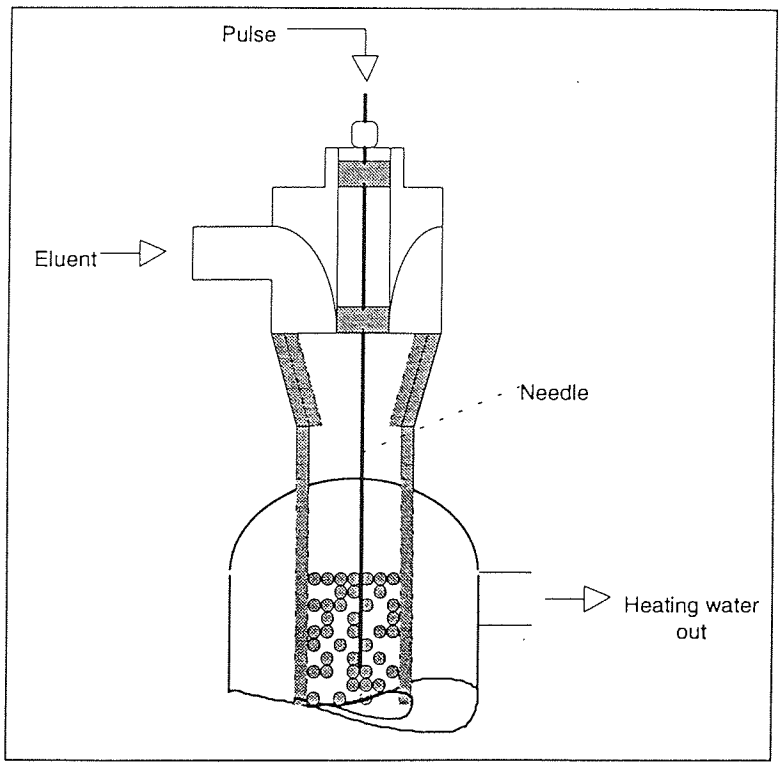
Figure - 3.1 - Schematic diagram of the Batch Chromatographic Bioreactor-Separator (BCBS)



3.3.3 Feed Port

The feed port was a glass connection "T" shape, catalogue no. S-2345 from Fisher Scientific Instruments UK Ltd, supplied by J. B. Cutforth Ltd, Birmingham, UK and adapted according to Figure 3.2. The eluent was introduced in a side inlet and the feed through a top inlet, by means of a long needle ending 2 cm below the resin level. The third inlet/outlet was fitted to the top of the chromatographic column.

Figure 3.2 - Feed port



3.3.4 Eluent and Feed Pumps

A dual head peristaltic pump, P-1 and P-2, catalogue no. 7554-30, manufactured by Cole-Parmer Instruments Co., Chicago, Ill., USA, equipped with a Solid State Masterflex Speed Controller, manufactured by Garnant Corp., Barrington, Illinois, USA, was used to deliver the eluent and feed to the column. The tubing used in the peristaltic pumps was type 96410-13, silicon, manufactured by Masterflex, Poole, UK.

For the physical separations, as the eluent was deionised and degassed water, a glass heat exchanger refrigerated by cold water was installed before the eluent pump to cool down the eluent to 20-25 °C to ensure a constant pumping rate by the pump. To remove insoluble particles a 50 micron filter was installed after the pump. The eluent used for the physical

separations was kept in a 10 dm^3 bottle T-1 heated by a hot plate, model Stirrer Hot Plate SS 3H, manufactured by Chem Lab, UK.

For the chromatographic bioreaction-separations, as some enzyme solutions contained insoluble materials, the filter was removed from the line and replaced by a resin trap consisting of a tube containing cation exchange resin, the same form as that used as the stationary phase. The eluent used for the chromatographic bioreaction-separations was kept in the volumetric flask used for its preparation.

The container T-2 used for the carbohydrate solutions or feed was a 100 cm^3 burette and a 50 micron filter was also installed after the feed pump to remove foreign materials from these solutions.

3.3.5 Heating Unit

The heating unit included a 40 dm^3 water tank, T-3 (stainless steel 316), heated by a thermostat heater model TE-7 Tempette, manufactured by Techne Ltd., Cambridge, UK. The circulation of the water through the reactor jacket was provided by a centrifugal pump P-5 manufactured by Grunfors Seletric, Warrington, UK.

3.3.6 Temperature Monitoring

Temperatures were monitored by thermocouples installed at five different points in the system and connected to an indicator model 1408-k, manufactured by Digitron Instruments Ltd., Hertford, UK. The temperature measurement at a chosen point was made with the help of a selector or multiple point switch. The following temperatures were monitored: heating tank, column-water inlet, column-water outlet, product outlet and the ambient temperature.

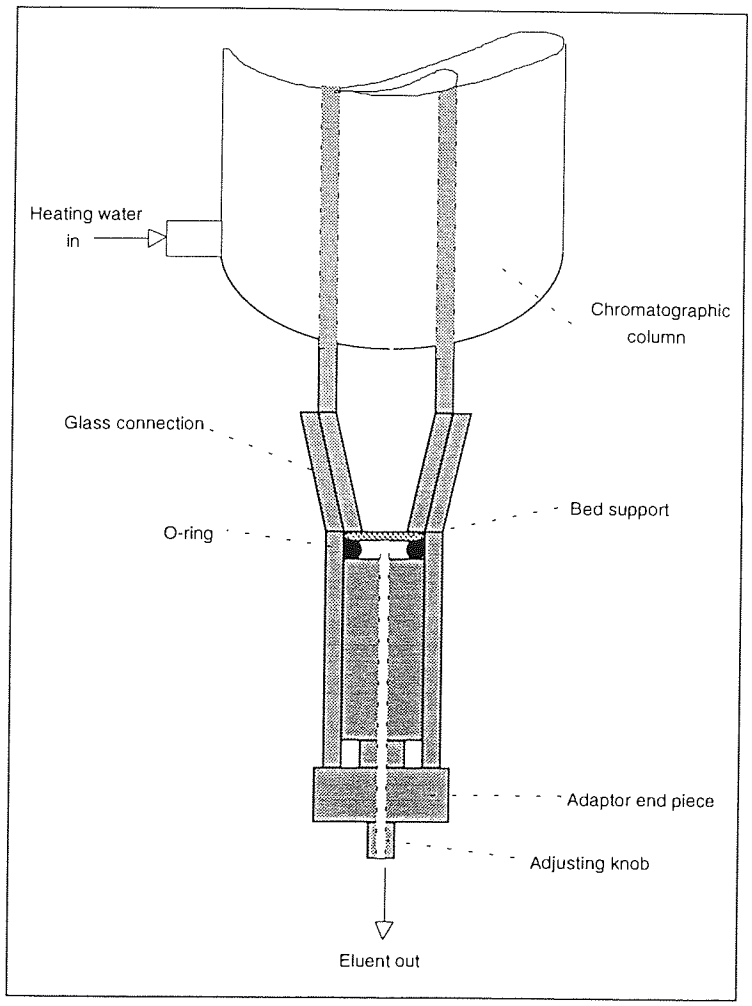
3.3.7 Detection Unit

This unit included a proportioning pump P-4 manufactured by Technicon Auto Analyser, London, UK, using a 0.2 cm^3/min polyethylene sampling tubing manufactured by Sterilin Instruments Ltd, Alton, Hampshire, UK, feeding a continuous differential refractive index monitor type Refracto Monitor, from Jobling Laboratory Division, London, UK. The refractive monitor was connected to the recorder-integrator unit RI-1, model HP 3390 A, manufactured by Hewlett Packard, Palo Alto, California, USA.

3.3.8 Column Outlet

The column outlet was an AC-10 type adaptor supplied by Pharmacia LKB Biotechnology, Uppsala, Sweden, according the schematic diagram shown in Figure 3.3. The materials in contact with the fluids were polyethylene and polypropylene.

Figure 3.3 - Column outlet



3.3.9 Product Collection

The liquid exiting from the column outlet passed into a burette, which was occasionally used to measure the elution flowrate and from there to the product collection bottles. Part of the liquid exiting the column was removed with the aid of a proportioning pump P-3 manufactured by Technicon Auto Analyser, London, UK, using a 0.2 cm^3/min

polyethylene sampling tubing manufactured by Sterilin Instruments Ltd, Alton, Hampshire, UK and collected in 10 cm^3 glass sample vials, supplied by Chromacol Ltd., London, UK.

During the chromatographic bioreaction-separation experiments a stainless steel coil was placed in the sampling line, after the proportioning pump P-3, and the coil immersed in boiling water to deactivate the enzyme. To inhibit further enzyme action, 0.1 cm^3 of 1M Na OH was added to the sample vials to bring the pH to 8-9. The vials were kept in an ice bath during the sampling period and immediately transferred to the refrigerator. The samples were analysed according to the procedure described in section 3.5.1.

3.4 Experimental Procedures used for the Physical Separations and the Bioreaction-separations

3.4.1 Eluent

Two types of eluent were used:

1. Deionised and degassed water for the physical separations

   The deionised water was prepared by deionisation of tap water using an Elgastat B 224 water deionisation unit manufactured by Elga Water Purification, High Wycombe, UK and stored in a 500 dm^3 stainless steel tank. It was then degassed by heating to a constant temperature of 65 ^oC.

2. Diluted enzyme solution for the bioreaction-separations

   This solution was made by the dilution of a known amount of the concentrated enzyme in deionised and degassed water. The deionised and degassed water used was cooled down to 35 ^oC prior to the preparation of the enzyme solution. After the addition of the enzyme, the pH was adjusted to the desired level by means of a buffer. The half life of the enzymes employed in this research program when maintained in dilute conditions at 35 ^oC was determined to be 36 hours. Therefore, the loss of activity was small over the period necessary to complete one or two runs which was 8 hours.

3.4.2 Feed

Six different feed materials were used in the physical separation experiments:

1. anhydrous glucose and fructose FF

2. sucrose, anhydrous glucose and fructose FF

3. anhydrous glucose and galactose

4. lactose monohydrate USP, anhydrous glucose and galactose

5. inverted sucrose (acid hydrolysis)

6. inverted sucrose (enzymatic hydrolysis)

Four materials were used in the in the batch chromatographic bioreaction-separations:

1. Sucrose

2. Lactose monohydrate USP

3. Inulin

4. Glucose anhydrous

For the physical separations and for the batch chromatographic bioreaction-separations the solutions were prepared by dissolving a pre calculated weight of the substrate in warm deionised and degassed water with constant agitation until complete dissolution. The solution was then cooled down to ambient temperature, the pH adjusted by means of a buffer and its volume completed to the mark.

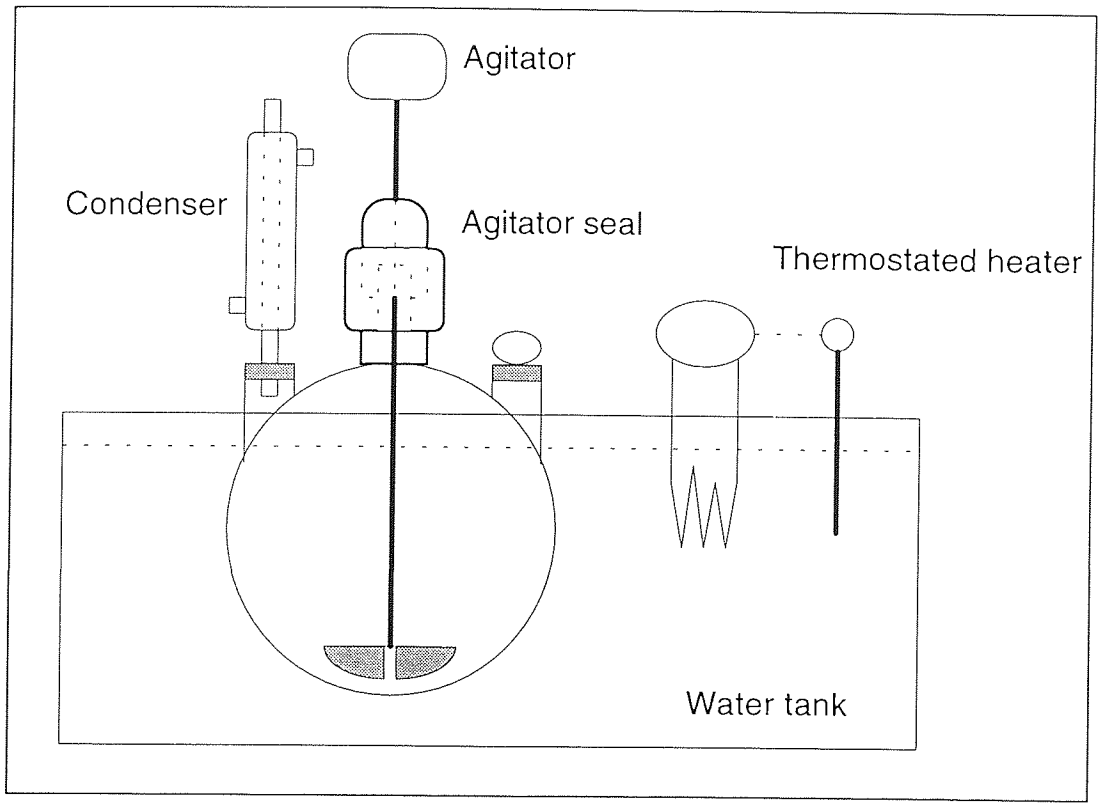
The inverted sucrose solutions were prepared by two different procedures, both using a batch reactor, a 2 dm^3 three necked PYREX flask equipped with a Liebig condenser, a speed controlled agitator manufactured by Gallenkamp Ltd, Dorset, UK, and heated by a thermostated bath as shown in Fig. 3.4.

3.4.2.1 Acid hydrolysis

1 dm^3 of a 50 % w/v sucrose solution was placed inside the batch reactor and heated to 80 °C with constant agitation. 1 cm^3 of 1 M of hydrochloric acid, was then added to the sucrose solution to adjust the pH to 2.0-2.2 using a pH meter model CD 720 manufactured by WPA Ltd, Cambridge, UK, and the solution kept for 3 hours at that temperature. After

the reaction period the reactor was removed from the bath, cooled down to ambient temperature by immersion in an ice bath and the pH adjusted to 5-6. These conditions were sufficient for a complete hydrolysis with formation of glucose and fructose, as verified by HPLC analysis as described in section 3.5.1. No colour formation was observed as a result of the thermal decomposition of the carbohydrates present.

Figure 3.4 - Batch reactor



3.4.2.2 Enzymatic hydrolysis

1000 cm^3 of a 50 % w/v sucrose solution buffered at pH 4.7 prepared with sodium acetate and acetic acid according to the Handbook of Chemistry and Physics (129) was placed inside the batch reactor and heated to 55 ^oC with constant agitation. 17 cm^3 of invertase containing 11600 U/cm^3 was then added to the sucrose solution to make up a 200 U/cm^3 solution, as recommended by the enzyme manufacturer, and kept for 8 hours at that temperature. The same care as described for the acid hydrolysis was taken, concerning cooling and pH adjustments after the reaction period. Total hydrolysis of sucrose was observed without formation of oligosaccharides.

3.4.3 Operation

The following operating procedure was adopted when working in the physical separation mode:

1. The heater H-1 of the heating unit was turned on, the thermostat adjusted to the selected operating temperature and the circulation pump P-5 activated.

2. When the temperature of the water circulating through the column jacket reached the pre set value, the eluent pump P-1 and the feed pump P-2 were then activated, simultaneously with the proportioning pumps P-3 and P-4 for the sampling collection and detection unit, respectively. The base line was adjusted to zero on the recorder integrator.

3. The valves V-1 and V-2 were operated in such way that the flow from the eluent tank T-1 was directed to the column through the three-way valve V-1 and the feed flow returned to tank T-2 through the valve V-2. The calibration of both eluent and feed flowrates was made using a 25 cm^3 burette. The volume of liquid pumped for a duration of 5 minutes was measured and the pumps settings adjusted until the required flowrate was obtained.

4. The feed was introduced in the column as a pulse, by closing the valve V-1 which forced the eluent to recycle back to the eluent tank T-1 and opening the valve V-2 until the desired volume of pulse, measured in the burette T-2 used as feed tank, was introduced into the column. The valves V-1 and V-2 were then returned to their original positions.

5 The sampling procedure started when the base line in the recorder integrator RI-1 started deflecting, a characteristic of product elution. The sampling was finished when the base line returned to the zero.

The following modifications to the above procedure were made operating in the chromatographic bioreaction-separation mode:

1. The feed was only introduced when the system reached a steady state as observed on the recorder integrator RI-1 of the detection unit. Steady state was defined as the state when the exiting eluent was the same as the entering eluent. At this stage it was assumed that the void space of the column was occupied by the enzyme solution.

2. The water in the reference cell of the differential refractive index monitor was replaced by a filtered enzyme solution, the same as that used as eluent.

3.4.4 Maintenance

After each operating day, the feed tank, feed pump and injection port were thoroughly cleaned. The sugar solution was completely removed from the system and hot water was flushed through the tubing, which was then drained and dried. The enzyme container was replaced by another container with deionised and degassed water kept at 65 ^{o}C and the eluent pump was left pumping overnight. The liquid in the reference cell of the refractive index monitor was replaced by bi-distilled water. Both sample collecting and detection unit pumps P-3 and P-4 were left running overnight.

During the weekends the system was filled with 2 % w/v sodium azide solution and, on the first working day after this treatment, the chromatographic bed was regenerated according to the procedure that will be described in section 4.2.2.3.

3.5 Analytical Procedures

3.5.1 Quantitative Determination of Carbohydrates by HPLC

The quantitative determination of carbohydrates was performed using an HPLC system fitted with a carbohydrate analysis column, HPX-87C 300 x 7.8 mm packed with 8 % cross linked cationic resin in the calcium form, particle size 7-10 μm, from Bio-Rad UK Ltd., Watford. The HPLC system consisted of:

- A sampling device Talbot ASI-3 Autosampler from Talbot Scientific, Alderley Edge, Cheshire, UK
- A metering pump model Bio-Rad 1330 from Bio-Rad UK Ltd, Watford
- A guard column type Hibar-Lichocart cartridge, pore size 1 μm, from BDH Chemicals Ltd, Atherstone, Warwickshire, UK
- A heating oven from Bio-Rad UK Ltd
- A differential refractometer model 1750 from Bio-Rad UK Ltd

- A Recorder Integrator model SP4270 from Spectra Physics, St. Albans, Herts., UK

3.5.1.1 HPLC Operation

The separation of the saccharides was made under isocratic elution mode at 85 °C and an eluent flowrate of 0.5 cm^3/min. Deionised, distilled and degassed water, filtered through a 10 μm slip-on filter, was used as eluent and the main separation mechanisms involved in the process were size exclusion and ligand exchange (130,131). The pressure in the column was always maintained below 10^5 kN/m^2 as suggested by the column manufacturer.

The samples collected from the bioreaction-separations runs were filtered using a 5 μm membrane filter from Gelman Science Ltd, Northampton, UK, into 2 cm^3 vials from Chromacol Ltd, London, UK and the vials placed in the HPLC autosampler. The method was calibrated using the external standard technique (132) and the standard carbohydrate solutions were made precisely 1 % w/v. As the samples collected from the chromatographic bioreaction-separations had been previously treated to deactivate the enzymes, no further treatment was necessary.

3.5.1.2 HPLC Maintenance

Various procedures were used to maintain an optimum performance of the HPLC system and they were employed occasionally whenever the performance dropped:

1. To reverse the column if the pressure drop exceeded 10^5 kN/m^2.

2. To clean the column with a 5 % w/v acetonitrile solution followed by a regenerating procedure using a 0.1 M calcium acetate solution at 65 °C with a flowrate of 0.2 cm^3/min for two days, if the resolution for the most overlapped peaks decreased by 20 %.

3. To clean the refractometer cell with a 6 M nitric acid general purpose reagent solution if the area of the 1 % w/v standards decreased by 50 %.

3.6 Enzyme Assays

3.6.1 Introduction to the Enzyme Assays

The action of the hydrolytic enzymes and the isomerase enzyme on the substrates used in the experimental work generate a mixture of reducing sugars. These can be quantitatively determined and related to the enzyme activity if the assay is done under standard conditions of pH and temperature. The procedures adopted by each enzyme manufacturer (133-136) differ, depending upon the substrate used in the assay and the quantitative analysis of the products produced. The methods are usually based on colorimetry where a chemical reagent reacts with the product or products formed, changing the colour of the reaction medium. Table 3.3 presents the main characteristics of the assays supplied by the enzyme manufacturers relating to the substrates used, the products released by the enzyme action and the chemical reagent used to produce an appropriate colour change.

The colour change produced is proportional to the amount of products released which in turn is proportional to the activity of the enzyme present in the sample. The optical density is read at a particular wavelength and converted into micromoles of substrate consumed using a standard curve. The activity of an enzyme or its capacity to transform a substrate into a product was defined by The Enzyme Commission of the International Union of Biochemistry in 1961, as the International Unit U, meaning the amount of enzyme which converts 1 μmol of substrate in one minute under optimised standard conditions of pH and temperature.

The calibration curve for the 3,5 Dinitro salicylic acid method employed in the isomerase assay supplied by the enzyme manufacturer (133) is presented in Appendix A-1.

3.6.2 Modified Assays

The colorimetric methods supplied by the enzyme manufacturers have the disadvantages of being extremely time consuming and requiring the recalibration of the absorbances curves every month (133) or whenever one of the reagents used in the colorimetric procedure is prepared. To overcome these difficulties the following modifications of the methods, discussed with the enzyme manufacturers (138-140), have been introduced.

1. The same substrates that were used in the bioreaction-separations were used in the assay.

2. The reducing sugars generated in the assays were quantified by HPLC techniques as described in section 3.5.1.

Table 3.3 Characteristics of the assays supplied by the enzymes manufacturers

Assay for	Substrate	Products formed	Chemical method
Invertase	Sucrose	Glucose and fructose	3,5 Dinitro salicylic acid
Lactase	O-nitrophenyl-beta-D-galactoside (ONPG)	O-nitrophenol and galactose	Sodium carbonate
Glucose-isomerase	Glucose	Fructose	Cysteine-sulphuric acid reagent
Inulinase	Inulin	Fructose and glucose	Resorcinol - Hydrochloric acid

The assay conditions used, such as the reaction or incubation time, temperature and pH for the test, the concentration of substrate, the relation enzyme/substrate and the pH and temperature conditions for the enzyme deactivation were the same as those suggested by the enzyme manufacturers and are presented in Table 3.4.

The general procedure for the determination of enzyme activity was as follows:

1. 3 cm^3 of the diluted substrate solution were transferred to a 10 cm^3 volumetric flask.

2. 1 cm^3 of buffer solution was added to the substrate. The following buffers were prepared (129):

   - sodium acetate - acetic acid for a buffer pH = 4.7

   - potassium dihydrogen orthophosphate - potassium hydrogen orthophosphate for a buffer pH = 7.5

3. 1 cm^3 of cofactor solution, magnesium sulphate heptahydrate, was added for the glucose-isomerase assay.

4. The flask was placed in a water bath and maintained at the test temperature and allowed to stabilise for 10 minutes.

5. 2 cm^3 of suitable diluted enzyme solution were added and a timer engaged for the required incubation time.

6. At the end of the incubation time the volumetric flask was removed from the bath and placed in a boiling water bath for 1 minute to deactivate the enzyme. This time was sufficient for the deactivation of the enzymes used.

7. The volumetric flask was then placed in an ice bath, 1 cm^3 of sodium hydroxide 0.5 M solution added and the volume completed with water to the mark. The sample was then analysed by HPLC according the method described in section 3.5.1.

The results obtained for the determination of invertase activities using the modified method were similar to the results obtained using the chemical colorimetric method, and so the modified procedures for the following assays: lactase, glucose-isomerase and inulinase, have been used in this study.

Table 3.4 - Conditions employed in the enzyme assays

Assay for	**Substrate**	**Temp (°C)**	**pH**	**Incubation time (min)**	**Products formed**
Invertase	Sucrose	55	4.7	10	Glucose and fructose
Lactase	Lactose	55	4.7	5	Glucose and galactose
Glucose-isomerase	Glucose	55	7.5	15	Fructose
Inulinase	Inulin	55	4.7	10	Glucose and fructose

The activity was calculated as follows:

$$Activity = \left(\frac{\mu \, moles \, of \, substrate \, consumed}{1\mu \, mole \, of \, substrate \, . \, t} \right) . d \quad U / min / cm^3 \qquad (3.1)$$

where t is the incubation time and d the dilution factor for the enzyme

3.7 Particle Size Measurement Applied to Ion-exchange Resins

A laser scattering dispersion technique using a Master Particle Sizer M 3.0 instrument, manufactured by Malvern Instruments, was used to determine the resin particle size (in the sodium form). This procedure was chosen to avoid the difficulties of the screening procedure of wet resin, which should be performed with flowing water, or the dry procedure where the resin is analysed in a shrunken condition (141). Both the wet and dry procedures present the difficulties of drying and weighing fractions, which are time consuming activities. Also, the possibility of beads breakage during the sieving step can result in false data, due to fines generation.

The literature does not mention particle size analysis of ion-exchange resins using the laser scattering dispersion technique: experience shows that care must be taken to avoid inconsistent results. Initially, successive determinations of particle size without removing the cell from the instrument gave results deviating about 30 % from the average value. The fluctuation of results for the same sample did not happen when a standard of known particle size was analysed.

The density of the standard material used was the same as the suspending medium (distilled water), but it was observed that the resin was not conveniently suspended in it. Consequently, the procedure of suspending the resin beads in a 25 % w/v sodium chloride solution of density = 1.16 g/cm^3, the same as the beads, was adopted.

3.8 Determination of V_{max} and the Michaelis-Menten Constant K_M

3.8.1 Introduction

The determination of V_{max}, the maximum reaction rate, and K_M, the Michaelis-Menten constant, are based on various graphical and computational procedures starting from the determination of the initial rates. These were obtained through a convenient treatment of the reaction rate data collected from batch reactions performed in the equipment shown in Figure 3.4, the same as that used for the preparation of inverted sucrose in section 3.4.2.

3.8.2 The Experimental Procedure for the Determination of Initial Velocities

To avoid enzyme inhibition effects either by the substrate or by the products released and also any deactivation of the enzyme during the progress of the reaction, the initial rate approach has been adopted (142). Using this procedure, batch reactions covering a range of initial substrate concentrations were conducted in diluted conditions of substrate and

enzyme at the optimum conditions of pH and temperature for the enzyme action. Samples of the reaction medium were withdrawn at 2 minutes intervals and analysed for reducing sugars, according to the method described in section 3.5.1.

The initial rate can be obtained graphically, drawing manually a tangent to the reaction progress curve at the origin. The method is regarded as imprecise (108,143) and other graphical (143-145) and numerical (143-146) methods for the calculation of the initial rate are presented in the literature.

To evaluate the initial rate a fourth order polynomial in the following was chosen (147):

$$f(x) = a_0 + a_1x + a_2x^2 + a_3x^3 + a_4x^4 \qquad (3.2)$$

Hence, the following polynomial in time can be written:

$$C(t) = a_0 + a_1t + a_2t^2 + a_3t^3 + a_4t^4 \qquad (3.3)$$

Differentiating equation (3.3) yields:

$$dC/dt = a_1 + a_2t + 2a_3t^2 + 3a_4t^2 \qquad (3.4)$$

For t = 0, equation 3.4 becomes:

$$v_0 \text{ (initial rate)} = a_1 \qquad (3.5)$$

The kinetic data collected during the batch experiments were treated using the software Mathematica® (148). The program plotted the curve concentration versus reaction time as a numerical function through a best fit procedure using an internal algorithm. The initial rate, which is the tangent to the curve at t = 0, was then calculated using this numerical function.

3.8.3 Quantification of V_{max} and K_M

After the calculation of the initial rates, graphical and numerical procedures can be used for the quantification of V_{max} and K_M for the Michaelis-Menten kinetic model (109,110,142,149,150).

The graphical methods are based on different ways of treatment applied to the initial velocities and their inherent initial concentrations. The direct linear plot developed by Eisenthal and Cornish-Bowden was regarded by them as subjected to error, although to a

lesser extent than the other methods such as the plots of Hanes, Eadie-Hofstee and Lineweaver and Burk (109).

Therefore, a computational procedure using the software Enzfitter® especially designed for enzymatic kinetic studies (151), which uses a numerical iterative procedure based on least squares to calculate V_{max} and the Michaelis-Menten constant K_M, has been used in this work.

CHAPTER FOUR

CHROMATOGRAPHIC SEPARATION AND BIOREACTION SYSTEMS

4.1 Introduction

Chromatographic columns have been employed as chromatographic reactors since the late 1950's (1) and the feasibility of their use as chromatographic bioreactor-separators was established by Zafar and Barker in 1986 (2,70). The behaviour of these systems are highly dependent on their two main components: the chromatographic column, as the separator, and the enzymatic reaction, sometimes referred to as bioreaction in this study.

The chromatographic column contains a stationary phase which performs a selective interaction with the chemical species involved in the process and should not inhibit the reaction. The interaction, in its turn, is dependent on the chemical nature of the stationary phase and on the chemical nature of the mobile phase or eluent.

The enzymes used in this research can only function in aqueous medium and therefore water was employed as eluent. A strong cationic ion exchange resin, also known as strong cationic resin, in the calcium form for the hydrolytic reactions or in the magnesium form for the isomerization reaction, was selected as the stationary phase.

4.2 Ion Exchange Resins as Stationary Phase for the Separation of Carbohydrates

4.2.1 Ion Exchange Resins - A General View

Ion exchange resins are synthetic polymers containing cationic or anionic functional groups on which are based their classification: that is either strong, medium or weak resins. Although natural and synthetic zeolites such as zeolite-Y have been used as stationary phase for the separation of carbohydrates (152,153), only cationic exchange resins are used industrially (154). Anionic exchange resins have been tested for the separation of carbohydrates (90), but are not generally used for carbohydrates separation due to the poor resolution they present, their susceptibility to organic fouling, their low resistance to thermal and chemical degradation and the fact that they are more expensive than the cationic resins.

Most of the cationic type ion exchange resins are polystyrene based materials cross linked with divinyl benzene containing a strong anionic sulphonic group $^{-}SO_3$, and produced either in a gel type or in a macroporous structure. Cations bonded to the anionic sulphonic group can be exchanged and the strength of the chemical bond is dependent on the electronegativity of the metal. They are produced in the form of spherical beads with controlled particle size which are usually kept in a fairly narrow range. The capacity of an ion exchange resin, is usually expressed in milliequivalents of calcium per unit volume of the resin and depends on the number of functional groups attached to the resin matrix, which in turn is proportional to the degree of cross linking of the resin. The crosslink, ranging from 4 to 12 %, determines the physical structure of the resin matrix. The greater the crosslink of a resin the smaller is its pore structure and its capacity to swell or shrink. A low degree of crosslink gives the resin a more open pore structure which will play an important role in the permeation of low molecular weight substances, such as di, tri and tetrasaccharides, in the size exclusion separation processes. Nevertheless, due to its lower exchange capacity, or its lower number of ionizable sites per unit volume of resin, it tends to be less selective.

4.2.2 Chromatography of Carbohydrates Using Cationic Resins as a Stationary Phase - The Effect of the Counter-ion.

4.2.2.1 Introduction

The use of cationic type ion exchange resins in the calcium form for the separation of carbohydrates is reported back in 1969 by S. A. Barker *et. al.* (130) who have also compared Ba^{+2} and Li^{+1} as a substitute cation. They observed that resins with lower cross linkage benefit the separation of carbohydrates. They also showed that calcium ions performed better separation compared to other metal ions and when the resins operate at low temperatures, they present favourable conditions for the separation of simple carbohydrates such as glucose, fructose, maltose and sucrose. The uses of cationic resins for the separation of carbohydrates by chromatography have also been investigated by Goulding (155) and extensively updated by Ben-Bassat *et. al.* (156).

The separation of carbohydrates by an ion exchange resin is not a true ion exchange process as there is no replacement of the calcium ions by the ions present in the aqueous solutions that percolate the resin bed. The process is based on the differences in molecular sizes, conformation of the compounds and the configuration of the hydroxyl groups. The mechanisms involved are size exclusion and ligand exchange.

4.2.2.2 Size Exclusion

Size exclusion is the mechanism of chromatography based on the sizes of the molecules. The mixture of solutes of different molecular sizes which flows through a chromatographic bed will travel in different pathways. The smaller molecules such as the monosaccharides glucose, fructose and galactose. will move most of the time diffusing in and out of the resin beads. The pores of the resin beads (16 angstroms) are slightly larger than those molecules (8-12 angstroms) (157). Therefore these products will tend to be retarded. Disaccharides such as sucrose and lactose, and higher saccharides will move faster because they do not penetrate into the resin matrix due to their molecular size. The separation of disaccharides, nevertheless, cannot be explained by the size exclusion mechanism.

4.2.2.3 Ligand Exchange Mechanism

The ligand exchange mechanism is responsible for the chromatographic separation of monosaccharides such as glucose, fructose, galactose and some disaccharides as sucrose and lactose by cation exchange resins in the calcium form (155). Calcium ions attached to cation exchange resins are hydrated in a structure known as the hydration sphere of calcium. The hydroxyl groups of a carbohydrate molecule can replace part of the water molecules of the calcium hydration sphere resulting in a co-ordination complex, whose strength depends on the conformation of the saccharide. Glucose, for instance, is an aldose and has an open chain structure, which can exist in two ring conformations or anomeric forms: α and β pyranose. Fructose, a ketose, exists in two anomeric forms: α and β furanose (158-160).

This phenomena is known as mutarotation and occurs when the carbohydrates undergoes solution. They inter-convert from an open chain to different ring or tautomeric forms, through the formation of open chain forms, until the equilibrium is reached (158). The proportion of the different anomeric forms for glucose and fructose is highly dependent on the temperature (160-162) .

The tautomeric forms of these carbohydrates adopt different conformations known as chair, boat and envelope (163), as shown in Figures 4.1 and 4.2, with the hydroxyls in axial and equatorial (planar) positions. The stability of the form is related to the position of the hydroxyls and the more stable conformation is when all the hydroxyl groups are in the equatorial position, which means that there is no steric hindrance between adjacent groups. Due to the tetrahedral geometry of the carbon atoms the possibility of all hydroxyls occupying the equatorial position is remote and the separation of two saccharides will

occur if one of the molecules provides more available sites for ligand exchange than the other.

The pairs of hydroxyl groups in axial-equatorial positions in the pyranose form are responsible for the ligand exchange with the water molecules held in the hydration sphere of the calcium ions attached to the ion exchange resin (155). Fructose can have one or two pairs depending on the tautomeric form adopted. Glucose can have one or none, also depending on the tautomeric form adopted. Furthermore, the Angyal hypothesis (163) states that the formation of stable complexes with cations demands an arrangement of hydroxyl groups such as axial-equatorial-axial which can be found in the β-D-fructose. Therefore, fructose is more strongly complexed by the cation exchange resin compared to glucose.

Galactose presents an arrangement of its hydroxyl groups of the type axial-equatorial and therefore is more retained by the cation exchange resin in the calcium form than glucose but less retained than fructose.

4.2.3 The Cationic Resin Purolite PCR 833

The stationary phase selected for the experimental work was a cationic resin, a copolymer styrene-divynyl benzene, gel type, 8 % cross linked, with a sulphonate functional group and supplied in the sodium form. The ion exchange capacity of this resin was 2 meq/cm^3 and the particle size range shows 10 % larger than 300 μm and 2 % smaller than 150 μm, according to the supplier (164). The resin is suitable to operate at 140 ^{o}C maximum and stable in the pH range from 3 to 9.

A Purolite PCR 833 resin, 8 % cross linked, was previously used by other workers in the laboratory for the semi-continuous chromatographic separation of glucose and fructose (89,91) and in the semi-continuous chromatographic bioreaction separation of sucrose into glucose and fructose using the enzyme invertase (4). The resin was manufactured by The Duolite Co. with the commercial name 225 SCR 14 (4).

Figure 4.1 - Tautomeric forms of glucose

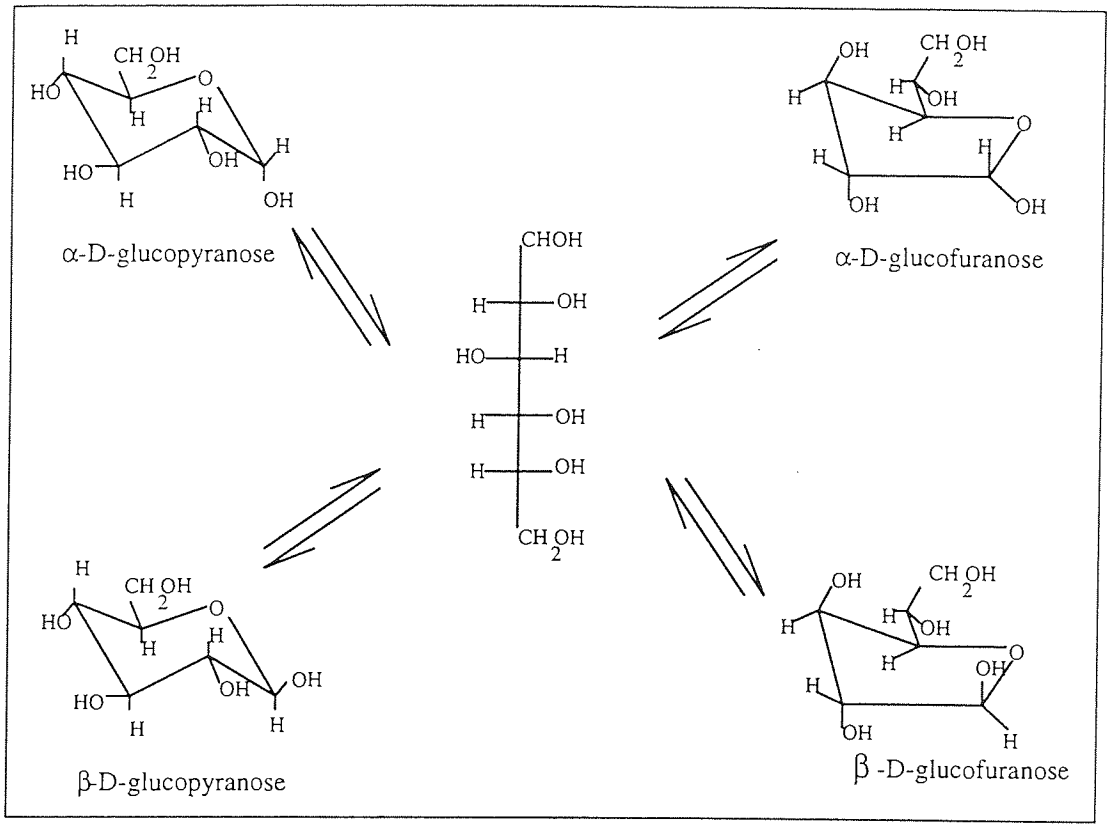
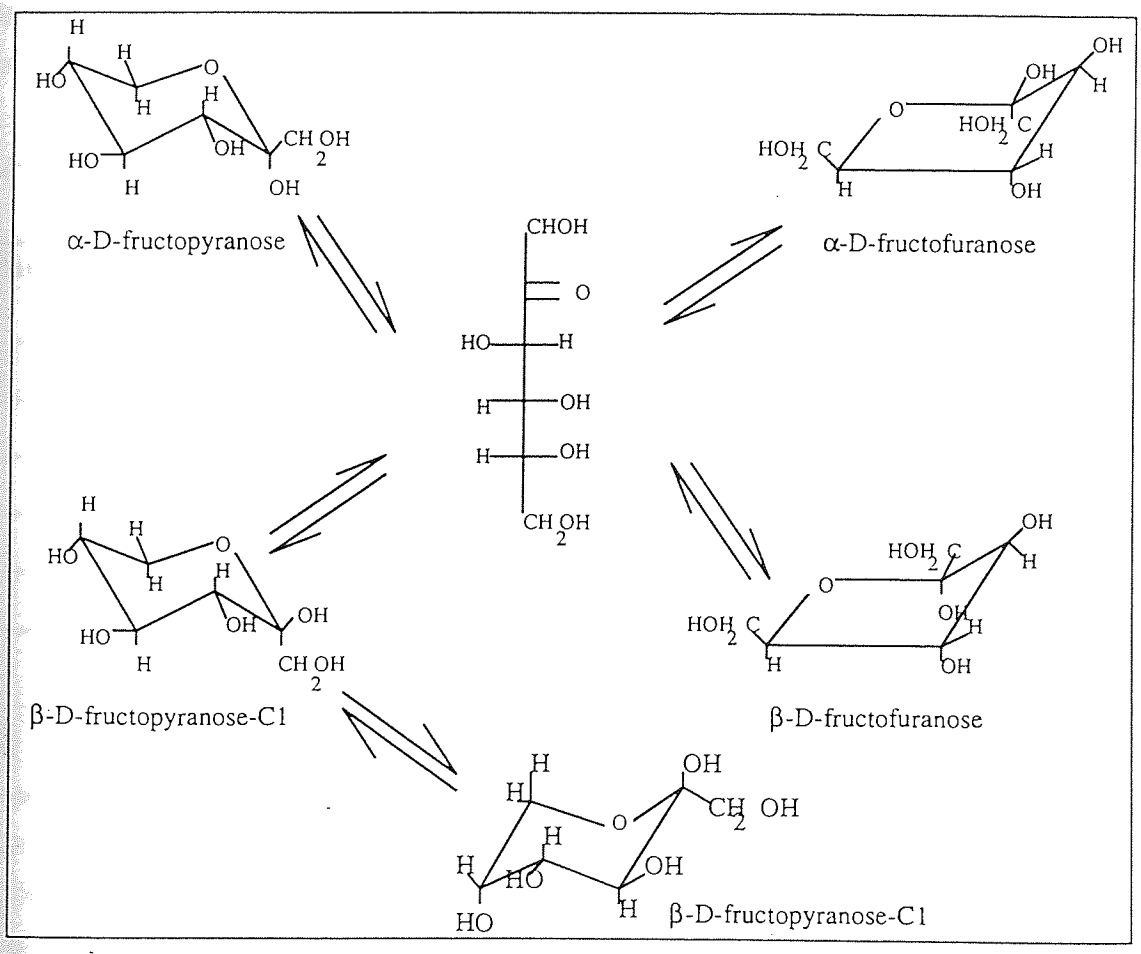


Figure 4.2 - Tautomeric forms of fructose



4.2.4 Resin Preparation

4.2.4.1 Elutriation

4.2.4.1.1 Introduction

It is a common knowledge that decreasing the size of the ion exchange particles employed in a chromatographic separation system will result in an increase in the resolution of that system, if the individual resin particles represent a significant resistance to internal mass transfer. On the other hand, the use of smaller particles results in an increase of operating pressure. However, this effect can be partially reduced by using particles in a narrow size range, which will ensure a minimum void volume in the packed resin bed. The larger resin beads make the separation less distinct. Some available industrial products on the market are known as monosphere resins. They have 90 % of the resin beads within 10-20 % of the mean bead size (165).

The size distribution analysis using the technique of laser scattering dispersion discussed in section 3.6 for the resin Purolite PCR 833 in the sodium form is presented in Table 4.1 and plotted in Figure 4.3. The results show a fairly wide range for the particle size distribution and a decision to remove both the fines and the coarse particles was made.

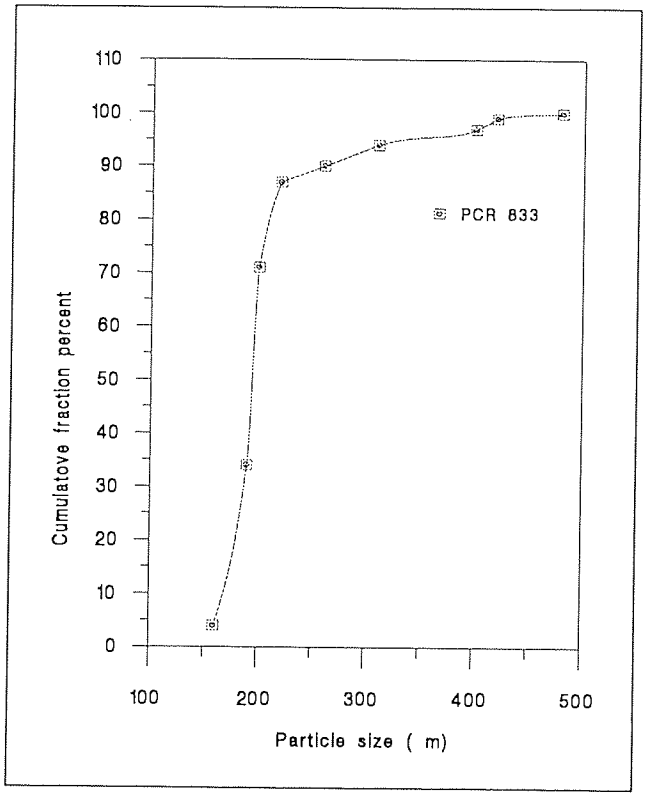
Table 4.1 Size distribution data on a cumulative basis for the cation exchange resin Purolite PCR 833

Particle size range µm	Resin PCR 833	Particle size range µm	Resin PCR 833
< 150	0	269-295	90
150-160	4	310-355	94
190-200	34	400-420	97
200-210	71	420-425	99
220-250	87	> 480	100

The technique usually employed involves suspending the resin particles in a fluid with subsequent decantation. The smallest particles decant slower and can be easily removed by a quick pouring of the supernatant liquid, before they settle down. This technique is not efficient or selective for a specific size cut. Other techniques involve the up flow of a liquid in a column or separation funnel, which operate in a similar principle to elutriators

(166,167). Elutriation was selected for the size grading of the cationic resin Purolite PCR 833.

Figure 4.3 - Size distribution curve for the cation exchange resin Purolite PCR 833



4.2.4.1.2 The Basic Principle of the Elutriation System

A spherical particle falling freely through a liquid column reaches a uniform downward linear velocity, or terminal velocity, according to the following relationship:

$$V_{Stokes} = 2\, g\, (d_1 - d_2) / 9\, \eta\, r_p^{\,2}\, 60 \text{ cm/min} \qquad (4.1)$$

where,

g = acceleration due to gravity = 980 cm/sec ^{2}

d_1 = density of the resin bead = 1.16 g/cm ^{3} (991)

d_2 = density of the fluid (water at 25 ^{o}C) = 1.0 g/cm ^{3}

r_p = radius of the particle in cm

η = viscosity of the fluid (water at 25 ^{o}C) = 0.00894 poise

Therefore,

$$V_{Stokes} = 2.339 \times 10^{5} \times r_p^{2} \text{ cm/sec} \quad (4.2)$$

Elutriation subjects the solid particle to an upward velocity. If the fluid velocity is slightly greater than the terminal velocity of the particle, this solid particle will be carried along with the fluid stream. For this assumption :

$$V_{up} \text{ (upward linear velocity)} = V_{Stokes} \quad (4.3)$$

and

$$V_{up} = \text{Input Flow Rate} / A_e \quad (4.4)$$

where,

A_e = Area of the elutriation compartment

Comparing 4.3 and 4.4:

$$\text{Input Flow Rate} = V_{Stokes} \times A_e \quad (4.5)$$

and, finally,

$$\text{Input Flow Rate} = 7.3482 \times 10^{5} \times r_p^{2} \times R_{elut}^{2} \quad (4.6)$$

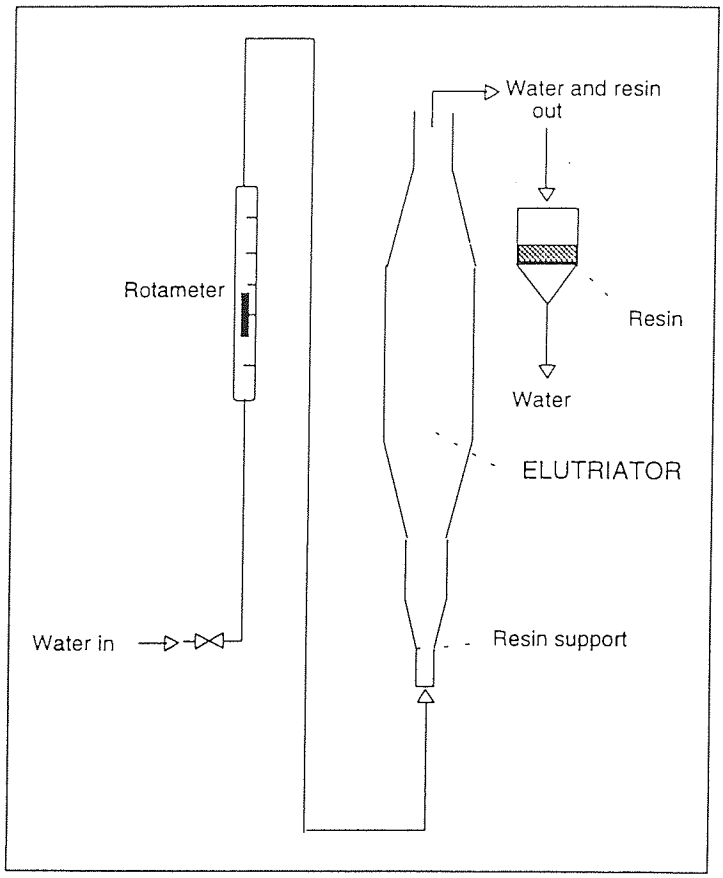
where,

R_{elut} = radius of the elutriator chamber

4.2.4.1.3 The Elutriation System

The elutriator was equipped with QVF pipeline components made of borosilicate glass, manufactured by QVF Glass Corning Ltd., Stone, Staffordshire, UK. A schematic diagram of the system is shown in Figure 4.4 and in Plate 3.1. The apparatus was made of expansion/reduction fittings that were connected to a glass cylinder 40 x 10.8 cm, which was the elutriator chamber. The gradual increase of the fittings section diameter corresponded to a gradual decrease of the fluid linear upward velocity. The inlet water flowrate was controlled by a needle valve and measured through a model Metric 14S rotameter, tube size 14 and stainless steel float type S (d = 14.20 mm, w = 16.50 g), manufactured by GEC - Eliot Process Instruments Ltd, UK.

Figure 4.4 - Schematic diagram of the elutriation system



4.2.4.1.4 Experimental Procedure

One charge of the chromatographic bed, approximately 600 cm^3 of resin, was submitted to elutriation. As can be seen from the data in Table 4.1, the resin Purolite PCR-833 selected for the present research project presented a very poor granulometric homogeneity: 4 % of the resin particles were below 190 μm and 13 % above 260 μm. The separation of these fractions was performed by setting the water flowrate to values obtained from equation 3.6 for R (radius of the upper cylindrical section of the elutriator) = 5.4 cm. Therefore, the flowrate of 2 dm^3/min elutriated the particles smaller than 190 μm and the flowrate of 3.35 dm^3/min left the particles larger than 250 μm in the elutriator.

4.2.4.1.5 Results and Conclusions

The data for size fractions and size distribution curves on a cumulative basis for the PCR 833 resin, before and after the elutriation, are presented in Table 4.2 and plotted in Figure 4.5. It is noticeable that the elutriation operation was conducted efficiently. The product

obtained was 100 % within 20 % of the mean bead size. Pictures of a representative sample of PCR-833 resin, before and after the elutriation, are shown in Plates 4.1 and 4.2.

Table 4.2 - Comparison of size distribution data for the cation exchange resin Purolite PCR 833 - before and after elutriation

Particle Size Range (μm)	Cationic Resin PCR 833		Particle Size Range (μm)	Cationic Resin PCR 833	
	Before elutriation	After elutriation		Before elutriation	After elutriation
< 150	0	0	260-295	90	0
150-160	4	0	310-355	94	0
190-200	34	38	400-420	97	0
200-210	71	82	420-480	99	0
220-250	87	100	> 480	100	0

Figure 4.5 - Size distribution curve for cation exchange resin Purolite PCR 833 - before and after elutriation

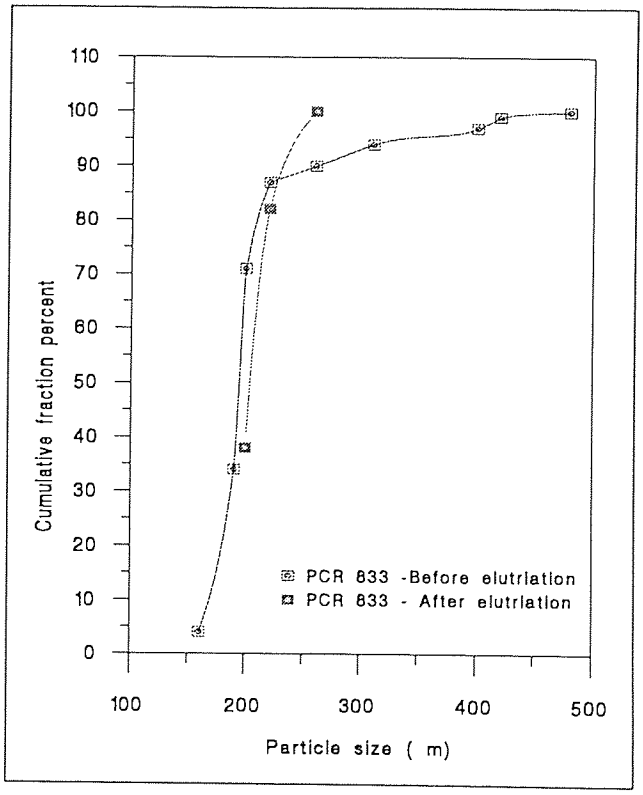


Plate 4.1 - Photograph of the cation exchange resin PCR-833 Na before elutriation

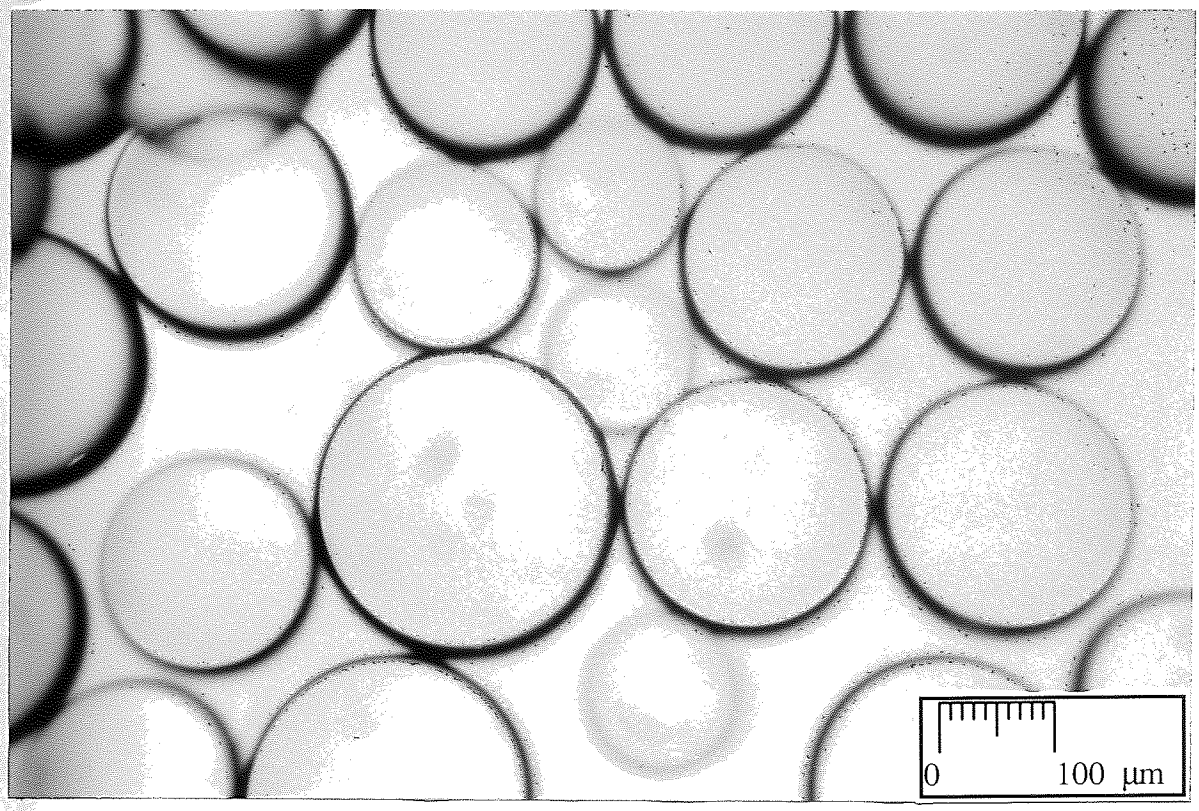
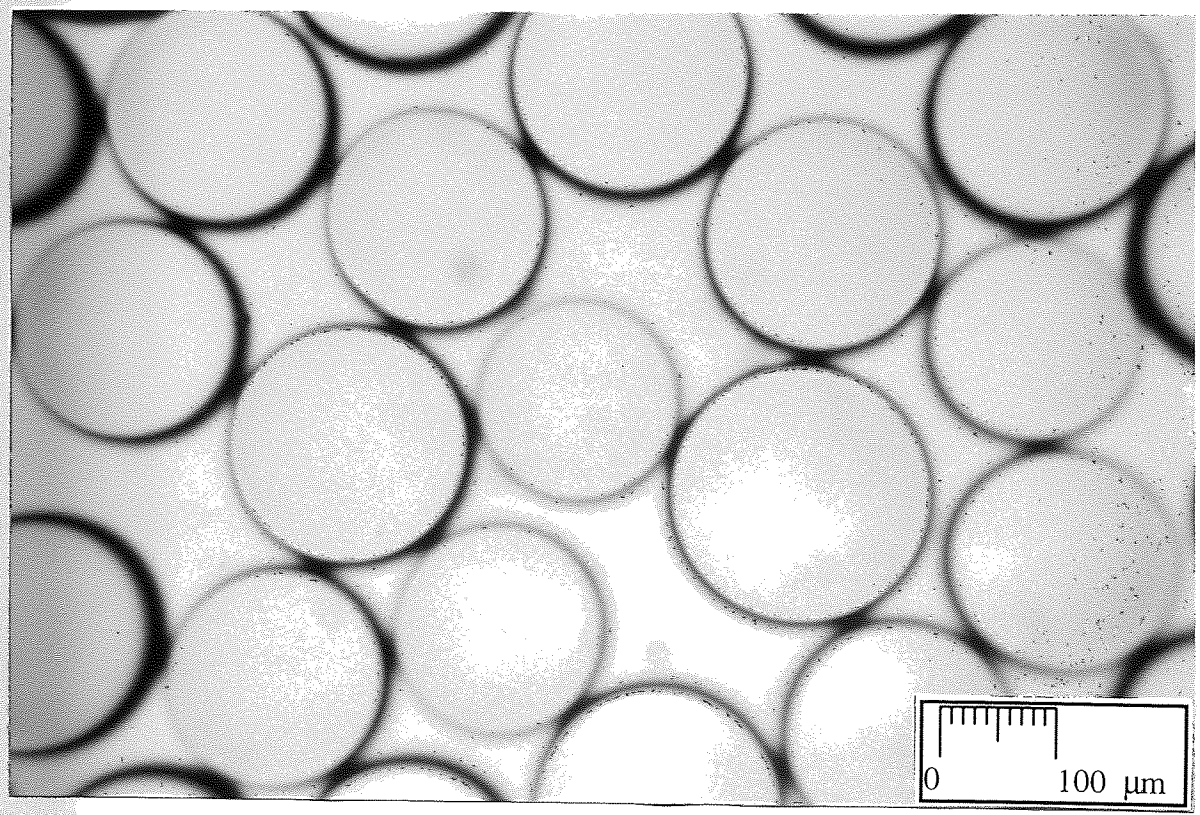


Plate 4.2 - Photograph of the cation exchange resin PCR-833 Na after elutriation



4.2.4.2 Packing the Bed

There are two different ways to fill a chromatographic column with material of coarse particle size such as the ion exchange resin employed in this experimental work: dry packing and the slurry packing technique. The first one leads to channelling due to any non homogeneity that occurs during the packing, which will promote uneven eluent flow distribution through the resin bed (132). The second should also be conducted with some care but it is a safer procedure which avoids channelling.

The technique consisted in pouring a 50 % w/v resin suspension in water on the top of the column, which had its feed port removed and, at the same time, applying suction at the bottom. The suction drained all the water from the poured slurry, leaving the wet resin on the bed support fitted in the column outlet. The feed port was then replaced and hot, deionised and degassed water was pumped through the bed for 24 hours, to remove air bubbles trapped in the voids. The column was then ready for the conditioning procedure.

4.2.4.3 Conditioning the Resin - On Site Procedure

According to instructions received from the manufacturer (168), to replace the sodium ions of the supplied resin by calcium ions it was first necessary to convert it into the hydrogen form. As cationic resins tend to expand 10 % of their volume when conditioned from sodium to the hydrogen form, a small column with the same diameter as the chromatographic reactor was fitted on top of it to allow the free expansion of the bed. This extension was removed when the resin shrunk back to its original volume due to its conversion to the calcium form.

The conditioning was performed in two steps. In the first, the resin was converted to the hydrogen form by elution of three bed volumes of a 5 % w/v solution of hydrochloric acid. The bed was then rinsed with deionised water until the pH of the eluent coming out of the column was the same as the eluent entering the column. The pH was measured with a pH meter manufactured by WPA Ltd. Cambridge, UK, and the instrument calibrated with standard buffer solution pH 7.0. In the second step, the resin was then converted to the calcium form by elution with three bed volumes of a 10 % w/v solution of calcium nitrate. After this second step, the resin was eluted overnight with deionised and degassed water at the recommended rinse flowrate.

The regenerating and rinse flowrates and the right amounts of the chemical substances used in both steps were calculated according to the ion-exchange capacity of the resin, based on

the technical catalogues supplied by the resin manufacturer (164). An excess of 50% of the theoretical amount was used.

For the system glucose-glucose isomerase the resin was converted to its magnesium form. In this case, the second step was carried out using three bed volumes of a 10 % w/v solution of magnesium sulphate heptahydrate, followed by the rinsing procedures described above.

4.3 Characterisation of the Chromatographic Columns Used with Ca and Mg Counter-ions

4.3.1 Determination of K_d's, NTP's, HETP's, Separation Factors, Resolution and Voidage

The characterisation of the columns allowed the investigation of the separation capacity of the stationary phases selected for the batch chromatographic bioreaction-separations. The parameters collected were later used in the mathematical modelling and simulation of the batch chromatographic bioreaction-separation (Chapter 9). The infinite dilution technique, similar to that employed by previous workers (2,4,88-91) was used, although the concentration of products during the bioreactions was much higher. Ganetsos (88,169) developed a correlation to accommodate this effect. The column parameters were determined from elution profiles of an inert material, which was used to measure the void volume of the column, and from the elution profiles of the carbohydrates involved in the experimental work. The elution volume, V_i, of the individual components was calculated by multiplying their respective retention times, t_{ri}, by the eluent flowrate.

The voidage (ε) of the bed was determined using a 1 % w/v solution of dextran T-2000, a glucose polymer with an average molecular weight of 2,000,000 daltons. Due to its very high molecular weight it percolates the chromatographic bed without penetrating into the resin pores. The calculation of the voidage was made using the equation presented in section 2.1.3.

The characterisation of the columns was achieved through the determination of the distribution coefficients (K_d), number of theoretical plates (NTP), height equivalent of a theoretical plate (HETP) for the carbohydrates, according to the equations established in section 2.1. The separation factors (α_{1-2}) and resolutions (R_{1-2}), were calculated for a pair of carbohydrates also using relationships presented in section 2.1.3.

4.3.2 Parameters for each Column

4.3.2.1 Introduction

All the enzymes selected for the experimental work in this thesis have their optimum working temperature around 55 °C. Therefore, this was the temperature chosen for characterisation of all chromatographic columns used either for the bioreaction-separations or for experiments related to pre-tests and the effect of flowrates on the distribution coefficients. The column used for the sucrose-invertase system was also characterised at 20 °C to enable a comparison to be made with the values obtained by other authors. The flowrate was set according to the diameter of the column used, in such a way that the superficial velocity would be in conformity with the value used by previous workers, who based their experiments on this value on a compromise basis of separation and throughput (90). Therefore, for the 1.96 cm I.D. diameter column the flowrate was set to 3.5 cm^3/min whereas for the 1.0 cm I.D. diameter column the flowrate was set to 0.91 cm^3/min, corresponding in both cases to a superficial velocity of 1.16 cm/min.

Calcium counter-ions were used for the systems sucrose-invertase, lactose-lactase and inulin-inulinase. As calcium acts as an inhibitor for the enzyme glucose-isomerase and magnesium is used as promoter for this enzyme (170,171), the latter was selected as counter-ion for the system glucose-glucose isomerase.

4.3.2.2 Batch Chromatographic Bioreactor-Separator I (BCBS-I) - Glass Column 200 x 1.96 cm I.D. Packed with Purolite Resin PCR 833 in the Calcium Form

The values obtained for the characterisation of the BCBS-I for the carbohydrates involved in the sucrose-invertase and lactose-lactase systems at 55 °C are presented in Table 4.3 and at 20 °C in Table 4.4 for the system sucrose-invertase. The separation factors ($\alpha_{1\text{-}2}$) for the system sucrose-invertase system at 55 °C and 20 °C are presented in Tables 4.5 and 4.6, respectively. The separation factors ($\alpha_{1\text{-}2}$) at 55 °C for the system lactose-lactase are presented in Table 4.7 and the resolutions ($R_{1\text{-}2}$) at 55 °C for the system sucrose-invertase, and for the system lactose-lactase are presented in Tables 4.8 and 4.9, respectively.

The values for the separation factors and resolutions were obtained at infinite dilution conditions, that is, pulsing small amounts of diluted solutions into the columns. The figures obtained are better than those that will be observed either in the chromatographic

separations or in the batch chromatographic bioreaction-separations, which employed higher pulse sizes and concentrations.

Table 4.3 Characterisation of the BCBS-I at 55 °C and 1.16 cm/min

Solute	t_{ri} (min)	K_{di}	NTP	HETP (cm)
Dextran	66	-	-	-
Sucrose	76	0.10	462	0.43
Lactose	74	0.08	421	0.47
Glucose	84	0.18	532	0.37
Galactose	97	0.30	481	0.41
Fructose	112	0.47	407	0.49

Table 4.4 Characterisation of the BCBS-I at 20 °C and 1.16 cm/min

Solute	K_{di}	NTP	HETP (cm)
Sucrose	0.11	198	1.00
Glucose	0.23	198	1.00
Fructose	0.58	129	1.54

Table 4.5 - Separation Factors on the BCBS-I for the system sucrose-invertase at 55 °C and 1.16 cm/min

α_{G-S} (Glucose-Sucrose)	α_{F-S} (Fructose-Sucrose)	α_{F-G} (Fructose-Glucose)
1.80	4.70	2.61

Table 4.6 - Separation Factors on the BCBS-I for the system sucrose-invertase at 20 °C and 1.16 cm/min

α_{G-S} (Glucose-Sucrose)	α_{F-S} (Fructose-Sucrose)	α_{F-G} (Fructose-Glucose)
2.09	5.27	2.52

Table 4.7 - Separation Factors on the BCBS-I for the system lactose-lactase at 55 °C and 1.16 cm/min

α_{G-L} (Glucose-Lactose)	α_{Gal-L} (Galactose-Lactose)	α_{Gal-G} (Galactose-Glucose)
2.25	3.75	1.67

Table 4.8 - Resolution on the BCBS-I for the system sucrose-invertase at 55 °C and 1.16 cm/min

R_{G-S} (Glucose-Sucrose)	R_{F-S} (Fructose-Sucrose)	R_{F-G} (Fructose-Glucose)
0.8	0.9	0.7

Table 4.9 - Resolution on the BCBS-I for the system lactose-lactase at 55 °C and 1.16 cm/min

R_{G-L} (Glucose-Lactose)	R_{Gal-L} (Galactose-Lactose)	R_{Gal-G} (Galactose-Glucose)
0.7	0.8	0.6

The resolution values obtained for the carbohydrates involved in the system sucrose-invertase show that these sugars can be partially separated with the stationary phase cation exchange resin in the calcium form, although the separation will tend to decrease when working with more concentrated solutions, which will overload the chromatographic column. The separation of the carbohydrates of the system lactose-lactase can also be partially separated, but to a lesser extent than the first system. This is due to a lower affinity of galactose for the calcium ion, compared to fructose, as seen in section 4.2.2.3.

The values for HETP and α_{1-2} found in this research were compared with the values found by other researchers for the system sucrose-invertase at 20 °C and 1.16 cm/min, using columns packed with cation exchange resin Purolite PCR 833 with calcium as counter-ions. They are presented in Tables 4.10 and 4.11.

Table 4.10 - Comparison of HETP values obtained in this work with those of previous workers in the laboratory, at 20 °C and 1.16 cm/min

Sucrose (cm)	Glucose (cm)	Fructose (cm)	Remarks
1.00	1.00	1.54	this work
2.18	2.16	1.68	reference 2
2.04	1.46	1.99	reference 4
-	1.46	1.99	reference 89
-	1.61	2.58	reference 91
-	-	2.65	reference 90

Table 4.11 - Comparison of separation factor values at 20 °C and 1.16 cm/min

α_{G-S}	α_{F-S}	Remarks
2.09	5.27	this work
1.63	3.88	reference 2
2.34	5.24	reference 4

The HETP values obtained for the column used as a bioreactor-separator for the system sucrose-invertase are lower than the values obtained by other researchers operating under the same conditions, for the same carbohydrates sucrose, glucose and fructose. Consequently, a higher number of theoretical plates per meter of column was obtained in this work. The improvement of the HETP value was probably due to:

1. the removal of fines by the elutriation procedure, which will tend to make the peaks narrower

2. the more efficient technique employed to replace the sodium by calcium ions, performed in two steps: the replacement of the sodium ions by hydrogen followed by the replacement of these by calcium ions. The elution of the regenerant made in a continuous way tends to diffuse more into the resin bead than the batch procedure used by previous researchers (5,164).

4.3.2.3 Batch Chromatographic Bioreactor-separator II (BCBS-II) - Glass Column 200 x 1.96 cm I.D. Packed with Purolite Resin PCR 833 in the Magnesium Form

The BCBS-II was characterised for the carbohydrates involved in the glucose-glucose isomerase system and the values are presented in Table 4.12. The temperature selected was 55 °C and the superficial velocity 1.16 cm/min, corresponding to a flowrate of 3.5 cm^3/min. The separation factor for glucose and fructose (α_{GF}) was 1.24 and the resolution (R_{FG}) for the same sugars 0.3.

Table 4.12 - Characterisation of the BCBS-II at 55 °C and 1.16 cm/min

Solute	t_{ri} (min)	K_{di}	NTP	HETP (cm)
Dextran	168	-	-	-
Glucose	210	0.162	282	0.70
Fructose	220	0.201	226	0.88

The values of α_{G-F} and R_{G-F} show that the separation of glucose and fructose using cation exchange resin PCR 833 with magnesium as counter-ion is very poor and will be affected further when working with more concentrated solutions.

4.3.2.4 Batch Chromatographic Bioreactor-separator III (BCBS-III) - Glass Column 200 x 1 cm I.D. Packed with Purolite Resin PCR 833 in the Calcium Form

This column was employed for the system inulin-inulinase and the characterisation was performed at 0.91 cm^3/min, corresponding to a superficial velocity of 1.16 cm/min, and at 55 °C. The values are presented in Tables 4.13 and 4.14

The resolution (R_{F-G}) for glucose and fructose was found to be 0.9 and the data obtained show that the column is adequate for the system inulin-inulinase, although the performance will be affected when working with more concentrated solutions.

Table 4.13 - Characterisation of the BCBS-III at 55 °C and 1.16 cm/min

Solute	t_{ri} (min)	K_{di}	NTP	HETP (cm)
Dextran	172	-	-	-
Inulin	178	0.19	471	0.42
Glucose	217	0.24	1322	0.15
Fructose	271	0.41	681	0.29

Table 4.14 - Separation Factors on the BCBS-III for the system inulin-inulinase at 55 °C and 1.16 cm/min

α_{G-I} (Glucose-Inulin)	α_{F-I} (Fructose-Inulin)	α_{F-G} (Fructose-Glucose)
1.26	2.16	1.71

4.3.2.5 Voidage

The voidage of the columns were found to be in the range 0.36-0.38, which is a low figure compared to the voidage value of 0.4 for spherical particles (172). Nevertheless, Samuelson (173) presents values of 0.33 for cationic resins with cross linking of 4 % and 0.38 for resins with cross linking of 8 %.

4.3.3 The Effects of Temperature and Flowrates on HETP's and the Effects of Background Concentration of Carbohydrates and Enzymes on the K_d's

4.3.3.1 Introduction

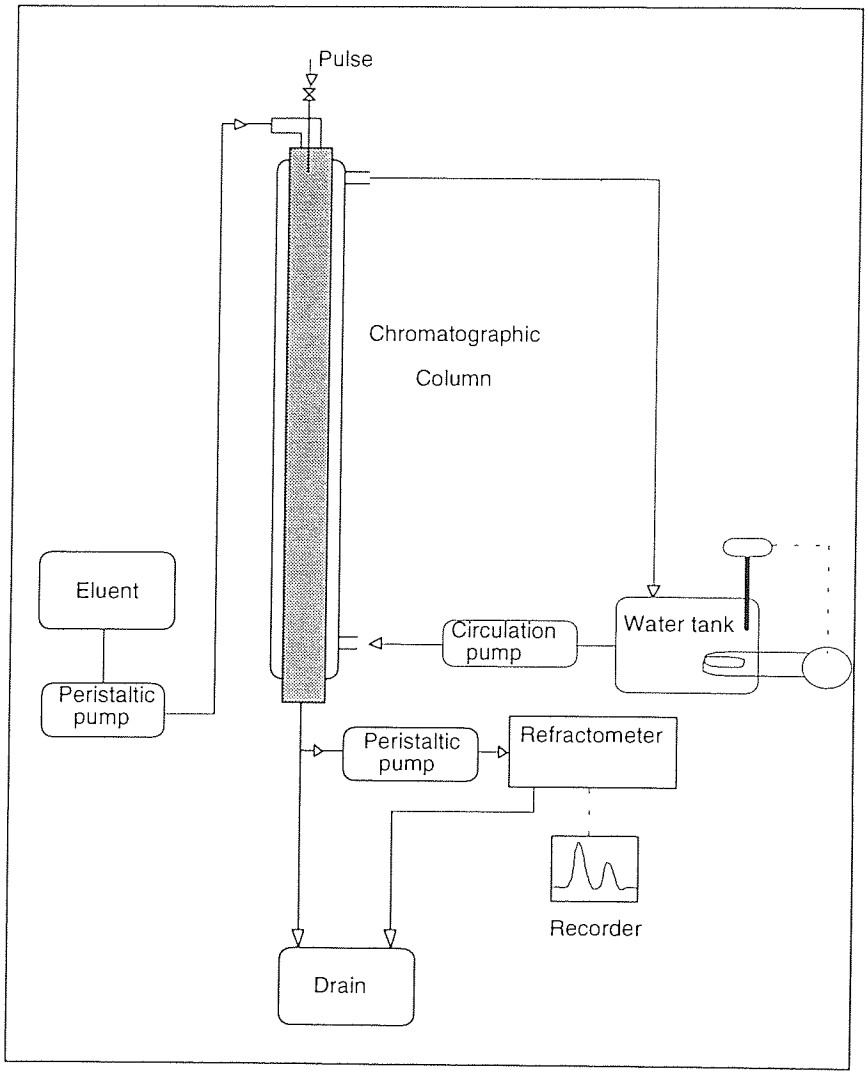
The distribution coefficients, the separation factors, the resolutions and the HETP's are all flow and temperature dependent parameters as explained elsewhere (21,24). The investigation of the effect of the temperature and flowrates on these parameters during the selection of the cationic exchange resin in the calcium form as stationary phase for the separation of carbohydrates has been well explored by previous researchers. Ching (90) and Thawait (89) studied the effects and extended the investigation analysing the effect of the background concentration on the distribution coefficients, in the presence of glucose and fructose.

It was observed during the early stages of this experimental work that the elution of concentrated carbohydrates solutions through the stationary phase contracted the resin bed. Another observation was related to the channelling of the top part of the resin bed due to the formation of air bubbles which also affected the volume of the bed. It is believed that the air came from the anhydrous glucose solution, which was not conveniently deaerated. To avoid the formation of air bubbles the carbohydrate solutions were deaerated at 85 °C.

As the calculation of the distribution coefficient is based on the total volume of the bed (see section 2.1.3), the correct determination of its value was necessary and the data re-evaluated. Therefore, a set of experiments determining the effect of temperature and background concentration on the HETP and distribution coefficients was performed and the results presented in the following sections.

The effects of temperature and flowrates on HETP's and the effects of background concentration of carbohydrates and enzymes on the K_d's were carried out in a batch chromatographic system as shown in Figure 4.6, using a jacketed glass column 1m long x 0.9 cm I.D. (section 3.3.2) packed with cationic exchange resin Purolite PCR 833 in the calcium form, with the particle size in the range 190 - 210 μm.

Figure 4.6 - Schematic diagram of the batch chromatographic system used for determination of effect of the temperature, flowrate and background concentrations on HETP's and K_d's



4.3.3.2 The Effects of Temperature on the HETP's

The effects of the temperature on the HETP's of a chromatographic column, for glucose, fructose and galactose, were studied at three levels of temperature: 45 °C, 55 °C and 65 °C. The following superficial velocities were chosen for the experiments: 0.07, 0.010, 0.014 and 0.017 cm/s.

The results presented in Figures 4.7, 4.8 and 4.9 show that the separation, based on the number of theoretical plates which increases as the size of the HETP decreases, favours working at lower flowrates and higher temperatures.

Enzymes in general have an optimum working temperature which dictated the value to be used in the batch chromatographic bioreaction-separations selected for this research. Nevertheless, the value chosen did not necessarily correspond to the best conditions for the

chromatographic separations. The flowrate selected was based on a compromise of throughput and separation and, therefore, an intermediate value was chosen in agreement with previous workers (4,89).

Figure 4.7 - Effect of temperature on HETP's for glucose on the cationic exchange resin Purolite PCR 833 in the calcium form

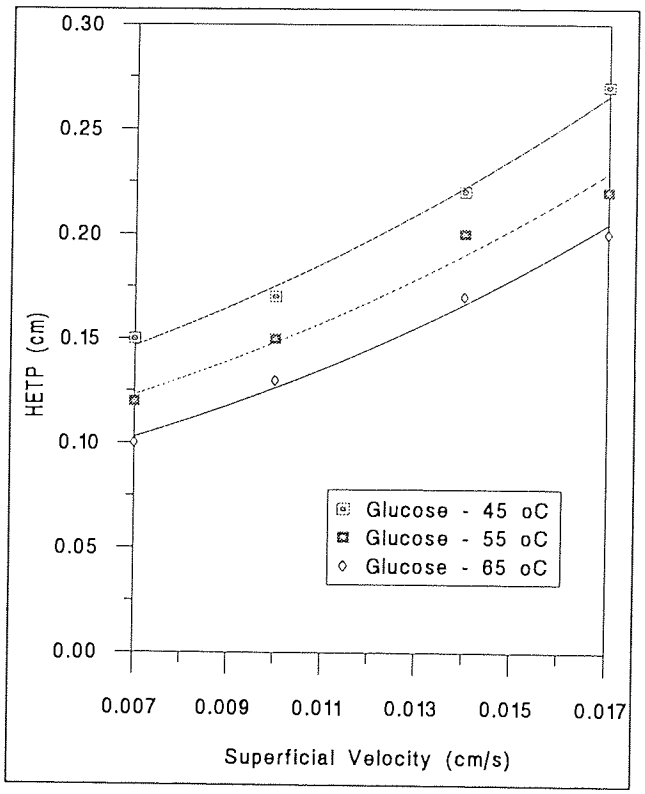


Figure 4.8 - Effect of temperature on HETP's for fructose on the cationic exchange resin Purolite PCR 833 in the calcium form

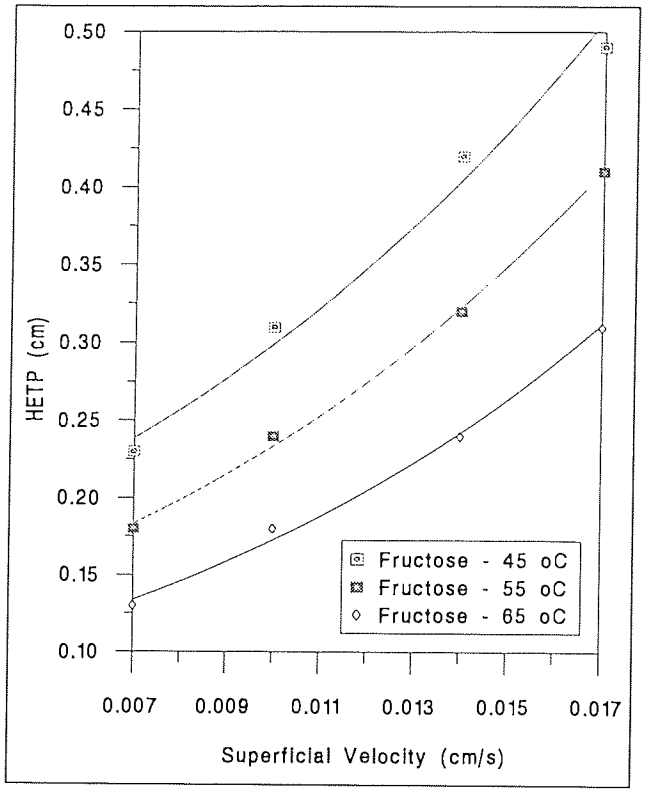
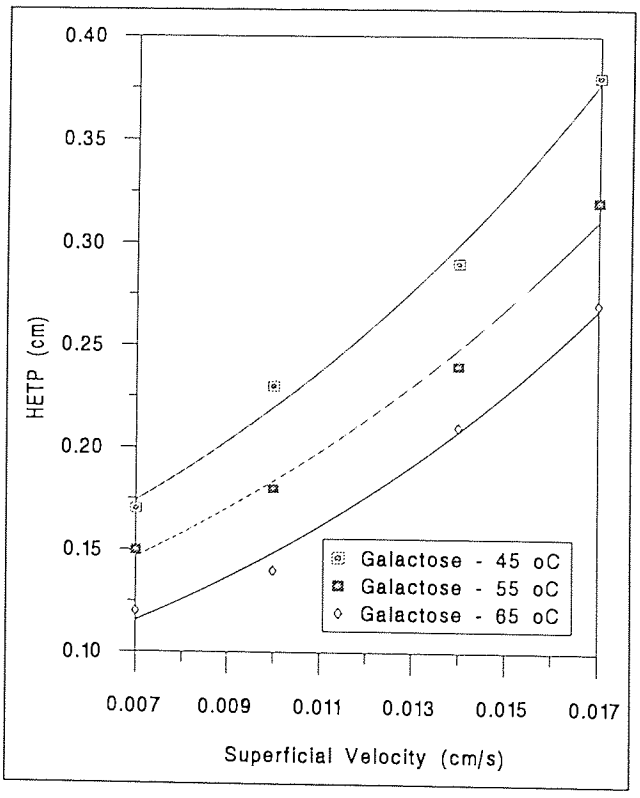


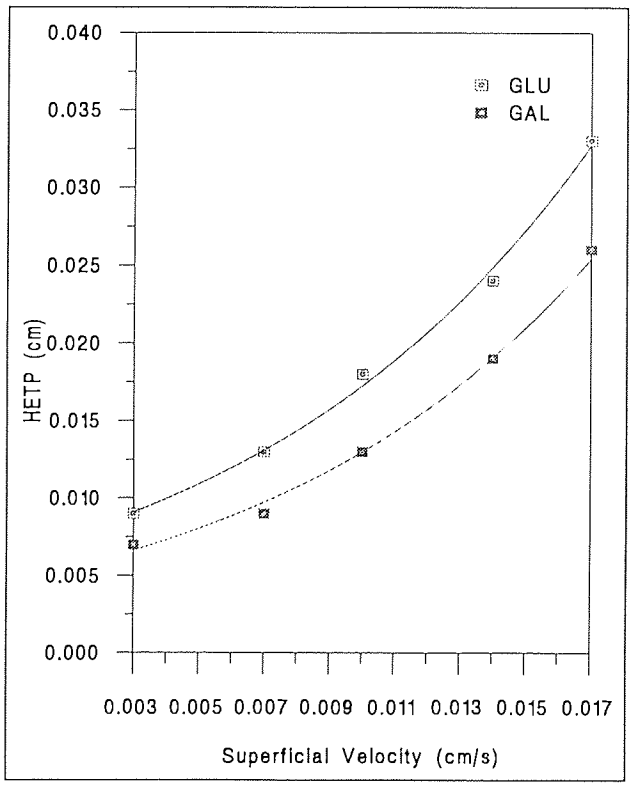
Figure 4.9 - Effect of temperature on HETP's for galactose on the cationic exchange resin Purolite PCR 833 in the calcium form



4.3.3.3 The Effect of Flowrate on the HETP's

The effect of the flowrate on the HETP's for the sugars glucose and galactose was studied on the analytical chromatographic column HPX-87C, manufactured by Bio-Rad UK Ltd., Waterford, containing a cation exchange resin in the calcium form (see section 3.3.2). The temperature selected for the set of experiments was 55 °C, the temperature required by the enzymes for the bioreactions, and the superficial velocities ranged from 0.03 - 0.017 cm/s. The results are presented in Figure 4.10 and they confirm that lower flowrates favour the separations, as the HETP decreases and consequently the number of theoretical plates of the column increases.

Figure 4.10 - Effect of flowrate on HETP's for glucose and galactose on the cationic exchange resin in the calcium form



4.3.3.4 The Effects of Background Concentration of Carbohydrates and Enzymes on the Distribution Coefficients

To study the effect of the background concentration of carbohydrates on the distribution coefficients, the following sets of experiments were carried out in the batch chromatographic system shown in Figure 4.6, using a jacketed glass column 1 m long x

0.9 cm I.D., packed with cation exchange resin Purolite PCR 833 in the calcium form, with the particle size in the range 190 - 210 μm:

- Effect of glucose background concentration on the K_d of fructose
- Effect of fructose background concentration on the K_d of glucose
- Effect of glucose background concentration on the K_d of galactose
- Effect of galactose background concentration on the K_d of glucose.

The experimental method is explained elsewhere (89). The technique consisted of the determination of the K_d's of carbohydrates species, using carbohydrate solutions as eluent, made up from other species at different concentrations. The results were interpolated in order to obtain a linear correlation. Therefore, eluent solutions of glucose, fructose and galactose with concentrations of 10, 20, 30, 40 and 50 % w/v were carefully prepared, dissolving a pre calculated amount of the carbohydrate in deionised and degassed water at 80 °C to remove any dissolved gas and then cooled down to ambient temperature. 1 % w/v pulse solutions of the carbohydrates whose distribution coefficients were to be determined were made by dissolving 1 g of the sugar in the eluent already prepared. The first point of the curve refers to the distribution coefficient determined at infinite dilution and presented in Table 4.3.

The experiments were conducted at 55 °C and with the superficial velocity of 0.0193 cm/s. The eluent was continuously pumped into the column until the baseline of the recorder showed a steady state, that is, the concentration of the eluent leaving the column had the same concentration as the eluent entering the column, meaning that the void space of the column was completely occupied by the eluent solution. The water in the reference cell of the refractometer was then replaced by the eluent solution and a pulse of carbohydrate solution injected into the column. The distribution coefficients were calculated according to the formula established in section 2.1.3 and the technique described in section 4.3.1. The results are presented in Tables 4.15, 4.16, 4.17 and 4.18. The data were plotted and the resulting graphs are presented in Appendix A-2.

Table 4.15 - Distribution coefficients of fructose with background concentration of glucose

Background concentration (% w/v)	Distribution coefficient of fructose ($K_{d\ Fru}$)
0	0.47
10	0.48
20	0.49
30	0.51
40	0.52
50	0.53

Table 4.16 - Distribution coefficients of glucose with background concentration of fructose

Background concentration (% w/v)	Distribution coefficient of glucose ($K_{d\ Glu}$)
0	0.18
10	0.20
20	0.23
30	0.26
40	0.28
50	0.30

Table 4.17 - Distribution coefficients of galactose with background concentration of glucose

Background concentration (% w/v)	Distribution coefficient of galactose ($K_{d\ Gal}$)
0	0.30
10	0.31
20	0.33
30	0.34
40	0.35
50	0.36

Table 4.18 - Distribution coefficients of glucose with background concentration of galactose

Background concentration (% w/v)	Distribution coefficient of glucose ($K_{d\ Glu}$)
0	0.18
10	0.21
20	0.23
30	0.25
40	0.26
50	0.31

The interpolation of the data presented in Tables 4.15, 4.16, 4,17 and 4.18 was done by the computer software CA-Cricket Graph® (174) which uses the Stineman interpolation method. From the curves presented in Figures 4.11, 4.12, 4.13 and 4.14 the following equations were obtained, representing the effects of background concentrations for each case studied, according to Ganetsos (88,169).

Effect of the background concentration of glucose on the distribution coefficient of fructose ($K_{d\ Fru}$):

$$K_{d\ Fru} = 0.47 + 0.0012\ C_{glu} \qquad (4.7)$$

Effect of the background concentration of fructose on the distribution coefficient of glucose ($K_{d\ Glu}$):

$$K_{d\ Glu} = 0.18 + 0.0014\ C_{fru} \qquad (4.8)$$

Effect of the background concentration of glucose on the distribution coefficient of galactose ($K_{d\ Gal}$):

$$K_{d\ Gal} = 0.30 + 0.00119\ C_{glu} \qquad (4.9)$$

Effect of the background concentration of galactose on the distribution coefficient of glucose ($K_{d\ Glu}$):

$$K_{d\ Glu} = 0.18 + 0.00092\ C_{gal} \qquad (4.10)$$

where C_{glu}, C_{fru} and C_{gal} represent the background concentration of glucose, fructose and galactose, respectively, in % w/v.

The equations 4.7, 4.8, 4.9 and 4.10 will be used later in the mathematical modelling and simulation of the batch chromatographic bioreaction-separation systems studied in this work (Chapter 9).

4.3.4 Analysis of the Flow Conditions - Reynolds Number and Pressure Drop

4.3.4.1 Reynolds Number

The Reynolds Number for the bed may be calculated as follows (172):

$$N_{Re} = \frac{d_p \upsilon_f \rho}{\mu} \qquad (4.11)$$

where,

d_p	=	particle diameter	=	200 μm
υ_f	=	superficial velocity of fluid	=	0.02 cm/s
ρ	=	density	=	1 g/cm^3
μ	=	viscosity	=	0.01 g/cm.s

Therefore, $N_{Re} = 0.04$.

The value obtained is characteristic of laminar flow (172).

4.3.4.2 Pressure Drop

From the Kozeny-Carman equation (175), the pressure drop, Δp, through a packed bed with spherical particles, under laminar flow conditions and assuming no wall effects, may be written as:

$$\Delta p = \frac{180\,(1-\varepsilon)^2\,\mu\,L\,u}{\varepsilon^3\,(d_p)^2} \qquad (4.12)$$

where:

ε	= voidage	= 0.36
μ	= viscosity of fluid	= 0.001 Pa.s

L = length of the bed = 198 cm

u = superficial velocity of fluid = 0.02 cm/s

d_p = particle diameter = 0.02 cm

Therefore, Δp = 15188.68 Pa (11.39 cm Hg)

The pressure drop measured with the manometer **M**, Figure 4.1, reached the level of 25 cm Hg during the experimental work. The high value obtained could be explained by the resin particle size distribution, according to Table 4.2, and by the higher viscosities from the more concentrated solutions used both for the physical separations and for the bioreaction-separations.

4.4 Coding of Experiments and Treatment of Data

The separation runs were coded according to the key presented in Table 4.19.

Table 4.19 - Codes and keys for the experimental runs

Code ⇒	**S (chronological number)**	**Syn**	**5**	**10 G**	**10 F**	**3.5**
Key ⇒	separation	nature of the pulse	Pulse size	10 % w/v glucose	10 % w/v fructose	flowrate (cm^3/min)

The first field, S, standing for separation is followed by the chronological number related to the experiment. The nature of the pulse was coded either as enz or ac, standing for products obtained through enzymatic hydrolysis or acid hydrolysis respectively. Synthetic mixtures were made by mixing more than two carbohydrates together and the following materials were used: sucrose (S), glucose (G), fructose (F), lactose (L), galactose (Gal) and inulin (I). Pulse size means the volume of the solution that was injected into the column, which was a percentage of the total empty column volume, or % TECV. For instance, the run S(13)-Syn-5-10G-10F-3.5 was the thirteenth physical separation using a synthetic mixture of glucose and fructose. A 5 % TECV pulse was used and a eluent flowrate of 3.5 cm^3/min was used.

The evaluation of the performance of the chromatographic column performing either physical separations or bioreaction-separations will be based on the following parameters: quantification of the rich fractions, the throughputs of these rich fractions and the asymmetry of the bandwidth.

The quantification of the rich products and their throughputs was based on the data extracted from the material balances for the runs, based on the 'time cut' method for obtaining pure compounds (89,176); this considers three possible fractions or cuts: an "A" rich product, with 100 % purity, a "B" rich product also with 100 % purity and a mixture, for two products "A" and "B" eluting from a chromatographic column, with some degree of overlapping, as exemplified by Figure 4.11.

Chromatographic profiles can deviate from a gaussian shape and the peak asymmetry, defined as asymmetry factor, As, can be measured by the ratio of the two halves of the bandwidth measured at 10 % of the peak height (131, 177). According to the literature, chromatographic peaks with asymmetry factor from 0.9 to 1.2 can be considered symmetrical (131). The graphical determination of the asymmetry factor is shown in Figure 4.12.

Figure 4.11 - Product fractions

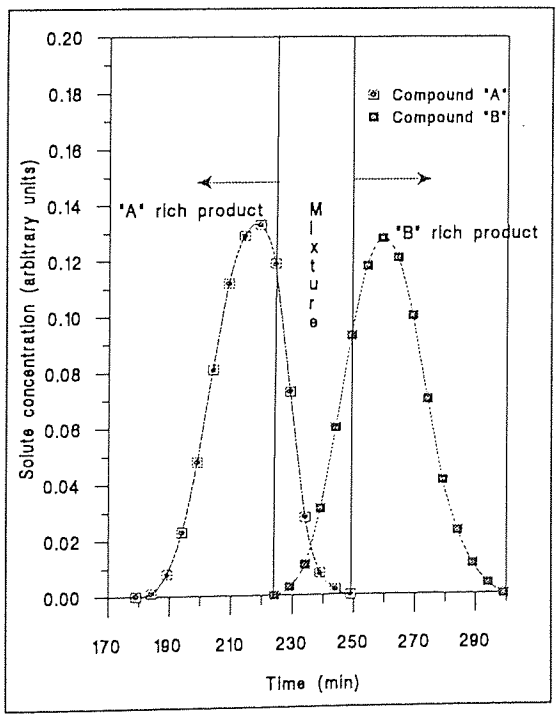
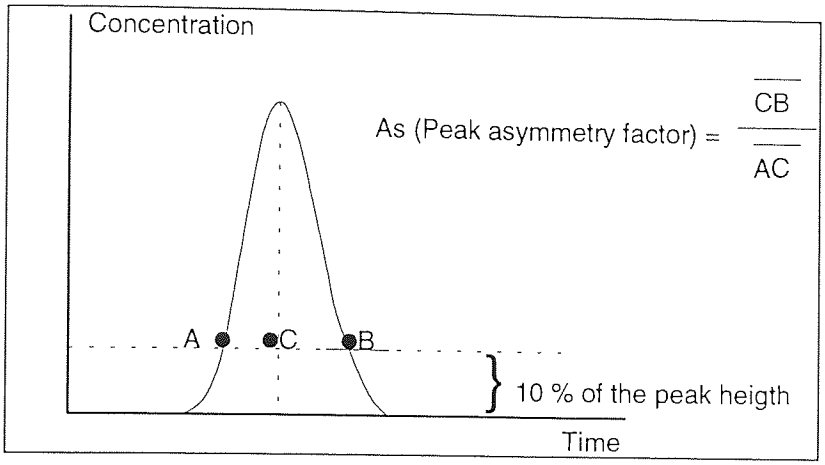


Figure 4.12 - Peak asymmetry factor



4.5 The Physical Separations of Carbohydrates Using the Glass Column 200 x 1.96 cm I.D.

4.5.1 Introduction

To investigate further the separation capacity of the chromatographic column to be used as bioreactor-separator, synthetic mixtures of the carbohydrates involved in the bioreaction-separations and products obtained either from the acid or the enzymatic hydrolysis of carbohydrates, as explained in section 3.2.2, were submitted to a chromatographic separation under flowrate conditions of 3.5 and 1.4 cm^3/min, corresponding to linear velocities of 1.16 cm/min and 0.53 cm/min, respectively. The temperature selected for the runs was 55 ^oC.

The objective of the experiments was to achieve the limit of the separation capacity of the column regarding the recovery of pure products.

The following data were used for the evaluation of the response of the system: percentage recovery of rich fractions or rich products (with 100 % purity) in relation to the amount injected as feed, the throughput of these pure products in g/h, the concentration of the fractions and some chromatographic figures of merit (178-180) such as the peak asymmetry factor and peak concentration.

4.5.2 Physical Separation of Sucrose, Glucose and Fructose with Calcium as Counter-ion

4.5.2.1 Synthetic Mixtures of Sucrose, Glucose and Fructose

Synthetic mixtures of sucrose, anhydrous glucose and analytical grade fructose were chromatographed using a jacketed glass column 200 x 1.96 I.D. packed with the cationic resin Purolite PCR-833 in the Ca^{2+} (BCBS-I). The results are presented in Tables 4.20 and 4.21. The elution profiles for sucrose, glucose and fructose are presented in Figure 4.13 and for glucose and fructose in Figure 4.14. The temperature used for the separations was 55 °C.

Table 4.20 - Separation of sucrose, glucose and fructose

Runs	Resolution	
	Sucrose/Glucose R_{S-G}	Glucose/Fructose R_{G-F}
S(13)-Syn-5-10S-10G-10F-3.5	1.12	0.95
S(14)-Syn-10-10S-10G-10F-3.5	0.88	0.71
S(15)-Syn-20-10S-10G-10F-3.5	0.77	0.45

The results presented in Table 4.21 indicated that working with the same pulse concentration, the increase of pulse size tended to increase the throughput. Nevertheless the resolution decreased and the recovery of rich fractions tended also to decrease. In decreasing the flowrate the number of theoretical plates increased and, as consequence, the resolution improved. For this condition, although the recovery of pure products tended to be higher, the throughput decreased considerably.

The asymmetry factor for the elution profiles of sucrose, glucose and fructose depended on the amount, % of TECV, and the concentration of the product injected, which means that mild conditions favour the formation of symmetrical peaks. In this case, the degree of overlapping is reduced and the separation and recovery of the products enhanced. The results from the experimental work confirmed that working under relatively mild conditions of pulse size and concentration, which kept the adsorption isotherm within the limits of the linear to slightly concave shape, the sucrose peaks were quite symmetrical.

The asymmetry factor for sucrose profiles ranged from 1.0 to 1.05 and the asymmetry factors for the glucose profiles ranged from 1.05 to 1.1, showing that the peaks were almost

symmetrical. The fructose and galactose profiles were quite symmetrical with their asymmetry factors ranging from 1.1 to 1.15.

The values for the asymmetry factor above 1.0 are characteristic of tailing peaks, which could be caused by the relatively large particle size of the stationary phase and by the convex shape of the adsorption isotherm, common in liquid chromatography (131).

Figure 4.13- Elution profiles for run S(01)-Syn-5-10S-10G-10F-3.5

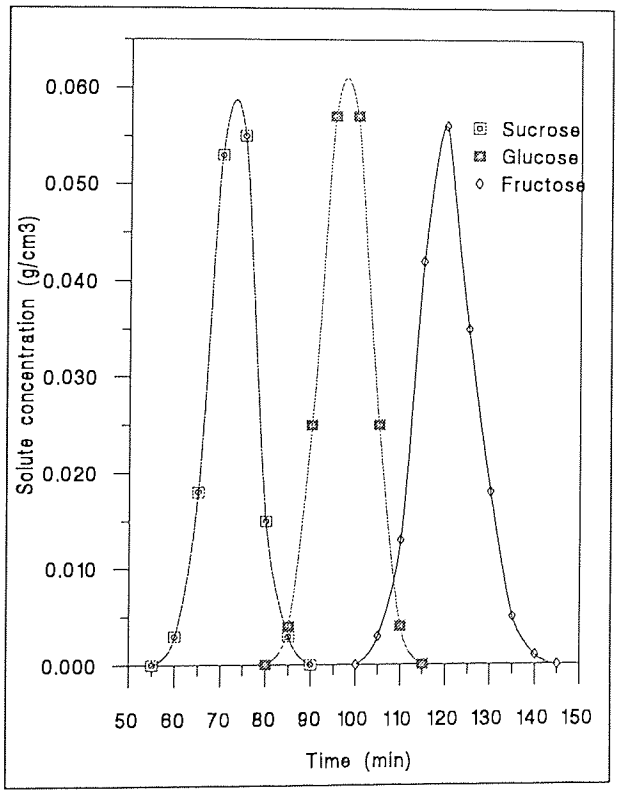


Figure 4.14 - Elution profiles for run S(14)-Syn-10-10G-10F-3.5

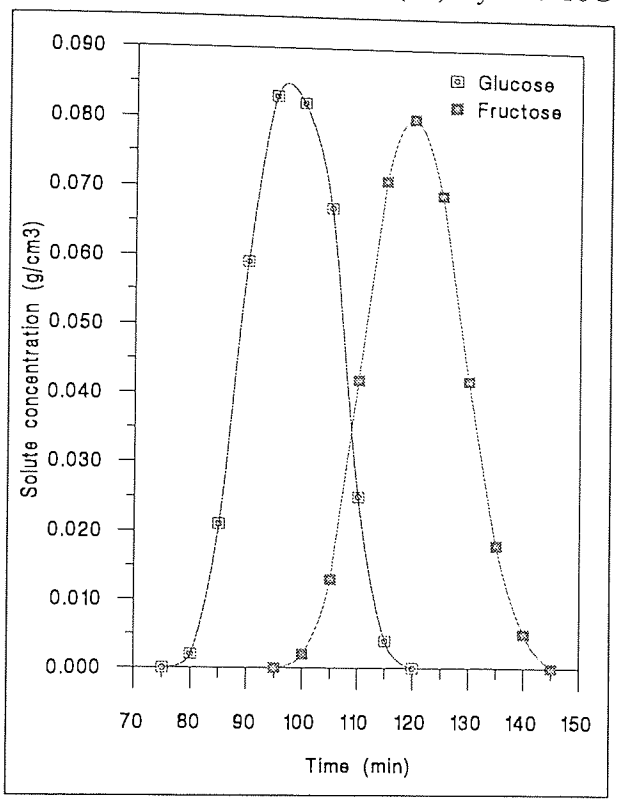


Table 4.21 - Separation of Glucose and Fructose

Runs	R_{GF}	Glucose Rich Fraction				Fructose Rich Fraction			
		% Glucose recovered	Through-put g/h	Peak conc. % w/v	Conc. of fraction % w/v	% Fructose recovered	Through-put g/h	Peak conc. % w/v	Conc. of fraction % w/v
S(13)-Syn-5-10G-10F-3.5	0.95	83.3	7.8	6.2	3.6	90.0	5.4	4.2	2.4
S(14)-Syn-10-10G-10F-3.5	0.71	48.3	8.4	8.5	4.1	61.7	9.0	8.0	4.2
S(15)-Syn-20-10G-10F-3.5	0.46	27.5	10.2	11.0	4.7	39.2	14.4	10.0	6.7
S(06)-Syn-5-20G-20F-3.5	0.75	33.3	7.8	9.0	11.4	31.7	4.2	7.5	5.7
S(17)-Syn-10-20G-20F-3.5	0.63	40.0	11.4	14.5	5.5	33.3	9.6	13.0	4.6
S(16)-Syn-20-20G-20F-3.5	0.53	25.0	18.0	19.8	8.6	23.8	17.4	18.5	8.1
S(04)-Syn-5-30G-30F-3.5	0.50	33.3	12.0	10.5	5.7	12.2	4.2	8.5	2.1
S(34)-Syn-10-30G-30F-3.5	0.45	26.0	18.6	16.0	9.0	18.3	10.2	14.0	4.7
S(35)-Syn-20-30G-30F-3.5	0.37	10.0	21.1	28.0	10.3	9.7	10.8	25	5.0
S(38)-Syn-5-10G-10F-1.4	0.95	88.0	5.9	5.4	8.7	85.0	6.9	4.1	2.3
S(36)-Syn-5-20G-20F-1.4	0.90	37.7	7.7	7.1	9.2	37.3	7.1	6.3	3.5
S(37)-Syn-5-30G-30F-1.4	0.95	36.3	10.1	8.7	12.0	33.3	7.5	8.7	4.9

4.5.2.2 Mixture of Glucose and Fructose Obtained by Acid Hydrolysis

Sucrose was hydrolysed by hydrochloric acid as described in section 3.3.2.1. The elution profiles for the reaction products are presented in Figure 4.15. It confirmed that sucrose was completely hydrolysed, as there was no indication of a sucrose peak. The formation of by-products was not detected either. The HPLC analysis for the acid hydrolysis reaction product, according to the method described in section 3.5.1, did not show any extra peaks and the glucose and fructose peaks corresponded to the standards D-glucose and β-D (-) fructose. The analysis of the fractions collected during the elution of the products also confirmed that each individual component was formed by a single carbohydrate, D-glucose and β-D (-) fructose, respectively.

4.5.2.3 Mixture of Glucose and Fructose Obtained by Enzymatic Hydrolysis

Sucrose was hydrolysed by the enzyme invertase (Bioinvert NL) as described in section 3.3.2.2. The elution profiles for the reaction products are presented in Figure 4.16. It shows that sucrose was almost completely hydrolysed and small amounts of by-products were detected. The material balance for the run is presented in Table 4.22. The HPLC analysis for the enzymatic reaction product, according to the method described in section 3.5.1, confirmed that the glucose and fructose peaks corresponded to the standards D-glucose and β-D (-) fructose. The analysis of the fractions collected during the elution of the two products, after the elution of sucrose and the oligosaccharide, also confirmed that each individual component was formed by a single carbohydrate, D-glucose and β-D (-) fructose, respectively.

Table 4.22 - Material balance for run S(10)-Enz-5-20G-20F-3.5

Product	Mass (g)	Composition (w/w)
Oligosaccharide	0.13	0.93
Sucrose	0.22	1.57
Glucose	6.96	49.61
Fructose	6.72	47.90

Figure 4.15 - Elution profiles for glucose and fructose obtained by acid hydrolysis
Run S(02)-Ac-20-10G-10F-3.5

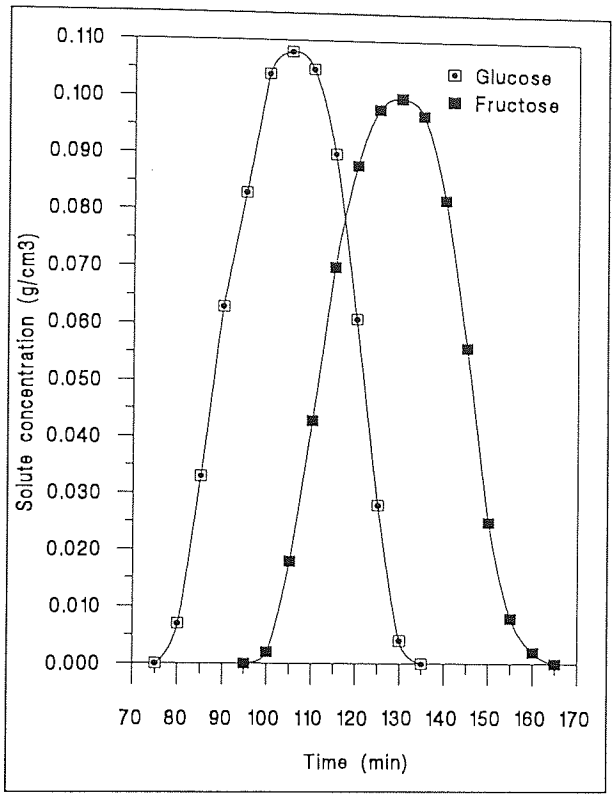
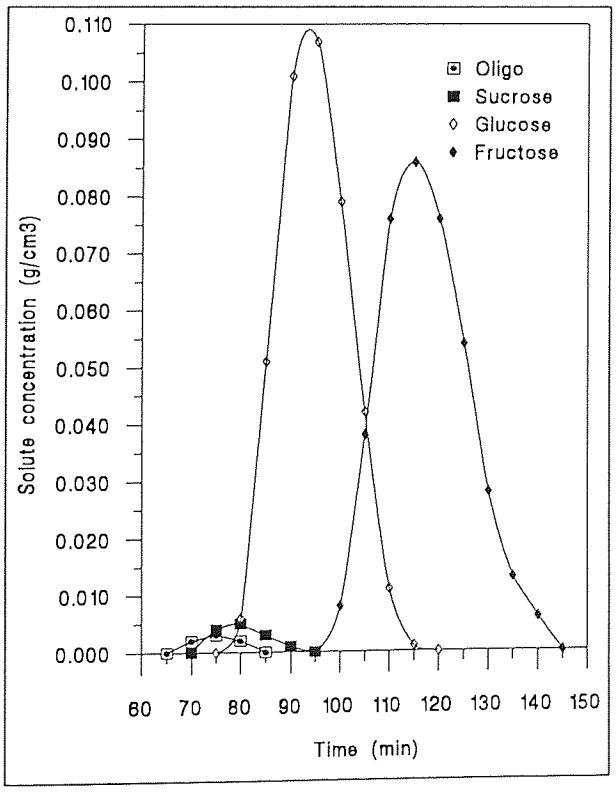


Figure 4.16 - Elution profiles for glucose and fructose obtained by enzymatic hydrolysis
Run S(10)-Enz-5-20G-20F-3.5



4.5.3 Physical Separation of Lactose, Glucose and Galactose with Calcium as Counter-ion

4.5.3.1 Synthetic Mixture of Lactose , Glucose and Galactose

Synthetic mixtures of lactose analar, anhydrous glucose and galactose were chromatographed using a jacketed glass column 200 x 1.96 I.D. packed with the cationic resin Purolite PCR-833 in the Ca^{2+} form (BCBS-I). The results are presented in Table 4.23. The elution profiles for the physical separation of those carbohydrates are presented in Figure 4.17. The temperature used for the separations was 55 ^{o}C.

The asymmetry factors (see section 4.4) for lactose and galactose were almost identical to the values found for sucrose and fructose, 1.0-1.15, showing that the profiles were quite symmetrical.

4.5.4 Physical Separation of Glucose and Fructose with Magnesium as Counter-ion

4.5.4.1 Synthetic Mixture of Glucose and Fructose

Synthetic mixtures of glucose anhydrous and fructose analytical grade were chromatographed using a jacketed glass column 200 x 1.96 I.D. packed with the cationic resin Purolite PCR-833 in the Mg^{2+} form (BCBS-II). The results are presented in Table 4.24 and the elution profiles are shown in Figure 4.18. The flowrate selected for the separations was 1.4 cm^3/min, corresponding to a linear velocity of 1.16 cm/min and the temperatures 55 ^{o}C and 65 ^{o}C. The asymmetry factor (see section 4.4), was always in the range 0.8 - 0.9 for the glucose peak, characteristic of a reasonable symmetrical profile. The asymmetry factor for the fructose profiles were in the range 0.6 - 0.8, denoting very asymmetrical peaks, probably due to a concave shape of the adsorption isotherm.

The fructose peaks when conducting physical separations from glucose on the cation exchange resin Purolite PCR 833 with magnesium as counter-ions presented a negative skew towards the glucose peak. This strong asymmetry could be explained by the much lower affinity of the magnesium ions for the fructose molecule, compared with calcium ions, based on the ligand exchange mechanism theory presented in section 4.2.2.3.

Table 4.23 - Separation of lactose, glucose and galactose

Runs	Resolution	
	Lactose/Glucose R_{L-G}	Glucose/Galactose R_{G-Gal}
S(21)-Syn-2-5L-5G-5Gal-3.5	0.50	0.30
S(22)-Syn-4-5L-5G-5Gal-3.5	0.38	0.22
S(23)-Syn-6-5L-5G-5Gal-3.5	0.26	0.14

Figure 4.17 - Elution profiles for run S(21)-Syn-2-5L-5G-5Gal-3.5

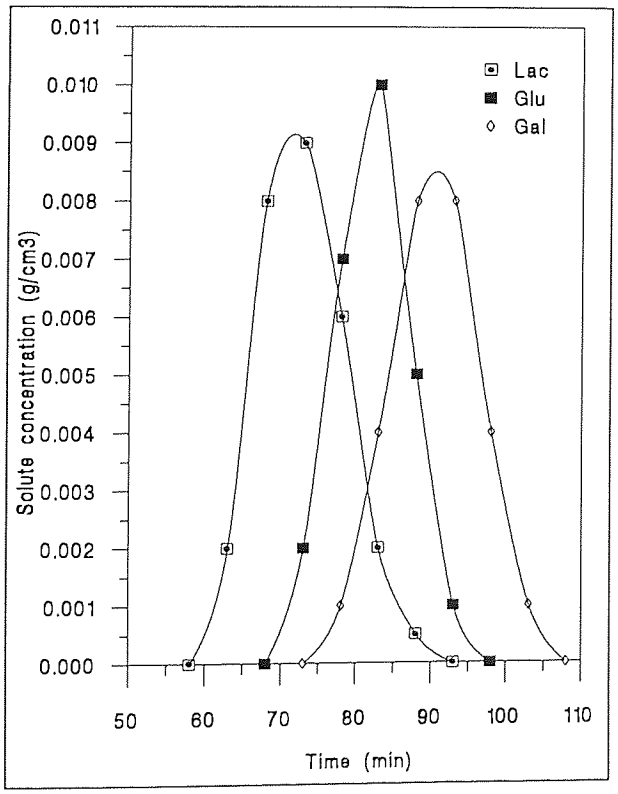
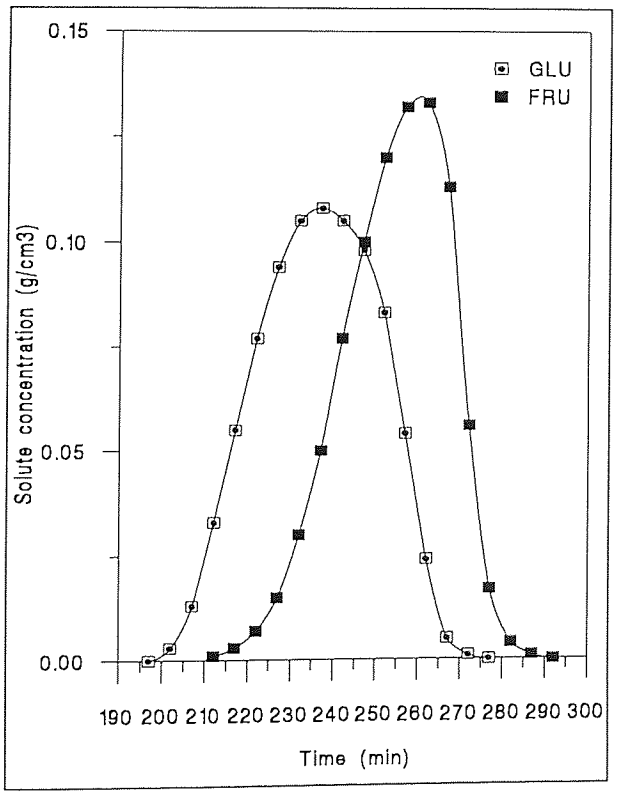


Table 4.24 - Separation of glucose and fructose using magnesium as counter-ions

Runs	Temperature ^{o}C	Resolution Fructose/Glucose R_{F-G}
S(38)-Syn-5-20G-20F-1.4	55	0.40
S(39)-Syn-5-20G-20F-1.4	65	0.36
S(40)-Syn-10-20G-20F-1.4	55	0.43
S(41)-Syn-10-20G-20F-1.4	65	0.39

Figure 4.18 - Elution profiles for glucose and fructose with magnesium as counter ions run S(38)-Syn-5-20G-20F-1.4



4.6 Kinetic Studies of the Bioreaction Systems Studied in this Thesis

4.6.1 Introduction

The four systems, three hydrolyses and one isomerization reaction, selected for this thesis were:

- sucrose hydrolysis by invertase
- inulin hydrolysis by inulinase
- lactose hydrolysis by lactase
- glucose isomerization by glucose-isomerase.

Kinetic studies of these systems, involving batch and continuous reactors and employing free and immobilised enzymes related to the species used in this research, have been extensively covered by many authors (69,114,115,117,119,121,155,181-192). Nevertheless, different substrates and enzymes were used in the experimental work of this research, which required different conditions of temperature and pH for the bioreactions. To evaluate the effect of these parameters on the extent of the reactions, combined with the effect of substrate and enzyme concentrations, batch kinetic studies was carried out in the equipment shown in Figure 3.4 and based on the initial rate procedure as presented in section 3.8.2. The objective of the studies was the determination of the kinetic parameters of the enzymatic reactions involved in this project, which will be used later in the mathematical modelling and simulation of the batch chromatographic bioreaction-separation process (see chapter 9).

4.6.2 Sucrose Hydrolysis by Invertase

The first system investigated was the hydrolysis of sucrose to a mixture of glucose and fructose by means of the enzyme invertase, produced from a strain of *S. cerevisiæ* and supplied by Biocon Biochemicals Ltd. with the commercial name of Bioinvert.

The data obtained for the initial reaction rate for the hydrolysis of sucrose by invertase Bioinvert, carried out at different substrate concentrations, at 55 ^{o}C and for an enzyme activity level of 60 U/cm^3, are plotted in Figure 4.19. The data showed that the maximum rate of reaction occurs approximately at a sucrose concentration of aprox. 0.1 g/cm^3. The kinetic constants V_{max}, the maximum reaction velocity, and K_M, the Michaelis-Menten constant, were calculated according to section 3.8.3 and are presented in Table 4.25. They

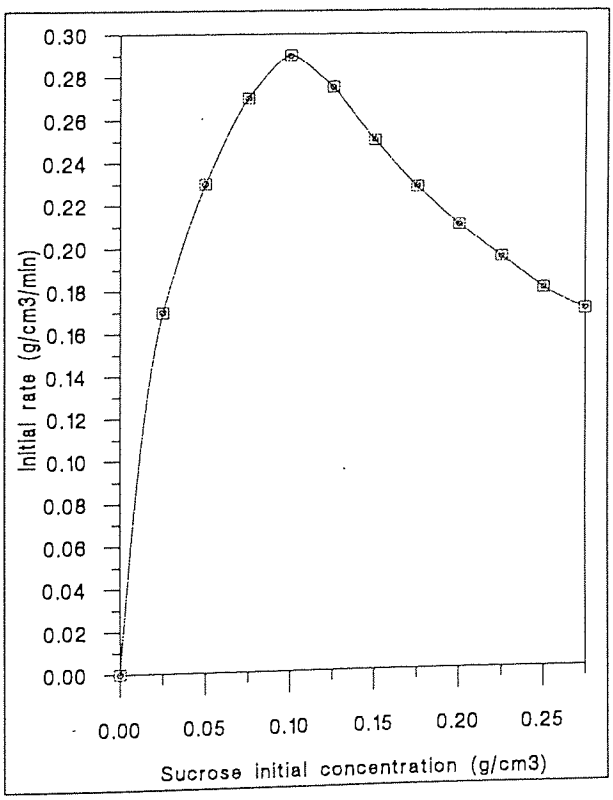
are compatible with the values found by Akintoye (4) and Sarmidi (5) and confirmed the work of Bowski *et al.* (115) on the modelling of the sucrose hydrolysis by invertase. Nevertheless, the Michaelis-Menten model does not fit the experimental data as also observed by Bowski *et al.* (115), who explained the deviation from the Michaelis-Menten model in terms of the combined effect of substrate inhibition and water concentration, as discussed in section 2.3.5.1. The value for the substrate inhibition constant, K_i, was calculated according to equation 2.20 (section 2.3.3) and is also presented in Table 4.25.

The investigation of the effect of the water concentration was not an object of this research. The slight discrepancy in the value found for K_M, as also observed by Akintoye (4), might be due to this type of inhibition.

Table 4.25 - Kinetic constants for the hydrolysis of sucrose by invertase Bioinvert at 55 °C and pH 4.7

Parameter	**V_{max} ($g/cm^3/min$)**	**K_M (g/cm^3)**	**K_i (g/cm^3)**
Value	0.26	0.02	0.47

Figure 4.19 - Initial rate of hydrolysis of sucrose by invertase vs. sucrose concentration



4.6.3 Lactose Hydrolysis by Lactase

The second system investigated was the hydrolysis of lactose to a mixture of glucose and galactose by means of the enzyme lactase, produced from a strain of *A. oryzæ* and supplied by Biocon Biochemicals Ltd. with the commercial name of Biolactase F.

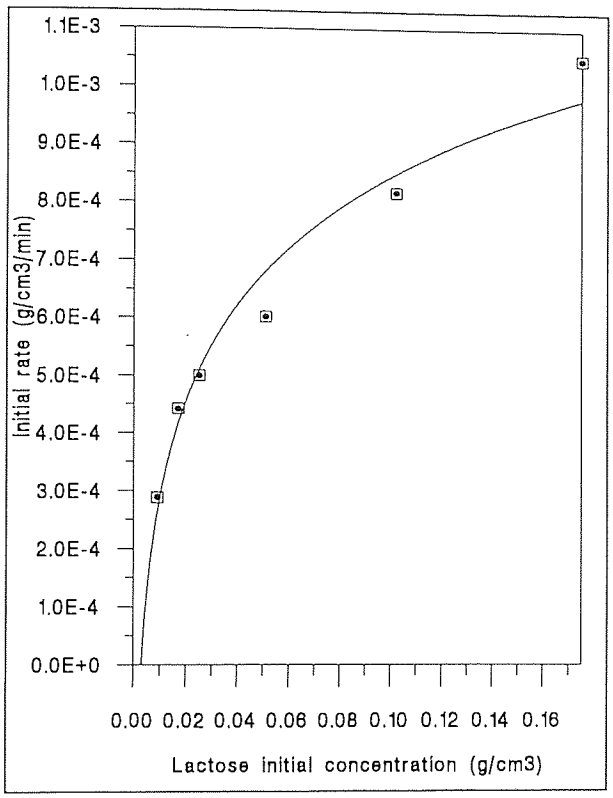
The batch hydrolysis of lactose by lactase Biolactase F was done at low substrate and enzyme concentrations, to overcome the strong competitive product inhibition as mentioned by Flaschel *et al.* (186). The experimental data of the initial reaction rate vs. lactose initial concentration are plotted in Figure 4.20. The work was carried out at 55 °C at an enzyme activity level of 40 U/cm^3.

The kinetic constants V_{max}, the maximum reaction velocity, K_M, the Michaelis-Menten constant were calculated according to section 3.7.3. The inhibition constant, k_i, was obtained from the literature (186). The values found, presented in Table 4.26, are very close to those obtained by Friend and Shainni (120) and by Shieh (6).

Table 4.26 - Kinetic constants for the hydrolysis of lactose by lactase Biolactase F at 55 °C and pH 4.7

Parameter	**V_{max} (g/cm^3/min)**	**K_M (g/cm^3)**	**K_i (g/cm^3)**
Value	5.8×10^{-3}	0.05	1.7×10^{-3}

Figure 4.20 - Initial rate of hydrolysis of lactose by lactase vs. lactose concentration



4.6.4 Inulin Hydrolysis by Inulinase

The third system investigated was the hydrolysis of inulin to a mixture of glucose and fructose by means of the enzyme inulinase, produced from a strain of Aspergillus niger and supplied by Novo Nordisk with the commercial name of Novozym 230.

The data obtained for the initial reaction rate for the hydrolysis of inulin by inulinase Novozym 230, at different substrate concentrations, are plotted in Figure 4.21. The work was carried out at 55 ^{o}C at an enzyme activity level of 1 U/cm^3. The results obtained were compatible with the work of Carniti *et al.* (119). The inulin hydrolysis by inulinase is not susceptible to any inhibition, either by substrate or product (119).

The values for V_{max} and K_M were calculated according to section 3.8.3 and are presented in Table 4.27.

Figure 4.21 - Initial rate of hydrolysis of inulin by inulinase vs. inulin concentration

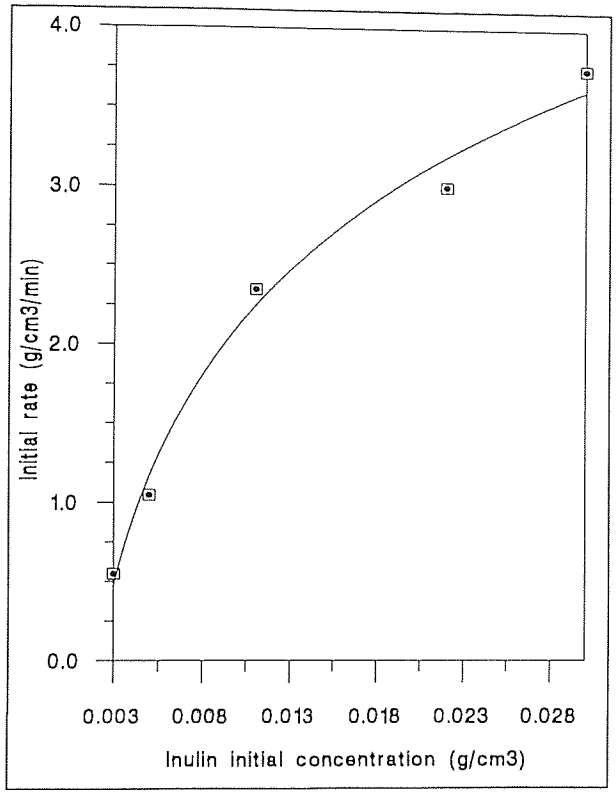


Table 4.27 - Kinetic constants for the hydrolysis of inulin by inulinase Novozym 230 at 55 °C and pH 4.7

Parameter	V_{max} ($g/cm^3/min$)	K_M (g/cm^3)
Value	0.26	0.02

4.6.5 Glucose Isomerization by Glucose-isomerase

The fourth system investigated was the isomerization of glucose to a mixture of glucose and fructose by means of the enzyme glucose-isomerase, produced from a strain of *A. missouriensis* and supplied by International Bio-Synthetics with the commercial name of Maxazyme GI-Liquid. This enzyme has been specially developed for the continuous production of high fructose syrups using regenerable support anion exchange resins,

although it can also be used in the liquid form, as free enzyme, in batch operation. As an immobilised catalyst the recommended working temperature ranges from 53 °C to 70 °C. Higher temperatures favour the displacement of the conversion towards fructose formation. The reaction is usually done at lower levels to avoid the instability of the enzyme at higher temperatures and its consequent formation of by-products. On the other hand, the work at lower temperatures also promotes microbial infection, which needs to be avoided.

The manufacturers of glucose-isomerase suggested the removal of oxygen from the glucose feed which acts as a catalyst for by-product formation and the addition of 2-5 μmol/l of magnesium as activator and stabiliser for the enzyme. Both suggestions were implemented during the experimental work. The glucose solutions were kept at 80 °C before the reactions and a 5 μmol/l solution of magnesium heptahydrate was mixed with the eluent, the diluted enzyme solution.

The work was carried out at 55 °C for an enzyme activity level of 2.7 U/cm^3 and the results obtained were compatible with the work developed by Ryu and Chung (188). The glucose isomerization, both the forward and the reverse reactions, by glucose-isomerase are not susceptible to any inhibition, either by substrate or product (119).

The data obtained for the initial reaction rate for the forward reaction, the isomerization of glucose to fructose and the reverse reaction, the isomerization of fructose to glucose, both by the enzyme Maxazyme, at different substrate concentrations, are plotted in Figures 4.22 and 4.23.

The Michaelis-Menten constant, K_M, determined by the procedure presented in section 3.8.3 and presented in Table 4.28, show that the affinity of the glucose to glucose-isomerase is almost the same as the fructose to the enzyme. The value for the maximum reaction rate for the forward reaction is close to the value found for the reverse reaction, as shown in Table 4.28. The opposing influences between the forward and reverse reaction result in an equilibrium with the equilibrium constant close to 1.0 (188).

Table 4.28 - Kinetic constants for the isomerization of glucose by glucose-isomerase at 55 °C and pH 7.5

	Forward reaction		Reverse reaction	
Parameter	V^S_{max} (g/cm^3/min)	K^S_M (g/cm^3)	V^P_{max} (g/cm^3/min)	K^P_M (g/cm^3)
Value	0.0132	0.26	0.0144	0.28

Figure 4.22 - Initial rate of isomerization of glucose to fructose by glucose-isomerase (forward reaction) vs. glucose concentration

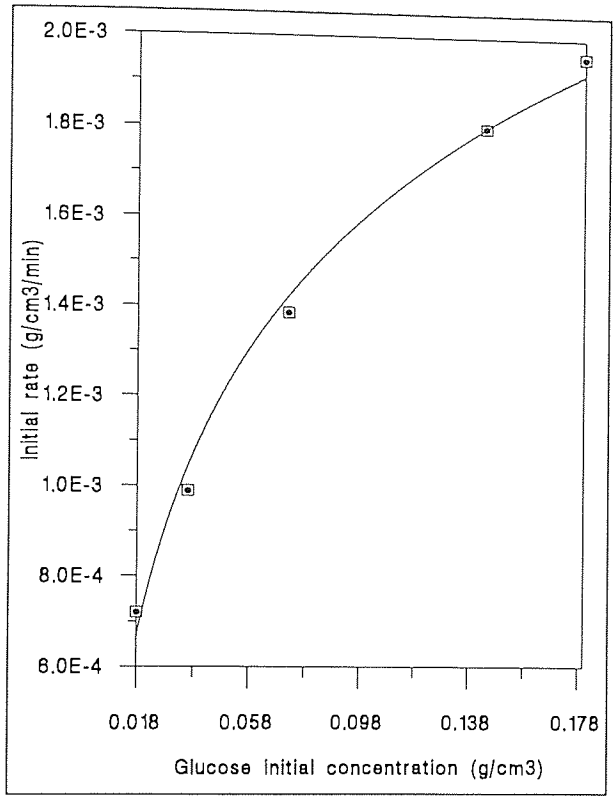
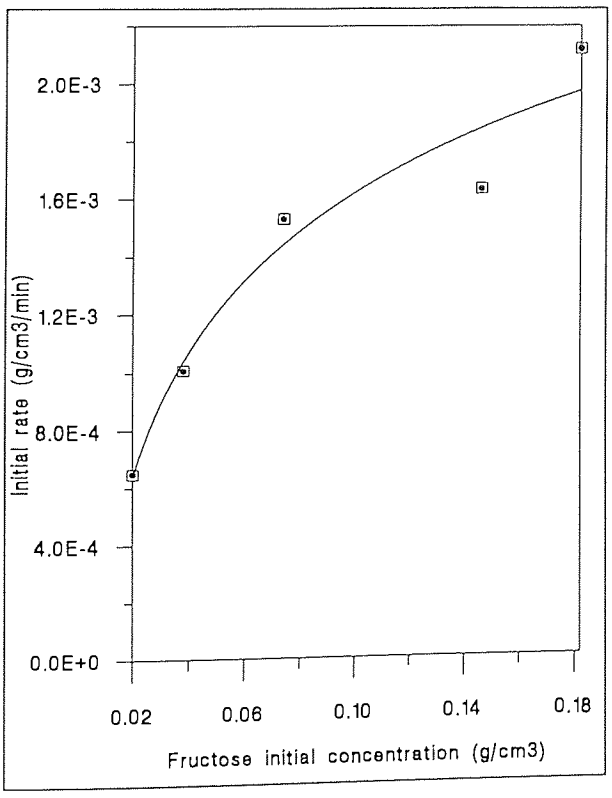


Figure 4.23 - Initial rate of isomerization of fructose to glucose by glucose-isomerase (reverse reaction) vs. fructose concentration



The kinetic constants were verified by comparing the value of the theoretical equilibrium constant with the value determined experimentally, according the following procedure.

The theoretical equilibrium constant is given by equation 2.22 (see section 2.3.3) as shown below.

$$\frac{[P]_{eq}}{[S]_{eq}} = \frac{V^{S}_{max}\, K^{P}_{M}}{V^{P}_{max}\, K^{S}_{M}} = K_{eq} \qquad (2.22)$$

By substituting the kinetic constants presented in Table 4.28 into equation 2.13,

$$K_{eq} = \frac{(0.0132)\ .\ (0.28)}{(0.0144)\ .\ (0.26)} = 0.9872 \qquad (4.13)$$

This result suggests that the maximum theoretical conversion of glucose to fructose attainable would be about 49 %, since at equilibrium,

$$\frac{\text{fraction of product}}{\text{fraction of residual substrate}} = \frac{P}{S} = \frac{P}{1 - P} = 0.9872 \qquad (4.14)$$

Thus, $P = 0.4968$ (or 49.68 %)

The maximum conversion attainable, was determined experimentally by the following procedure. The reactions performed for the determination of the initial rates were left to react until the isomerization of glucose to fructose reached a steady state, meaning that the conversion reached its maximum conversion. The experimental value found for the conversion was 49.96%.

The kinetic constants were also determined at 65 °C and the values found are presented in Table 4.29. The value for the equilibrium constant, K_{eq}, and for the fraction of product, P, were 1.0422 and 51.03, respectively. The equilibrium concentration of the product, or the maximum conversion attainable, was confirmed experimentally; the value found was 51.13 %.

Table 4.29 - Kinetic constants for the isomerization of glucose by glucose-isomerase at 65 °C and pH 7.5

	Forward reaction		Reverse reaction	
Parameter	V^{S}_{max} (g/cm³/min)	K^{S}_{M} (g/cm³)	V^{P}_{max} (g/cm³/min)	K^{P}_{M} (g/cm³)
Value	0.0208	0.22	0.0254	0.28

CHAPTER FIVE

BATCH CHROMATOGRAPHIC BIO-REACTION SEPARATION USING THE SYSTEM SUCROSE-INVERTASE

5.1 Introduction

To understand the performance of the chromatographic column operating under concerted conditions of reaction and separation, a well known enzymatic reaction - the hydrolysis of sucrose by the enzyme invertase to a mixture of glucose and fructose - was studied in the system BCBS - I (see section 4.3.2.2). The enzyme invertase was produced from a strain of *S. cerevisiæ* supplied by Biocon. This selected bioreaction has already been studied in the SCCR-1 (Semi-Continuous Chromatographic Reactor) by Akintoye (4) and in the CRAC (Continuous Rotating Annular Chromatograph) by Sarmidi (5). The results of the experiments on the batch system are presented in this chapter and the following parameters were considered in the experimental work: pulse size, pulse concentration, enzyme activity and eluent flowrate. The objective of the experiments was to study the behaviour of the BCBS under simultaneous bioreaction-separation conditions and to identify the more important parameters affecting its performance.

The mathematical modelling of the BCBS performing the enzymatic inversion of sucrose will be presented in chapter 9.

5.2 Exploratory Experiments

Exploratory experiments were carried out with the objective of establishing the limits of feed pulse size and concentration which would make the separation of the products obtained impractical, due either to the overloaded condition of the column or to the low conversion of substrate caused by an insufficient enzyme activity. The conversion of substrate was calculated according to equation 5.1. As a starting point, the conditions employed were the same as those used by Akintoye (4), who observed that on the SCCR-S system the optimum level of invertase activity was 60 U/cm^3, working with substrate concentrations in the range 25 - 55 % w/v. For this set of experiments the eluent flowrate was maintained constant, at 3.5 cm^3/min, corresponding to a superficial velocity of 1.16 cm/min. The eluent and the feed solutions were prepared as described in sections 3.3.1 part 2 and 3.3.2, respectively.

The evaluation and characterisation of the elution profiles were performed by using chromatographic figures of merit (180) such as peak concentration and asymmetry factor,

although the shape of the peaks and the conversion of substrate were taken into account as well.

5.3 Conversion of Substrate

The conversion of substrate in the BCBS, considering that the hydrolysis reaction is a complete reaction, was calculated according to the following equation:

$$\% \text{ Conversion} = \left(1 - \frac{m_{suc}(res)}{m_{suc}(inj)}\right) \text{ x } 100 \qquad (5.1)$$

where,

m_{suc} (res) = residual mass of sucrose = $\sum_{i=1}^{n} C_i \cdot \Delta t_{samp} \cdot Q_e$

m_{suc} (inj) = mass of sucrose injected = [Pulse volume (cm^3) **x** Pulse concentration (g/cm^3)]

C_i = concentration of sample in g/cm^3

Δt_{samp} = sampling time interval in min

Q_e = eluent flowrate in cm^3/min

5.4 Codes and Keys for the Experimental Runs

The codes and keys for the experimental runs, presented in section 4.4, have been modified to incorporate some of the basic parameters of the batch chromatographic bioreaction-separations and are presented in Table 5.1.

Table 5.1 - Codes and keys for the bioreaction-separations

Code ⇒	**RS (Chronological number)**	**SUC**	**5**	**10**	**100**	**3.5**
Key ⇒	Reaction- separation	Substrate used	Pulse size % TECV	Pulse concentration % w/v	Enzyme Activity U/cm^3	Flowrate cm^3/min

The first field, RS, standing for reaction-separation, is followed by the chronological number related to the experiment. The substrate was coded SUC, standing for sucrose.

Pulse size means the volume of the substrate solution that was injected into the column, which was expressed as percentage of the total empty column volume or % TECV.

For instance, the run RS(06)-SUC-20-40-60-3.5 was the sixth reaction-separation experiment carried out using sucrose as raw material (inversion by the enzyme invertase). A 20 % pulse of a 40 % w/v solution was injected into the reactor-separator and the reaction was performed at an enzyme activity of 60 U/cm^3 flowing at 3.5 cm^3/min.

5.5 General Results of the Preliminary Experiments

Two levels of enzyme activity were used in the preliminary set of experiments: 60 U/cm^3 and 80 U/cm^3. The results presented in Table 5.2 showed that the reactions were not complete as unreacted sucrose and oligosaccharides were detected by the HPLC analysis performed on the fractions collected at the column outlet. A typical elution profile from the preliminary set of experiments, the run RS(06)-SUC-20-40-60-3.5, is shown in Figure 5.1. This figure shows the unreacted sucrose peak, the peaks related to the oligosaccharides, codified as Oligo 1 and Oligo 2, and the peaks for glucose and fructose. The profiles for the remaining runs are presented in Appendix A-3.

Table 5.2 - Exploratory experiments for the Sucrose-Invertase system

Experimental run	Sucrose conversion %	Resolution (R_{1-2})	
		R_{S-G}	R_{G-F}
RS(05)-SUC-10-40-60-3.5	98.1	0.9	0.6
RS(06)-SUC-20-40-60-3.5	51.2	0.9	0.6
RS(02)-SUC-10-60-80-3.5	99.9	-	0.3
RS(03)-SUC-20-60-80-3.5	92.2	0.6	0.2

The following characteristics, similar to those found by Sarmidi working on the CRAC system (5), were observed when analysing the experimental profiles and the results presented in Table 5.2.

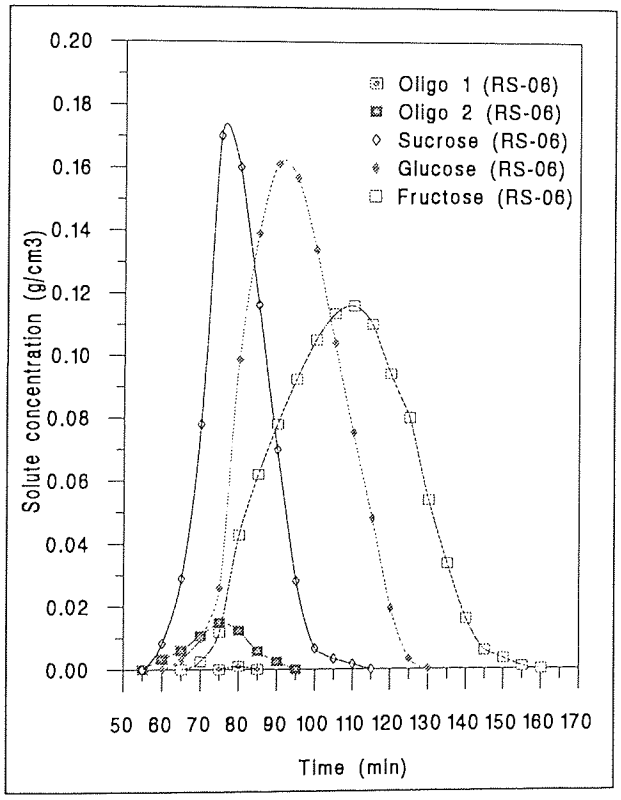
1. For the same level of enzyme activity the conversion of sucrose and the resolution between glucose and fructose decreased with an increase of the amount of substrate pulsed into the column.

2. Higher resolutions between glucose and fructose were observed for higher conversions of sucrose.

3. For lower conversions of sucrose the three products eluted, unreacted sucrose, glucose and fructose, presented overlapped peaks and, in the case of the recovery of pure products, only a small amount of fructose could be obtained as such.

4. The asymmetry factors found were in the range 0.9-1.0 for sucrose, 0.8-0.9 for glucose and 0.6-0.8 for fructose.

Figure 5.1 - Elution profile for run RS(06)-SUC-20-40-60-3.5



The simultaneous reaction-separation effects on the development of the profiles are well exemplified in the runs which presented unreacted sucrose peaks. The reaction rate of the hydrolysis of sucrose into glucose and fructose was low and occurred along the complete length of the column. The reaction products were formed at the front end of the sucrose peak and co-eluted under it. As a consequence, a 'tag-along effect' *i.e.* the spreading of a band by the band which is eluted just before it (99,193), occurred which contributed to the asymmetry factors for both the glucose and fructose profiles reaching very low values.

In the preliminary experimental runs where higher conversions were achieved, most of the reaction occurred in the upper part of the column and the remaining length of the reactor was used for the separation. The tag-along effect for those runs was less effective and the asymmetry factor for the reaction products was slightly higher, meaning that the profiles tended to be symmetric. Therefore, in order to obtain pure products, as a result of a more efficient separation or higher resolution, and also to avoid the tag-along effect, it is necessary, at least, to have a complete conversion of the substrate in the upper part of the column. This can be achieved by working at a higher enzyme activity level.

5.6 Analysis of the Results of the Preliminary Experiments Regarding the Behaviour of the System as a Batch Chromatographic Bioreactor-Separator

Although the preliminary runs were incomplete reactions, only the figures obtained from the material balance for fructose were analysed regarding the behaviour of the system as a BCBS. The following figures, tabulated in Table 5.3, were considered: percentage of recovery of rich fructose fraction (see section 4.4, Treatment of Data), the throughput of fructose in g/h, the concentration of the fraction and the peak concentration and the enzyme usage. This last figure was obtained according to the calculations presented in Appendix A-4. Enzyme usage referred to the theoretical amount of enzyme required to convert the same amount of substrate in a batch reactor over the same period of time under optimised standard conditions for the enzyme action.

Although the reactions were incomplete, the results from Table 5.3 showed that, for the same level of enzyme activity, the throughput of the fructose recovered with 100 % purity, increased with an increase of the pulse concentration. The same was true for the peak concentration of glucose, but to a lesser extent. Nevertheless, the fructose recovered, with 100 % purity, in relation to the fructose generated, decreased with the pulse size. The concentration of the fraction recovered also increased with an increase of pulse concentration. The performance of the system regarding the changes of the level of enzyme activity could not be fully elucidated as the feed conditions, pulse size and concentration were also changed.

On examination of the results presented in Tables 5.2 and 5.3 and the general shape of the profiles from the set of preliminary runs, to increase the productivity of the BCBS it is first necessary to complete the reaction. The second point of importance is the selection of the optimum conditions of pulse size and pulse concentration which will allow the best resolution of the mixture glucose and fructose and decrease tag-along effects. However, for the practical application of the technique, the final concentration for the pure fractions should be as high as possible.

Table 5.3 - Behaviour of the BCBS for incomplete reactions

Experimental Run	Fructose 100 % purity			Fructose peak conc.	Enzyme usage
	% recovery of total fructose generated	Through-put g/h	Conc. of fraction recovered % w/v	% w/v	% of theoretical
RS(05)-SUC-10-40-60-3.5	20.3	5.2	2.5	10.5	23.9
RS(06)-SUC-20-40-60-3.5	11.3	6.7	2.3	11.5	12.0
RS(02)-SUC-10-60-80-3.5	13.0	7.4	3.5	10.5	21.3
RS(03)-SUC-20-60-80-3.5	4.8	19.2	3.5	14.0	10.6

5.7 The Systematic Investigation of the Performance of the System as a Batch Chromatographic Bioreactor-Separator

5.7.1 Introduction

The experiments presented in this section was designed using the factorial experiment method outlined by Davies (194). The objective of the methodology applied was to determine the effects of a number of variables, or factors, and their interaction on the performance of the system working as a Batch Chromatographic Bioreactor-Separator. The factorial experiment minimises the amount of experimental work required to assess a given number of variables and was designed in such a way that the effect of changing any one variable could be assessed independently of the others. The term factor, used in this context, means any variable experimental parameter such as pulse size, for example.

Four factors, pulse size, pulse concentration, level of enzyme activity and eluent flowrate were studied. Two levels for each factor were selected, giving a total of 16 (= 2^4) sets of experimental conditions. Each set of experimental conditions is termed the treatment combination and the results of each treatment combination, such as resolution between the products, or pure product throughput, for instance, are called the response.

The effect of a factor is the change in response produced by a change in the level of the factor. When a factor is examined at two levels only, the effect is simply the difference between the average response values of all trials carried out at the first level of the factor and that of all trials at the second level. In the factorial experiments the variance between the trials carried out at different levels was required and was calculated in a similar way to the effect values. Variance is defined as the mean value of the squares of the deviations of a set of observations from their mean value. The variance ratio test (F-test) was used to evaluate statistically the mean value for significance (194,195).

5.7.2 Choice of Factors and Factor Levels

The factors chosen, denoted by a capital letter, were the same as used in the preliminary runs and the eluent flowrate was also included to broaden the range of the factorial experiment. The feed flowrate was kept the same as the eluent throughout the experimental procedure.

Two set of values, or levels, represented by a numbered subscript, were selected for each of the four factors to be studied. The chosen factor and factors levels were:

A (pulse size)	% TECV	$A_1 = 5$	$A_2 = 10$
B (pulse concentration)	% w/v	$B_1 = 40$	$B_2 = 60$
C (enzyme activity)	U	$C_1 = 100$	$C_2 = 150$
D (flowrate)	cm^3/min	$D_1 = 1.4$	$D_2 = 3.5$

The selection of the factor levels are also based on the preliminary runs which showed that pulse sizes of 20 % TECV for sucrose concentrations higher than 60 % w/v, which is the operational limit imposed by the viscosity of the sucrose solutions, generated overloaded conditions for the column, and made the separation of the products incomplete. The results from the preliminary runs also showed that the amount of enzyme used was crucial, as unconverted sucrose was found in some runs in the eluent collected from the column. Therefore, higher levels of enzyme activity were required for the factorial experiment.

Each treatment combination was carried out once and in a random order, to eliminate any underlying time dependent trends. All the possible treatment combinations and the order in which they were performed, with the number in brackets, are presented in Table 5.4.

Table 5.4 - The treatment combination in the experimental programme

Enzyme activity (U/cm^3)	Flowrate (cm^3/min)	Pulse size (% TECV) A			
		5		10	
		Pulse concentration (% w/v) B		Pulse concentration (% w/v) B	
C	D	40	60	40	60
100	1.4	$A_1B_1C_1D_1$ (5)	$A_1B_2C_1D_1$ (3)	$A_2B_1C_1D$ (14)	$A_2B_2C_1D_1$ (10)
	3.5	$A_1B_1C_1D_2$ (1)	$A_1B_2C_1D_2$ (7)	$A_2B_1C_1D_2$ (16)	$A_2B_2C_1D_1$ (12)
150	1.4	$A_1B_1C_2D_1$ (11)	$A_1B_2C_2D_1$ (15)	$A_2B_1C_2D_1$ (8)	$A_2B_2C_2D_1$ (4)
	3.5	$A_1B_1C_2D_2$ (9)	$A_1B_2C_2D_2$ (13)	$A_2B_1C_2D_2$ (2)	$A_2B_2C_2D_2$ (6)

5.7.3 Results and Statistical Analysis

There were seven responses of interest for each of the 16 treatment combinations and they are presented in Table 5.5. Response (1) is the resolution between glucose and fructose, R_{G-F}, responses (2) and (3) are the peak concentration of glucose, Pk_G, and fructose, Pk_F, responses (4) and (5) are the percentage of the products recovered with 100 % purity, G and F. There are other responses, such as rich fraction throughputs of glucose and fructose, T_G and T_F respectively, which were not analysed statistically since they were derived from responses (4) and (5).

The calculation of the variance or mean square values for each factor and each interaction was based on a systematic tabular method, the Yates's method, described by Davies (194) and reported in Appendix A-5.

Each treatment combination was only carried out once and no prior estimation of the experimental error was available. The error variance was estimated from the higher order interactions so that the significance of the variance values of each treatment combination

could be assessed. The estimate of error was tested by Bartlett's criterion (196) which is detailed in Appendix A-5.

Table 5.5 - Experimental results for the factorial experiment

Runs	Response						
	(1) R_{G-F}	(2) Pk_G % w/v	(3) Pk_F % w/v	(4) G %	(5) F %	(6) T_G g/h	(7) T_F g/h
$A_1B_1C_1D_1$	0.52	7.0	5.8	16.5	41.6	3.1	3.1
$A_2B_1C_1D_1$	0.52	12.1	8.8	7.2	25.9	1.4	3.9
$A_1B_2C_1D_1$	0.60	8.6	7.2	6.5	23.5	0.9	2.7
$A_2B_2C_1D_1$	0.48	12.1	9.2	4.9	13.3	1.4	2.4
$A_1B_1C_2D_1$	0.55	8.3	7.0	33.0	42.8	3.1	3.2
$A_2B_1C_2D_1$	0.44	12.8	10.9	20.1	31.1	3.8	3.9
$A_1B_2C_2D_1$	0.45	10.8	9.0	21.0	42.3	4.0	3.4
$A_2B_2C_2D_1$	0.40	15.0	12.5	8.7	17.8	3.2	2.8
$A_1B_1C_1D_2$	0.41	8.0	6.3	6.2	35.7	2.3	4.6
$A_2B_1C_1D_2$	0.31	13.9	10.2	0.4	20.4	0.6	6.3
$A_1B_2C_1D_2$	0.47	9.6	8.0	1.9	22.9	1.1	8.7
$A_2B_2C_1D_2$	0.31	13.5	10.5	2.3	12.0	2.6	9.0
$A_1B_1C_2D_2$	0.43	9.6	7.7	15.0	42.9	3.8	8.1
$A_2B_1C_2D_2$	0.39	15.3	12.5	6.2	25.5	3.2	9.6
$A_1B_2C_2D_2$	0.64	12.9	10.2	14.4	25.5	5.4	7.2
$A_2B_2C_2D_2$	0.43	16.2	13.9	7.7	10.6	5.7	5.9

The knowledge of the error variance allows the variance ratio test for significance, the F-test (190,191), to be calculated for each treatment combination. The values obtained are presented in Appendix A-5. The F-value is the ratio of a response variance of a treatment

combination divided by the error variance of that particular set of responses. The standard F-value, which corresponds to the number of degrees of freedom used to calculate both the variance and the error variance values based on a particular level of significance, was obtained from standard F-tables (194).

The level of significance, which should be stipulated at the start of a trial and then adhered to, was set at 5 %. As either positive or negative changes in the observed F-values were of interest, a double sided-test was appropriate, giving a standard F-value of 6.61. In this case, the observed variance was based on 1 degree of freedom and the error variance was calculated using 5 degrees of freedom. Therefore, if any F-value obtained from the treatment combination was greater than 6.61, the value was considered to be significant. If the F-values obtained were lower than the critical value, then there was a greater than 5 % chance that the observed variance was due to random variation and therefore was not significant. Discretion must be observed for F-values close to the critical value.

5.7.4 Discussion of the Statistical Results

5.7.4.1 Introduction

The statistical results regarding the four main effects and the main interaction effect will be presented in this section. The effects and the F-values found for the responses are given in Tables 5.6 to 5.9. For better visualisation of the effect of each factor, two chromatograms, representing a pair of treatment combinations, are presented in each of the following sections. The remaining chromatograms from the factorial design are presented in Appendix A-6.

5.7.4.2 Effect of Pulse Size

Table 5.6 presents the effects and F-values obtained when increasing the pulse size (factor A) from 5 % TECV to 10 % TECV. The effect of increasing the pulse size can be better visualised analysing the profiles for the treatment combinations $A_1B_1C_1D_1$, for a pulse size of 30 cm^3 and $A_2B_1C_1D_1$, for a pulse size of 60 cm^3, shown in Figures 5.2 and 5.3, respectively.

Table 5.6 - Effects and F-values for the increase of pulse size (factor A)

Response (Number)	Effect	F-value
Resolution between glucose and fructose (1)	-0.10	12.13
Peak concentration of glucose (2)	4.51	605.02
Peak concentration of fructose (3)	3.41	1686.18
% of glucose rich product recovered, with 100 % purity (4)	-7.13	45.20
% of fructose rich product recovered , with 100 % purity (5)	-15.08	298.56

The results from Table 5.6 showed that the increase of the level for the pulse size significantly reduced the performance of the BCBS, although the effect on the resolution was not significant. The effect of -0.1 for the resolution indicated that the increase of the pulse size from 5 % TECV to 10 % TECV decreased the resolution by 0.1.

The elution profiles for fructose showed very asymmetrical peaks for both runs, although the effect was more significant for the higher pulse size. For the treatment combination $A_1B_1C_1D_1$, the lower pulse size, the asymmetry factor for fructose was 0.67 and for the treatment combination $A_2B_1C_1D_1$, the higher pulse size, the same factor was 0.41.

The effect of the increase of the pulse size caused an increase of the negative skew of the elution fructose profile towards the glucose peak. There was a proportional increase of the band broadening for both products, which caused a greater overlapping; this, together with the tag-along effect, decreased the resolution between glucose and fructose.

This effect can be explained by the increase of the amount of substrate in relation to the enzyme available for the reaction by about 100 %, which affected the reaction rate. As explained in section 4.5.2, the hydrolysis of sucrose by invertase is inhibited both by the initial substrate concentration, for values above 0.1 g/cm^3, and by the water inhibition, as observed by Bowski *et al* (115). As the reaction tended to be retarded by the excess of substrate, less reactor length was available for the separation of the products formed. This effect caused the strong negative skew for the fructose elution profile.

Figure 5.2 - Elution profile for run RS(14)-SUC-5-40-100-1.4

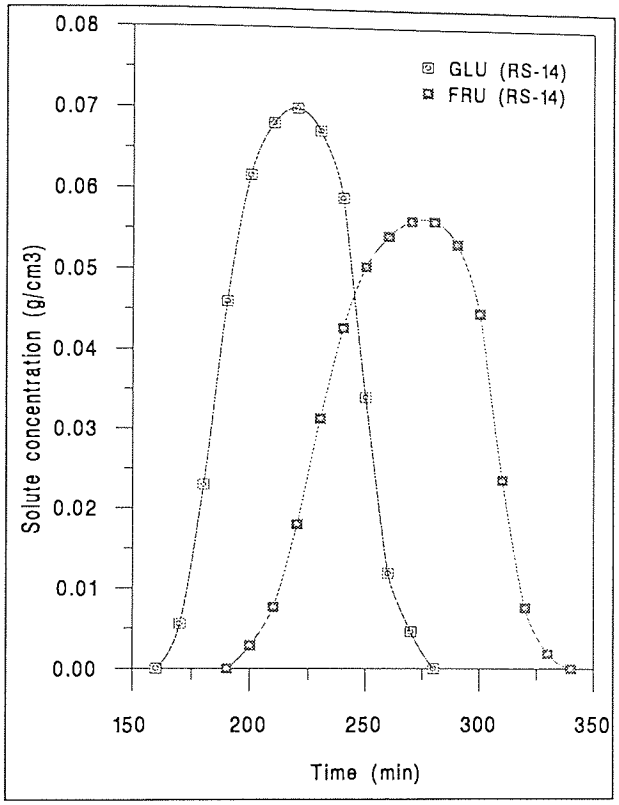
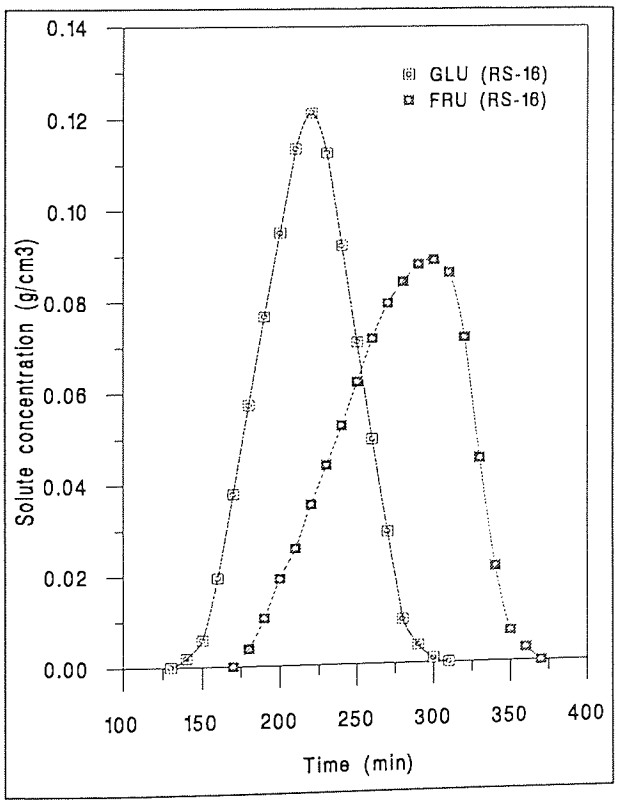


Figure 5.3 - Elution profile for run RS(16)-SUC-10-40-100-1.4



The effect of the band broadening observed for each product and the consequent overlapping of the two elution profiles, which was not enough to decrease significantly the resolution between glucose and fructose, contributed to the decrease in the performance of the BCBS, measured by the percentage of products recovered with 100 % purity. The values found for both fractions, as shown in Table 5.5, were confirmed by the statistical analysis presented in Table 5.6. The analysis indicated that the effect of the increase of pulse size on the pure products recovery were statistically significant.

The increase of the pulse size was confirmed by the positive effects observed on both peak concentrations of glucose and fructose, as shown in Table 5.6. The statistical analysis of the effect of the increase of pulse size on the peak concentrations showed that there was a 4.5 % and 3.4 % increase for glucose and fructose, respectively.

5.7.4.3 Effect of Pulse Concentration

The effects caused by the increase of the pulse concentration (factor B), from 40 % w/v to 60 % w/v, on the responses are discussed in this section. The effects and the F-values are presented in Table 5.7. Figures 5.4 and 5.5 present the elution profiles for both products, for the treatment combinations $A_1B_1C_2D_1$ and $A_1B_2C_2D_1$. The first combination was set at the lower pulse concentration and the second at the higher level.

Table 5.7 - Effects and F-values for the increase of pulse concentration (factor B)

Response (Number)	Effect	F-value
Resolution between glucose and fructose (1)	0.03	0.86
Peak concentration of glucose (2)	1.46	63.55
Peak concentration of fructose (3)	1.41	288.89
% of glucose rich product recovered, with 100 % purity (4)	-4.65	19.25
% of fructose rich product recovered , with 100 % purity (5)	-12.25	197.15

The values of the statistical analysis presented in Table 5.7 showed that the effect of the pulse concentration significantly decreased the performance of the BCBS as the recovery of both fractions with 100 % purity decreased 4.6 % and 12.2 %, respectively.

The effect on the resolution, although not significant, was relatively higher compared to the effect on this same response resulting from the increase of the pulse size. This was also

true for the effects on the recovery of fractions with 100 % purity, meaning that it is preferable to operate the system under mass overload than under volume overload.

The effect of the increase of the pulse concentration increased the amount of substrate injected by 50 %. The results indicated that there was a significant increase in the concentration for both peaks.

Working with more concentrated solutions will avoid high evaporation costs to recover the products formed. On the other hand, the fraction representing the mixture of products will increase, meaning that the amount of recycle will also increase.

The fructose elution profile for the treatment combination $A_1B_2C_2D_1$, run RS(15)-SUC-5-60-150-1.4, shown in Figure 5.5, presented an asymmetry factor of 0.85 denoting a slightly asymmetrical profile. The increase of pulse concentration caused an increase of the band broadening for this product and the tag along effect (193) was noticed. However, this asymmetry factor was comparable to the value found for the fructose profile for the treatment combination $A_1B_2C_1D_1$, run RS(17)-SUC-5-60-100-1.4. The lower value for the asymmetry factor can be explained by the higher enzyme activity level employed for the first treatment combination.

The band broadening was caused by the increase of the amount of substrate as explained in section 5.7.4.2, regarding the effect of pulse size. In the present case the reaction was also retarded by the increase of the amount of substrate in relation to the enzyme available for the reaction. Consequently, the reaction occurred along the chromatographic column rather than in the upper parts of it.

Figure 5.4 - Elution profile for run RS(19)-SUC-5-40-150-1.4

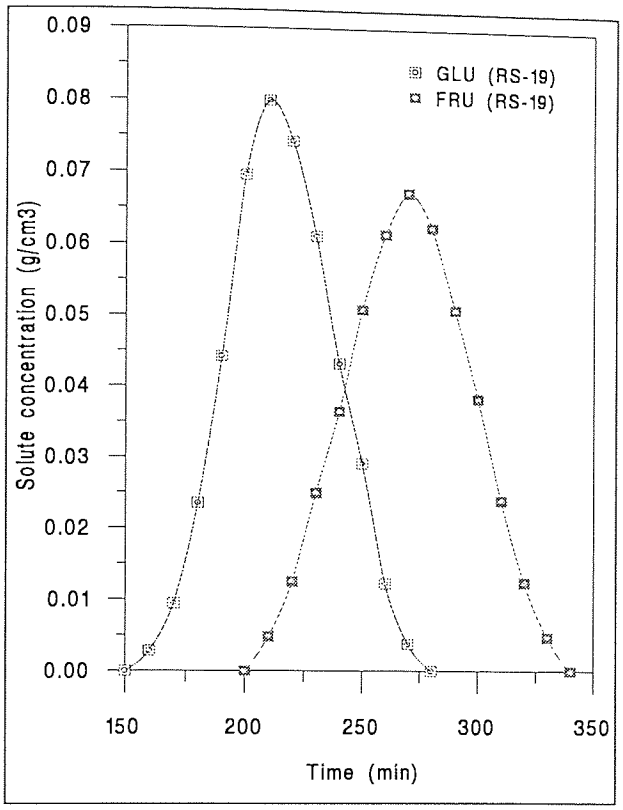
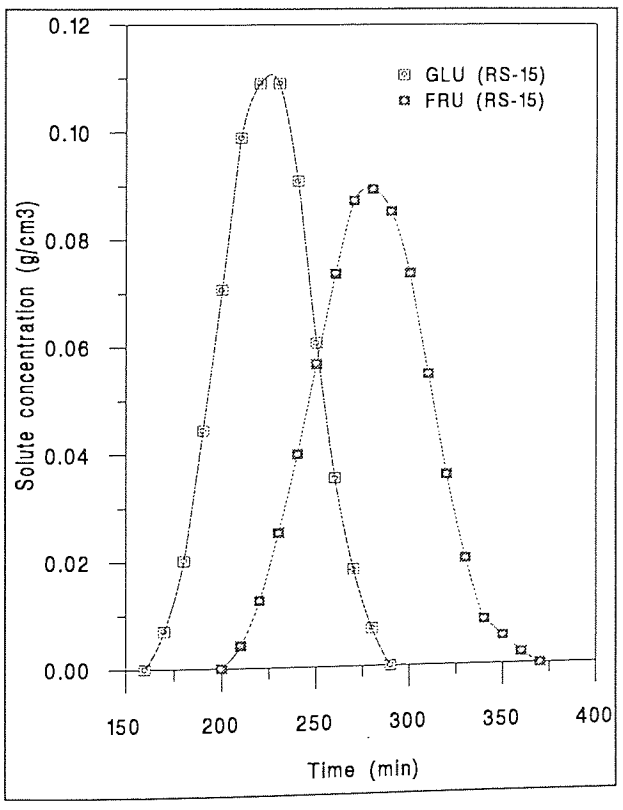


Figure 5.5 - Elution profile for run RS(15)-SUC-5-60-150-1.4



5.7.4.4 Effect of Enzyme Activity

The effects and F-values obtained increasing the enzyme activity (factor C) from 100 to 150 U/cm^3 are presented in Table 5.8. The effect of increasing the enzyme activity can be better visualised analysing the profiles for the treatment combinations $A_2B_2C_1D_1$, for the enzyme activity of 100 U/cm^3, and $A_2B_2C_2D_1$, for the enzyme activity of 150 U/cm^3, shown in Figures 5.6 and 5.7, respectively.

Table 5.8 - Effects and F-values for the increase of enzyme activity (factor C)

Response (Number)	Effect	F-value
Resolution between glucose and fructose (1)	0.01	0.23
Peak concentration of glucose (2)	2.01	120.34
Peak concentration of fructose (3)	2.21	798.80
% of glucose rich product recovered, with 100 % purity (4)	10.03	89.47
% of fructose rich product recovered , with 100 % purity (5)	5.40	38.31

Figure 5.6 - Elution Profile for run RS(21)-SUC-10-60-100-1.4

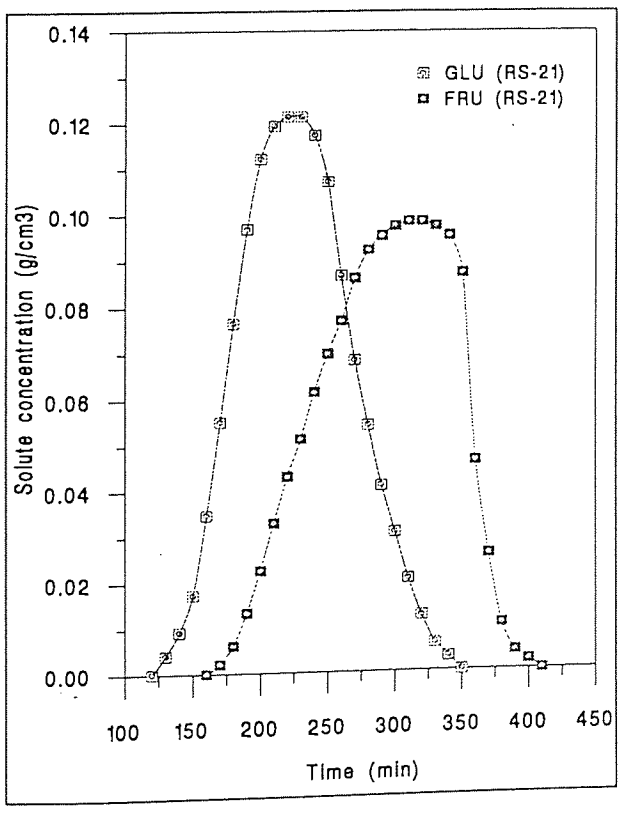
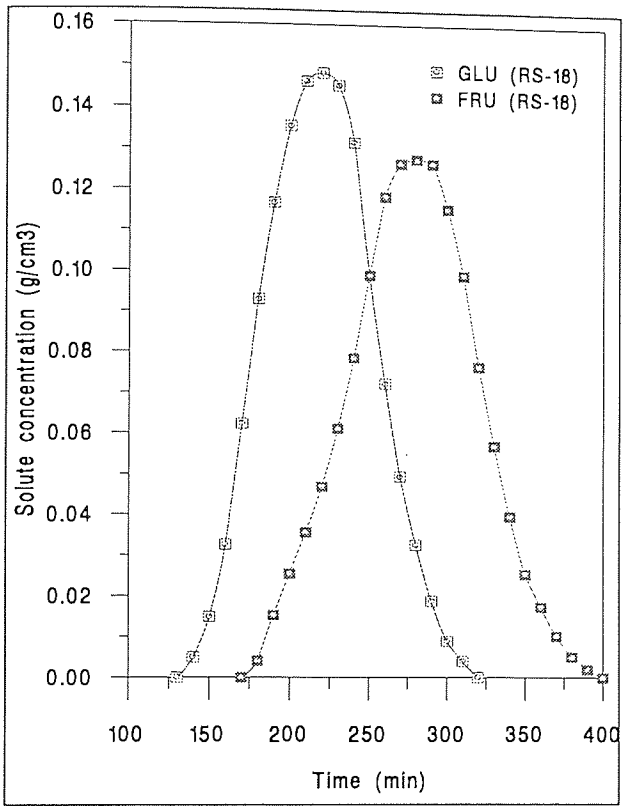


Figure 5.7 - Elution Profile for run RS(18)-SUC-10-60-150-1.4



The results from the statistical analysis presented in Table 5.8 showed that the effect of factor C, the enzyme activity, was statistically insignificant on the resolution. On the other hand, the effect was very significant on the other responses, namely on the performance of the BCBS, expressed by the recovery of the fractions with 100 % purity. The performance of the system increased and the peak concentrations increased, as well.

The fructose elution profile shown in Figure 5.6 presented a broad negative skew peak towards the glucose peak causing greater band overlapping. A tag along effect was also observed which contributed to a decrease of the resolution between the two products. The glucose peak presented a slight tailing towards fructose, characteristic of product overload. The value found for the asymmetry factor for fructose was 0.45 and for glucose aprox. 1.0. The pulse size and the pulse concentration were set at the higher levels and also contributed to a further decrease of the resolution.

Figure 5.7 shows the run carried out at the higher enzyme activity, the treatment combination $A_2B_2C_2D_1$. Working at higher enzyme activity level the fructose elution profile presented an asymmetry factor of 0.94 and the glucose profile maintained the same

previous value of 1.0, a typical value for symmetrical peaks. The tag along effect for fructose was not noticed (193).

The larger amount of enzyme employed per unit volume of eluent, maintaining all the other factors at a constant level, increased the availability of active sites for the bioreaction to proceed. The reaction rate increased and most of the substrate was consumed in the upper parts of the column, leaving more column length for the separation of the products formed. Therefore, the chromatograms were more symmetrical with less band broadening. The sharpening of the bands caused an increase of the peak concentrations and the recovery of rich fractions.

5.7.4.5 Effect of Flowrate

Table 5.9 presents the effects and F-values obtained when increasing the flowrate (factor D) from 1.4 cm^3/min to 3.5 cm^3/min. The effect can be better visualised when analysing the profiles shown in Figures 5.8 and 5.9, for the treatment combinations $A_1B_2C_1D_1$ and $A_1B_2C_1D_2$, for the flowrate set at 1.4 cm^3/min and 3.5 cm^3/min, respectively.

The results presented in Table 5.9 showed that increasing the flowrate from 1.4 cm^3/min to 3.5 cm^3/min did not produce a significant effect on the resolution. However, the values indicated that the effect was statistically significant on the performance of the BCBS and on the peak concentrations for both products as well.

The performance of the system decreased as indicated by the decrease of 7.9 % and 5.3 % on the percentage of glucose and fructose rich fractions, respectively.

By increasing the flowrate the residence time decreased and, consequently, the HETP for each product decreased resulting in a lower degree of separation. This conclusion was confirmed by the negative value observed for the resolution. The same observation was also made by Zafar (2) studying the biosynthesis of dextran in a batch chromatographic column.

The peak concentrations of both products also increased significantly as indicated by the values presented in Table 5.9.

Table 5.9 - Effects and F-values for the increase of flowrate (factor D)

Response (Number)	Effect	F-value
Resolution between glucose and fructose (1)	-0.07	0.23
Peak concentration of glucose (2)	1.54	70.24
Peak concentration of fructose (3)	1.11	179.21
% of glucose rich product recovered, with 100 % purity (4)	-7.98	56.62
% of fructose rich product recovered , with 100 % purity (5)	-5.35	37.60

Figure 5.8 - Elution profile for run RS(17)-SUC-5-60-100-1.4

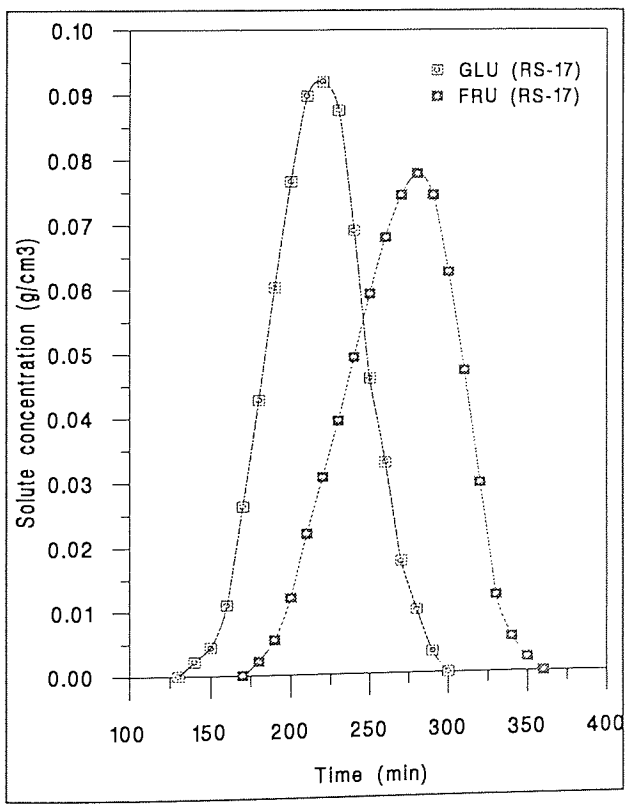
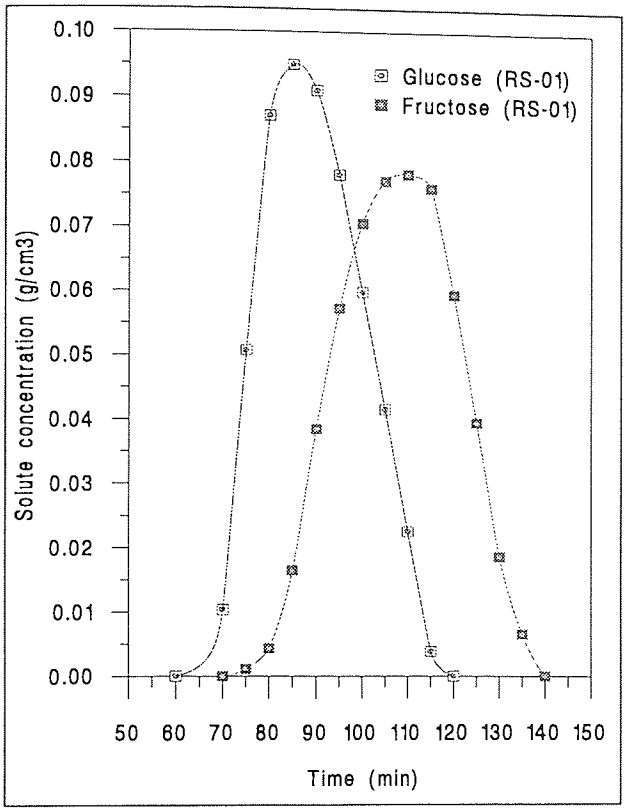


Figure 5.9 - Elution profile for run RS(01)-SUC-5-60-100-3.5



5.7.4.6 Effect of Significant Interactions

The significant interactions found for the five responses were collected from Tables A-5-3-4 to A-5-3-7 and are presented in Table 5.10.

Table 5.10 - F-values for the significant interactions

Treatment Combination	Response		
	Pk_G (2)	Pk_F (3)	G (4)
$A_2B_2C_1D_1$	(-)18.43	(-)34.41	-
$A_2B_1C_2D_1$	-	(+)45.81	-
$A_1B_2C_2D_1$	(+)17.27	(+)30.97	-
$A_2B_1C_1D_2$	-	(+)14.14	-
$A_1B_2C_1D_2$	-	-	(+)16.27

The values indicated that the pulse concentration (B) and the enzyme concentration (C) interacted and produced the most positive significant effects for response (2) and (3), glucose and fructose peak concentration, respectively. The F-values of 17.27 and 30.97 for each response were recorded. This combined effect suggests that the increase of the enzyme concentration had the main effect of rapidly bringing down the sucrose concentration below the level where substrate inhibition was insignificant. The individual effects of each factor increased the levels of both responses, as observed in Tables 5.6 and 5.8. The combined effects of the factors enhanced the values for both responses further.

The most negative effects were observed for the interaction of pulse size (A) and pulse concentration (B) for the same responses (2) and (3). Increasing both factors and maintaining the remaining ones at a constant level, increased the substrate concentration to the point where inhibition was significant. As the reaction rate decreased, the sucrose hydrolysis occurred along the column and, consequently, the band broadening increased and the peak concentration for both products decreased.

Positive interactions of pulse size (A) and enzyme activity (C) were observed for response (3). Again, this can be explained by the increase of the reaction rate which forced the products to be formed in the upper parts of the column. Positive interactions of pulse size (A) and eluent flowrate (D) were also observed for response (3).

The interaction of pulse concentration (B) and eluent flowrate (D) produced a positive significant effect for response (4), the percentage of glucose rich product recovered with 100 % purity. The effect of the increase of eluent flowrate was more noticeable for the fructose profile, as it tended to be always broader than the glucose profile.

5.8 Performance of the Batch Chromatographic Bioreactor-Separator System Producing a Fructose Rich Fraction with 90 % Purity

The systematic investigation of the BCBS system performance presented in section 5.7 considered the throughput of both 100 % rich glucose and fructose fractions. In this section evaluation of the performance of the BCBS for the system sucrose-invertase will be based on the production of a fructose rich fraction with 90 % purity. According to the data treatment presented in section 4.4 two other fractions can also be collected, prior to the elution of the fructose 90 % rich fraction: a glucose rich fraction and a recycle. The runs RS(19)SUC-5-40-150-1.4 and RS(08)SUC-10-60-150-3.5 were selected for the evaluation as they produced the highest and the lowest fructose rich fractions with 100 % purity. The results for the evaluation are presented in Table 5.11.

Figure 5.11 - Performance of the BCBS system

Runs	Fraction 1		Fraction 2		Recycle		Through -put (*)
	Glucose (g)	Fructose (g)	Glucose (g)	Fructose (g)	Glucose (g)	Fructose (g)	
RS(19)	3.19	0.52	0.22	2.70	2.89	3.38	8
RS(08)	9.43	1.40	0.78	6.88	8.26	10.14	27

* expressed in kg sucrose/h.m^3 of resin

By analysing the data presented in Table 5.11, the following observations can be drawn concerning run RS(19)SUC-5-40-150-1.4. Fraction number 1 is a glucose rich fraction with 90 % purity in glucose. Fraction number 2 is a fructose rich fraction with 90 % purity in fructose. The third fraction, the recycle, is a mixed fraction containing 53.9 % fructose and 46.1 % glucose. Each fraction represented 28.7 %, 22.6 % and 51.3 % of the total product formed, respectively. Both fractions 1 and 2 are potentially of commercial interest as both represented 90 % pure products as compared to those obtained in the high fructose syrup sector. The third fraction is close to a commercial 55 % high fructose syrup and could also be of commercial interest.

With respect to run RS(08)SUC-10-60-150-3.5 the following observations can be made. Fraction number 1 is a glucose rich fraction with 87 % purity in glucose. Fraction number 2 is a fructose rich fraction with 90 % purity in fructose. The third fraction, the recycle, is a mixed fraction containing 55.1 % fructose and 45.9 % glucose. Each fraction represented 29.3 %, 20.7 % and 49.9 % of the total throughput, respectively. Both fractions 1 and 2 are potentially of commercial interest as both represented pure products as compared to those obtained in the high fructose syrup sector. The third fraction is similar to a commercial 55 % high fructose syrup and may also be of commercial interest to this sector.

The substrate throughput in relation to the bed packing was found to be 8 kg sucrose/h.m^3 of resin for run RS(19)SUC-5-40-150-1.4. This low figure could be explained by the low pulse size and pulse concentration used compared to run RS(08)SUC-10-60-150-3.5, which had a throughput of 40 kg sucrose/h.m^3 of resin.

The enzyme consumption for both runs was found to be 48 % of the amount theoretically required to produce the same amount of product in a conventional batch reactor.

5.9 Practical Conclusions

Each factor studied was found to affect the performance of the BCBS. An increase in pulse size, pulse concentration and eluent flowrate levels diminished the performance of the system, measured by the effects on the responses (5) and (6), the percentage of glucose and fructose recovered with 100 % purity, respectively. The increase in enzyme activity increased the performance of the system as indicated by the same responses.

Pulse concentration, factor (B), and enzyme concentration, factor (C), interacted to produce the most significant effects for response (2) and (3), glucose and fructose peak concentration, respectively.

Although further economical evaluation is required the BCBS can clearly be operated to produce fructose and glucose rich fractions that are of potential interest to the high fructose syrup industry.

CHAPTER SIX

BATCH CHROMATOGRAPHIC BIOREACTION-SEPARATION USING THE SYSTEM INULIN-INULINASE

6.1 Introduction

The systematic study of the hydrolysis of inulin from chicory root using the enzyme inulinase from a strain of *A. niger* into a mixture of glucose and fructose was carried out in the BCBS-III system (see section 4.3.2.4). The enzyme was supplied by Novo Nordisk under the trade name of NOVOZYM 230. Two techniques were employed for the chromatographic bioreactions: in the first one a continuous flow of an enzyme solution was used as eluent and the substrate was injected into the column as a pulse. In the second, a mixture of enzyme-substrate was injected as a pulse and deionised and degassed water was used as eluent. This technique was used with the objective of decreasing the amount of enzyme used for the bioreaction-separations and also for collecting products free of enzyme.

Batch hydrolysis experiments were conducted to determine the effect of the enzyme activity on the hydrolysis of inulin. The inulin was also characterised concerning its number-averaged degree of polymerisation, $(DP)_n$.

Preliminary batch chromatographic bioreaction-separations were carried out in a column 1 m long x 0.9 cm i.d. to confirm the effect of enzyme activity level on the performance of the chromatographic reactor. The same activity levels as those employed in the batch reactions were used in the preliminary set of experiments.

The main objective of the work presented in this chapter was to evaluate the performance of the batch chromatographic bioreactor-separator operating under concerted enzymatic hydrolysis and separation conditions.

The modelling of the BCBS performing the hydrolysis of inulin by inulinase will be presented in chapter 9.

6.2 Characterisation of the Inulin Used

The number-averaged degree of polymerisation $(DP)_n$ of inulin was evaluated on the basis of the ratio of fructose to glucose residues in the molecule, considering that each molecule of inulin contains one glucose residue. This ratio was determined after complete hydrolysis of inulin carried out during the experiments for the determination of the initial reaction

rate, carried out in dilute conditions as presented in section 4.5.4. The average value of 32 was found for the $(DP)_n$ of inulin, confirming the data already discussed in chapter 2.

6.3 Batch Hydrolysis of Inulin with the Enzyme Inulinase

It is important to carry out the batch chromatographic bioreaction-separations in such a way that the formation of the products should occur in the upper parts of the column, leaving most of the column length to be used for the separation of the products. Therefore, a fast reaction time is required.

To determine the best enzyme activity to use in the BCBS-III system, batch hydrolyses of inulin with inulinase were carried out in the batch reactor as described in section 3.3.2. The reaction volume for each batch was 100 cm^3 of 10 % w/v inulin solution. Three reactions were carried out, each one containing 25 U/cm^3, 50 U/cm^3 and 100 U/cm^3 of inulinase, respectively. The reactions were followed by analysing the reducing sugars formed by the HPLC technique presented in section 3.4.1 and evaluating the conversion as the ratio between these reducing sugars and the reducing sugars corresponding to the complete hydrolysis of the substrate. The progress curves for the reactions are shown in Figure 6.1 which demonstrate the effect of inulinase activity on the conversion of inulin to reducing sugars.

Figure 6.1 showed that on doubling the enzyme activity from 25 U/cm^3 to 50 U/cm^3 the reaction rate increased. Nevertheless on doubling the enzyme activity from 50 U/cm^3 to 100 U/cm^3, no further improvement of the reaction rate was achieved.

The activity of 50 U/cm^3 was selected as the higher level of the eluent activity to be used in the batch chromatographic bioreaction-separations.

6.4 Batch Chromatographic Bioreaction-separations for the Inulin-Inulinase System Using the Flowing Enzyme Technique

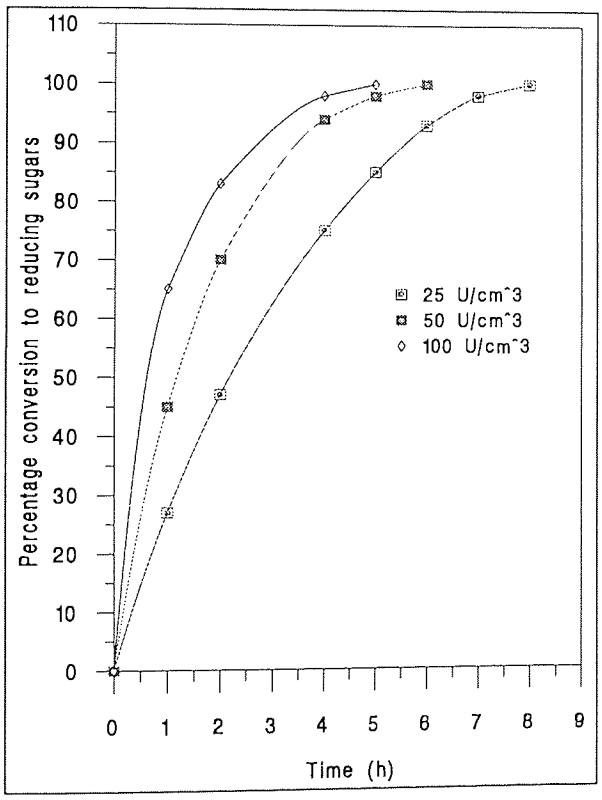
6.4.1 Preliminary Runs

6.4.1.1 Introduction

The effects of several factors on the performance of the batch chromatographic bioreactor-separator were established in Chapter 5. It was determined that lower flowrates and an increase of enzyme activity tended to improve the performance of the system. An investigation of the effect of enzyme activity and pulse size, maintaining the other factors

at a constant level, was first performed in a column 1 m long x 0.9 cm i.d., packed with Purolite resin PCR-833 in the calcium form. This column contained approximately 20 % less theoretical plates per meter than the 2m column (BCBS-III). This was possibly due to two reasons: firstly, an uneven bed distribution, as the column was difficult to pack and, secondly, an improper resin conditioning operation as the sodium ions had been replaced by calcium ions in a one step procedure. Since the intermediate replacement of sodium ions by hydrogen ions had not been done, less calcium ions were introduced per unit volume of resin.

Figure 6.1 - Effect of inulinase activity on the hydrolysis of inulin



As the solubility of pure inulin at 55 °C, the optimum temperature for the enzyme action, is approximately 10 % w/v, this was the pulse concentration used for the experiments. The eluent flowrate was kept at 0.74 cm^3/min, corresponding to the higher level of the superficial velocity of 1.16 cm/min employed in the investigation of the sucrose-invertase system. Three levels of enzyme activity were tested: 10 U/cm^3, 25 U/cm^3 and 50 U/cm^3. Two levels of pulse size were used: 1 % TECV and 5 % TECV.

The eluent and the feed solutions were prepared according to the techniques described in sections 3.3.1 part 2 and 3.3.2, respectively.

6.4.1.2 Results of the Preliminary Experiments

The codes and keys for the experimental runs followed a similar notation as that presented in section 5.4 and the letters INU characterise the runs for the inulin-inulinase system. The preliminary experiments were found to be complete reactions as no unreacted inulin was detected by the HPLC analysis performed on the fractions collected. The figures for the resolution between glucose and fructose, the percentage of fructose rich product as defined in section 4.4, the throughput of this product, the peak concentration and the enzyme usage, calculated according to Appendix A-4, are presented in Table 6.1. A typical chromatogram from the preliminary set of experiments (run RS(04)-INU-1-10-50-0.74) is shown in Figure 6.2. The profiles for the remaining runs are presented in Appendix A-7.

Table 6.1 - Results of the preliminary runs with the 1 m x 0.9 cm column

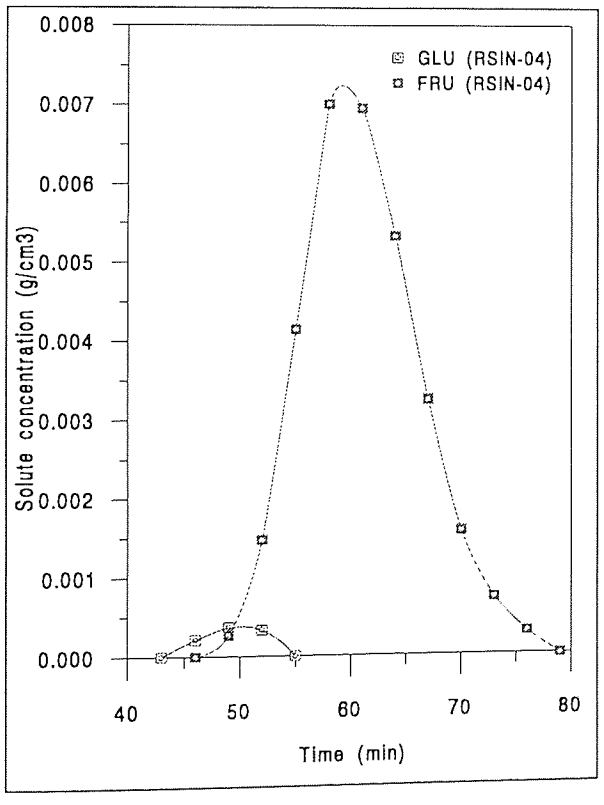
Experimental runs	Resolution (glucose-fructose) RS_{G-F}	Fructose 100 % purity % recovery of total fructose generated	Fructose through-put g/h	Peak conc. % w/v	Enzyme usage % of theoretical
RS(04)-INU-1-10-50-0.74	0.46	94.4	0.18	0.73	133.6
RS(05)-INU-5-10-50-0.74	0.51	45.8	0.32	2.82	36.6
RS(06)-INU-1-10-25-0.74	0.63	84.6	0.2	0.89	63.0
RS(07)-INU-5-10-25-0.74	0.40	51.9	0.43	3.14	12.6
RS(08)-INU-1-10-10-0.74	0.64	78.0	0.19	0.86	22.9
RS(09)-INU-5-10-10-0.74	0.35	53.0	0.54	2.87	115.9

The results from table 6.1 showed that, for the same level of pulse size and concentration, the increase of the enzyme activity increased the recovery of the rich fructose fraction confirming the effects of this factor on the performance of the system, as observed in chapter 5.

Working with the same level of enzyme activity and increasing the pulse size, the resolution between the products formed decreased and, consequently, the recovery of the rich fructose fraction also decreased. The data also showed that the concentration of the fructose rich fractions increased with an increase of the pulse size.

The asymmetry factor for the glucose profile showed three different characteristics: symmetrical peaks, asymmetrical peak with positive skew towards the fructose peak and asymmetrical peak with negative skew towards the fructose peak. The shapes of the glucose peaks for the preliminary runs do not seem to be related either to the pulse size or to the enzyme activity level, although the runs employing the higher pulse size levels showed the glucose profile co-eluting under the fructose profile. The fructose profiles had very symmetrical peaks in all the runs.

Figure 6.2 - Elution profiles for run RS(04)-INU-1-10-50-0.74



6.4.2 The Systematic Investigation of the Inulin-Inulinase System Using a 2 m x 1 cm I.D. Column (BCBS-III) - The Flowing Enzyme Approach

6.4.2.1 Introduction

The objective of this set of experiments was to establish the volume overload conditions above which the performance of the batch chromatographic bioreactor-separator would

deteriorate in performance. The experiments were carried out at two levels of enzyme activity and the pulse sizes were gradually increased for each run.

The following conditions were employed in this set of experiments:

1. The eluent flowrate was adjusted to 0.91 cm^3/min corresponding to a superficial velocity of 1.16 cm/min.

2. The pulse concentration was kept at the limit of the inulin solubility at 55 ^oC, which was 10 % w/v.

3. Three levels of pulse size were used: 5 % TECV, 10 % TECV and 20 % TECV.

4. Two levels of enzyme activity were used: 10 U/cm^3 and 25 U/cm^3.

6.4.2.2 Results of the Systematic Investigation

The results from these experiments are presented in Table 6.2 and the profiles for the runs RS(15)-INU-5-10-25-0.91, RS(14)-INU-10-10-25-0.91 and RS(11)-INU-10-10-10-0.91 are presented in Figures 6.3, 6.4 and 6.5.

The data confirmed that the reactions for this set of experiments were also complete as no inulin was detected in the fractions collected, meaning that the lowest level of enzyme activity was enough to completely hydrolyse the substrate .

The results from Table 6.2 showed that by keeping the enzyme activity at a constant level the resolution decreased with an increase of pulse size. The increase of the pulse size, which was doubled for each reaction, caused a considerable increase in the amount of substrate in relation to the enzyme available for the reaction. As the reaction rate decreased, the products were formed along the column rather than in the upper parts of it and less reactor length was available for the separation of the products formed. This effect caused the broadening and the overlapping of the elution profiles which contributed to a decrease in the fructose rich fraction recovery.

By increasing the pulse size from 5 % TECV to 10 % TECV, the percentage of fructose rich product recovered decreased by approximately 20 %. By increasing the pulse size from 10 % TECV to 20 % TECV, the recovery of the rich fructose fraction decreased by a further 70 % . This effect was observed for both levels of enzyme activity.

The fructose peak concentration increased with an increase of the pulse size and this effect was also noted for the throughput of the rich fructose fraction.

The increase of the level of the enzyme activity tended to improve the overall performance of the BCBS with respect to the resolution between glucose and fructose, the recovery of the fructose rich fraction and the throughput of this fraction.

Increasing the activity level by 150 %, from 10 U/cm^3 to 25 U/cm^3, only improved the recovery of the fructose rich fraction by about 35 %, indicating that the best level for the enzyme activity should be kept within the range used in this set of experiments.

The judgement regarding the optimum levels for pulse size and enzyme activity to be used in a practical situation, where high throughputs and purities are required, should be linked to process economics. Another point to be considered is that, on increasing the throughput of the fructose rich fraction, the recovery of pure fructose in relation to the fructose formed decreased and the amount of glucose rich fraction to be recycled increased.

Table 6.2 - Results for the systematic investigation of the inulin-inulinase system using the BCBS-III

Experimental run	**Resolution (glucose-fructose)** RS_{G-F}	**Fructose 100 % purity % recovery of total fructose generated**	**Fructose through-put** g/h	**Peak conc.** % w/v	**Enzyme usage** % of **theoretical**
RS(12)-INU-5-10-10-0.91	0.87	79.5	1.0	3.9	12.4
RS(11)-INU-10-10-10-0.91	0.68	62.7	1.6	7.0	8.4
RS(10)-INU-20-10-10-0.91	0.33	21.7	1.8	7.5	4.1
RS(15)-INU-5-10-25-0.91	0.95	99.4	1.2	4.1	32.4
RS(14)-INU-10-10-25-0.91	0.82	78.7	2.3	6.2	21.6
RS(13)-INU-20-10-25-0.91	0.57	29.4	2.4	8.0	10.2

Figure 6.3 - Elution profiles for run RS(15)-INU-5-10-25-0.91

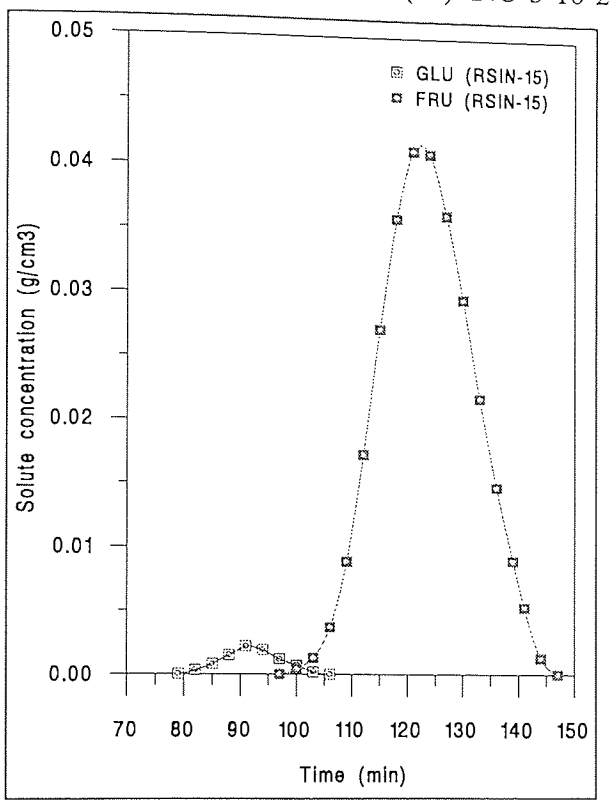


Figure 6.4 - Elution profiles for run RS(14)-INU-10-10-25-0.91

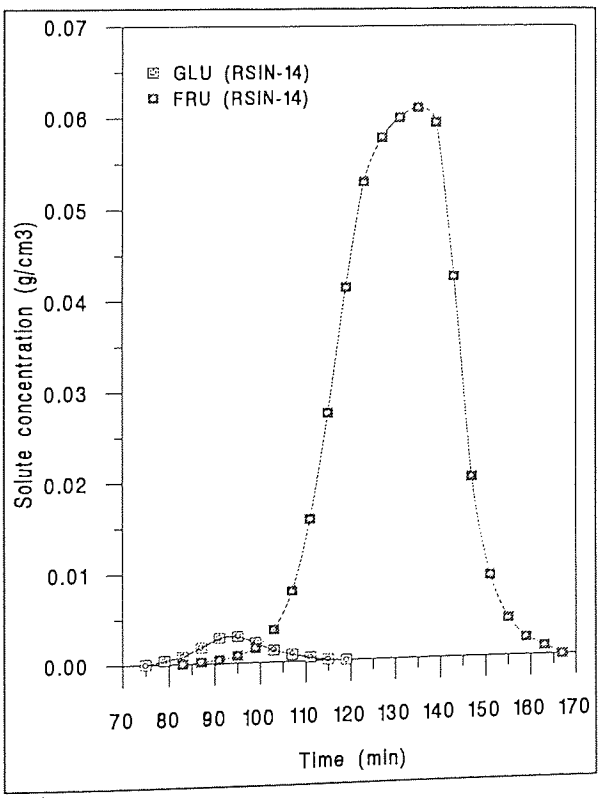
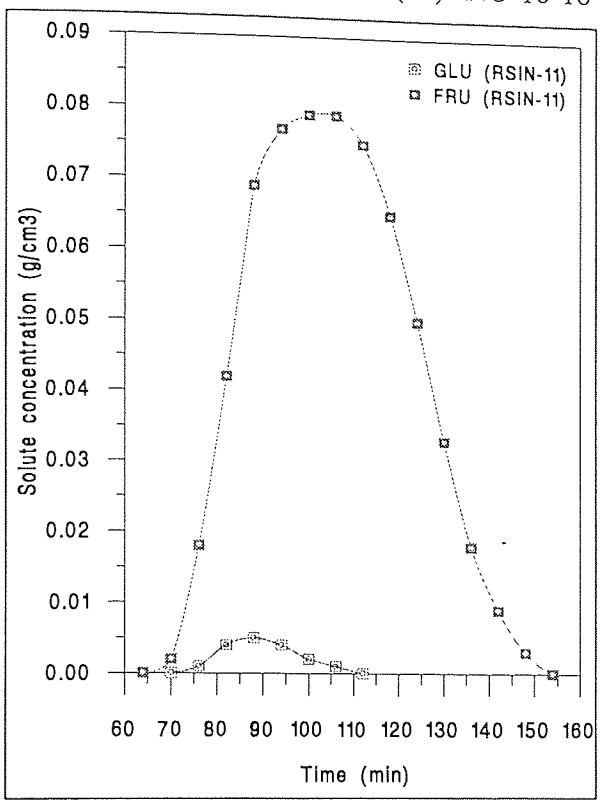


Figure 6.5 - Elution profiles for run RS(11)-INU-10-10-10-0.91



6.5 A Further Experiment on the BCBS-III Using the System Inulin-Inulinase - Experiments under Overload Conditions

The set of experiments presented in section 6.4.2 showed the effect of pulse size on the performance of the BCBS-III for different levels of enzyme activity. The profiles did not indicate either broad band widths, gross asymmetries or peak shoulders as those observed by Sarmidi working with the system modified starch-maltogenase (5) .

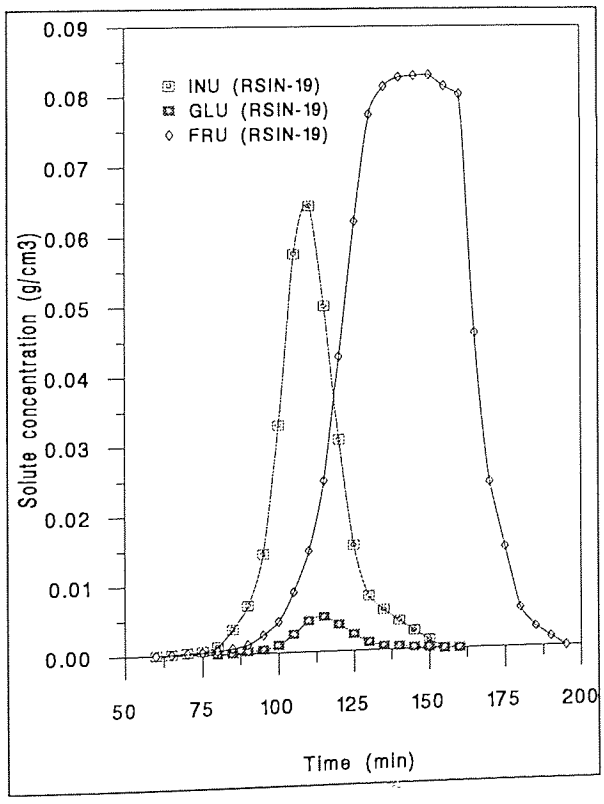
A further experiment was conducted in the BCBS-III, which consisted of operating the system under fixed conditions of enzyme activity and pulse concentration, and gradually increasing the pulse size. The enzyme activity was kept at 25 U/cm^3 and the pulse concentration at 10 % w/v.

The results of the experiments are tabulated in Table 6.3 and the profile for run RS(19) -INU-35-10-25-0.91 is shown in Figure 6.6. As the glucose fraction is mixed with inulin and fructose, the data presented referred only to the fructose fraction.

Table 6.3 - Results from the volume overload experiment

Experimental run	Resolution glucose-fructose RS_{G-F}	Unreacted inulin g	Fructose 100 % purity % recovery of total fructose generated	Through-put g/h	Peak conc. % w/v	Enzyme usage % of theoretical
RS(16)-INU-22.5-10-25-0.91	0.50	0	27.2	2.4	8.0	7.3
RS(17)-INU-25-10-25-0.91	0.43	0	26.4	2.6	8.4	7.5
RS(18)-INU-30-10-25-0.91	0.38	0.7	25.3	2.6	8.3	8.6
RS(19)-INU-35-10-25-0.91	0.25	1.6	21.4	2.8	8.4	8.7

Figure 6.6 - Elution profiles for run RS(19)-INU-35-10-25-0.91



The results presented in Table 6.3 showed that working with pulse sizes above 30 % TECV, the level of enzyme activity used was not enough to completely hydrolyse the inulin. The amount of unreacted inulin increased with the pulse size as less enzyme was

available for the hydrolysis reaction. For the pulses of 30 % TECV and 35 % TECV a residual of 16 % and 36 % of the total amount of inulin injected, respectively, was left unreacted.

The run RS(19)-INU-35-10-25-0.91 showed a strong band broadening caused by the higher level of the pulse size. The increased amount of substrate in relation to the enzyme available for this reaction contributed furthermore to the band broadening effect which caused the recovery of the rich fructose fraction to reach the lowest value for this set of experiments. The fructose profile for this run also showed an asymmetry with a negative skew towards the inulin peak, which was a characteristic observed for the incompleted batch chromatographic bioreaction-separations for the sucrose-invertase system presented in chapter 5. Both glucose and fructose profiles presented their front end peaks coinciding with the inulin front end peak which is a characteristic of incompleted batch bioreaction separations.

The profiles for the run RS(19)-INU-35-10-25-0.91 shown in Figure 6.6 did not show the separation of the lower $(DP)_n$ intermediate hydrolysis products and it was assumed that they co-eluted under the inulin profile. The band broadening of the inulin peak could corroborate this assumption although the co-elution of the glucose under the inulin band could also be responsible for the enlargement of the profile. The HPLC analysis employed for the determination of reducing sugars was not able to detect the smaller inulin chains.

The value of 1.7 kg of inulin/h.m^3 of resin for the throughput of the system operating with a diluted enzyme solution as eluent was found.

6.6 Batch Chromatographic Bioreaction-separation with a Pulse of a Mixture of Enzyme and Substrate into the Column

6.6.1 Introduction

The flowing enzyme approach has one major drawback. The products collected at the column outlet are contaminated with the enzymes used for the reactions. In a practical situation these solutions will demand further purification steps to remove these proteinic materials such as by the use of ultra filtration membranes.

To avoid this inconvenience the technique of injecting the substrate mixed with the enzyme solution into the column in the form of a pulse was investigated. A set of four experiments at four levels of enzyme activity with the pulse size and eluent flowrate at a constant level were carried out. The pulse size and the eluent flowrate were kept at 5 % TECV and 0.91

cm^3/min, respectively. The eluent flowrate was adjusted to correspond to the same superficial velocity of 1.16 cm/min employed in the investigation of the batch chromatographic bioreaction-separation for the system inulin-inulinase using the flowing enzyme method presented in section 6.4.2.

The feed for this set of experiments was prepared using the following procedure. 50 cm^3 of a 10 % w/v inulin solution was prepared according to section 3.3.2. Just before the start of a run the inulin solution was cooled to 10 °C, mixed with an exact amount of concentrated inulinase solution for the level of activity required for the run and immediately injected into the column as a pulse.

The inulin solution was freshly prepared for each run and, during the pulse time, no turbidity was noticed in the feed meaning that the inulin was still in solution. After the pulse, a sample of the feed was analysed using the HPLC method and it did not detect either glucose or fructose in the sample, indicating that the reaction occurred inside the column.

6.6.2 General Results

The results from the batch chromatographic bioreaction-separation experiments when using a mixed pulse of enzyme and substrate into the BCBS-III are presented in Table 6.4. The chromatographic profiles representing runs RS(01)-INU-5-10-10-0.91 and RS(02)-INU-5-10-25-0.91 are shown in Figures 6.7 and 6.8 indicating that the runs gave incomplete reactions.

Table 6.4 - Results of the runs employing a mixed pulse of enzyme and substrate

Experimental run	**RS_{G-F}**	**Unreacted inulin** g	**Fructose 100 % purity % recovery of total fructose generated**	**Fructose through-put** g/h	**Peak conc.** % w/v	**Enzyme usage** % of theoretical
RS(01)-INU-5-10-10-0.91	0.62	0.35	42.4	1.2	4.0	1.6
RS(02)-INU-5-10-25-0.91	0.67	0.33	44.7	1.3	3.9	4.0
RS(03)-INU-5-10-50-0.91	0.68	0.29	49.8	1.5	4.2	8.1
RS(20)-INU-5-10-100-0.91	0.67	0.27	52.1	1.6	4.5	16.2

Figure 6.7 - Elution profiles for run RS(01)-INU-5-10-10-0.91

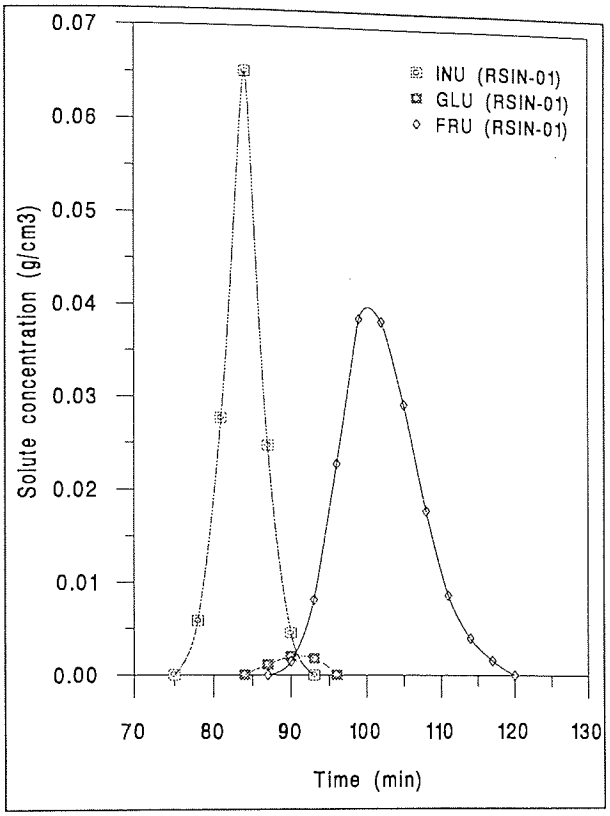
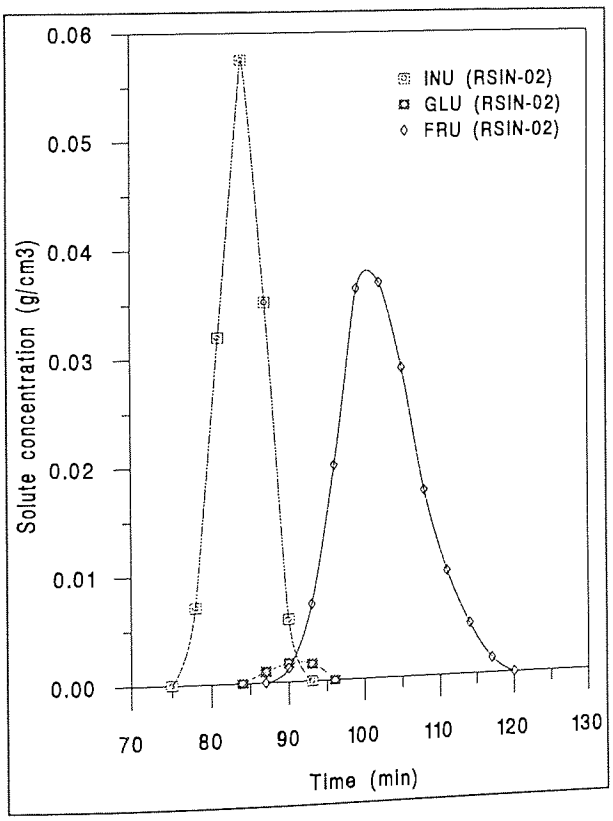


Figure 6.8 - Elution profiles for run RS(02)-INU-5-10-25-0.91



The profiles also indicated that the glucose and fructose profiles were nearly symmetrical and the fructose profile presented the characteristic tailing for products with longer retention times.

From Table 6.4 it can be seen that, even for the higher level of enzyme activity of 100 U/cm^3, it was not possible to complete the hydrolysis reaction. The conversion of inulin and the recovery of the fructose rich fraction increased with an increase of the level of enzyme activity. The increase of the fructose rich fraction throughput increased proportionally with the inulin conversion. The results showed, however, that by increasing the activity from 10 U/cm^3 to 100 U/cm^3, the recovery of the fructose rich fraction increased by only 23 %.

The incomplete reactions can be explained by the difference in the elution rate found for inulin and inulinase. Inulinase migrated faster than inulin along the column and the reaction only occurred when both materials eluted together. The results from the elution profiles indicated that the reaction occurred in the upper parts of the column as the glucose and fructose presented similar resolution values compared to those presented for the higher pulse size.

On comparing the results from this set of experiments with the results from the previous ones, presented in sections 6.4.1.2 and 6.4.2.2, the decrease of the enzyme usage was confirmed. This was verified comparing the results from the following runs: RS(01)-INU-5-10-10-0.91 and RS(12)-INU-5-10-10-0.91, runs RS(02)-INU-5-10-25-0.91 and RS(15)-INU-5-10-25-0.91, and runs RS(03)-INU-5-10-50-0.91 and RS(05)-INU-5-10-50-0.91.

Although the technique of batch chromatographic bioreaction-separations with a pulse of a mixture of enzyme and substrate into the column generated enzyme-free fractions with a lower consumption of enzyme, the results from this set of experiments showed that the reactions were incomplete and the recovery of fructose rich fractions, the throughput and the peak concentrations were lower than the flowing enzyme approach. The value of 3.4 kg of inulin/h.m^3 of resin for the throughput of the system operating with a mixed pulse of enzyme and substrate was found.

6.7 Practical Conclusions

Inulin can be hydrolysed by the action of the enzyme inulinase in a BCBS with the reactions products being partially separated. Two techniques can be employed. The first uses a diluted enzyme solution as eluent. In the second or a mixture of substrate and enzyme is pulsed into the chromatographic reactor. By using the first technique the

fractions collected at the outlet of the chromatographic reactor are contaminated with the enzyme. A further treatment is therefore necessary to remove the proteinic material if the technique is to be used practically.

On working with a diluted enzyme solution as eluent, fructose rich fractions with 100 % purity representing up to 99.4 % of the total fructose generated can be recovered. In this case the enzyme usage was 32.4 % of that theoretically required to produce the same amount of product in a conventional batch reactor.

It was observed that the maximum pulse size concentration was related to the solubility of inulin which is about 10 % w/v at 55 ^{o}C, the optimum temperature for the enzyme action. The operating limit for the system using a 10 % w/v inulin solution was achieved for pulses sizes of 30 % TECV working with the enzyme activity level of 25 U/cm^3. Beyond these conditions the inulin is left partially hydrolysed.

On pulsing a mixture of substrate and enzyme into the chromatographic reactor it was not possible to complete the hydrolysis of inulin. This was possibly due to the insufficient contact time between the enzyme and the substrate, as the first one migrated through the column ahead the inulin. By using this technique, 50 % of the substrate is left unreacted and 50 % of the fructose generated was collected as a rich fructose fraction with 100 % purity.

Throughputs of 1.7 and 3.4 kg of inulin/h.m^3 of resin were achieved when using a diluted enzyme solution as eluent and using a mixed pulse of enzyme and substrate respectively.

Finally, a judgement regarding the application of both techniques to practical situations demands further economic evaluation. It should take into account, at least, the following points: the recycle of unconverted inulin, the throughput and concentration of the fructose rich fraction and its inherent water evaporation costs and the enzyme consumption.

CHAPTER SEVEN

BATCH CHROMATOGRAPHIC BIOREACTION-SEPARATION USING THE SYSTEM LACTOSE-LACTASE

7.1 Introduction

A further study of the batch chromatographic column as a batch chromatographic bioreactor-separator was carried out using the system lactose-lactase to produce a mixture of glucose and galactose. The enzyme lactase was produced from a strain of *A. oryzæ* supplied by Biocon under the trade name Biolactase. The number of theoretical plates in the 2 m x 1.96 cm column (BCBS - I) was not enough for a satisfactory separation of these two carbohydrates, compared with glucose and fructose (see sections 4.4.1.1 and 4.4.2). It was therefore decided to connect two 2 m columns in series to obtain a higher number of theoretical plates. The performance of the system was slightly increased but still inferior to the performance achieved for the systems sucrose invertase and inulin-inulinase when working with columns with less theoretical plates.

A second set of experiments was carried out using the Semi Continuous Chromatographic Reactor (SCCR-S) adapted for batch chromatographic bioreaction-separation. The modified system presented a higher number of theoretical plates per meter compared to the 4 m column. These particular experiments were made in collaboration with M. T. Shieh, a fellow researcher.

The main objective of the experiments described in this chapter was to use an available and relatively inexpensive raw material, such as lactose which is obtained from whey, and to transform it into more valuable products, namely glucose and galactose, using the batch chromatographic bioreactor-separator.

7.2 Preliminary Batch Bioreaction-separation Experiments Using a 4 m x 1.96 cm I.D. Column

7.2.1 Introduction

The experiments for the physical separation of glucose and galactose presented in section 4.4.2 showed that resolution between these two carbohydrates was inferior to the values obtained for glucose and fructose as presented in section 4.4.1.1. The explanation is based on the difference in retention for galactose, compared to fructose, by the ion-exchange resin PCR-833 in the calcium form. This effect is related to the axial-equatorial-axial sequence

of the hydroxyl groups of the galactose molecule, as discussed in section 4.2.2.3. The insufficient number of theoretical plates has also contributed to the incomplete separation of glucose and galactose.

To partially eliminate the relative low retention of the galactose by the stationary phase, it was decided to work with a column having a higher number of theoretical plates. The increased number of theoretical plates could increase the retention times for both sugars and therefore enhance the separation. To accomplish this objective, two 2 m x 1.96 cm columns giving a total length of 4 m were connected in series. The stationary phase used was the cationic Purolite PCR 833 resin in the calcium form, the same as used for the systems sucrose-invertase and inulin-inulinase. The 4 m column had 960 NTP's for glucose and 1060 NTP's for galactose, double the value found for the 2 m column.

The experiments were carried out at the levels of 5 % TECV and using 5 % w/v and 10 % w/v lactose solutions as feed. The enzyme activity level was fixed at 60 U/cm^3, based on the work of Shieh (6) and two levels of flowrate were employed: 3.5 cm^3/min, and 1.75 cm^3/min, corresponding to a superficial eluent velocity of 1.16 cm/min and 0.58 cm/min, respectively. The use of a lower flowrate increased even further the NTP's available. Both eluent and feed solutions were prepared according to the procedures described in sections 3.3.1 part 2 and 3.3.2, respectively.

7.2.2 Results of the Preliminary Experiments

The codes and keys for the experimental runs followed those presented in section 5.4. The letters LAC, standing for lactose, referred to the lactose-lactase system.

The experiments showed that it was possible to completely hydrolyse lactose by the enzyme lactase in a batch chromatographic bioreactor-separator as no unreacted lactose was detected by the HPLC analysis performed on the fractions collected. The figures for the percentage of glucose and galactose rich fractions as defined in section 4.4.1.1, their throughputs and the enzyme usage, calculated according Appendix A-4, are tabulated in Table 7.1.

The results presented in Table 7.1 confirmed the observations for the systems sucrose-invertase and inulin-inulinase. The increase of pulse concentration, keeping the flowrate and enzyme activity at constants levels, decreased the performance of the system. The figures for the percentage of recovery of 100 % pure glucose and galactose fractions and the throughputs of these products were used as performance indicators. It was also confirmed that either a decrease of the flowrate level or an increase in the enzyme activity levels increased the performance of the system. However, the enzyme usage was found to

be larger compared to the systems sucrose-invertase and inulin-inulinase as the pulse size and the pulse concentrations were kept at a low level.

The chromatograms for runs RS(01)-LAC-5-5-60-3.5 and RS(02)-LAC-5-10-60-3.5 presented in Figures 7.1 and 7.2 showed a tag-along effect for the glucose and galactose profiles indicating that the reaction occurred along the column rather than in the upper parts of it, resulting in a lower degree of separation and affecting the performance of the system. The effect of a decrease in the flowrate and an increase of the enzyme activity levels can be better visualised through the profiles for runs RS(03)-LAC-5-5-60-1.75, RS(04)-LAC-5-10-60-1.75 and RS(05)-LAC-5-5-100-3.5 presented in Appendix A-8.

Table 7.1 - Results of the preliminary experiments

Experimental runs	Glucose with 100 % purity		Galactose with 100 % purity		Enzyme usage
	% recovered of total available	Through-put g/min	% recovered of total available	Through-put g/min	% of theoretical
RS(01)-LAC-5-5-60-3.5	0.6	0.06	4.5	0.44	211.0
RS(02)-LAC-5-10-60-3.5	0.26	0.11	5.5	0.44	99.7
RS(03)-LAC-5-5-60-1.75	37.9	0.73	38.8	0.40	310.7
RS(04)-LAC-5-10-60-1.75	17.2	0.80	18.6	0.50	140.7
RS(05)-LAC-5-5-100-3.5	14.0	0.67	16.6	0.64	351.7

7.3 Bioreaction-separation in the Semi Continuous Chromatographic Reactor (SCCR-S) Adapted for Batch Chromatography

7.3.1 Introduction

The Semi Continuous Chromatographic Reactor employed by Shieh (Figure 7.26 reference 6) was modified to operate in the batch mode by installing direct small bore connections to reduce the liquid hold-up between the 12 adjacent columns. The columns 1 and 12 were separated by closing the valve between them. The feed and the eluent were introduced directly into column 1 via valves which allowed the input of either the lactose feed or the eluent to the column. The products were collected at the column 12 outlet. Figure 7.3 shows the arrangement of the SCCR-S system operating in the batch mode.

Figure 7.1 - Elution profile for run RS(01)-LAC-5-5-60-3.5

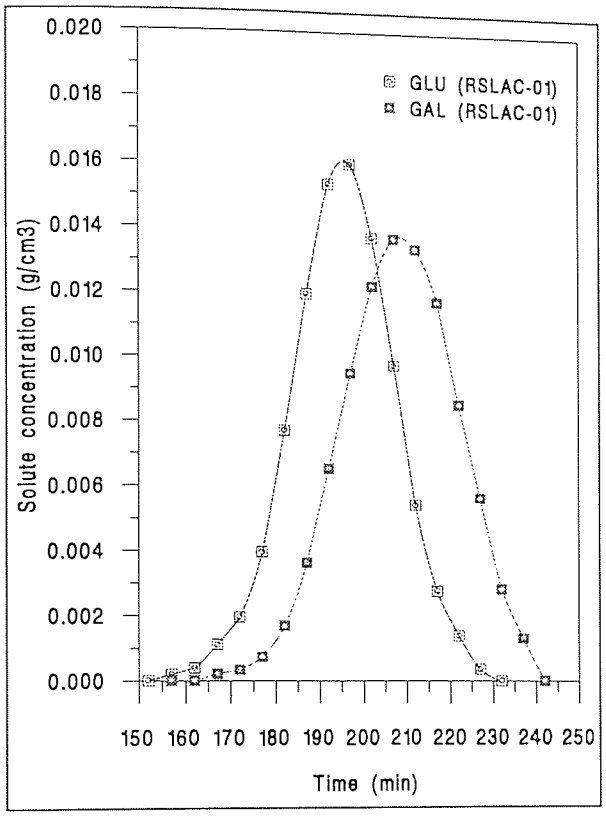


Figure 7.2 - Elution profile for run RS(02)-LAC-5-10-60-3.5

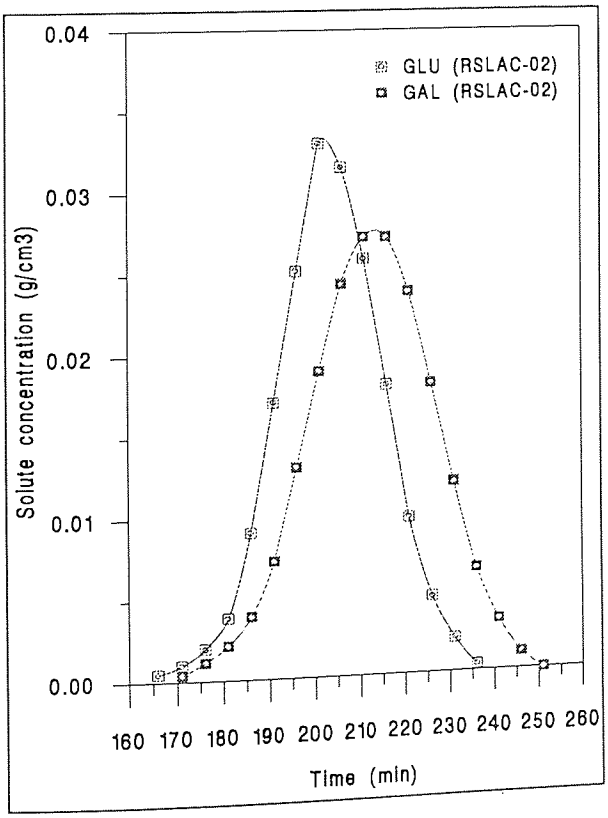
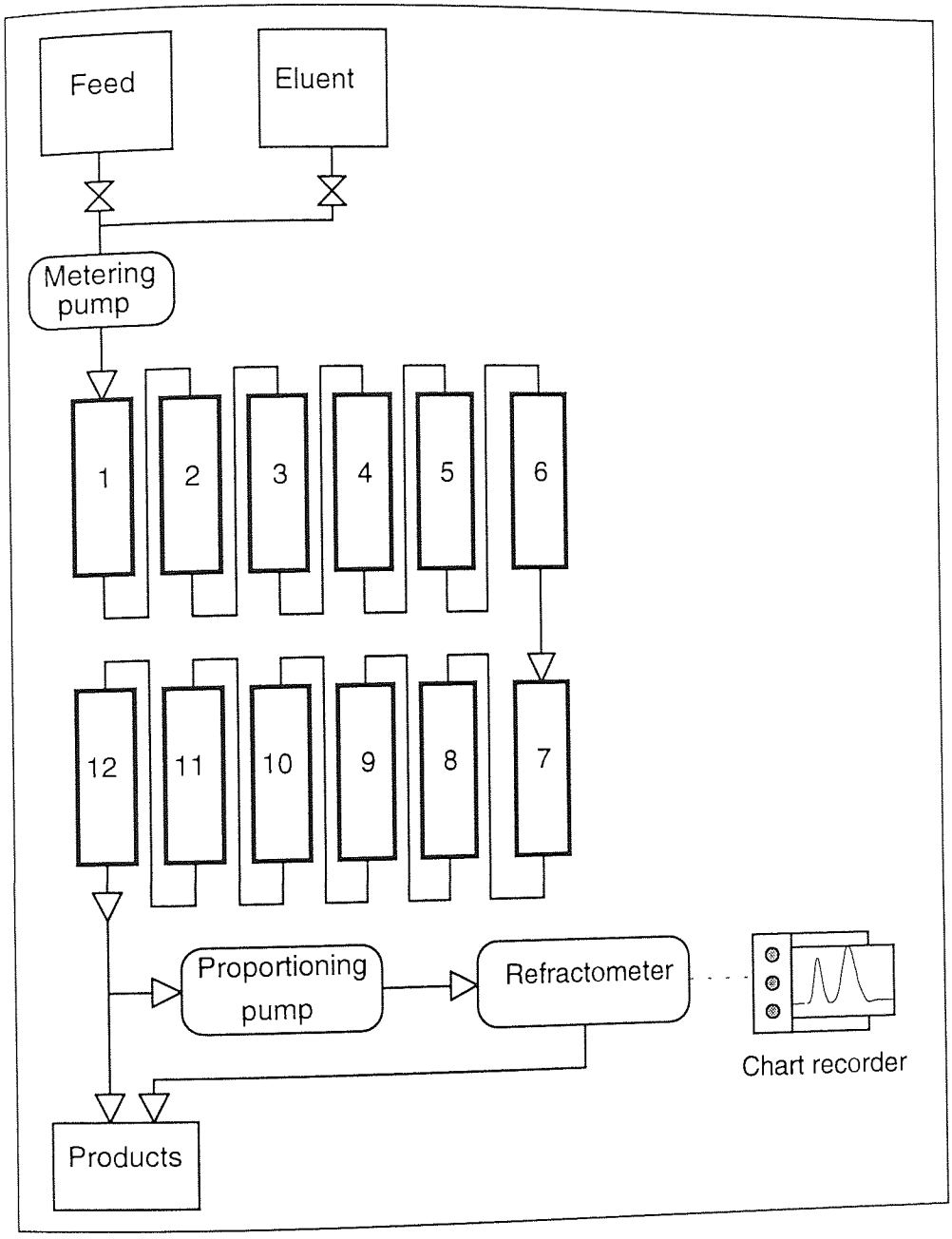


Figure 7.3 - The SCCR-S system adapted to operate in the batch mode



The system was packed with DOWEX 50W-X4 (150 μm mean particle size) cationic resin in the calcium form. Each column was characterised following the procedure described in section 4.3.1 for the carbohydrates glucose and galactose and the number of theoretical plates found for each species was 1200 and 1164, respectively. The total packed column length was 7.8 m.

As in the BCBS systems, a detection unit to indicate the elution of the products was installed at the column 12 outlet. It consisted of a peristaltic pump feeding a refractometer which was connected to a chart recorder

Before each run, the SCCR-S was first flushed with the eluent for 6 hours to ensure that the system had the same enzyme activity as in the incoming eluent.

7.3.2 Experimental Conditions

This set of experiments were conducted in such way that the effect of different levels of factors could be analysed individually. The conditions for the runs are presented in Table 7.2. The temperature was kept at 55 °C, the optimum temperature for the lactase action. Two levels of eluent flowrate were used: 25 cm^3/min and 30 cm^3/min, corresponding to superficial velocities of 0.96 cm/min and 1.31 cm/min, respectively.

Table 7.2 - Conditions for the runs employing the modified SCCR-S system

Effect studied	**Pulse size** % TECV	**Pulse concentration** % w/v	**Enzyme activity** U/cm^3	**Flowrate** cm^3/min
Pulse size	2 and 5	10	45	30
Pulse concentration	2	5 and 15	45	30
	2	10 and 20	45	25
Enzyme activity	2	20	45 and 60	30
Eluent flowrate	1	30	45	25 and 30

7.3.3 Experimental Results and Discussion

7.3.3.1 Introduction

The results for the percentage of glucose and galactose rich fractions, their throughputs and the enzyme usage for the set of experiments using the SCCR-S adapted for batch chromatographic bioreaction-separation are tabulated in Tables 7.3, 7.4, 7.5 and 7.6.

The results confirmed the effects of pulse size, pulse concentration, enzyme activity and eluent flowrate on the performance of the batch chromatographic bioreactor-separator as already discussed in Chapters 5 and 6 for the systems sucrose-invertase and inulin-inulinase.

7.3.3.2 Effect of Pulse Size

Table 7.3 presents the experimental results for the study of the effect of pulse size on the performance of the SCCR-S operating in the batch mode. For this set of experiments the concentration of the feed was 10 % w/v, the flowrate was kept at the level of 30 cm^3/min and the enzyme activity of the eluent was 45 U/ cm^3.

Table 7.3 - Effect of pulse size

Experimental runs	Glucose with 100 % purity		Galactose with 100 % purity		Enzyme usage
	% recovered of total available	Through -put g/min	% recovered of total available	Through -put g/min	% of theoretical
RS(07)-LAC-2-10-45-30	45.1	14.66	9.2	4.00	171
RS(06)-LAC-5-10-45-30	10.7	8.65	1.3	2.14	81

By increasing the pulse size from 2 % TECV to 5 % TECV (250 % increase) the percentage recovery of 100 % pure glucose and 100 % pure galactose decreased by 76 % and 86 % and the throughput of glucose and galactose decreased by 41 % and 46 %, respectively.

The effect of increasing the pulse size from 2 % TECV to 5 % TECV can be observed analysing the elution profiles for run RS(07)-LAC-2-10-45-30, for a pulse size of 360 cm^3 and for run RS(06)-LAC-5-10-45-30, for a pulse size of 900 cm^3, presented in Figures 7.4 and 7.5, respectively.

The increase in the pulse size increased the amount of substrate in relation to the amount of enzyme available for the reaction, which resulted in a lower reaction rate. Consequently, the reaction occurred along the chromatographic column and less length was used for the separation of the products formed.

The profiles for glucose and galactose for both runs indicated that the peaks were very symmetrical. The glucose profile showed a slight tail which is characteristic of the behaviour of this carbohydrate eluting through a chromatographic bed made of cationic resin in the calcium form. The asymmetry factor for glucose was found to be closer to the values generally found for the systems sucrose-invertase and inulin-inulinase. This could

be explained by the role of the particle size employed. The systems sucrose-invertase and inulin-inulinase used larger particle size (190-210 μm) compared to the lactose-lactase system (150 μm mean).

The profiles for galactose presented less asymmetry compared to the fructose profiles for the systems sucrose-invertase and inulin-inulinase. The mechanism of separation of galactose is less dependent on the axial hydroxyls of the molecule as observed for the fructose molecule. This carbohydrate tends therefore to travel through the resin pores with a higher rate as the calcium co-ordination complex tends to be less effective as explained in section 4.2.2. Consequently, the profiles tended to be more symmetrical and gave much less tailing. The tag along effect (193) or exaggerated band broadening for the glucose and galactose peaks was not obtained.

Figure 7.4 - Elution profile for run RS(07)-LAC-2-10-45-30

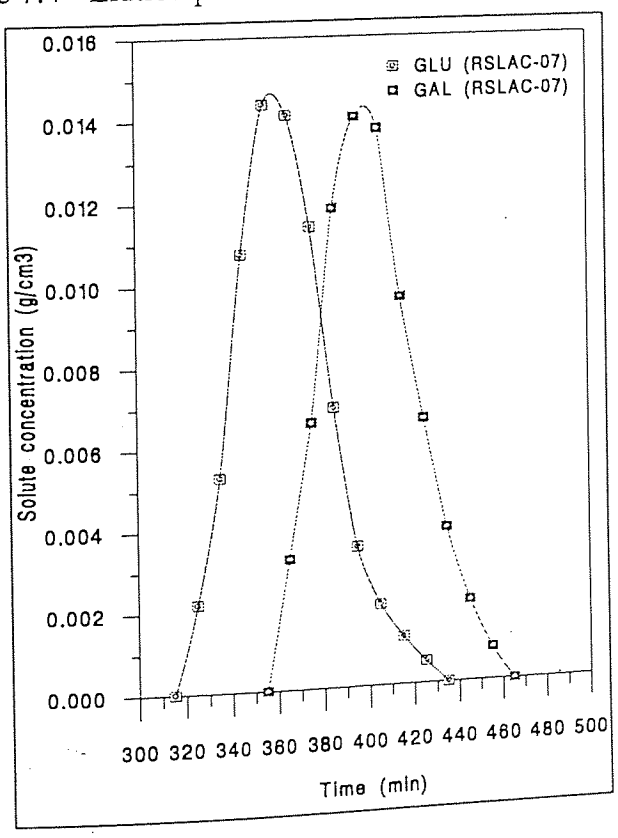
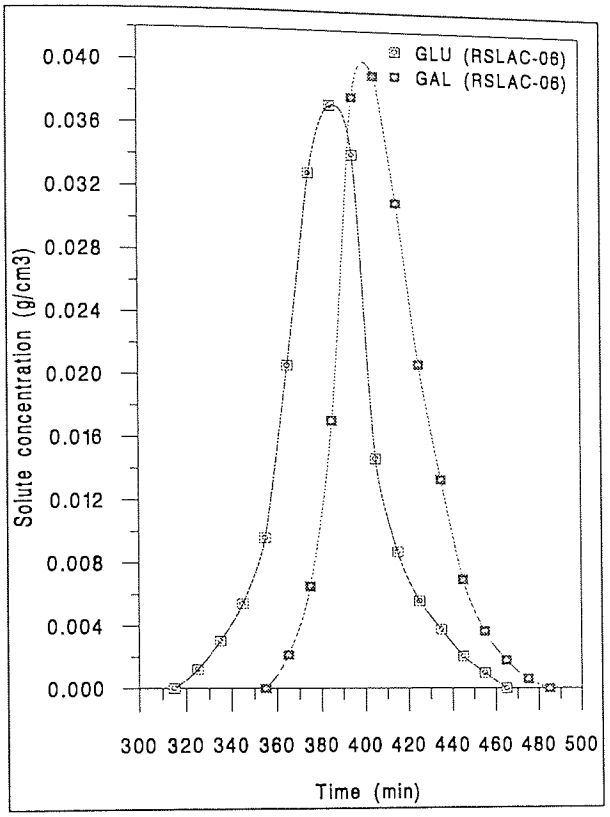


Figure 7.5 - Elution profile for run RS(06)-LAC-5-10-45-30



7.3.3.3 Effect of Pulse Concentration

Table 7.4 presents the experimental results for the study of the effect of pulse concentration on the performance of the SCCR-S operating in the batch mode. Two set of experiments were carried out. The pulse size level was kept at 2 % TECV and the enzyme activity level at 45 U/ cm^3, for both sets. Lactose solutions at 5 % w/v and 15 % w/v and at 10 % w/v and 20 % w/v were employed as substrate for the first and the second set of experiments, respectively.

For the first set of experiments, by increasing the pulse concentration from 5 % w/v to 15 % w/v the percentage recovery of 100 % pure glucose and 100 % pure galactose decreased by 1.5 % and 24.0 %, respectively. The throughput for glucose increased by 100 % and galactose decreased by 24 %.

For the second set of experiments, by increasing the pulse concentration from 10 % w/v to 20 % w/v the percentage recovery of 100 % pure glucose and 100 % pure galactose decreased by 69 % and 63 %, respectively. The throughput for both glucose and galactose decreased by 80 % and 9 %, respectively.

However, it is not possible to compare the results for the two set of experiments to explain the difference observed in the performance of the system as the flowrates were set at different levels.

Table 7.4 - Effect of pulse concentration

Experimental runs	Glucose with 100 % purity		Galactose with 100 % purity		Enzyme usage
	% recovered of total available	Through -put g/min	% recovered of total available	Through -put g/min	% of theoretical
First set of experiments					
RS(08)-LAC-2-5-45-30	39.4	6.43	23.9	3.91	381
RS(09)-LAC-2-15-45-30	38.8	12.62	6.8	2.94	127
Second set of experiments					
RS(14)-LAC-2-10-45-25	49.3	10.71	19.7	5.12	177
RS(16)-LAC-2-20-45-25	15.1	9.83	7.2	4.67	98

Comparing the increase of mass injected into the system as a pulse, either by means of an increase of pulse size or the increase of pulse concentration, the results indicated that it is preferable to operate the system under mass overload rather than under volume overload. These results are in agreement with the results found for the systems sucrose-invertase and inulin-inulinase (see Chapters 5 and 6). The results also corroborate the conclusions of Sarmidi (5) who worked with the system sucrose-invertase in the continuous rotating annular chromatograph. Furthermore, the system behaviour is in agreement with that obtained by Zafar (2) who studied the biosynthesis of dextran in a batch chromatographic bioreactor-separator.

The increase of the pulse size increased the amount of substrate in relation to the amount of enzyme available for the reaction, which resulted in a lower reaction rate. Consequently, the reaction occurred along the chromatographic column and less length was used for the separation of the products formed.

The effect of increasing the pulse concentration from 5 % w/v to 15 % w/v can be visualised when studying the elution profiles for runs RS(08)-LAC-2-5-45-30 and

RS(09)-LAC-2-15-45-30 presented in Figures 7.6 and 7.7, respectively. Figures 7.8 and 7.9 present the profiles for runs RS(14)-LAC-2-10-45-25 and RS(16)-LAC-2-20-45-25 showing the effect of increasing the pulse concentration from 10 % w/v to 20 % w/v.

The same observations concerning the shapes and symmetries for both glucose and galactose profiles presented in section 7.3.3.2 are valid for the present case.

Figure 7.6 - Elution profile for run RS(08)-LAC-2-5-45-30

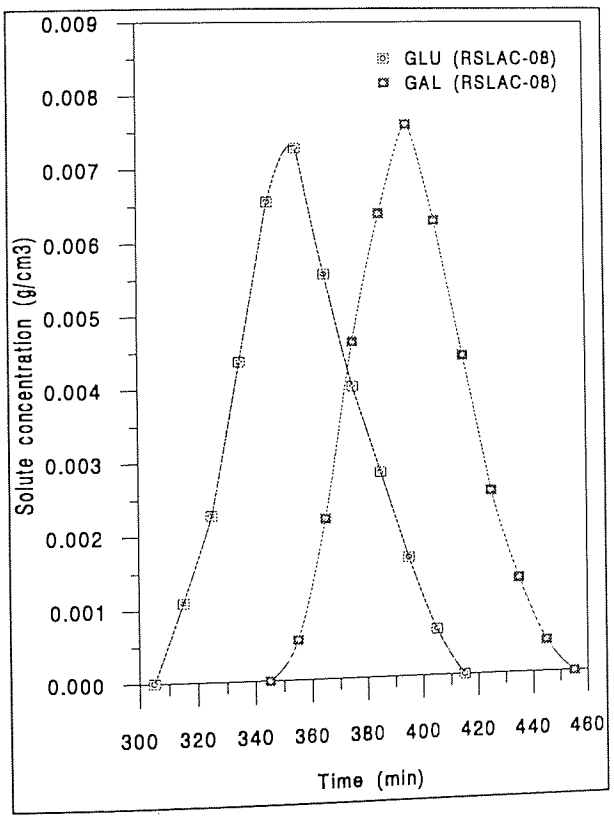


Figure 7.7 - Elution profile for run RS(09)-LAC-2-15-45-30

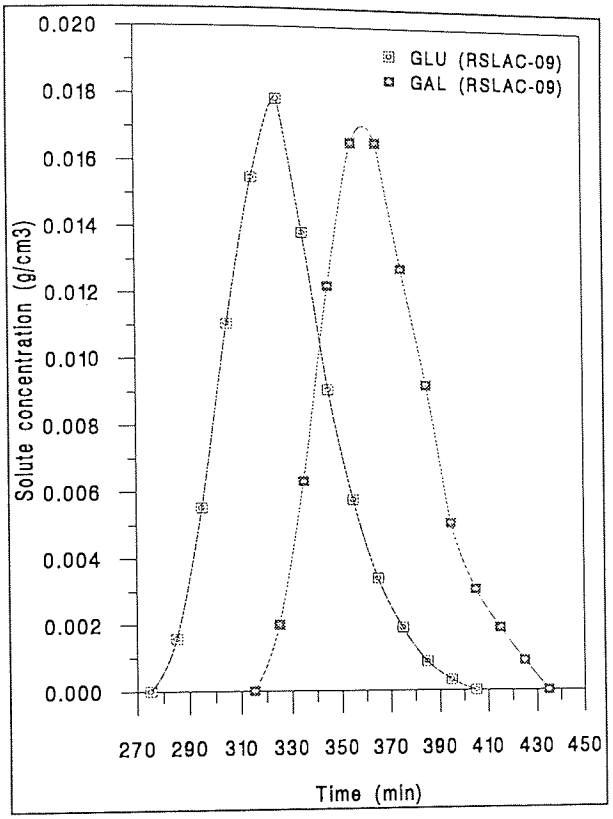


Figure 7.8 - Elution profile for run RS(14)-LAC-2-10-45-25

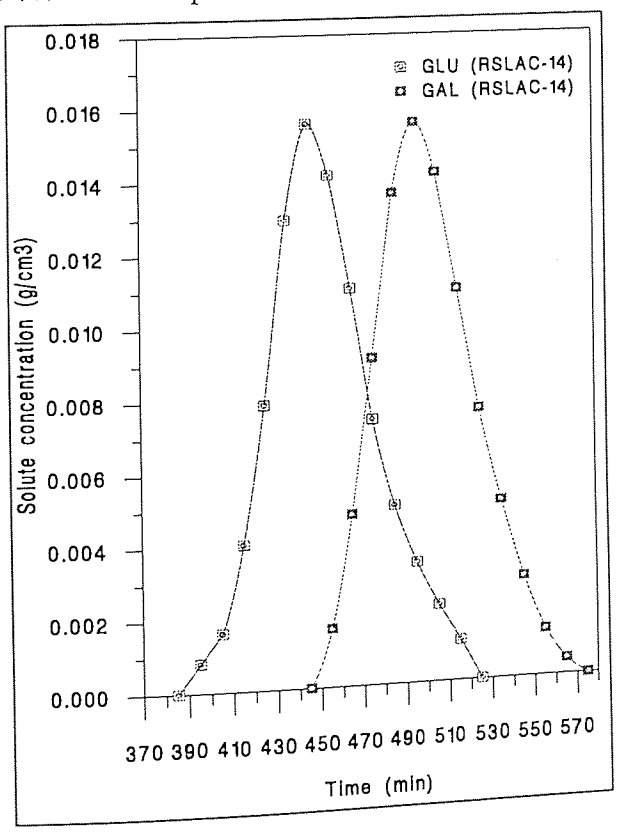
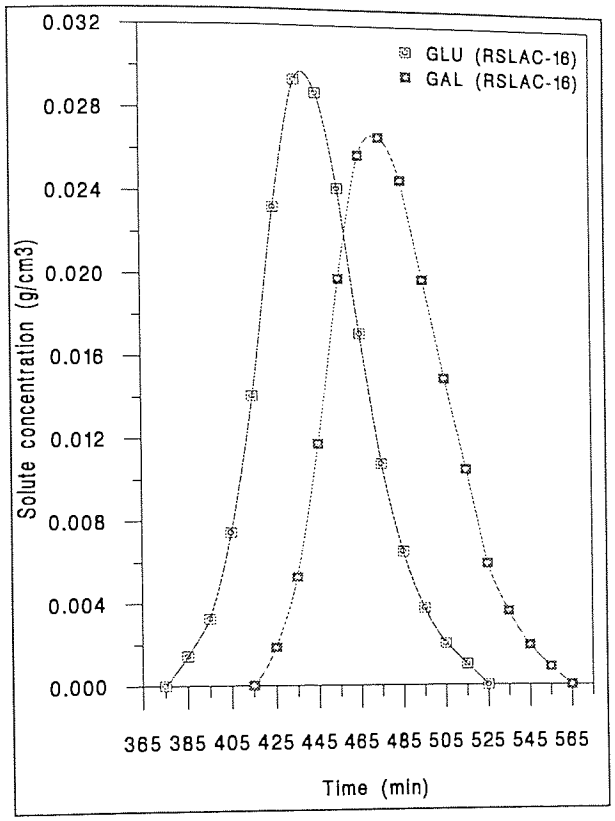


Figure 7.9 - Elution profile for run RS(16)-LAC-2-20-45-25



7.3.3.4 Effect of Enzyme Activity

Table 7.5 presents the experimental results for the study of the effect of enzyme activity on the performance of the SCCR-S operating in the batch mode. Two levels of enzyme activity were used for these runs: 45 U/cm^3 and 60 U/cm^3 and the runs were carried out employing a 20 % w/v lactose solution as feed. The pulse size and eluent flowrate were kept at a constant level of 2 % TECV and 30 cm^3/min, respectively.

The effect of increasing the enzyme activity from 45 U/cm^3 to 60 U/cm^3 can be observed through the elution profiles for run RS(10)-LAC-2-20-45-30 for an enzyme activity of 45 U/cm^3, and for run RS(12)-LAC-2-20-60-30 for an enzyme activity of 60 U/cm^3, presented in Figures 7.10 and 7.11, respectively.

From the results presented in Table 7.5 it can be seen that the performance of the system increased when the enzyme activity was increased. This confirms the results obtained for the systems sucrose-invertase and inulin-inulinase. These results also are in agreement with the results obtained by Sarmidi (5) working with the system sucrose-invertase in the continuous rotating annular chromatograph (CRAC) and those obtained by Zafar (2) working with the system sucrose-dextransucrase for the biosynthesis of dextran.

Table 7.5 - Effect of enzyme activity

Experimental runs	Glucose with 100 % purity		Galactose with 100 % purity		Enzyme usage
	% recovered of total available	Through -put g/min	% recovered of total available	Through -put g/min	% of theoretical
RS(10)-LAC-2-20-45-30	29.2	10.05	4.2	3.64	101
RS(12)-LAC-2-20-60-30	41.6	10.71	6.9	5.94	134

By increasing the enzyme activity, the reaction rate increased causing the substrate lactose to be converted to glucose and galactose in a shorter time. Therefore less of the column length was used for the reaction, leaving the remaining length available for the separation of the products.

Figure 7.10 - Elution profile for run RS(10)-LAC-2-20-45-30

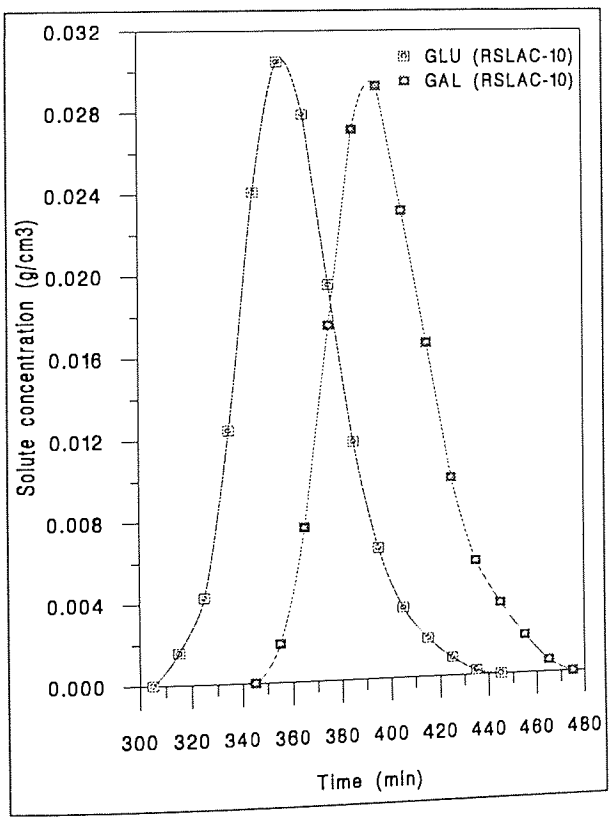
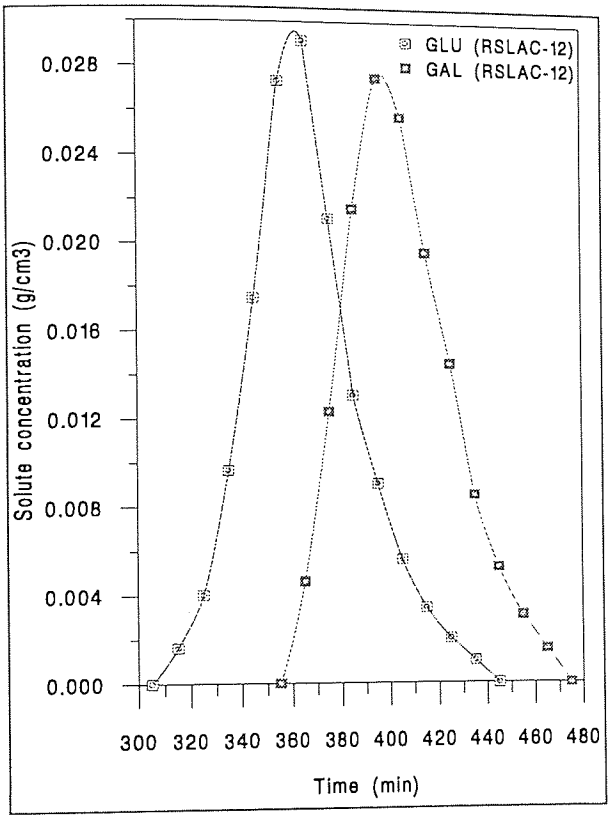


Figure 7.11 - Elution profile for run RS(12)-LAC-2-20-60-30



7.3.3.5 Effect of Eluent Flowrate

Table 7.6 presents the experimental results for the study of the effect of eluent flowrate on the performance of the SCCR-S operating in the batch mode. Two set of experiments were carried out using eluent flowrates at 25 cm^3/min and 30 cm^3/min. For both sets the enzyme activity was kept at 45 U/ cm^3. Pulses sizes of 2 % TECV and 1 % TECV for lactose solutions of 20 % w/v and 30 % w/v were employed in the first set and in the second, respectively.

The effect of increasing the flowrate from 25 cm^3/min to 30 cm^3/min can better be visualised when studying the elution profiles for run RS(11)-LAC-1-30-45-30 for the flowrate of 30 cm^3/min and for run RS(15)-LAC-1-30-45-25 for the flowrate of 25 cm^3/min, presented in Figures 7.12 and 7.13, respectively.

Table 7.6 - Effect of eluent flowrate

Experimental runs	Glucose with 100 % purity		Galactose with 100 % purity		Enzyme usage
	% recovered of total available	Through-put g/min	% recovered of total available	Through-put g/min	% of theoretical
First set of experiments					
RS(10)-LAC-2-20-45-30	29.2	10.05	4.2	3.64	101
RS(16)-LAC-2-20-45-25	15.1	9.83	7.2	4.67	98
Second set of experiments					
RS(11)-LAC-1-30-45-30	51.8	25.44	18.6	9.10	112
RS(15)-LAC-1-30-45-25	34.3	16.73	31.4	8.76	118

The results presented in Table 7.6 showed that by reducing the eluent flowrate the residence time in the system was increased, resulting in better separation (Figures 7.12 and 7.13) and better purities for the galactose rich product.

On reducing the flowrate the elution time for the products (Figures 7.12 and 7.13) increased. This resulted in a reduction of the throughputs observed at lower eluent flowrates. The same observation was made by Zafar (2).

7.4 Comparison of the SCCR-S Operating in Continuous Mode and Operating in Batch Mode for the System Lactose-Lactase

7.4.1 General Results and Analysis

The SCCR-S operating in the semi-continuous mode was used by Shieh (6) for the bioreaction-separation of lactose to glucose and galactose using the enzyme lactase. The results obtained by operating the equipment in the batch mode presented in this chapter were compared with the results obtained by Shieh in the semicontinuous mode. The figures for throughput, enzyme usage and concentration of products were used as parameters for comparison.

Figure 7.12 - Elution profile for run RS(11)-LAC-1-30-45-30

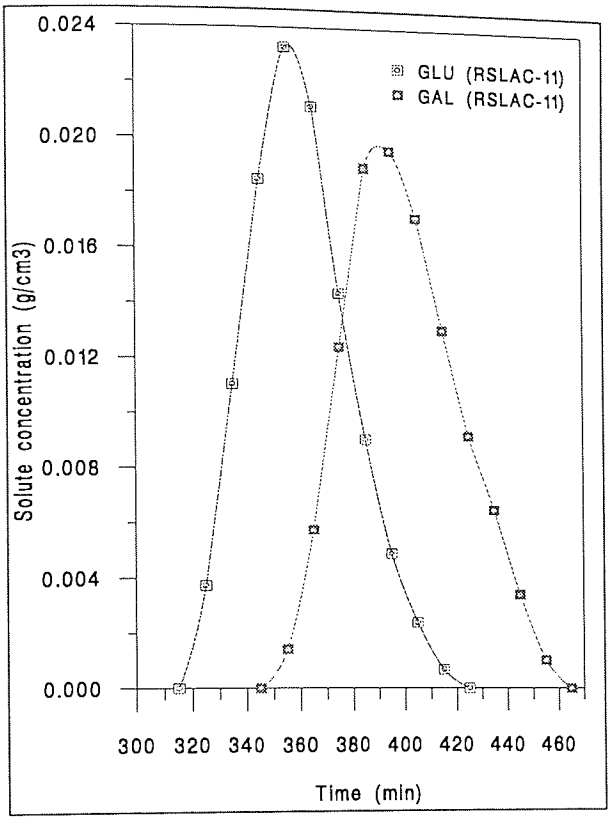
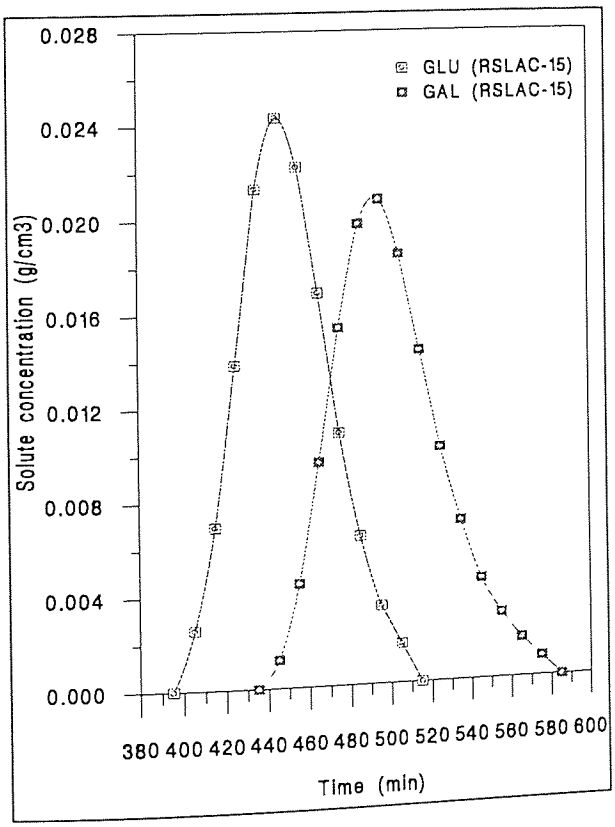


Figure 7.13 - Elution profile for run RS(15)-LAC-1-30-45-25



To facilitate the comparison, the product rich fraction concept (see section 4.4.1) for the second product or product "B", galactose in this case, was redefined. The definition for the first fraction, fraction "A" (the glucose rich fraction or Glu RF) was kept the same and meant the pure glucose which eluted from the beginning of the peak (time T1), until the galactose peak started co-eluting (time T2). The galactose rich fraction (Gal RF) was considered the mixture of products collected starting from time T2 until the end of the galactose elution, time T3. No product recycle was considered. The data are tabulated in Tables 7.7, 7.8 and 7.9 and were, in part, compiled from reference 6.

The figures presented in Table 7.7 compare the performance of the system based on throughput. For feed concentrations of 10 % w/v to 20 % w/v, the throughputs for the system operating in the continuous mode were 2.3 to 2.6 times those of the batch operation mode. For the feed concentration of 30 % w/v the ratio increased to 5.0. More glucose was recovered in the glucose rich product during the continuous operation compared to the operation in the batch mode.

The SCCR-S operation in the continuous mode also required less enzyme than in the batch mode according to the figures presented in Table 7.8. The continuous mode required only between 32-51 % of the amount of enzyme required for batch mode operation. The enzyme usage ratio decreased as the feed concentration was increased for both modes of operation, although the figures were more favourable for the continuous mode of operation. Consequently, the operating costs for the continuous operation of the SCCR-S will be lower.

On the other hand, in terms of product concentrations, the operation of the unit in the batch mode produced more concentrated fractions as indicated by the values shown in Table 7.9.

The continuous operation of the SCCR-S presents some advantages over that of the batch mode. Firstly, there is no need for recycle: the products continuously exit from the two product lines from the SCCR-S, while in the batch operation there is a need to recycle the overlaping section between the peaks. Secondly, there is a repeatable product quality from cycle to cycle in the SCCR-S operation while the batch product differs slightly in quality from batch to batch.

Table 7.7 - Comparison of batch and continuous operation of the SCCR-S

Feed conc. % w/v	Continuous operation				Batch operation				Relative through-put continuous/ batch
	Experimental run (*)	Product purities %		% Glucose recovered in Glu RP	Experimental run	Product purities %		% Glucose recovered in Glu RP	
		Glu RP	Gal RP			Glu RP	Gal RP		
10	10-4.5-4.5-28-300-28	99.9	63.6	49.3	2-10-45-25	99.9	64.9	49.4	2.38
15	15-4.5-4.5-28-300-28	99.9	60.0	52.6	2-15-45-25	99.9	66.2	46.6	2.38
20	20-4.5-4.5-28-300-29	91.9	68.8	57.4	2-20-45-25	99.9	59.3	45.2	2.63
30	30-4.5-4.5-30-400-28	99.9	77.1	70.4	1-30-45-25	99.9	64.4	34.3	5.0

(*) The following coding for the reactions carried out using the SCCR-S system in the continuous mode was used: the first number (10, 15, 20 or 30) refers to the concentration, the two following numbers (4.5 and 4.5) refer to the feed and eluent flowrates, respectively, the following number (28 or 30) to the enzyme flowrate, the following (300 or 400) to the enzyme activity and the last number (28 or 29) to the switch time.

Table 7.8 - Comparison of the enzyme usage for the batch and continuous operation of the SCCR-S

Feed conc. % w/v	Enzyme consumption (U/g of lactose)		Relative enzyme consumption
	Continuous	Batch	continuous/batch
10	3000	5937	0.51
15	2000	4250	0.47
20	1500	3281	0.46
30	1333	4167	0.32

Table 7.9 - Comparison of the product concentration for the batch and continuous operation of the SCCR-S

Feed conc. (% w/v)	Continuous (product concentration) (% w/v)		Batch (product concentration) (% w/v)	
	Gal RP	Glu RP	Gal RP	Glu RP
10	0.44	0.30	1.03	0.61
15	0.66	0.48	1.55	1.00
20	0.83	0.76	1.93	1.10
30	1.14	1.22	1.44	0.80

7.5 Practical Conclusions

The BCBS operating under concerted bioreaction and separation conditions for the hydrolysis of lactose by lactase did not yield promising results. The stationary phase was not adequate for an efficient separation of glucose and galactose, as shown in the experiments of physical separation presented in section 4.3.2.2. Contrary to fructose, the galactose molecule does not present a proper arrangement of the axial-equatorial-axial sequence of the hydroxyl groups to be complexed by the calcium ions. This is the probable cause for the galactose not being retarded in a chromatographic development.

The BCBS operation showed that is possible to recover up to 38 % of the galactose generated as a galactose rich fraction with 100 % purity. It is also possible to collect a glucose rich fraction containing 37.9 % of the total glucose generated with 100 % purity. These results were obtained using pulse conditions of 5 % TECV, a lactose solution of 5 % w/v and an eluent with enzyme activity of 60 U/cm^3.

The work under more favourable separation conditions, as those offered by the SCCR adapted for batch operation, presented better results. The effect of pulse size, pulse concentration, enzyme activity and flowrate on the performance of the SCCR were comparable to the results obtained for the sucrose-invertase system presented in Chapter 5.

The data show that the SCCR-S performing the hydrolysis of lactose by lactase is more suitable for the production of glucose rich product operating in the continuous mode than when operating in the batch mode. The data used for comparison took into account the production of only one pure fraction, the glucose rich fraction. Unfortunately, the SCCR-S system did not have a sufficient number of columns to allow the production of a galactose rich fraction, which is the more valuable product. The results of the comparison showed that when operating in continuous mode, a stream of glucose rich product with purities of up to 77 % can be obtained. The batch mode operation produced glucose rich fractions with purities of up to 50 %. By operating in a continuous mode the throughput of the system can be almost tripled as compared to batch operation.

CHAPTER EIGHT

A STUDY OF THE BATCH CHROMATOGRAPHIC BIOREACTOR-SEPARATOR PERFORMING AN ISOMERIZATION REACTION - THE ISOMERIZATION OF GLUCOSE TO FRUCTOSE

8.1 Introduction

The enzymatic isomerization of glucose to fructose was carried out in a batch chromatographic bioreactor-separator for the first time. The enzyme used was a glucose-isomerase produced from a strain of *A. missouriensis* supplied by International Bio-Synthetics under the trade name of Maxazyme GI-Liquid. The runs were performed using the BCBS-II system, a 2 m x 1.96 cm I.D. column, containing cation exchange resin in the magnesium form as stationary phase. The use of magnesium was chosen from the knowledge that calcium inhibits the glucose-isomerase action whilst magnesium acts as a promoter and stabiliser for it (69,171,197) .

Two techniques were employed for the chromatographic bioreactions: the flowing eluent enzyme mode and the mixture enzyme-substrate pulse approach (see Chapter 6).

The main objective of the work presented in this chapter was to verify whether the principles that governed the hydrolysis bioreaction-separations presented in the previous chapters could also be applied to an isomerization reaction performed in a chromatographic column. The second objective was to compare the performance of the chromatographic column operating under simultaneous isomerization bioreaction and separation conditions compared with the hydrolysis bioreactions studied in this thesis.

8.2 Batch Chromatographic Bioreaction-separations for the Glucose-Glucose Isomerase System - The Flowing Enzyme Approach.

8.2.1 The Conditions Used in the Experimental Work

The conditions employed for the runs were based on the suggested information supplied by the enzyme manufacturer concerning the optimum temperature for the enzyme action, pH and the ratio of enzyme to substrate (171). Magnesium sulphate was added to the enzyme solution to maintain the cation concentration at the level required for the biocatalyst action.

The experiments was carried out with the pulse size ranging from 1 % TECV to 5 % TECV using glucose solutions with concentrations of 10 % w/v to 40 % w/v as feed.

The enzyme activity level were varied from 50 U/cm^3 to 300 U/cm^3 and the flowrate adjusted to correspond to the superficial velocity of 0.45 cm/min, as employed for the previous systems. Two levels of temperature were used: 55 ^oC and 65 ^oC. Both eluent and feed solutions were prepared according to the procedures described in section 3.3.1 part 2 and 3.3.2, respectively.

8.2.2 Experimental Results and Discussion

The codes and keys for the experimental runs followed those presented in section 5.4. The term ISO, standing for isomerization, was introduced to relate the experiments to the Glucose-Glucose isomerase system.

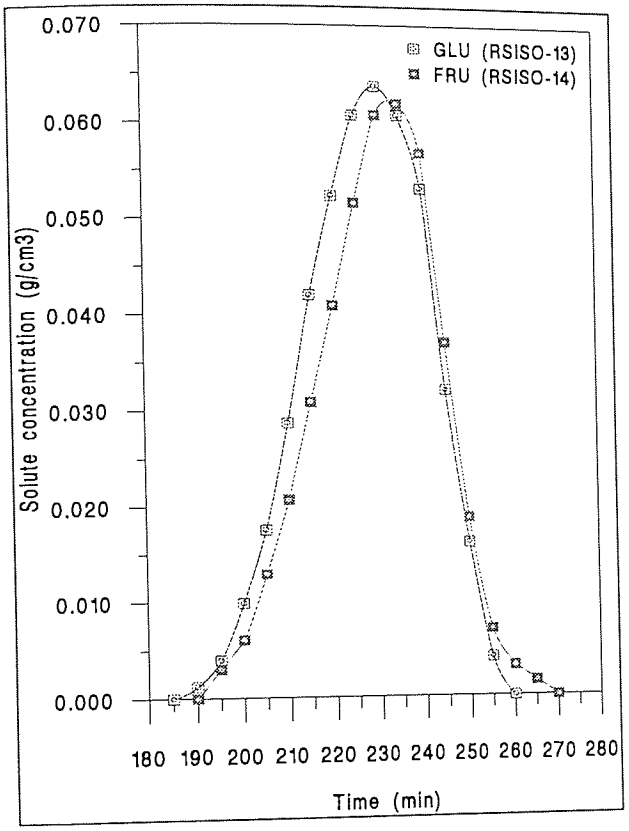
Table 8.1 presents the following figures: amount of glucose injected, percentage of unconverted glucose, percentage of fructose formed in relation to the glucose pulsed into the system, the isomerization equilibrium related to fructose (see section 4.5.5), the percentage of the rich fractions recovered and the enzyme usage, calculated according to Appendix A-4. The elution profile for run RS(13)-ISO-5-20-120-1.4 is shown in Figure 8.1.

Table 8.1 - Results of the runs employing the conventional approach for the system glucose-glucose isomerase

Experimental runs	Glucose injected (g)	Glucose unconverted (g)	Fructose formed (g)	Conversion (% fructose/ total solids)	Glucose with 100 % purity (% recovery of total glucose unconverted)	Fructose with 100 % purity (% recovery of total fructose generated)	Enzyme Usage % of theoretical
RS(13)-ISO-5-20-120-1.4	6	3.1	2.9	47.9	1.16	1.16	42.8
RS(14)-ISO-5-20-120-1.4*	6	3.0	3.0	50.0	1.01	1.02	42.8
RS(15)-ISO-2.5-40-120-1.4	6	3.1	2.9	47.6	1.00	1.02	42.8
RS(26)-ISO-1-40-300-1.4	2.4	1.3	1.1	45.8	1.14	1.12	267.7
RS(25)-ISO-5-40-300-1.4	12	6.1	5.9	49.2	1.00	1.11	53.5
RS(27)-ISO-1-40-50-1.4	2.4	1.3	1.1	45.8	1.16	1.03	44.6

(*) run performed at 65 °C

Figure 8.1 - Elution profile for run RS(13)-ISO-5-20-120-1.4



The number of theoretical plates of the chromatographic column using the cation exchange resin PCR-833 in the magnesium form as stationary phase was 282 for glucose and 226 for fructose. Using the resin in the calcium form the number of theoretical plates was 532 and 407 for glucose and fructose, respectively (see sections 4.3.2.2 and 4.3.2.3). Using magnesium as counter ions the resolution for both products decreased and the chromatograms for the products presented quite asymmetric profiles and negatively skewed peaks.

The data in Table 8.1 and the profiles presented in Figure 8.1 showed that the recovery of rich fractions decreased as a result of two combined effects. The first effect was due to the magnesium counter-ion which did not present the same complexing properties as calcium ions. The second one was related to the characteristic of an isomerization reaction of the type A $\leftrightarrow$ B, performed in a chromatographic column as pointed out by Sardin *et al.*(77): wherever B (fructose) is formed in the column and whatever the separation between A (glucose) and B (fructose), the backward reaction converts fructose back to glucose along the chromatographic column. This is true because the enzyme is active along the column.

The conversions, expressed by the relation between the fructose formed and the total solids generated, were in the range 45 % to 50 %. These figures are identical to the conversion obtained at steady state for the temperatures of 55 °C and 65 °C (187,188,189) . It seemed not possible to improve these figures in the batch chromatographic bioreactor-separator operating isothermally and using the enzyme solution as eluent.

8.3 Batch Chromatographic Bioreaction-separations with a Pulse of a Mixture of Enzyme and Substrate into the Column

8.3.1 Introduction

To improve the conversion of fructose, Sardin (73) suggested the use of a heating zone moving at the same velocity as the glucose pulse. It was not possible to modify the equipment to generate this effect. The alternative of pulsing the mixture of enzyme and substrate was then investigated.

The three components of the system are the substrate glucose, the fructose generated by the reaction and the enzyme. They percolated through the chromatographic column at different elution rates. The enzyme is a macromolecule which does not penetrate into the resin pores. It moves along the column occupying the voids and therefore travels faster than either the glucose which does pass through the pores or fructose which forms a loose complex with the magnesium ions on the resin.

By injecting a pulse mixture of substrate and enzyme, it was anticipated that the enzyme band while co-eluting with the glucose band would generate fructose which would than be held back by the stationary phase. As the enzyme moves ahead of the glucose band, the reaction will no longer take place. The remaining column length will then be available for the separation of the reaction products.

The technique of injecting a pulse of substrate mixed with the enzyme solution was performed according to the procedure presented in section 6.6.

8.3.2 Experimental Conditions

Eight experiments with the flowrate set at a constant level of 1.4 cm^3/min, corresponding to a superficial velocity of 0.45 cm/min were carried out. The temperature was kept at 55 °C, the optimum temperature for the enzyme action. Two levels for the pulse size, pulse concentration and enzyme activity were used: 1% TECV and 5 % TECV, 10 % w/v and 40 % w/v and 150 U/cm^3 and 300 U/cm^3, respectively.

The feed for this set of experiments was prepared according to section 3.3.2. Just before the start of a run the glucose solution was cooled down to 10 °C, mixed with an exact amount of concentrated glucose-isomerase solution for the level of activity required for the run and immediately injected into the column as a pulse. After the pulse, a sample of the feed was quenched and analysed using the HPLC method. Fructose was not detected in the sample, indicating that the reaction occurred inside the column.

8.3.2 Results and Discussion

8.3.2.1 Introduction

The results from this set of experiments are tabulated in Table 8.2. The analysis of the effects of each variable in the performance of the batch chromatographic bioreactor-separator using cationic resin with magnesium as counter-ion under a mixed pulse of enzyme and substrate is presented in the following sections.

8.3.2.2 Effect of Pulse Size

The effect of the increase of pulse size can be observed by analysing the figures presented in Table 8.2 for each pair of reactions carried out at 1 % TECV and 5 % TECV. A pair of elution profiles for runs RS(17)-ISO-1-10-150-1.4 and RS(18)-ISO-5-10-150-1.4 are shown in Figures 8.2 and 8.3, respectively.

Table 8.2 - Results for the runs employing a mixed pulse of enzyme and substrate

Experimental runs	Glucose injected (g)	Unconverted Glucose (g)	Fructose formed (g)	Conversion (% fructose/ total solids)	Glucose with 100 % purity (% recovered of total glucose unconverted)	Glucose through-put (g/h)	Fructose with 100 % purity (% recovered of total fructose generated)	Fructose through-put (g/h)	Enzyme Usage % of theoretical
RS(17)-ISO-1-10-150-1.4	0.6	0.4	0.2	30.4	39.9	0.7	74.6	0.7	346.5
RS(18)-ISO-5-10-150-1.4	3.0	1.6	1.4	45.7	11.6	0.5	28.0	1.9	100.8
RS(21)-ISO-1-40-150-1.4	2.4	1.8	0.6	25.0	12.9	0.6	21.7	1.3	86.6
RS(22)-ISO-5-40-150-1.4	12.0	6.2	5.8	48.2	5.2	1.3	13.3	5.1	25.2
RS(19)-ISO-1-10-300-1.4	0.6	0.4	0.2	38.7	69.0	0.8	82.5	0.8	693.0
RS(20)-ISO-5-10-300-1.4	3.0	1.6	1.4	48.4	3.2	0.6	15.1	1.5	200.0
RS(23)-ISO-1-40-300-1.4	2.4	1.6	0.8	31.9	6.9	0.7	22.0	1.7	173.2
RS(24)-ISO-5-40-300-1.4	12.0	6.0	6.1	50.3	8.9	2.1	12.9	5.2	50.4

Figure 8.2 - Elution profile for run RS(17)-ISO-1-10-150-1.4

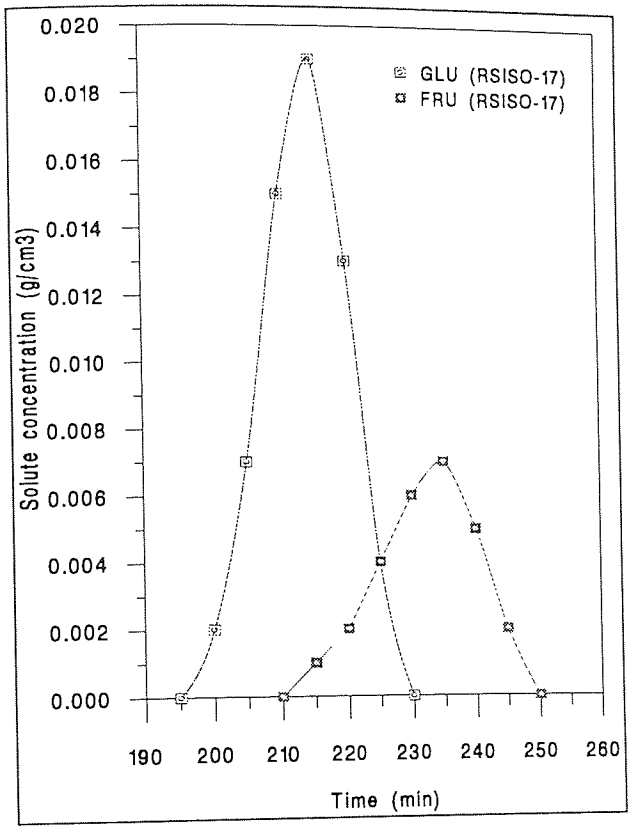
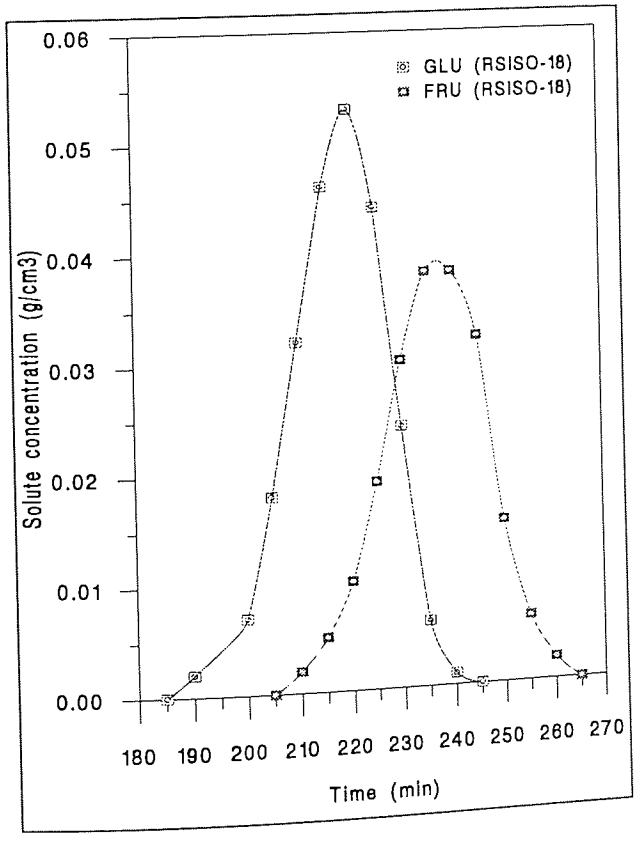


Figure 8.3 - Elution profile for run RS(18)-ISO-5-10-150-1.4



The results for each pair of runs show that by increasing the pulse size the conversion of glucose into fructose and the throughputs of the pure fractions increased and the percentage of pure products decreased.

The increase of conversion is due to the increase of retention time for the larger band corresponding to the larger pulse size, which, in its turn, corresponded to a larger amount of substrate available for the reaction. The enzyme band coeluting over a larger band of substrate increased the amount of product formed. On the other hand, less of the column length was available for the separation. By increasing the pulse size the profiles also presented larger bands. These two combined effects contributed to a decrease in the performance of the system.

8.3.2.3 Effect of Pulse Concentration

The effect of an increase of pulse concentration can be observed by analysing the figures presented in Table 8.2 for each pair of reactions carried out at 10 % w/v and 40 % w/v. The results from each pair of reactions showed that by increasing the pulse concentration the percentage recovery of 100 % pure glucose and 100 % pure fructose decreased, although the throughputs for these products increased. These results are in agreement with the results obtained from the other systems studied in this thesis.

8.3.2.4 Effect of Enzyme Activity

The results from each pair of reactions carried out at 150 U/cm^3 and 300 U/cm^3 are presented in Table 8.2. It can be observed that the conversion of glucose into fructose and the throughputs for the pure fractions increased with an increase of the enzyme activity level.

By increasing the enzyme activity level the reaction equilibrium is reached faster in the upper parts of the column leaving more column length for the separation of the products formed. The resolution of the products was enhanced and the performance of the system consequently improved.

8.4 Practical Conclusions

The isomerization reaction of glucose into fructose by means of glucose isomerase did not yield good results. In the presence of the enzyme the backward reaction continuously occurred in the column. The profiles obtained for the reactions showed that the glucose peak co-eluted with the fructose peak and a very poor separation for the products was

obtained (see Figure 8.1). This set of experiments confirmed the theory presented in section 2.2.2, regarding the use of liquid chromatographic reactors performing isomerization reactions.

Conversions ranging from 47.9 % to 50 % were obtained with enzyme usage ranging from 42 % to 267 % of that theoretically required to produce the same amount of product in a conventional batch reactor. As the resolution, R_{G-F}, was very low it was not possible to collect either rich glucose or fructose fractions with enhanced glucose and fructose values. However it was possible to reach the conversion obtained at steady state working at of 55 ^{o}C.

When using the mixed substrate-enzyme approach conversions ranging from 25.0 % to 50.3 % were recorded for substrate concentration of 40 % w/v. The enzyme usage ranged from 25 % to 346 % to that theoretically required to produce the same amount of product in a conventional batch reactor.

CHAPTER NINE

MATHEMATICAL MODELLING AND SIMULATION OF THE BATCH CHROMATOGRAPHIC BIOREACTION-SEPARATION PROCESS

9.1 Introduction

The first simplified model proposed for the course of a chemical reaction in a chromatographic column was introduced by Magee (52) in 1963. The theory was based on a power law model and it did not take into account either the axial dispersion or the role of the stationary phase in the process. Gore (64) introduced the participation of the stationary phase in his model developed in 1967. The models presented by Langer (60) *et al.* in 1969 and by Schweich and Villermaux (99) in 1978 included a parameter to account for the dispersion process, although it was first recognised and treated by Lapidus and Amundsen in 1952 (25).

In this thesis, a mathematical model based on the work developed by Zafar (2) was improved by including the axial dispersion term in the continuity equation. The set of differential equations was solved using a finite difference method. A FORTRAN program was developed and the simulations were performed by using a 386 micro computer with a mathematical coprocessor.

The main objective in developing the model was to study the effect of the biochemical reaction on the performance of the system and to optimise the operating conditions, namely the eluent flowrate and the enzyme activity. The simulations were performed for the hydrolysis reactions of sucrose, inulin and lactose studied in this thesis. The simulation for the isomerisation reaction was not accomplished.

The system sucrose-invertase was used to test the model. Simulated profiles were obtained by changing the flowrate and the enzyme activity and compared to the profiles obtained for the experimental runs. Furthermore, the hydrolysis of sucrose was simulated for three different axial distances from the feed port. The simulated profiles obtained showed the development of the chromatographic bands inside the BCBS.

9.2 The Mathematical Model

9.2.1 Introduction

On modelling a batch chromatographic bioreaction-separation process, a difficulty arises from the complexities of the physical and thermodynamical phenomena involved. The

bioreaction itself plays an important role in the process and there is not, at present, a proper model to describe the interaction of all possible variables. A second difficulty arises from the resolution of the equations, especially in the case of non-linear adsorption. The model developed for the batch chromatographic bioreactor-separator included a bioreaction term which will contribute further to the non-linearity of the equations involved.

The modelling approach used the continuity equation derived from a differential mass balance for the mobile phase flowing through a chromatographic column. It corresponded to the semi-ideal differential model which can be considered appropriate for the development of batch chromatographic bioreaction-separation modelling.

9.2.2 Development of the Model

The formulation of the model used simplified hypotheses to handle the problem more easily. These were as follows:

- the chromatographic bed is made of uniformly packed porous, spherical mono-sized, particles
- the bed operates under isothermical conditions
- the radial velocity is assumed to be negligible
- the adsorption of a component is linear and defined by the relation

$$K_d = q/C \tag{9.1}$$

  which is also independent of the concentration of the other components, where q = concentration of solute in the stationary phase and C = concentration of solute in the mobile phase
- the adsorption equilibrium is assumed to be instantaneous
- there is a flat velocity profile (plug flow)
- the reaction will only proceed in the mobile phase and the kinetics follows the inhibited Michaelis-Menten model (see section 2.3).

A material balance for a substrate eluting through a differential section Δz of an ideal chromatographic column, as shown in Figure 9.1, can be expressed as:

Accumulation of solutes in the liquid and stationary phases	-	Net change of solutes due to flow	=	Dispersion of solutes	-	Consumption of substrate or generation of products

where:

Accumulation of solutes in the liquid and stationary phases = $\Delta z\, \varepsilon\, A \frac{\partial C_s}{\partial t} + \Delta z (1-\varepsilon) A \frac{\partial q_s}{\partial t}$

Net change of solutes due to flow = $A \varepsilon U C_z - A \varepsilon U C_{z+\Delta z}$

Dispersion of solutes = $D_{iax} \frac{\partial^2 C_s}{\partial z^2}$

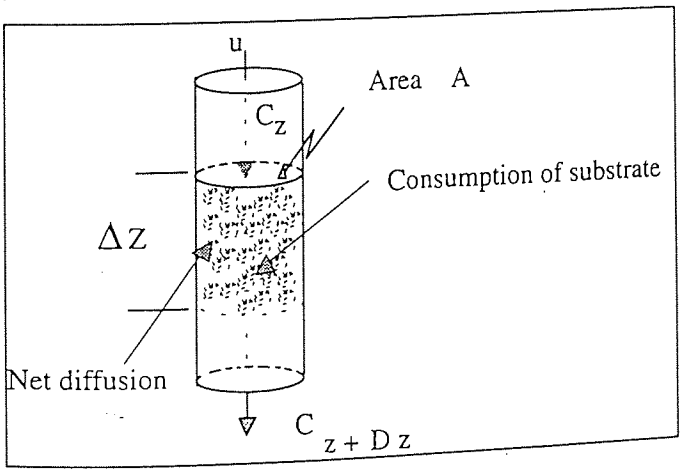
Consumption of substrate or generation of products = $r\, A\, \varepsilon\, \Delta z$, where r represents the reaction rate, expressed by:

$$r = \frac{C_s V_{max}}{C_s + K_M + \frac{C_s^2}{K_i}}$$

From the material balance, the following continuity equation can be written:

$$\left(\frac{1-\varepsilon}{\varepsilon}\right)\frac{\partial q_s}{\partial t} + \frac{\partial C_s}{\partial t} + u_0 \frac{\partial C_s}{\partial z} = D_{iax} \frac{\partial^2 C_s}{\partial z^2} - \frac{C_s V_{max}}{C_s + K_M + \frac{C_s^2}{K_i}} \qquad (9.2)$$

Figure 9.1 - Differential section of an ideal chromatographic column



Equation 9.2 is a second order non-linear partial differential equation and it describes the following phenomena:

- transport by axial dispersion in the mobile phase: $D_{i\,ax}\frac{\partial^2 C_s}{\partial z^2}$
- convective transport in the mobile phase: $u_0\frac{\partial C_s}{\partial z}$
- accumulation in the mobile phase: $\frac{\partial C_s}{\partial t}$
- accumulation in the stationary phase: $(\frac{1-\varepsilon}{\varepsilon})\frac{\partial q_s}{\partial t}$
- consumption of substrate (reaction term): $\frac{C_s V_{max}}{C_s + K_M + \frac{C_s^2}{K_i}}$

Equation 9.2 without the reaction term corresponds to the mass balance equation for a solute flowing through a chromatographic bed (198). The value of V_{max} is directly proportional to the enzyme concentration used for the bioreaction and the following correlation was used to express it (199):

$$V_{max} = k_3 . E_0 \qquad (9.3)$$

k_3 is a proportionality constant and E_0 the initial enzyme concentration. The values for k_3 for the systems sucrose-invertase, inulin-inulinase and lactose-lactase are shown in Table 9.1

The axial dispersion coefficient $D_{i\,ax}$ was related to the Peclet number through the following correlation:

$$N_{Pe} = \frac{D_p . u_0}{D_{i\,ax}} \quad (ref.165) \quad , \quad u = \frac{u_0}{\varepsilon} \quad \text{and} \quad \varepsilon . N_{Pe} = 0.2031 \ (ref.165) \qquad (9.4)$$

For an average particle size of 200 μm,

$$D_{i\,ax} = 9.487 \text{ x } 10\text{-}4 . u \qquad (9.5)$$

The eluent interstitial velocity u_0 in the convective transport term in the continuity equation 9.2 was expressed by the following correlation:

$$u_0 = \frac{Q_e}{S \cdot \varepsilon} \tag{9.6}$$

Using 9.1, 9.3, 9.5 and 9.6 in equation 9.2, yields:

$$\alpha_S \frac{\partial C_S}{\partial t} + u_0 \frac{\partial C_S}{\partial z} + \frac{C_S V_{max}}{C_S + K_M + \frac{C_S^2}{K_i}} - D_{iax} \frac{\partial^2 C_S}{\partial z^2} = 0 \tag{9.7}$$

where $$\alpha_S = \left(\frac{\varepsilon + (1-\varepsilon)}{\varepsilon} K_{dS}\right) \tag{9.8}$$

Similar equations can be written for the formation of glucose and fructose.

For glucose:

$$\alpha_G \frac{\partial C_G}{\partial t} + u_0 \frac{\partial C_G}{\partial z} - Coef_G \frac{C_G V_{max}}{C_G + K_M + \frac{C_G^2}{K_i}} - D_{iax} \frac{\partial^2 C_G}{\partial z^2} = 0 \tag{9.9}$$

where $$\alpha_G = \left(\frac{\varepsilon + (1-\varepsilon)}{\varepsilon} K_{dG}\right) \tag{9.10}$$

and $Coef_G$ is the stoichometric coefficient for glucose.

For fructose:

$$\alpha_F \frac{\partial C_F}{\partial t} + u_0 \frac{\partial C_F}{\partial z} - Coef_F \frac{C_F V_{max}}{C_F + K_M + \frac{C_F^2}{K_i}} - D_{iax} \frac{\partial^2 C_F}{\partial z^2} = 0 \tag{9.11}$$

where $$\alpha_F = \left(\frac{\varepsilon + (1-\varepsilon)}{\varepsilon} K_{dF}\right) \tag{9.12}$$

and $Coef_F$ is the stoichometric coefficient for fructose.

The model assumed that the substrate pulse entering the batch chromatographic bioreactor-separator contains enzyme with the same activity as the eluent. In practice, however, the pulse does not contain any enzyme. As the pulse enters the reactor the enzyme already present in the column will gradually mix with it. To account for the variation of the enzyme concentration, the following correlation was introduced in the model (2):

$$V'_{max} = V_{max} \left(1 - \frac{Vol}{Void}\right) \tag{9.13}$$

The following initial and boundary conditions were used:

Initial conditions: C_S (0,t) = pulse concentration, C_G (0,t) = 0 and C_F (0,t) = 0

Boundary conditions: C_S (z,0) = 0, C_G (z,0) = 0 and C_F (z,0) = 0

9.2.3 Numerical Solution of the Model

The finite difference analysis method has proved to be a suitable way of solving the ideal model equation. However, it results in chromatographic profiles affected by a so called numerical diffusion, due to the finite value of the time and space increments of the discretization as observed by Bellot and Condoret (198). This artificial diffusion may be used to simulate the band broadening due to the resistance to mass transfer and axial dispersion. The procedure consists in adjusting the length of the space and time increments of the integration, so that the numerical diffusion thus created equals an axial dispersion reported by Lenhoff (200).

The concept of the propagation through a grid as presented by Czok and Guiochon (201) was adopted. There are two different ways to replace each of the first order partial derivatives in equation 9.7 by finite difference backward and forward quotients (202). The diffusion term, the second order partial derivative, is replaced by a central difference type. For this purpose three points in the grid should be used. They are located at the following positions: (z-1, t), (z, t-1) and (z-1, t-1). Since two positions in the column are used, the change in concentration can be given for both locations. Likewise, for the gradient concentration, both times can also be used:

$$(a)\ \frac{\partial C_s}{\partial z} = \frac{C_s(z,t) - C_s(z-1,t)}{\Delta z} \qquad (b)\ \frac{\partial C_s}{\partial z} = \frac{C_s(z,t-1) - C_s(z-1,t-1)}{\Delta z} \tag{9.14}$$

$$(c)\ \frac{\partial C_s}{\partial t} = \frac{C_s(z,t) - C_s(z,t-1)}{\Delta t} \qquad (d)\ \frac{\partial C_s}{\partial t} = \frac{C_s(z-1,t) - C_s(z-1,t-1)}{\Delta t} \tag{9.15}$$

This allows three different combinations that can be inserted into the differential equation. The equation is then rearranged to isolate the new concentration in terms of C_S (z, t). The combination (b) and (d) is to be avoided since neither terms contains C_S (z, t).

The central difference used to express the diffusion term was:

$$\frac{\partial^2 C_s}{\partial z^2} = \frac{C_s(z+1,t) - 2C_s(z,t) + C_s(z-1,t)}{\Delta x^2} \tag{9.16}$$

Using the relations (b) from 9.14, (c) from 9.15 and 9.16 and expressing the sucrose concentration in terms of C_S (z, t + 1) the equation for the sucrose profile becomes:

$$C_S(z,t+1) = C_S(z,t) - \varphi_S\,(C_S(z,t) - C_S(z-1,t))$$

$$-\beta_S \cdot \frac{V_{max} \cdot C_S(z,t)}{K_M + C_S(z,t) + \dfrac{(C_S(z,t))^2}{K_i}}$$

$$+\frac{\Delta t \cdot 9.847 \cdot 10^{-4} \cdot u_0}{\alpha_s} \cdot (C_S(z+1,t) + 2\,C_S(z,t) - C_S(z-1,t)) \qquad (9.17)$$

where $$\varphi_S = \frac{\Delta t \cdot u_0}{\Delta z \cdot \alpha_S} \qquad (9.18)$$

$$\beta_S = \frac{\Delta t}{\alpha_S} \qquad (9.19)$$

For glucose:

$$C_G(z,t+1) = C_G(z,t) - \varphi_G\,(C_G(z,t) - C_G(z-1,t))$$

$$-\beta_G \cdot Coef_G \cdot \frac{V_{max} \cdot C_S(z,t)}{K_M + C_S(z,t) + \dfrac{(C_S(z,t))^2}{K_i}}$$

$$+\frac{\Delta t \cdot 9.847 \cdot 10^{-4} \cdot u_0}{\alpha_G} \cdot (C_G(z+1,t) + 2\,C_G(z,t) - C_G(z-1,t)) \qquad (9.20)$$

where $$\varphi_G = \frac{\Delta t \cdot u_0}{\Delta z \cdot \alpha_G} \qquad (9.21)$$

and $$\beta_G = \frac{\Delta t}{\alpha_G} \qquad (9.22)$$

And for fructose:

$$C_F(z,t+1) = C_F(z,t) - \varphi_F\,(C_F(z,t) - C_G(z-1,t))$$

$$-\beta_F \cdot Coef_F \cdot \frac{V_{max} \cdot C_S(z,t)}{K_M + C_S(z,t) + \dfrac{(C_S(z,t))^2}{K_i}}$$

$$+\frac{\Delta t \cdot 9.847 \cdot 10^{-4} \cdot u_0}{\alpha_F} \cdot (C_F(z+1,t) + 2\,C_F(z,t) - C_F(z-1,t)) \qquad (9.23)$$

where $$\varphi_F = \frac{\Delta t \,.\, u_0}{\Delta z \,.\, \alpha_F} \qquad (9.24)$$

and $$\beta_F = \frac{\Delta t}{\alpha_F} \qquad (9.25)$$

9.3 Computer Program

A FORTRAN program was written to calculate the concentrations of sucrose, glucose and fructose in relation to time along the column length. The FORTRAN compiler used was developed at Salford University (203). The program can run on personal computers based on the 80386 (or higher) processors. The flowchart of the program is presented in Appendix A-9, the list of symbols used in the program is presented in Appendix A-10 and the listing of the program is presented in Appendix A-11. The program included graphic routines that allow the profiles to be ploted on the screen of the computer monitor and a typical profile obtained is shown in Appendix A-12.

The program uses three grids of z rows by t columns, where z and t stand for distance and time, respectively. Each grid relates to a solute as reported by Bellot and Condoret (198). The unknown concentrations of the solutes sucrose, glucose and fructose are calculated by the equations 9.17, 9.20 and 9.23, starting from the values assigned to all cells located in the first row for all columns: cells $C_S(0,t)$, $C_G(0,t)$ and $C_F(0,t)$. The values used corresponded to the initial and boundary conditions. The first calculation step was performed for the second row of cells, from each grid, regarding an increment Δz for the distance and Δt for the time interval. The calculation proceeded until the value for the last row, representing the column outlet was known.

As observed by Zafar (2), Akintoye (3) and Sarmidi (5) the sucrose conversion calculated through the simulation did not match the actual conversion obtained experimentally. The distortion can be explained in terms of physical adsorption and denaturation of the enzyme. To accommodate these effects the value for the proportionally constant, k_3, was corrected. The value used throughout the simulations was 5.0×10^{-4} g U^{-1} s^{-1}, instead of 5.77×10^{-4} as originally calculated. The value assigned for the proportionally constant k_3 has not been corrected for simulations performed for the systems inulin-inulinase and lactose-lactase.

For the simulations of the systems inulin-inulinase and lactose-lactase the same program was used because the development of the model considered an enzymatic reaction transforming one substrate in two products (see section 9.2.2). The typical basic operating data used for the simulation are presented in Table 9.1. The distribution coefficients

presented are those obtained at infinite dilution reported in section 4.3.2.2. The computer program, however, corrects these values for the background concentrations according to the relations presented in section 4.3.3.4.

Table 9.1 - Typical basic operating parameters used for the simulation

Parameters	Units	Value		
		System sucrose-invertase	System inulin-inulinase	System lactose-lactase
k_{dS}	-	0.10	-	-
k_{dInu}	-	-	0.19	-
k_{dL}	-	-	-	0.08
k_{dG}	-	0.18	0.24	0.18
k_{dF}	-	0.47	0.41	-
k_{dGal}		-	-	0.30
V_{max}	g cm^{-3} s^{-1}	4.33×10^{-3}	4.33×10^{-3}	9.67×10^{-5}
K_M	g cm^{-3}	0.02	0.02	0.05
K_i	g cm^{-3}	variable	variable	variable
$Coef_G$	-	0.526	0.017	0.526
$Coef_F$	-	0.526	0.594	-
$Coef_{Gal}$	-	-	-	0.526
k_3	g U^{-1} s^{-1}	5.0×10^{-4}	1.44×10^{-3}	1.93×10^{-5}
H	cm	198	198	198
Φ	cm	1.96	1.0	1.96

9.4 Simulation Results

9.4.1 Introduction

Although the model did not include the mass transfer effect, this could be generated by the proper selection for the distance and time increment values (204-206). A numerical diffusion effect was created by the distance and time increments introduced in the equations 9.19, 9.22 and 9.25 as observed by Bellot and Condoret (201).The numerical diffusion was then controlled in such way that the profiles generated by the mathematical solution could match the experimental profiles. Sucrose has the lowest mass transfer coefficient compared to glucose and fructose (79). The experimental profile for sucrose was then selected to estimate the dispersion effect due to mass transfer resistance.

For this purpose, the simulation program for the reaction-separation was transformed into a model for the physical separation. This was accomplished by removing the reaction term

and including the initial and boundary conditions for both glucose and fructose. The next step was the selection for the proper discretised values.

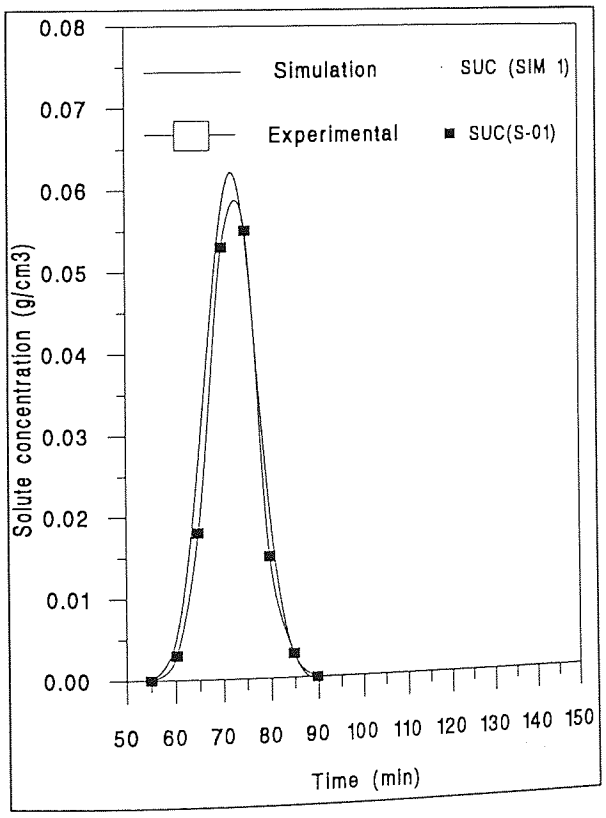
On considering that the mobile phase is moved by exactly one Δz for every time unit Δt, the following relation can be written (197):

$$u_0 = \frac{\Delta z}{\Delta t} \tag{9.26}$$

The height equivalent of a theoretical plate (HETP) for sucrose was selected as the distance increment Δz. Figure 9.2 shows a very good agreement between the experimental and the simulated profiles at Δz = 0.5 cm and Δt = **10** s. The simulations performed through the mathematical model developed in this chapter used these values for the discretised values.

Simulation runs were performed to investigate the effects of enzyme activity on the performance of the batch chromatographic bioreactor-separator and also to study its behaviour at different flowrates.

Figure 9.2 - Comparison of the simulated profile with the experimental profile for run S(01)-Syn-5-10S-10G-10F-3.5



9.4.2 Effect of Enzyme Activity

The comparison of the experimental and simulated elution profiles for the reactions RS(12)-SUC-10-40-100-3.5 and RS(09)-SUC-10-40-150-3.5 are presented in Figures 9.3 and 9.4, respectively. The simulated elution profiles showed good agreement with the experimental profiles. This can be observed by analysing the shape of the peaks and the resolutions obtained for both products, both for the simulated and the experimental runs. The resolutions R_{G-F} between glucose and fructose for the simulation profiles for runs RS(12)-SUC-10-40-100-3.5 and RS(09)-SUC-10-40-150-3.5 were 0.35 and 0.44 respectively, as compared to the experimental R_{G-F} values of 0.31 and 0.39. The values obtained confirmed that the correction introduced for the value of V_{max} in the mathematical model was satisfactory (see section 9.2.2)

The model confirmed that by increasing the enzyme activity the reaction rate increased and the products are formed in the upper parts of the chromatographic column rather than in the lower parts of it. Less column length was used for the reaction to take place and, therefore, most of it was used for the separation. The resulting peaks were narrower and sharper and the resolution R_{G-F} between glucose and fructose enhanced.

Figure 9.3 - Comparison of experimental and simulated elution profiles for run RS(12)-SUC-10-40-100-3.5

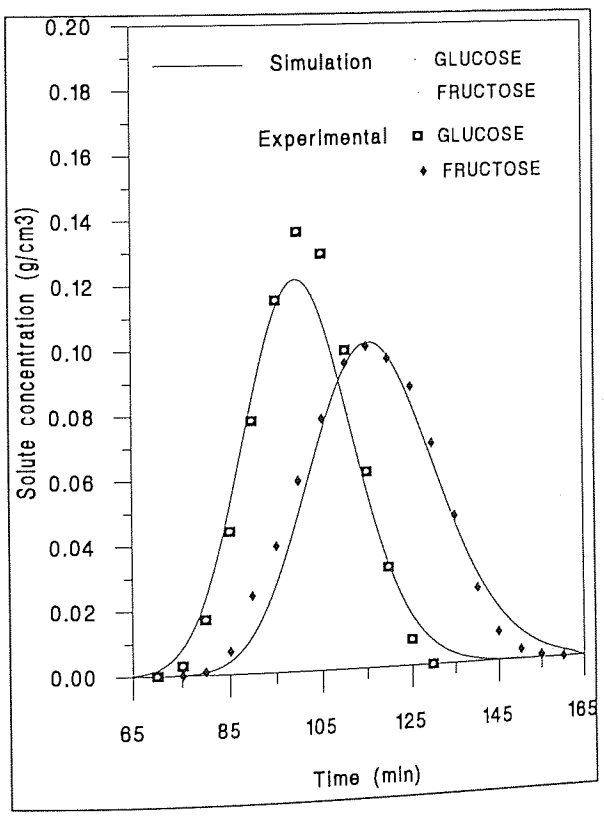
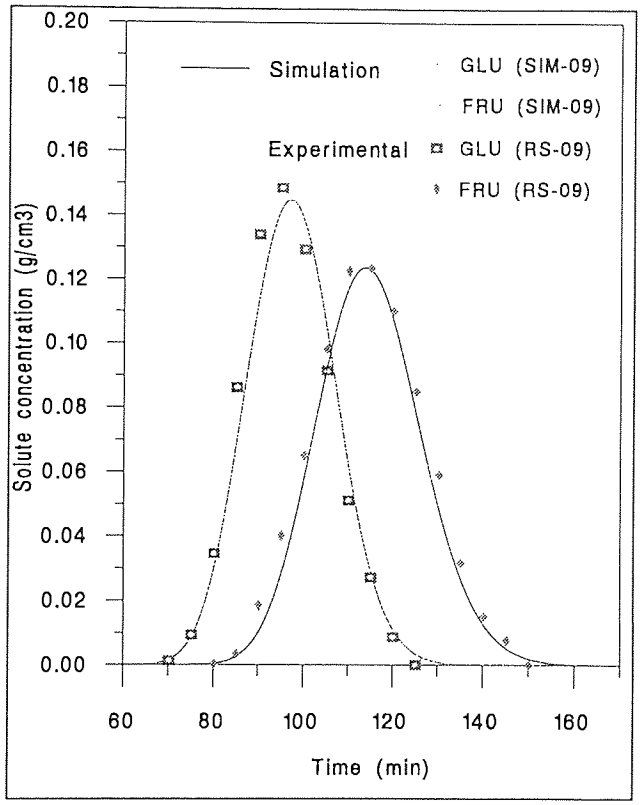


Figure 9.4 - Comparison of experimental and simulated elution profiles for run RS(09)-SUC-10-40-150-3.5



The model was also used to simulate a batch chromatographic bioreaction-separation for three levels of enzyme activity, using a sucrose solution with a concentration of 40 % w/v. The pulse size employed was 10 % TECV. The superficial velocity was fixed at 1.16 cm/min corresponding to a flowrate of 3.5 cm^3/min. The simulation was performed using eluents with enzyme activity of 50 U/cm^3, 100 U/cm^3 and 150 U/cm^3, respectively. The resulting profiles for the simulation are presented in Figure 9.5 for the enzyme levels of 50 U/cm^3 and 100 U/cm^3 and in Figure 9.6 for the enzyme levels of 100 U/cm^3 and 150 U/cm^3. The R_{G-F} values for the resolution between glucose and fructose were 0.22, 0.35 and 0.44 for each level of the enzyme activity used. As expected, at lower enzyme activities the profiles were broader. On the other hand, lower resolutions and product concentrations were obtained.

Figure 9.5 - Profiles for the simulation using eluent with enzyme activity of 50 U/cm^3 and 100 U/cm^3

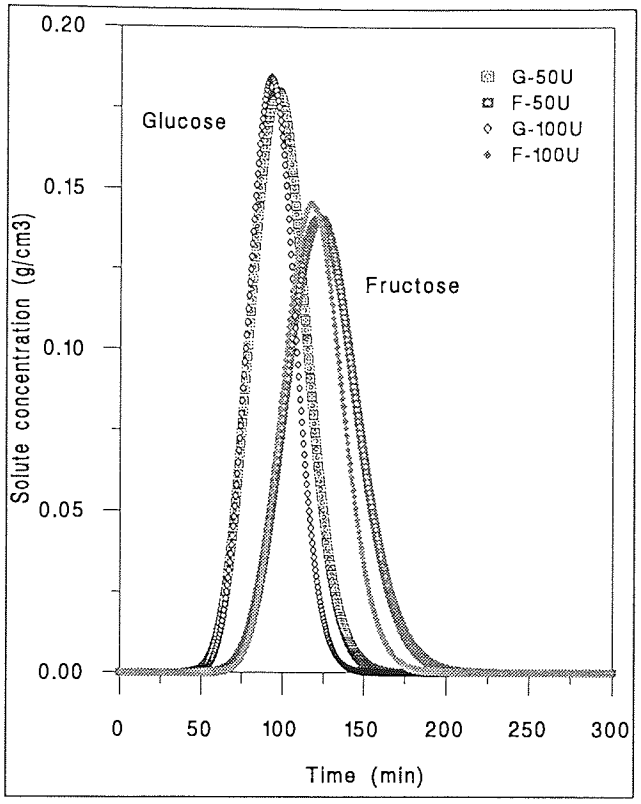
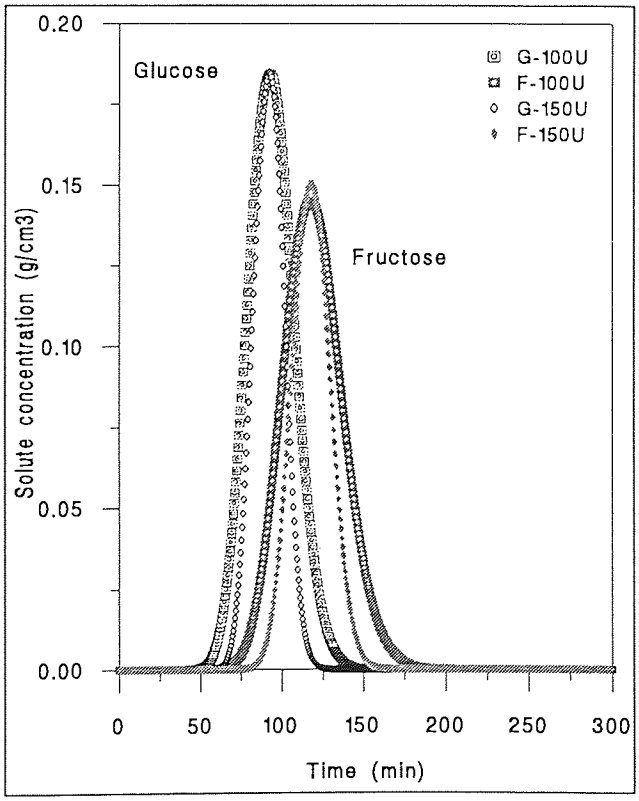


Figure 9.6 - Profiles for the simulation using eluent with enzyme activity of 100 U/cm^3 and 150 U/cm^3

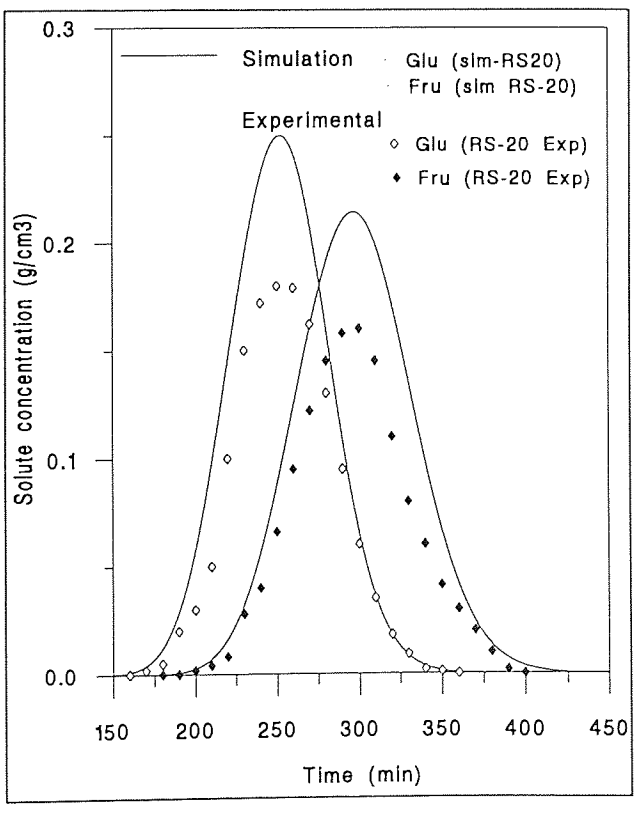


9.4.3 Effect of Flowrate

The effect of flowrate was simulated and the results for the levels of 1.4 cm^3/min and 3.5 cm^3/min are presented in Figures 9.4 and 9.7, respectively, for runs RS(09)-SUC-10-40-150-3.5 and RS(20)-SUC-10-40-150-1.4. The results showed enlargement of the profiles at lower flowrates confirming the experimental results. The effect of decreasing the flowrate produced an enhancement on the resolution R_{G-F} between glucose and fructose. This could be due to the decrease of the HETP for both products, resulting in an increase of the number of theoretical plates.

On comparing the experimental profiles from runs RS(09)-SUC-10-40-150-3.5 and RS(20)-SUC-10-40-150-1.4 with the simulated profiles the model indicates poor predictions at lower flowrates concerning the peak heights. This could be due to the inadequate mixing of the substrate with the enzyme assumed by the model. The chromatograms showed that the model's predictions of retention times for both flowrates are very accurate especially at high eluent flowrates. Also, the predicted peak shapes match quite well with the experimental ones.

Figure 9.7- Simulation and experimental profiles for run RS(20)-SUC-10-40-150-1.4



9.4.4 Simulation of the Performance of the Batch Chromatographic Bioreactor-separator at Different Column Heights

The simulation profiles for run RS(12)-SUC-10-40-100-3.5 at three different column heights from the injection port are shown in Figure 9.8 and 9.9. The behaviour of the bioreaction-separation inside the chromatographic column can be observed by analysing the profiles for the products at column heights of 20 cm, 60 cm, 100 cm and 198 cm. The column height of 198 cm corresponds to the column outlet.

At a distance of 20 cm from the feed port, most of the substrate was still present but diluted as it had been mixed with the eluent. The conversion of substrate was 18 % at this point. The products, glucose and fructose, were being formed in the front end of the sucrose profile. A slight separation of glucose from fructose was noticed.

As the substrate travelled down the column, swept by the eluent, the reaction proceeded further and at a distance of 60 cm from the feed port, shown in Figure 9.9, the conversion of sucrose reached 74 %. At this point a better separation of glucose from fructose was observed and it was confirmed that sucrose eluted first as its retention time was slightly lower than that of the other products. There was not enough column length for an efficient separation of glucose from fructose.

Further down the column, at a distance of 100 cm from the feed port, the conversion reached 94 % (see Figure 9.8). At this point the separation of glucose from fructose was noticeable. The sucrose profile developed under the profiles of glucose and fructose. The sucrose profile presented a triangular shape and the glucose and fructose peaks were broader. The fructose profile presented a very slight tail.

At the column outlet, the reaction was complete as sucrose was not detected and the separation of the products reached its maximum resolution. As expected, the glucose profile was narrower and presented a smaller bandwidth than the fructose profile.

Figure 9.8 - Simulated concentration profiles for run RS(12)-SUC-10-40-100-3.5 at column lengths of 20 cm, 100 cm and 198 cm

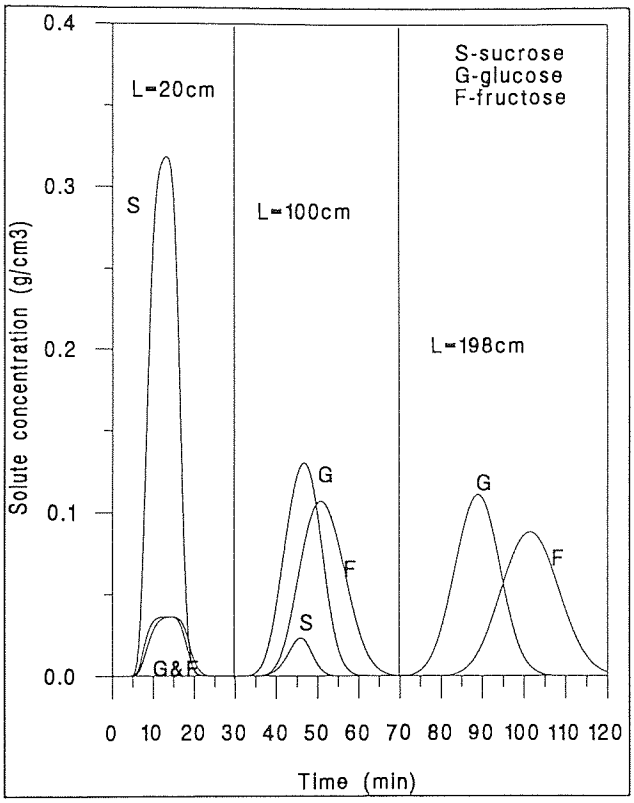
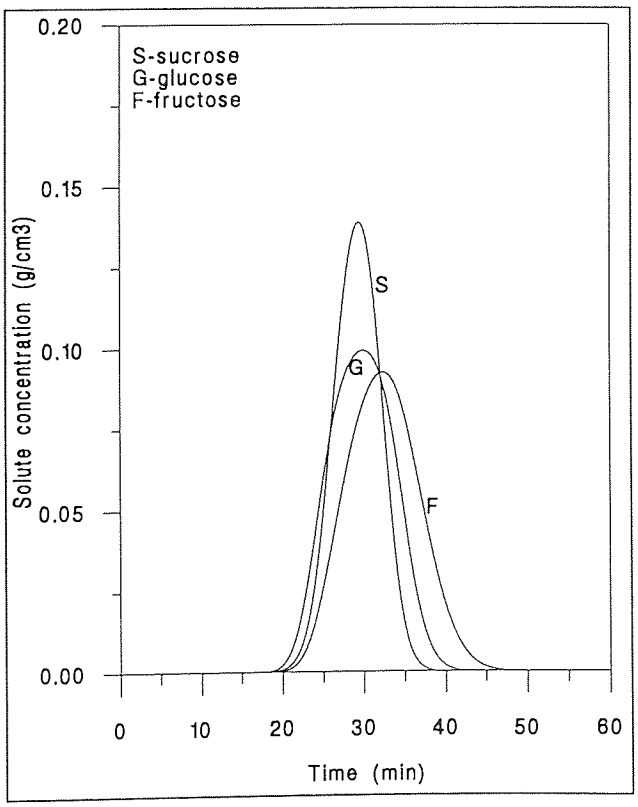


Figure 9.9 - Simulated concentration profiles for run RS(12)-SUC-10-40-100-3.5 at a column length of 60 cm



9.4.5 Simulation of the Systems Inulin-Inulinase and Lactose-Lactase

The simulation of the systems inulin-inulinase and lactose-lactase were performed using the same program developed for the system sucrose-invertase as explained in section 9.3. The comparison of the experimental and simulated elution profiles for the reactions RS(15)INU-5-10-25-0.91 and RS(01)LAC-5-5-60-3.5 are shown in Figures 9.10 and 9.11 respectively.

Figure 9.10- Simulation and experimental profiles for run RS(15)INU-5-10-25-0.91

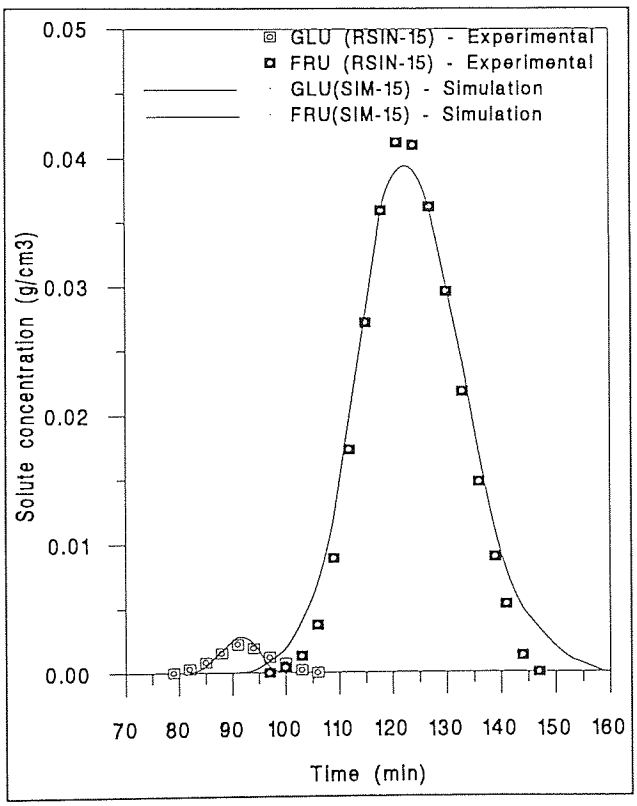
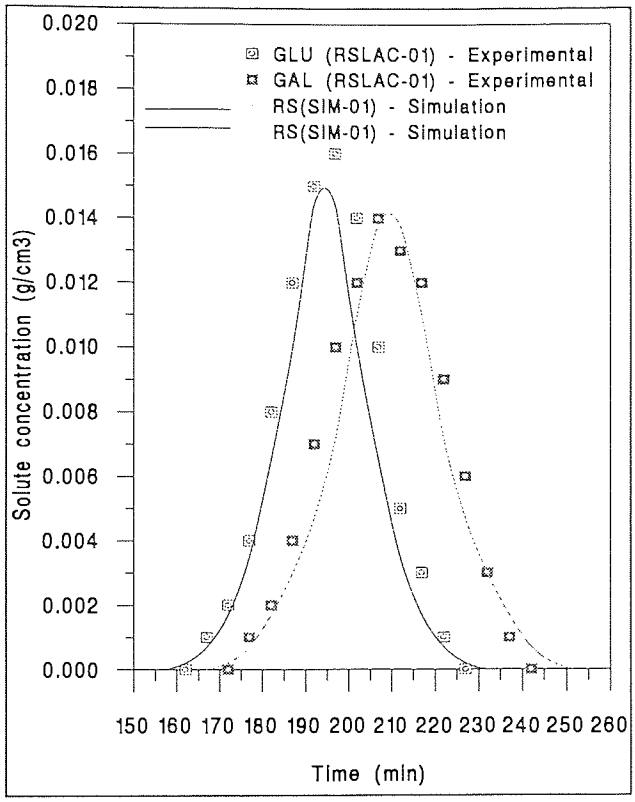


Figure 9.11- Simulation and experimental profiles for run RS(01)LAC-5-5-60-3.5



CHAPTER TEN

CONCLUSIONS AND RECOMMENDATIONS

10.1 Conclusions

This research has confirmed that it is possible to conduct simultaneous bioreaction and separation in batch chromatographic columns and obtain several product fractions of known composition, depending on the time interval selected for the collection.

It was observed, however, that the separation performance of the system when operating as a bioreactor-separator was diminished as compared to the performance of the column when operating in the separation mode. By operating in the bioreaction-separation mode part of the column was used for the bioreaction and the remaining part for the separation of the products formed.

System Sucrose-Invertase

On working on the inversion of sucrose by means of the enzyme invertase, complete conversions were achieved employing pulse size and pulse concentration up to 20 % TECV and 60 % w/v, respectively. Under these conditions a glucose rich fraction and a fructose rich fraction, with purities up to 90 % and representing about 50 % of the products formed were obtained. The enzyme consumption was 45 % of the amount theoretically required to produce the same quantity of product in a conventional batch reactor. Values of up to 27 kg sucrose/m^3 resin/h for the throughput of the system were achieved. Both rich fractions produced could be of interest to the high fructose syrup industry.

The system was thoroughly investigated by means of a factorial experimental procedure and it was observed that an increase in pulse size, pulse concentration and flowrate affected significantly the performance of the BCBS. The interaction of pulse concentration and enzyme concentration had the most significant effect on the peak concentration for the products.

System Inulin-Inulinase

In studying the hydrolysis of inulin by the enzyme inulinase, complete conversions were achieved employing pulse size and pulse concentration of up to 20 % TECV and 10 % w/v, respectively. Under these conditions two rich fractions were obtained: a glucose fraction with 95 % purity containing 100 % of the total glucose formed and a fructose fraction with

100 % purity containing 95 % of the total fructose formed. The enzyme consumption was 32 % of the amount theoretically required to produce the same amount of product in a conventional batch reactor. A value of up to 3.4 kg inulin/m^3 resin/h for the throughput of the system was achieved. Both rich fractions produced could also be of interest to the high fructose syrup industry.

The bioreaction-separation of inulin by inulinase was also performed by pulsing a mixture of enzyme and substrate into the chromatographic column. When using this approach 50 % of the substrate was left unreacted. In this case, only 50 % of the total fructose formed was collected as a 100 % fructose rich fraction whilst 100 % of the glucose produced was collected as a 95 % rich fraction. The enzyme consumption was 16.2 % of the amount theoretically required to produce the same amount of product in a conventional batch reactor. A throughput of up to 3.4 kg inulin/m^3 resin/h for the system was achieved.

System Lactose-Lactase

The BCBS operating under concerted bioreaction and separation conditions for the hydrolysis of lactose by lactase did not achieve a good separation for the reaction products glucose and galactose. The galactose molecules do not present a proper arrangement of the axial-equatorial-axial sequence of the hydroxyl groups to be efficiently complexed by the calcium ions as compared to fructose. On analysing the data obtained for the system lactose-lactase it was observed that a rich galactose fraction with 100 % purity, representing 38 % of the galactose generated, could be obtained. A glucose rich fraction with 100 % purity, representing 37.9% of the glucose generated could also be obtained. This confirms that the process makes it possible to recover both reaction products, in contrast with traditional galactose production. In the traditional process, glucose is removed by further fermentation with yeasts and is therefore not recovered.

System Glucose-Glucose Isomerase

In studying the system glucose-glucose isomerase, which is a reversible reaction, the separation obtained with the stationary phase conditioned in the magnesium form was very poor although the conversion obtained was compatible with that achieved in conventional batch reactors. As the resolution for the products glucose and fructose, R_{G-F}, was very low, it was not possible to collect either a glucose rich fraction or a fructose rich fraction with enhanced glucose and fructose values.

Mathematical Modelling and Computer Simulation

A mathematical model to simulate batch chromatographic bioreaction-separations has been developed. The modelling approach used the continuity equation derived from a differential mass balance for the mobile phase flowing through a chromatographic column. The axial dispersion and the Michaelis-Menten kinetic model with substrate inhibition was incorporated in the model. A finite difference method was used to solve the set of partial differential equations and a FORTRAN program developed for the algorithm. The computer simulation was performed on a personal computer and the results showed good agreement with some of the experimental results for the systems sucrose-invertase, inulin-inulinase and lactose-lactase. The simulation was not performed for the isomerization reaction.

10.2 Recommendations for future Work

Selection of Enzyme-Substrate Systems

The selection of systems to be carried out in the BCBS should be the subject of a systematic screening. Cooperation with other research groups within the University and with enzymes manufacturers should be considered. Work with non-aqueous systems, other stationary phases and the use of immobilized enzymes should be investigated.

Suggestions for Other Systems Worthy of Study

- Hydrolysis of pentosans with xylanases for the production of xylose, using a cation exchange resin in the Ca^{2+} form.
- Hydrolysis of modified starch with cyclodextrin transglycosylase for the production of cyclodextrin, using strongly crosslinked dextran as stationary phase.
- Hydrolysis of triglycerides with lipases.
- Oxidation of sorbitol by oxidase for the production of sorbose, an important intermediate of vitamin C synthesis.
- Isomerization of lactose to lactulose by means of the enzyme lactulase. Lactulose is used as a raw material in the pharmaceutical sector
- Isomerization of sucrose by isomaltulase for the production of isomaltulose. This product is also employed in the pharmaceutical industry.

New Approaches for Conducting Batch Chromatographic Bioreaction-Separations

The chromatographic bioreaction-separations performed in this research have been carried out by maintaining the pulse flowrate constant and at the same level as the flowrate used for the elution. The work with higher flowrate values for the pulse should be investigated. In doing so, the enzyme and substrate could be more efficiently mixed, with the possibility of the reaction being enhanced. In consequence, more column length could be available for separation purposes.

The mixed pulse of substrate and enzyme approach used for the systems inulin-inulinase and glucose-glucose isomerase should be investigated further.

Isomerization of Glucose to Fructose

Investigation of the isomerization of glucose in a BCBS using the moving reaction zone approach should be performed (see section 2.2.2 relating to isomerization reactions). Another possible approach to conduct isomerizations would be repetitive injections of enzyme after each pulse of substrate.

Evaluation of performance

Criteria for comparison of the performance of chromatographic bioreactor-separators systems should be developed. Attention should be given to economic evaluation, including raw material costs (substrates and enzymes) and evaporation costs.

Modelling

The model should be revised taking into account the intraparticle diffusion in the micropores of the stationary phase. The interaction effects of multicomponent systems should be included in the model.

NOMENCLATURE

A	cross section area of the chromatographic column
C'	dimensionless concentration
C_i	concentration of a component *i* in the mobile phase
C_k*	concentration of a component *k* equilibrated to C_k in Hashimoto's intermittent moving-bed model
Coef	Stoichometric coefficients for the products formed in the hydrolysis reactions (the indexes g, f and gal stand for glucose, fructose and galactose, respectively)
d_1	density of the resin bead
d_2	density of the fluid used for the elutriation
$D_{i\,ax}$	axial dispersion coefficient
e	base of the natural logarithm
E_o	initial enzyme concentration
F	percentage of Fructose recovered with 100 % purity
$F_{s/m}$	relationship between concentration of solute in the mobile phase and the stationary phase
FRP	fructose rich product
G	percentage of Glucose recovered with 100 % purity
g	acceleration due to gravity
GRP	glucose rich product
h	height of a chromatographic peak
HETP	height equivalent to a theoretical plate
J	number of cells in Martin and Synge model
K^{∞}_{dA}	distribution coefficient at infinite dilution for solute A
K^{∞}_{dB}	distribution coefficient at infinite dilution for solutes B
K_2	capacity factor of the most retarded component
k_0	mass transfer coefficient in Martin and Synge model
k_3	proportionality constant relating maximum initial reaction velocity and enzyme concentration
K_{di}	distribution coefficient for a component i
K_{eq}	equilibrium constant for isomerization reactions
K_i	inhibition constant
K_M	Michaelis constant

K^{P}_{M}	Michaelis constant for the backward (or reverse) reaction
K^{S}_{M}	Michaelis constant for the forward reaction
L	column length
m_k	distribution coefficient for component k in Hashimoto's intermittent moving-bed model
$m_{suc}(inj)$	mass of sucrose injected
$m_{suc}(res)$	residual mass of sucrose
N*	"apparent" number of plates for a chromatographic column
N_{Pe}	Peclet number
NTP	number of theoretical plates
[P]	product concentration
$[P]_{eq}$	product concentration at equilibrium for isomerization reactions
Pk_F	peak concentration of fructose
Pk_G	peak concentration of glucose
Q	volumetric flowrate
Q_e	eluent flowrate
q_i	concentration of a component in the stationary phase
r	reaction rate (sometimes referred to ν)
R_S (or R_{1-2})	resolution between components 1 and 2
Re	Reynolds number
R_{elut}	radius of the elutriator chamber
r_p	radius of the particle (ion exchange bead)
[S]	substrate concentration
$[S]_{eq}$	substrate concentration at equilibrium for isomerization reactions
t	time interval in a grid
TECV	total empty column volume
T_F	rich fructose fraction throughput
T_G	rich glucose fraction throughput
t_{ri}	retention times for the component i
u	eluent superficial velocity
u_0	eluent interstitial velocity
u_s	hypothetical rate movement of the adsorbent particles in Hashimoto's continuous moving bed model
V	volume of the mobile phase
V_{up}	upward linear velocity in an elutriator chamber

V_0	total void volume in the column occupied by the mobile phase
V_i	volume of mobile phase required for the complete elution of a component
V_{max}	maximum reaction rate
V^P_{max}	maximum reaction rate for the substrate formation (reverse or backward reaction)
V_s	volume of the stationary phase
V^S_{max}	maximum reaction rate for product formation (forward reaction)
V_T	total volume of the column
W_i	peak width measured at the base of the elution curve for component i
$W_{h/e}$	peak or band width measured at a height equal the relation h/e
x	dimensionless distance
z	position in a grid

Greek letters

μ	viscosity of the fluid
τ	dimensionless time
α_{A-B}	separation factor for products A and B
ε	voidage of the chromatographic bed
ρ	fluid density in Reynolds number relation
θ	angular co-ordinate for annular chromatographs
ω	rotation rate of annular chromatographs
ν	reaction rate
ν_f	superficial velocity of fluid in Reynolds number relation
ν_n	superficial velocity in Hashimoto's intermittent moving bed model
Δp	pressure drop of a chromatographic bed
Δt_{samp}	sampling time interval
Δz	differential section of an ideal chromatographic column

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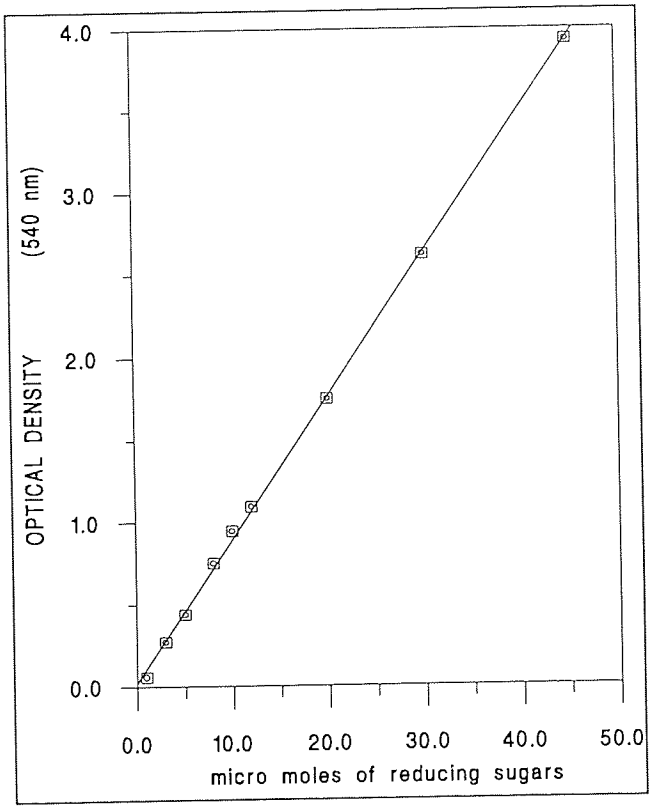
APPENDIX A-1

Adsorbance Curve for the DNS Method for Invertase Assay



APPENDIX A-2

Effects of Background Concentrations on Distribution Coefficients

Figure A-2-1 - Effect of glucose background concentration on the distribution coefficient of fructose

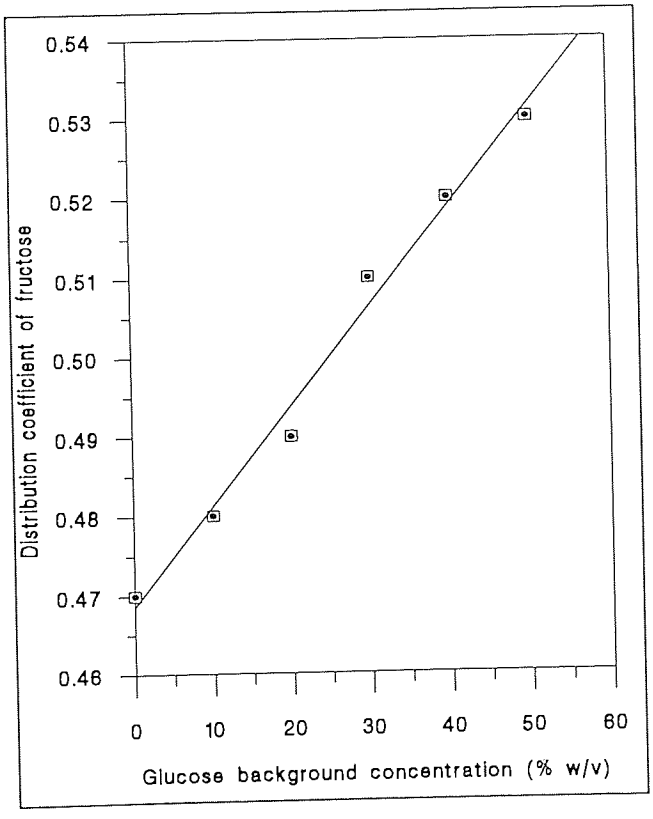


Figure A-2-2 - Effect of fructose background concentration on the distribution coefficient of glucose

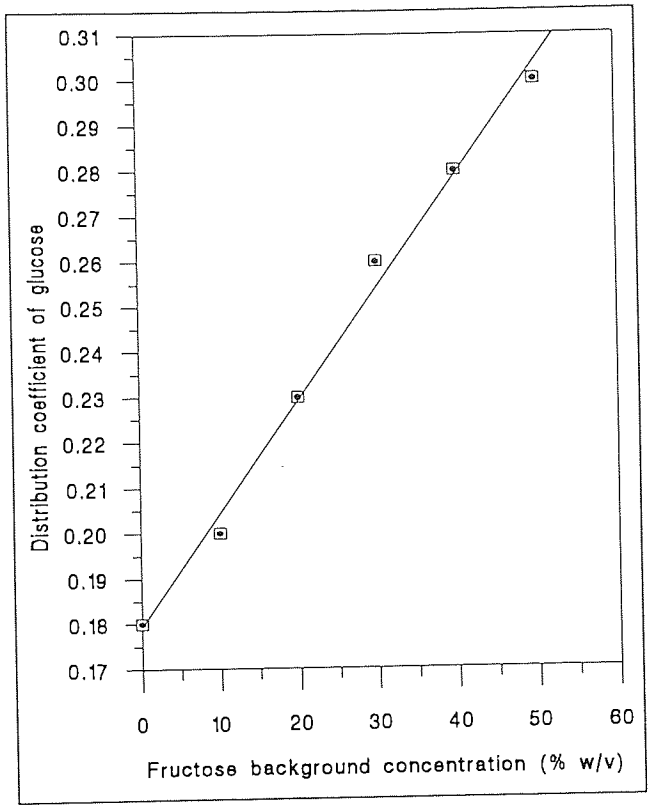


Figure A-2-3 - Effect of glucose background concentration on the distribution coefficient of galactose

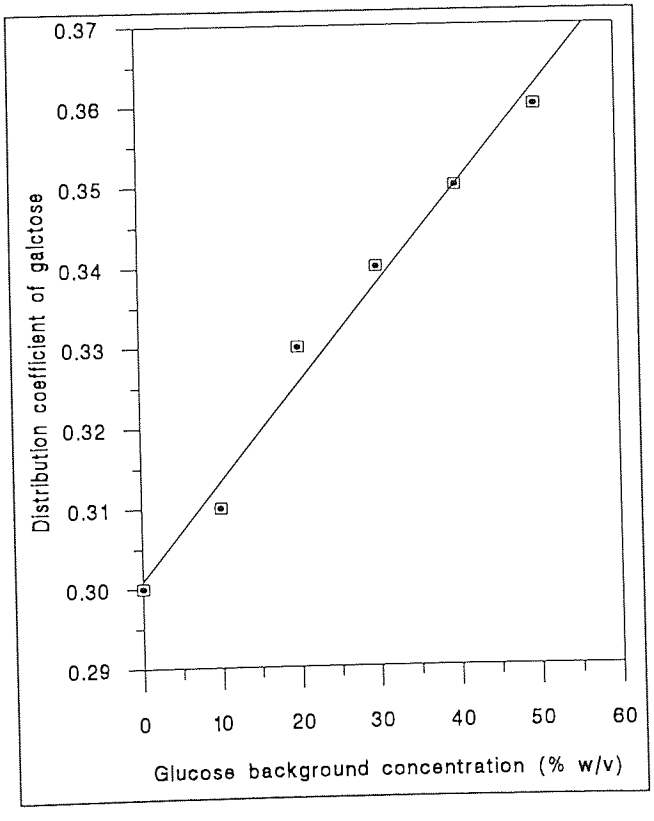
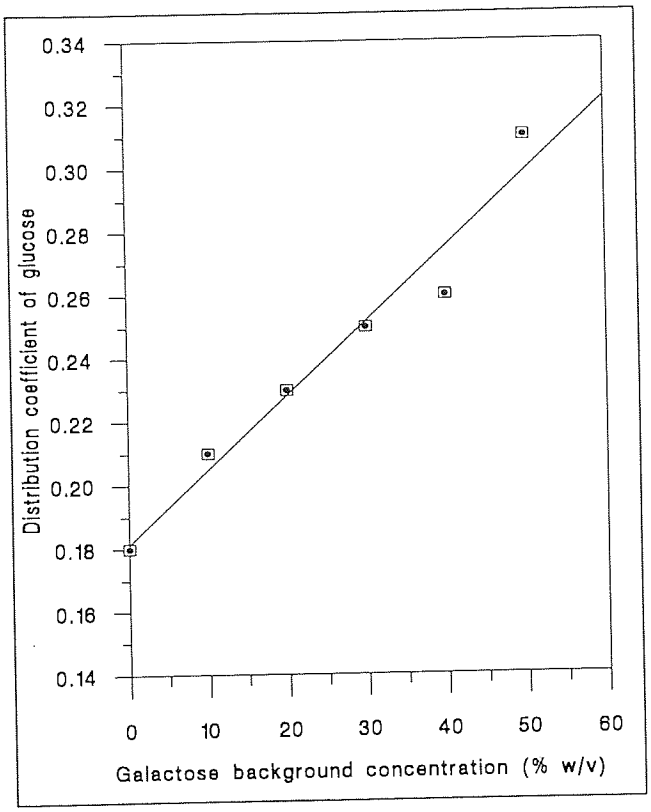


Figure A-2-4 - Effect of galactose background concentration on the distribution coefficient of glucose



APPENDIX A-3

Profiles from the Exploratory Experiments for the Sucrose-Invertase System

Figure A-3-1 - Elution profile for run RS(02)-SUC-10-60-80-3.5

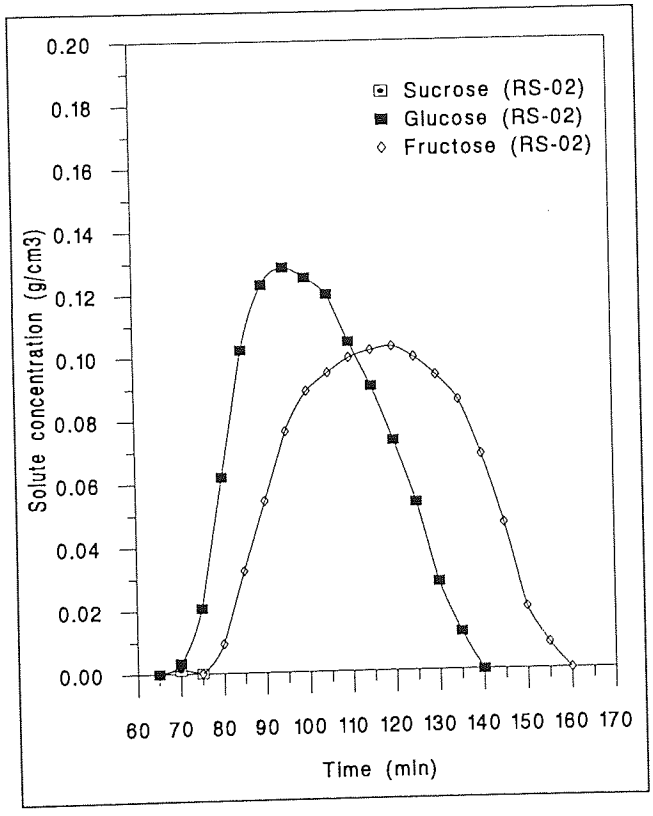


Figure A-3-2 - Elution profile for run RS(03)-SUC-20-60-80-3.5

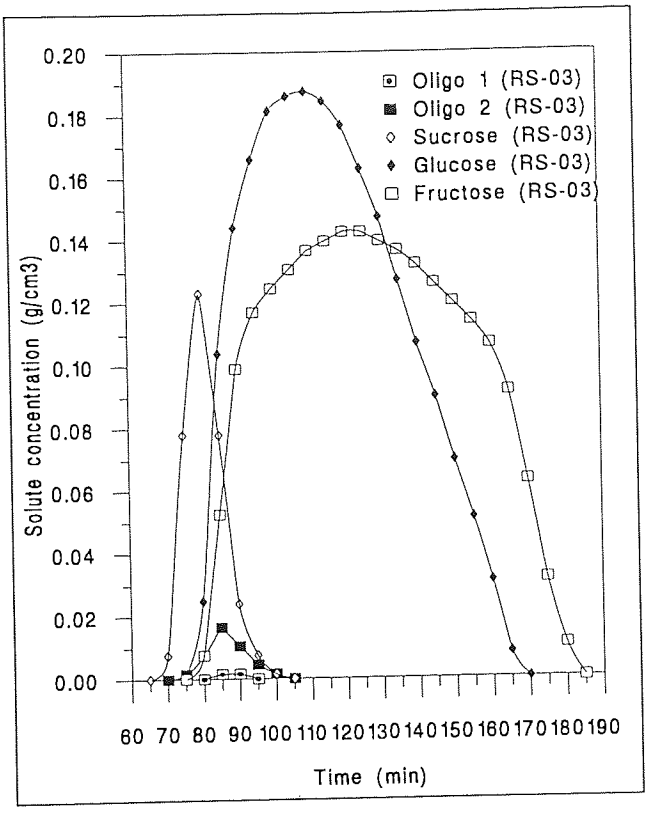
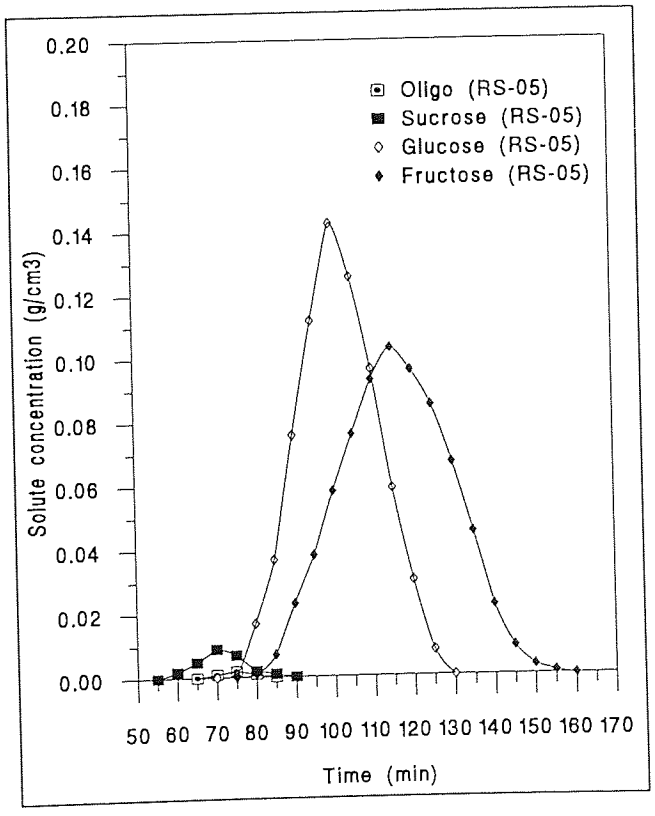


Figure A-3-3 - Elution profile for run RS(05)-SUC-10-40-60-3.5



APPENDIX A-4

Enzyme Usage Calculations For a Standard Run

Invertase Enzyme Unit Definition:

1 Unit (U) of enzyme is the amount of enzyme that converts 1 μmol of sucrose in one minute at the standards conditions of pH and temperature.

1 U converts 3.42×10^{-4} g of sucrose/min

2924 U convert 1 g of sucrose/min

Experimental conditions

Run RS(02)-SUC-10-60-80-3.5

Enzyme activity:	80 U/cm^3
Time for the elution of glucose and fructose:	80 min
Volumetric flowrate of enzyme fed into the system:	3.5 cm^3/min
Total enzyme activity used:	80 x 3.5 x 80 = 22400 U
Concentration of the sucrose solution fed into the system:	60 % w/v
Pulse size = 10 % TECV = 0.1 x 600 cm^3 =	60 cm^3
Mass of sucrose fed into the system =	60 x 0.6 = 36 g

Theoretical amount of enzyme required to convert 36 g of sucrose/min = 2924 x 36 = 105263 U

$$\text{Enzyme usage} = \frac{\text{enzyme used}}{\text{theoretical amount required}} = \frac{22400}{105263} \times 100 = 21.3\ \%$$

APPENDIX A-5

The Statistical Analysis of the Experimental Data Presented in Section 5.7

The statistical treatment of the response values reported in Table 5.5 was based on a tabular procedure outlined by Davies (194). The first step was the calculation of the effect value and the mean square value, or variance, for each treatment combination according to the factorial experiment. The procedure will be exemplified with the response (1), the resolution between glucose and fructose (R_{G-F}). The treatment combinations and response values (1) are listed in a standard order as presented in Table A-5-1. The first entry in column (1) is the sum of the first two response values (0.38+0.27), the second entry equals the sum of the second pair (0.34+0.23) and so on until the top half of the column (1) is complete. The values in the lower half of column (1) are calculated by subtracting the first value in each of the of the same pairs from the second. Column (2) is derived in a similar way, by summing and differencing the successive pairs of values listed in column (1). Columns (3) and (4) are derived from the preceding column in a similar manner.

Table A-5-1 Calculation of effect and mean square data for response (1)

Treatment combination	Response (1) R_{G-F}	(1)	(2)	(3)	(4)	Effect Value	Mean square
A1B1C1D1	0.38	0.65	1.22	2.55	5.94	-	-
A2B1C1D1	0.27	0.57	1.33	3.39	-0.74	-0.09	0.0342
A1B2C1D1	0.34	0.64	1.50	-0.23	0.28	0.04	0.0049
A2B2C1D1	0.23	0.69	1.89	-0.51	-0.08	-0.01	0.0004
A1B1C2D1	0.36	0.72	-0.22	-0.03	0.50	0.06	0.0156
A2B1C2D1	0.28	0.78	-0.01	0.31	0.22	0.03	0.0030
A1B2C2D1	0.31	0.82	-0.26	0.15	0.32	0.04	0.0064
A2B2C2D1	0.38	1.07	-0.25	-0.23	0.04	0.00	0.0001
A1B1C1D2	0.41	-0.11	-0.08	0.11	0.84	0.11	0.0441
A2B1C1D2	0.31	-0.11	0.05	0.39	-0.28	-0.04	0.0049
A1B2C1D2	0.47	-0.08	0.06	0.21	0.34	0.04	0.0072
A2B2C1D2	0.31	0.07	0.25	0.01	-0.38	-0.05	0.0090
A1B1C2D2	0.43	-0.10	0.00	0.13	0.28	0.04	0.0049
A2B1C2D2	0.39	-0.16	0.15	0.19	-0.20	-0.03	0.0025
A1B2C2D2	0.64	-0.04	-0.06	0.15	0.06	0.01	0.0002
A2B2C2D2	0.43	-0.21	-0.17	-0.11	-0.26	-0.03	0.0042

The effect value was calculated by dividing each of the values in column (4) by the number of treatment combinations carried out at each factor level, in this case 8. The mean square (variance) was calculated by squaring each of the entry of column (4) and dividing by the number of the total treatment combinations carried out, in this case 16.

Estimate of error variance (V)

There was no true internal estimate of the error variance and only a single replication of the experiment was carried out. In factorial experiments of such nature it is customary to employ the an estimate of error variance based on higher-order of interactions (194). The practice may be justified on the ground that often higher-order effects of this kind are non-existent, or at least negligible. On this assumption the mean square values corresponding to the higher-order interactions may be added together and divided by the total of their degree of freedom to give an estimate of the error variance. Table A-5-2 presents the estimated error variance for the data shown in Table A-5-1.

Table A-5-2 - Calculation of the estimate error variance

Interaction	Mean square (variance)
ABC	0.0001
ABD	0.0090
ACB	0.0025
BCD	0.0002
ABCD	0.0042
Total	0.0161

Considering the number of degrees of freedom as 5, the error variance (V) is 0.0161/5 = 0.0032.

It is also possible that one or more of the mean square values may appear considerably larger than the rest, leading the observer to the conclusion that the value should be ignored to obtain a more accurate estimate of the error variance. The hypothesis that the mean squares found are in fact estimates of the same variance and may therefore be used as an estimate of error can be tested by Bartellett's criterion (196).

The criterion is based on the measurement of the divergence, M, of a group of mean square values, based on one degree of freedom. M is calculated according to the relation:

$$M = \phi \ln V - \Sigma \ln V_i \qquad \text{(A-5-1)}$$

where, ϕ equals the total number of degrees of freedom, V is the error variance and V_i an individual mean square value.

Therefore, for the data presented in Table A-5-1 the calculations are as follows:

Interaction	Mean square		ln (Mean square)
ABC	0.0001		-9.210
ABD	0.0090		-4.711
ACB	0.0025		-5.991
BCD	0.0002		-8.517
ABCD	0.0042		-5.473
$\Sigma V =$	0.016	$\Sigma \ln V_i =$	-33.776

$$\begin{aligned} \phi &= 5 \\ V &= 0.003 \\ \ln V &= -5.740 \\ \phi \ln V &= -28.700 \end{aligned}$$

Thus, substituting the values found for $\phi \ln V$ and for $\Sigma \ln V_i$ in the expression A-5-1, M can be calculated:

$$M = -28.700 - (-33.776) = 5.076$$

A significance test is then applied to the values of M found. To apply this test, the assumption, (called the null-hypothesis) is made that only random error variation is responsible for a change occurring in the response. If the observed quantity, M in this case, has only a small probability (α) of being attained or exceeded if the null-hypothesis were true, then it is concluded that the hypothesis is untrue. α is called the level of significance and the conclusion is usually expressed: 'a statistical test indicates a significant departure from the given assumption at the 100 α % level (e.g. 10 % level if α=0.1)'.

The significance points for M were obtained by Nair (207) for various degree of freedom. For the case when each mean square has a single degree of freedom and for a value of α = 0.05, the value of 12.0 is required for significance. As the calculated value (M = 5.076) is less than the tabulated one, then there is a greater than 5 % chance that the observed divergence of the mean square values in Table A-5-2 is due to random variation. Therefore these values can be used to obtain an estimate of the error variance for the response (1) in this factorial experiment.

The other responses were also tested for the divergence of the mean square values for higher order of interaction and the calculated M values are presented in Table A-5-3. The values show that the error variation is not significant.

Table A-5-3 - M values for the factorial experiment responses

Response (number)	M value
Resolution between glucose and fructose (1)	5.08
Peak concentration of glucose (2)	5.97
Peak concentration of fructose (3)	5.36
% of glucose rich product recovered, with 100 % purity (4)	1.45
% of fructose rich product recovered , with 100 % purity (5)	2.38

The Variance Ratio for Significance - The 'F-test'

The higher order mean square values was used to give an estimate of error variance. Thus the variance (mean square) listed in Table A-5-1 can be used to test for significance and the F-test was selected to compare the estimate of the error variance. If the observed deviation is significantly greater than the error can account for, then the cause of the deviation is significant. To verify if a factor affects the response, the value of the response obtained at the lower level is subtracted from the average at the higher level, squared and divided by the number of treatment combinations. The value found is the required variance which must be compared with the variance due to the error to give an F-value, which measures the interaction between the factors. The F-value is expressed by the relation:

$$F = \text{Mean square} / \text{Error variance} \qquad \text{A-5-2}$$

The Table A-5-4 presents the F-values for each treatment combination listed in Table A-5-1.

Table A-5-4 - F-Values for the mean square values of response (1)

Response	Mean square	F-value
A1B1C1D1	-	-
A2B1C1D1	0.0342	10.64
A1B2C1D1	0.0049	1.52
A2B2C1D1	0.0004	0.12
A1B1C2D1	0.0156	4.86
A2B1C2D1	0.0030	0.94
A1B2C2D1	0.0064	1.99
A2B2C2D1	0.0001	0.03
A1B1C1D2	0.0441	13.72
A2B1C1D2	0.0049	1.52
A1B2C1D2	0.0072	2.25
A2B2C1D2	0.0090	2.81
A1B1C2D2	0.0049	1.52
A2B1C2D2	0.0025	0.78
A1B2C2D2	0.0002	0.07
A2B2C2D2	0.0042	1.31

Any of the F-value presented above which there is a greater than 100 % α chance that the observed variance is not consistent with the random variation was, again, extracted from an standard F-table, based on one and five degrees of freedom for the observed variance and for the error variance, respectively. The level of α, stipulated before the statistical analysis, was set at the 5 % level ($\alpha = 0.05$). A double sided test was adopted. The F-value of 6.61 was obtained from the standard table. Therefore, if an F-value of less than 6.61 is found, then there is a greater chance that the observed variance is due to random variation. On the

other hand, if the value is greater than 6.61, then the observed F-value was significant. Discretion should be exercised for F-values close to 6.61.

For instance, the change on the level of pulse concentration (A) from 5 to 10 % results in an F-value of 10.64. The effect value shown in Figure A-5-1 for the corresponding treatment combination is negative, indicating that the response value decreases as the factor level increases. It shows that there is a greater than 95 % chance that in increase in pulse concentration decreases the resolution between glucose and fructose. An increase in enzyme activity (D) also increases the resolution but the interative effect of pulse size (A) and enzyme activity (D) presents an F-value of 1.52, indicating that this effect is not significant.

The calculation of the F-values for the remaining responses are given in Tables A-5-5, A-5-6, A-5-7 and A-5-8.

Table A-5-5 Calculation of F-values for response (2) - Glucose peak concentration

Treatment combination	Response (2) Pk_G	(1)	(2)	(3)	(4)	Effect value	Mean square	F-value
A1B1C1D1	7.00	19.10	39.80	86.70	185.70	-	-	-
A2B1C1D1	12.10	20.70	46.90	99.00	36.10	4.51	81.4506	605.02
A1B2C1D1	8.60	21.10	45.00	17.30	11.70	1.46	8.5556	63.55
A2B2C1D1	12.10	25.80	54.00	18.80	-6.30	-0.79	2.4806	18.43
A1B1C2D1	8.30	21.90	8.60	6.30	16.10	2.01	16.2006	120.34
A2B1C2D1	12.80	23.10	8.70	5.40	-0.70	-0.09	0.0306	0.23
A1B2C2D1	10.80	24.90	9.80	-1.90	6.10	0.76	2.3256	17.27
A2B2C2D1	15.00	29.10	9.00	-4.40	0.90	0.11	0.0506	0.38
A1B1C1D2	8.00	5.10	1.60	7.10	12.30	1.54	9.4556	70.24
A2B1C1D2	13.90	3.50	4.70	9.00	1.50	0.19	0.1406	1.04
A1B2C1D2	9.60	4.50	1.20	0.10	-0.90	-0.11	0.0506	0.38
A2B2C1D2	13.50	4.20	4.20	-0.80	-2.50	-0.31	0.3906	2.90
A1B1C2D2	9.60	5.90	-1.60	3.10	1.90	0.24	0.2256	1.68
A2B1C2D2	15.30	3.90	-0.30	3.00	-0.90	-0.11	0.0506	0.38
A1B2C2D2	12.90	5.70	-2.00	1.30	-0.10	-0.01	0.0006	0.00
A2B2C2D2	16.20	3.30	-2.40	-0.40	-1.70	-0.21	0.1806	1.34

Table A-5-6 Calculation of F-values for response (3) - Fructose peak concentration

Treatment combination	Response (3) Pk_F	(1)	(2)	(3)	(4)	Effect value	Mean square	F-value
A1B1C1D1	5.8	14.60	31.00	70.40	149.70	-	-	-
A2B1C1D1	8.8	16.40	39.40	79.30	27.30	3.41	46.5806	1686.18
A1B2C1D1	7.2	17.90	35.00	12.40	11.30	1.41	7.9806	288.89
A2B2C1D1	9.2	21.50	44.30	14.90	-3.90	-0.49	0.9506	34.41
A1B1C2D1	7	16.50	5.00	5.40	17.70	2.21	19.5806	708.80
A2B1C2D1	10.9	18.50	7.40	5.90	4.50	0.56	1.2656	45.81
A1B2C2D1	9	20.20	6.40	-1.40	3.70	0.46	0.8556	30.97
A2B2C2D1	12.5	24.10	8.50	-2.50	0.90	0.11	0.0506	1.83
A1B1C1D2	6.3	3.00	1.80	8.40	8.90	1.11	4.9506	179.21
A2B1C1D2	10.2	2.00	3.60	9.30	2.50	0.31	0.3906	14.14
A1B2C1D2	8	3.90	2.00	2.40	0.50	0.06	0.0156	0.57
A2B2C1D2	10.5	3.50	3.90	2.10	-1.10	-0.14	0.0756	2.74
A1B1C2D2	7.7	3.90	-1.00	1.80	0.90	0.11	0.0506	1.83
A2B1C2D2	12.5	2.50	-0.40	1.90	-0.30	-0.04	0.0056	0.20
A1B2C2D2	10.2	4.80	-1.40	0.60	0.10	0.01	0.0006	0.02
A2B2C2D2	13.9	3.70	-1.10	0.30	-0.30	-0.04	0.0056	0.20

Table A-5-7 Calculation of F-values for response (4) - Recovery of glucose pure product (with 100 % purity)

Treatment combination	Response (4) C_G	(1)	(2)	(3)	(4)	Effect value	Mean square	F-value
A1B1C1D1	1.3	2.20	4.10	23.10	77.40	-	-	-
A2B1C1D1	0.9	1.90	19.00	54.30	-26.00	-3.25	42.2500	13.46
A1B2C1D1	0.9	13.50	10.80	-5.10	-9.80	-1.23	6.0025	1.91
A2B2C1D1	1	5.50	43.50	-20.90	8.40	1.05	4.4100	1.40
A1B1C2D1	7.9	6.60	-0.30	-8.30	47.60	5.95	141.6100	45.11
A2B1C2D1	5.6	4.20	-4.80	-1.50	-14.60	-1.83	13.3225	4.24
A1B2C2D1	4	21.30	-5.40	0.30	-4.40	-0.55	1.2100	0.39
A2B2C2D1	1.5	22.20	-15.50	8.10	-5.00	-0.63	1.5625	0.50
A1B1C1D2	6.2	-0.40	-0.30	14.90	31.20	3.90	60.8400	19.38
A2B1C1D2	0.4	0.10	-8.00	32.70	-15.80	-1.98	15.6025	4.97
A1B2C1D2	1.9	-2.30	-2.40	-4.50	6.80	0.85	2.8900	0.92
A2B2C1D2	2.3	-2.50	0.90	-10.10	7.80	0.98	3.8025	1.21
A1B1C2D2	15	-5.80	0.50	-7.70	17.80	2.23	19.8025	6.31
A2B1C2D2	6.3	0.40	-0.20	3.30	-5.60	-0.70	1.9600	0.62
A1B2C2D2	14.5	-8.70	6.20	-0.70	11.00	1.38	7.5625	2.41
A2B2C2D2	7.7	-6.80	1.90	-4.30	-3.60	-0.45	0.8100	0.26

Table A-5-7 Calculation of F-values for response (5) - Recovery of fructose pure product (with 100 % purity)

Treatment combination	Response (5) C_F	(1)	(2)	(3)	(4)	Effect value	Mean square	F-value
A1B1C1D1	17.3	25.70	36.40	93.40	289.80	-	-	-
A2B1C1D1	8.4	10.70	57.00	196.40	-87.00	-10.88	473.0625	199.34
A1B2C1D1	7.5	30.70	91.50	-28.60	-72.00	-9.00	324.0000	136.53
A2B2C1D1	3.2	26.30	104.90	-58.40	4.80	0.60	1.4400	0.61
A1B1C2D1	17.5	56.10	-13.20	-19.40	34.00	4.25	72.2500	30.44
A2B1C2D1	13.2	35.40	-15.40	-52.60	-9.20	-1.15	5.2900	2.23
A1B2C2D1	18.7	68.40	-25.70	-2.20	-0.60	-0.08	0.0225	0.01
A2B2C2D1	7.6	36.50	-32.70	7.00	-14.20	-1.78	12.6025	5.31
A1B1C1D2	35.7	-8.90	-15.00	20.60	103.00	12.88	663.0625	279.40
A2B1C1D2	20.4	-4.30	-4.40	13.40	-29.80	-3.73	55.5025	23.39
A1B2C1D2	22.9	-4.30	-20.70	-2.20	-33.20	-4.15	68.8900	29.03
A2B2C1D2	12.5	-11.10	-31.90	-7.00	9.20	1.15	5.2900	2.23
A1B1C2D2	42.9	-15.30	4.60	10.60	-7.20	-0.90	3.2400	1.37
A2B1C2D2	25.5	-10.40	-6.80	-11.20	-4.80	-0.60	1.4400	0.61
A1B2C2D2	25.9	-17.40	4.90	-11.40	-21.80	-2.73	29.7025	12.52
A2B2C2D2	10.6	-15.30	2.10	-2.80	8.60	1.08	4.6225	1.95

APPENDIX A-6

Elution Profiles of the Factorial Experiments

Figure A-6-1 - Elution Profile for run RS(10)-SUC-10-60-100-3.5

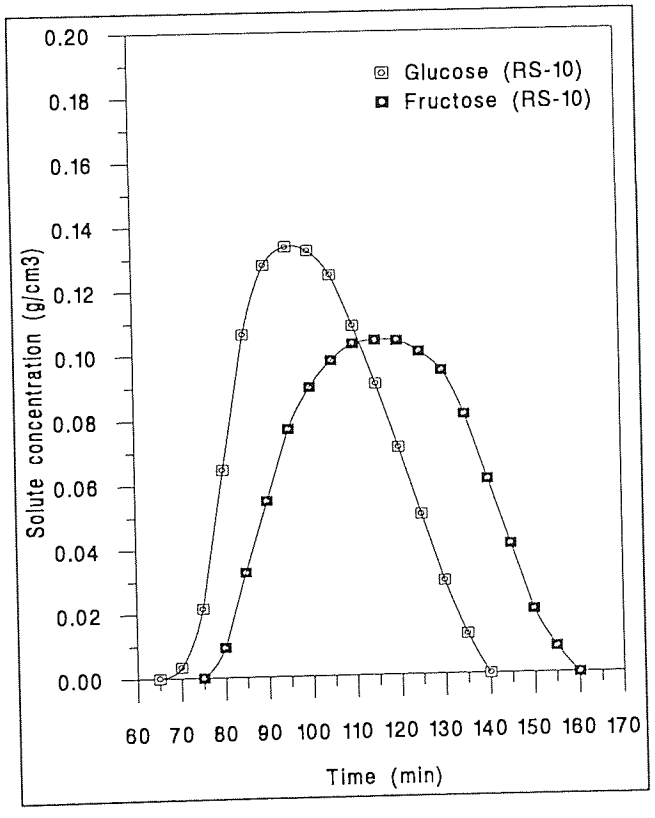


Figure A-6-2 - Elution Profile for run RS(11)-SUC-5-40-100-3.5

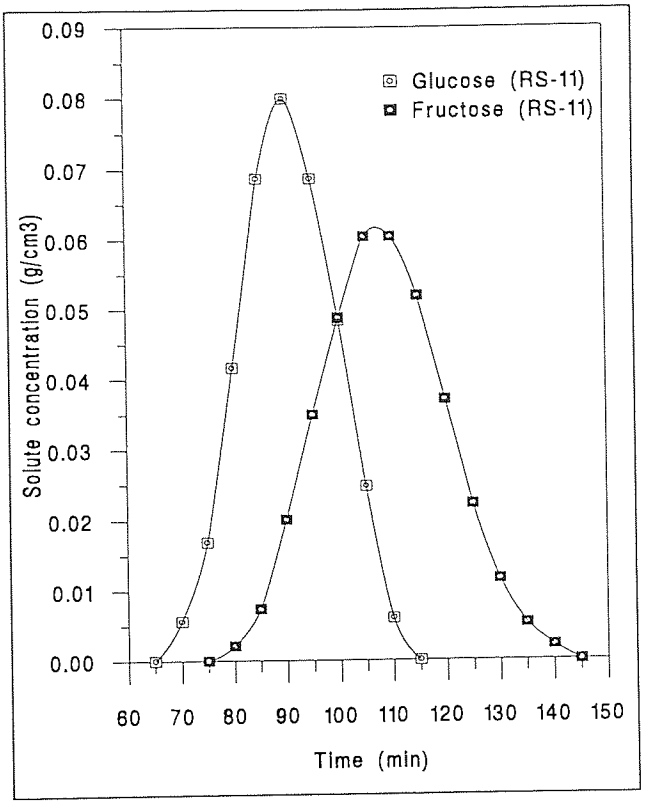


Figure A-6-3 - Elution Profile for run RS(12)-SUC-10-40-100-3.5

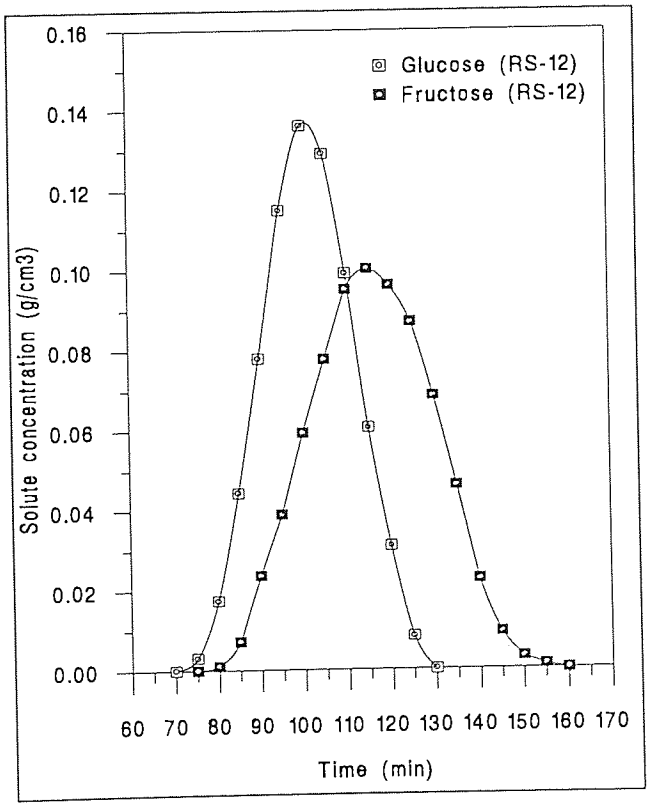


Figure A-6-4 - Elution Profile for run RS(07)-SUC-5-60-150-3.5

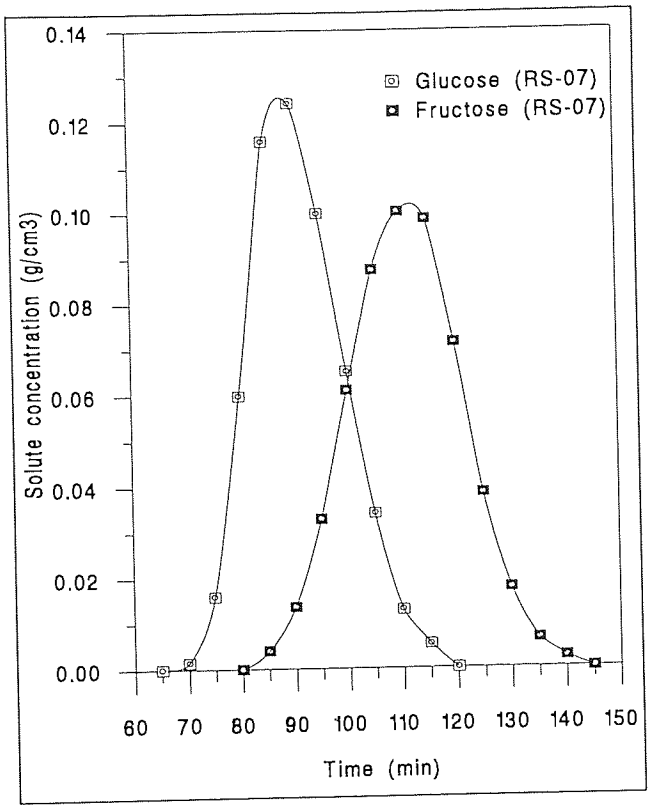


Figure A-6-5 - Elution Profile for run RS(08)-SUC-10-60-150-3.5

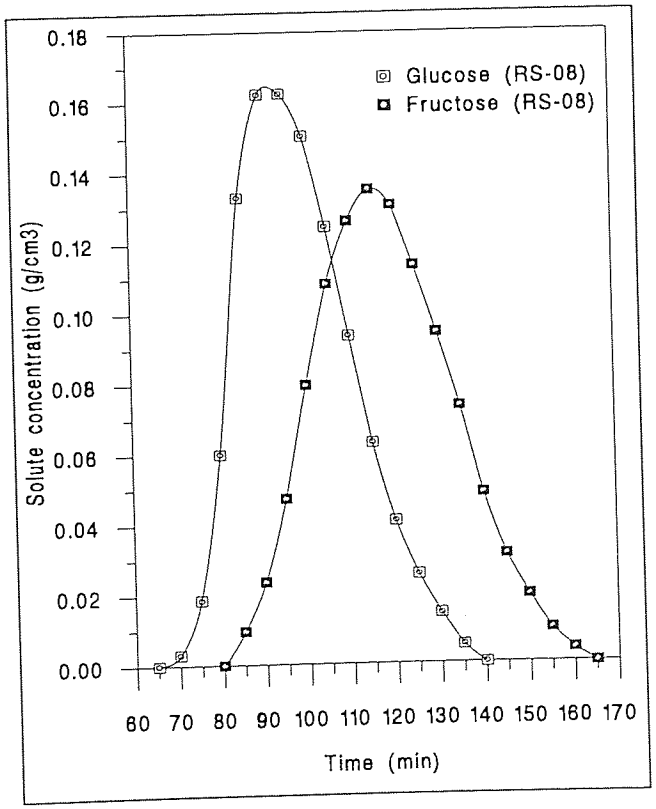


Figure A-6-6 - Elution Profile for run RS(09)-SUC-10-40-150-3.5

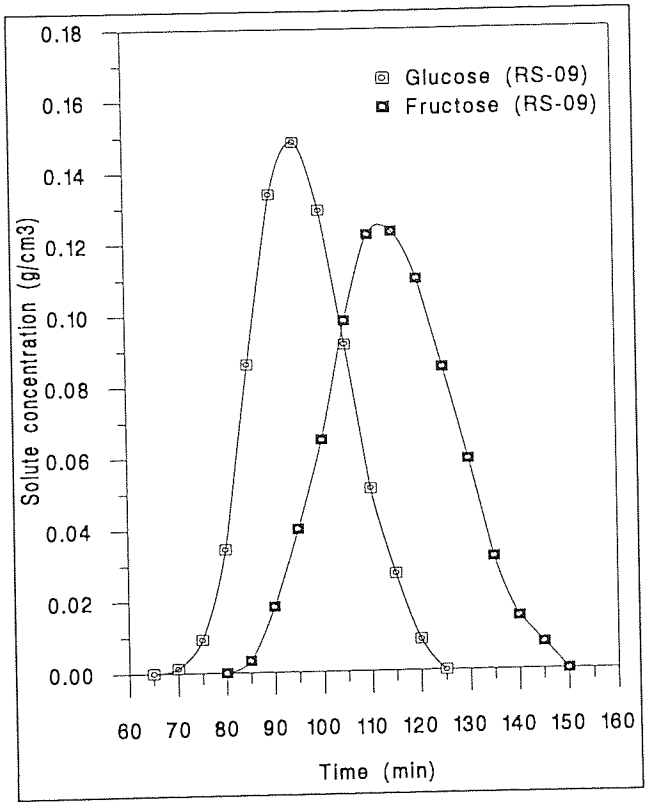


Figure A-6-7 - Elution Profile for run RS(13)-SUC-5-40-150-3.5

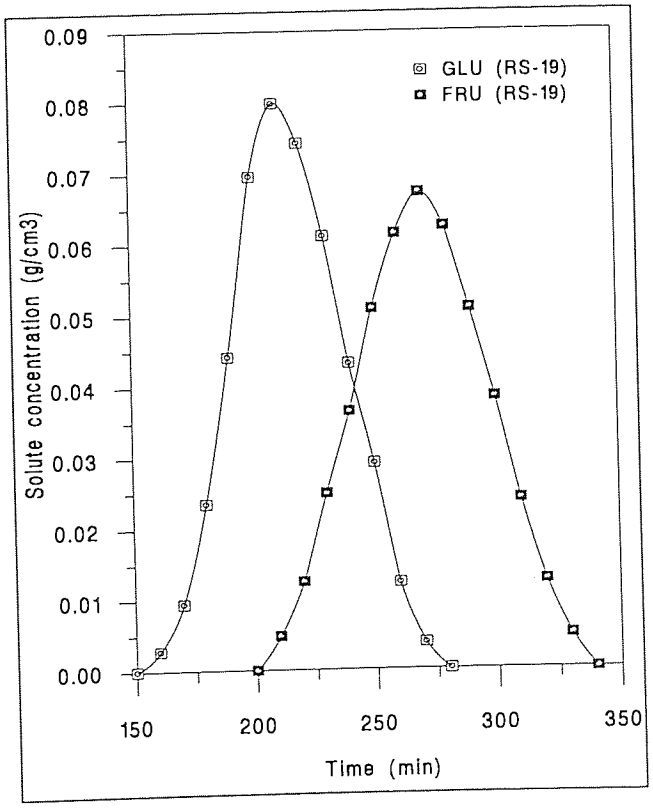
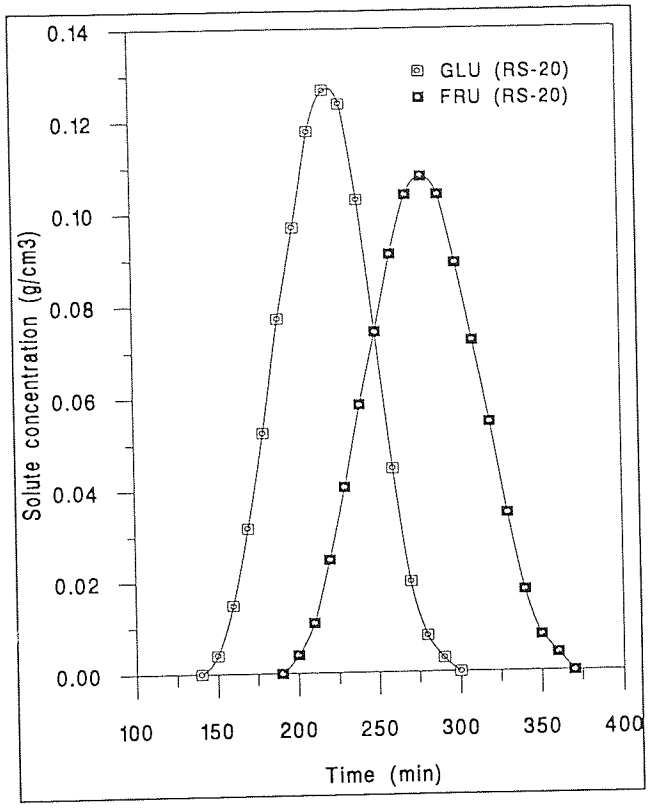


Figure A-6-8 - Elution Profile for run RS(20)-SUC-10-40-150-1.4



APPENDIX A-7

Elution Profiles from the Exploratory Experiments for the Inulin-Inulinase System

Figure A-7-1 - Elution profile for run RS(05)-INU-5-10-50-0.74

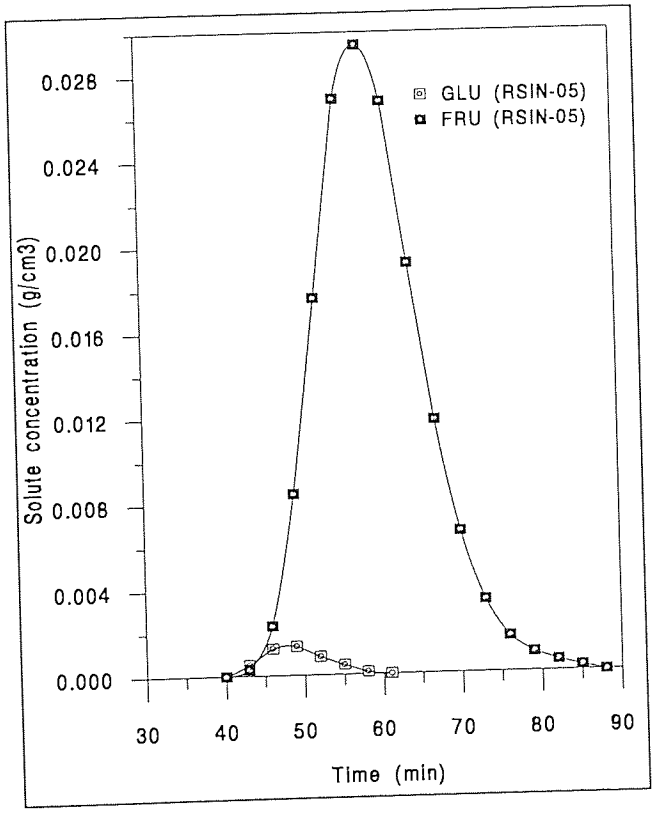


Figure A-7-2 - Elution profile for run RS(06)-INU-1-10-25-0.74

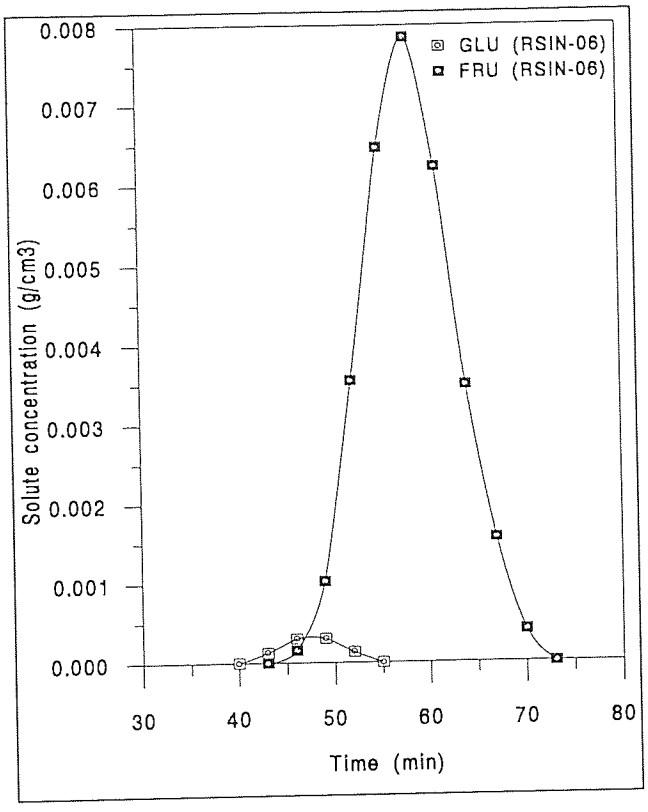


Figure A-7-3 - Elution profile for run RS(07)-INU-5-10-25-0.74

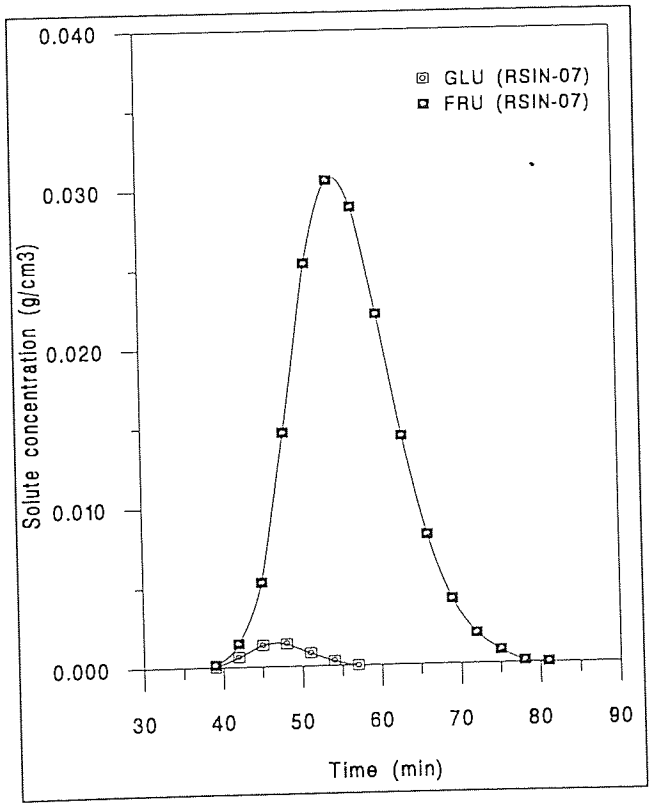


Figure A-7-4 - Elution profile for run RS(08)-INU-1-10-10-0.74

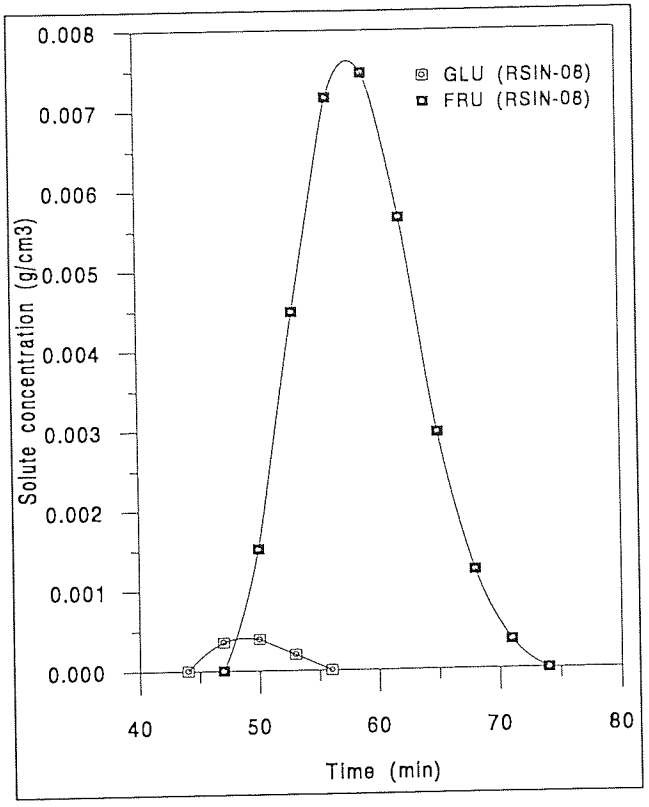
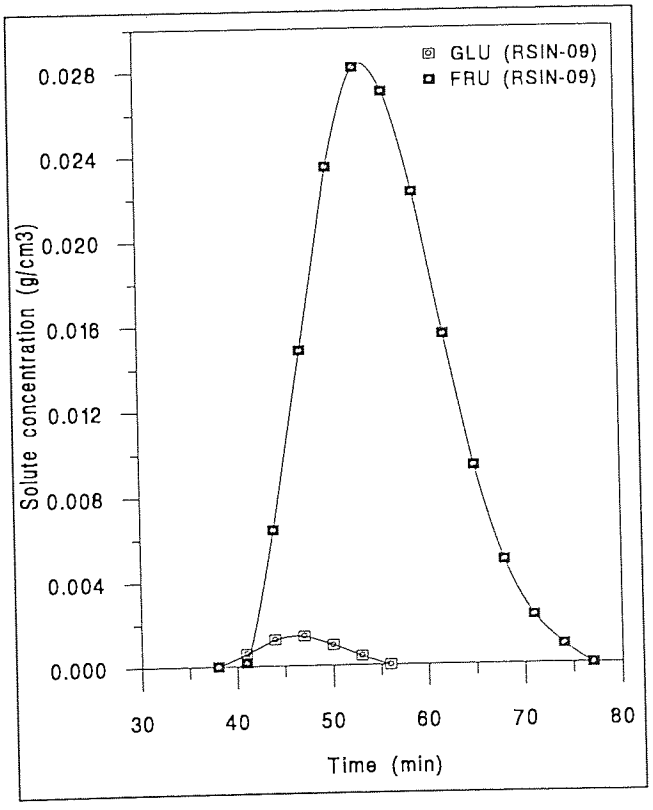


Figure A-7-5 - Elution profile for run RS(09)-INU-5-10-10-0.74



APPENDIX A-8

Elution Profiles from the Exploratory Experiments for the Lactose-Lactase System

Figure A-8-1 - Elution profile for run RS(03)-LAC-5-5-60-1.75

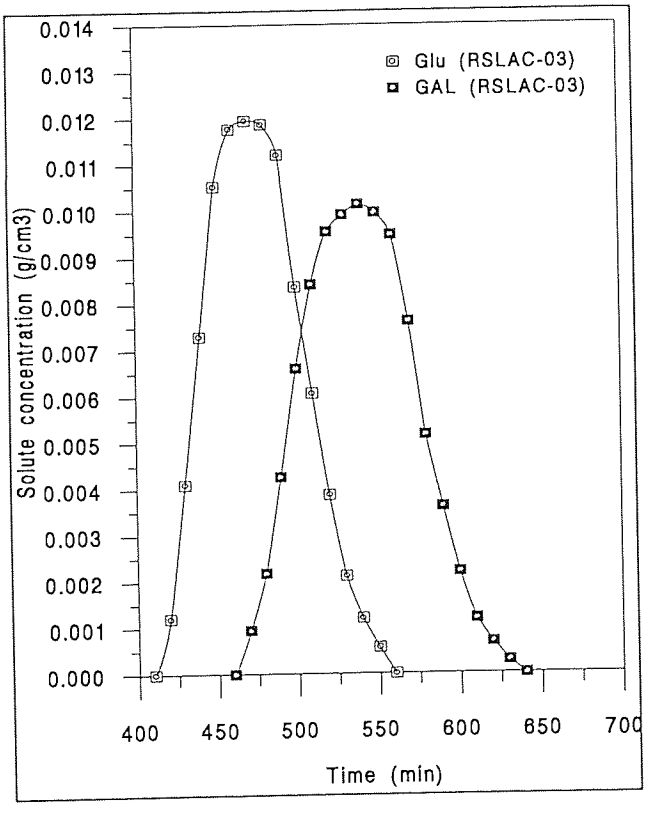


Figure A-8-2 - Elution profile for run RS(04)-LAC-5-10-60-1.75

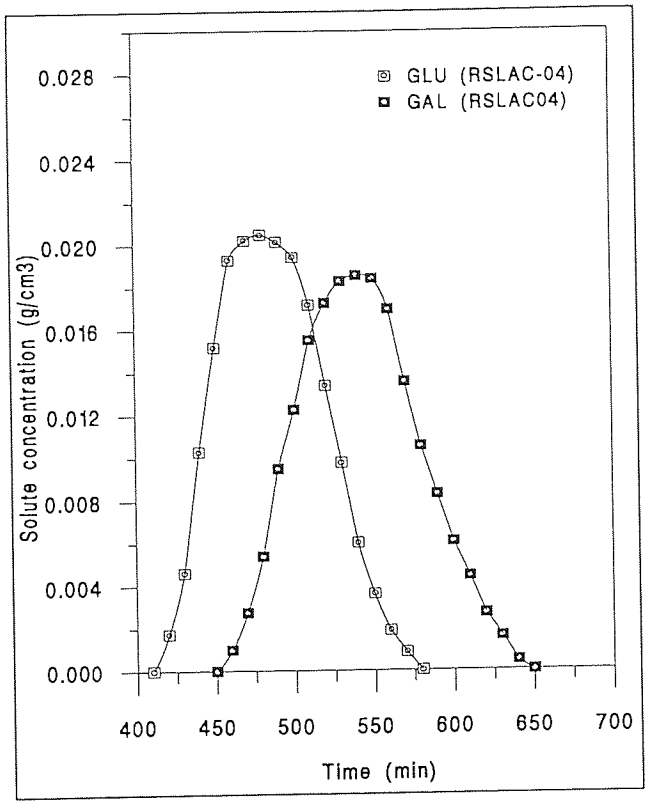
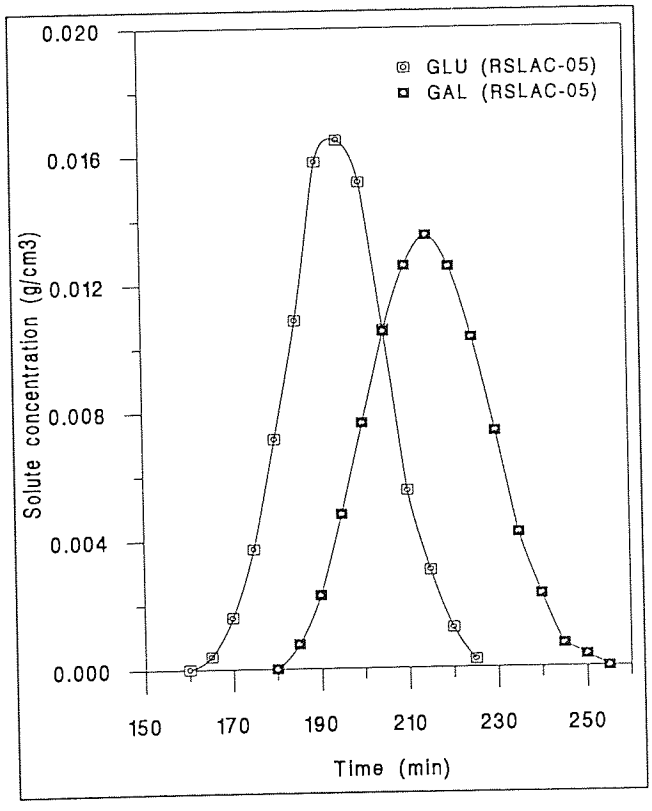
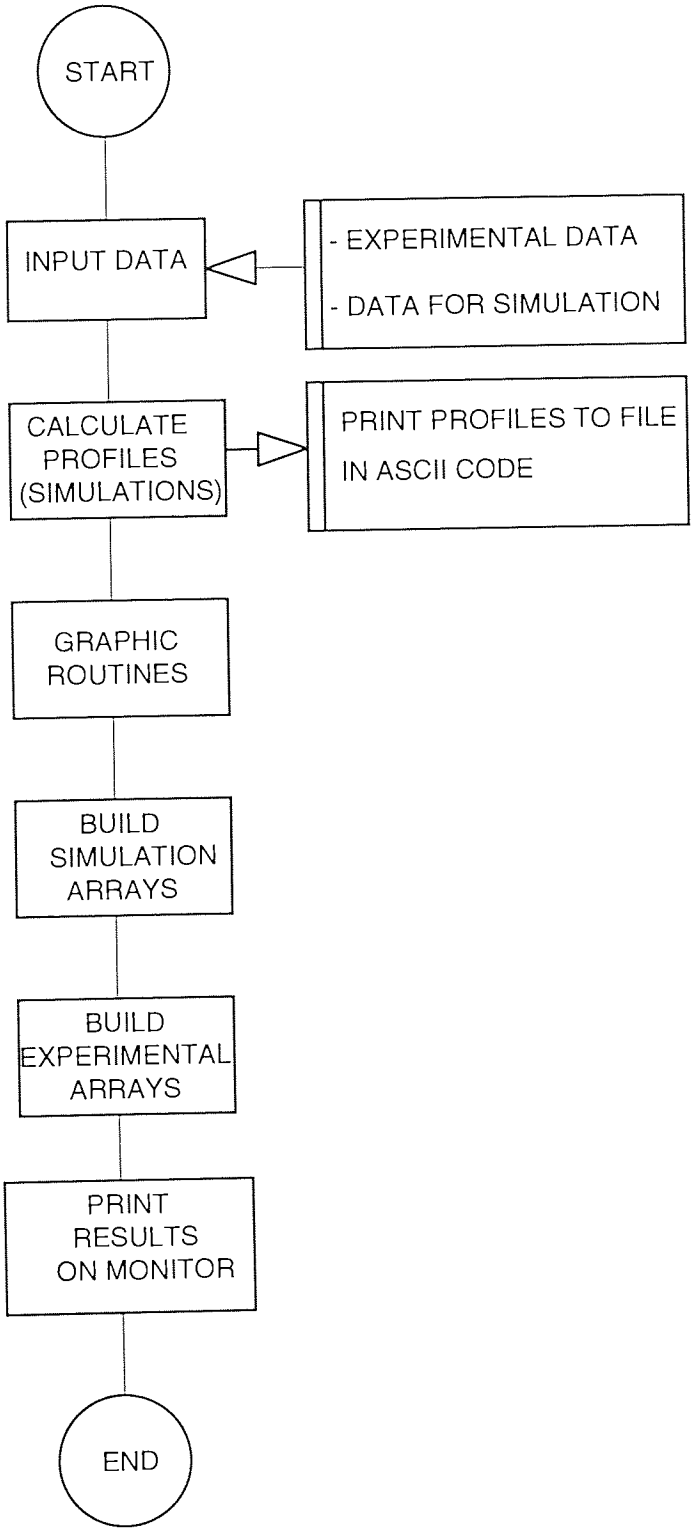


Figure A-8-3 - Elution profile for run RS(04)-LAC-5-5-100-3.5



APPENDIX A-9

Flowchart for the Computer Program for the Simulation of the Batch Chromatographic Bioreactor-Separator



APPENDIX A-10

List of Symbols Used in the FORTAN Program (Appendix A-11)

ALPHAF	relation of voidage and distribution coefficient for fructose concentration equation
ALPHAG	relation of voidage and distribution coefficient for glucose concentration equation
ALPHAS	relation of voidage and distribution coefficient for sucrose concentration equation
AREA	area of the column cross section
BETAF	relation of parameters for fructose concentration equation
BETAG	relation of parameters for glucose concentration equation
BETAS	relation of parameters for sucrose concentration equation
DIA	diameter of the chromatographic column
DT	time increment
DT1	time interval for profile results
DZ	distance interval
EFR1	eluent flowrate in cm^3/min
EFR2	eluent flowrate in cm^3/s
ETA	voidage
EZERO	initial enzyme concentration
H	column lenght
INJT	injection time
K3	proportionality constant relating maximum initial reaction velocity and enzyme concentration
KDF	distribution coefficient for fructose
KDF0	distribution coefficient for fructose at infinite dilution
KDG	distribution coefficient for glucose
KDG0	distribution coefficient for glucose at infinite dilution
KDS	distribution coefficient for sucrose
KDS0	distribution coefficient for sucrose at infinite dilution
KI	inhibition constant
KM	Michaelis constant
PHIF	relation of parameters for fructose concentration equation
PHIG	relation of parameters for glucose concentration equation
PHIS	relation of parameters for sucrose concentration equation
RR	loop controller
SCONC	sucrose concentration
SMIN	minimum sucrose concentration to be used in the calculations
TT	total time
U	eluent superficial velocity
USUP	eluent intersticial velocity
VMAX	maximum reaction velocity
VMAX1	corrected value for maximum reaction velocity
VOID	void volume
VOL	puse volume

APPENDIX A-11

Computer Program Used to Model the Behaviour of the Batch Chromatographic Bioreactor-Separator

```
      program RSMODEL

C     LUIZ EDUARDO M. TADDEI/1994/ASTON UNIVERSITY/CEAC//UFRJ-BR
C     BATCH CHROMATOGRAPHIC BIOREACTOR-SEPARATOR MODEL

C     PROGRAM DEVELOPED FOR 386 PC COMPATIBLES USING THE FTN386
C     COMPILER  FROM UNIVERSITY OF SALFORD.

C     THE PROGRAM INCLUDES GRAPHIC ROUTINES FOR VGA MONITOR (VIDEO)


C     Array dimentions for the simulation

      DIMENSION S(0:6,0:12000),G(0:6,0:12000),F(0:6,0:12000)

C     Array dimentions for the Salford video routines

      DIMENSION Y1(1000),XEXPG(1000),X(1000),YG(1000),YF(1000),YEXPF(1000),
     +XEXPF(1000),YEXPg(1000),Y2(1000)

C     Integers for the simulation

      INTEGER *2 M,T,Z,R1,BCF,BC

C     Integers for the Salford video routines

      INTEGER *2 J,NPTS,NPOINTS,,ih,iv,icol,fo,HSIZE,VSIZE,NUCOL,xj,yj,ix,
     +NCOL0,NCOL,y1m,x0,y0,x1,y11,xc0,yc0,xc1,yc1,TP,TP0,ICOLOR,IICOLOR

C     Real numbers for the simulation

      REAL*4 ETA,VOL,SCONC,DT,DZ,H,DIA,INJT,DT1,EFR1,EFR2,VOID,AREA,U,
     +TT,PHIS,PHIG,PHIF,BETAS,BETAG,BETAF,RR,VMAX,VMAX1,KM,SMIN,KDS,KDG,
     +KDF,KDS0,KDG0,KDF0,USUP,ALPHAS,ALPHAG,ALPHAF,KI,EZERO,K3

C     Real numbers for the Salford video routines

      REAL*4 xinf,xsup,yinf,ysup,ymax,si,ro


C ---------- 1 st part of Salford routines starts ----------
C If the Salford compiler it is not intended to be used then the folowing lines shoud be omitted
C Unformated records
      character*28 str
      character*25 txt1
      character*23 txt2,txt3
```

```
      character*2 TP1
C Parameters for video
      tp1='.'
      iicolor=2
      inorm=1
      ymax=y1m
      icolor=15
      xinf=0
      xsup=300
      yinf=0
      ysup=1.2
C  Colours
C Concentration and time marks
      clrmks=2
C Box
      clrbx=12
C Profiles from simulation
      clrprof=15
C Experimental profiles
      clrprofx=2

C ---------- 1st part of Salford routines ends ----------

      OPEN(UNIT=7,FILE='DAT_MOD2',STATUS='OLD')

C Inputs data from file DAT_MOD2

C Characteristics of pulse
C Pulse Volume (cm3) and Pulse Concentration (g/cm3)
      READ (7,*) VOL, SCONC

C Kinetics data
C Michaelis constant (KM), enzyme initial concentration (Ezero) and K3
C (KM in g/cm3, Enzyme Initial conc. in U and K3 in g/U.s)
      READ (7,*) KM, EZERO, K3

C Distribution Coeficient for Sucrose, Glucose and Fructose
      READ (7,*) KDS0, KDG0, KDF0

C Characteristics of chromatographic column
C Column Height (cm) and Diameter (cm)
      READ (7,*) H, DIA

C Voidage and void volume
      READ (7,*) ETA, VOID

C Eluent flowrate (cm3/min)
      READ (7,*) EFR1

C Distance and Time Increments (cm and s)
      READ (7,*) DZ, DT

      CLOSE (7)
```

```
C Parameter for calculation of concentrations

      SMIN=0.0001

C Program starts preliminary calculations

      AREA=3.1416*DIA**2/(4.0)
      EFR2=EFR1/60
      USUP=EFR2/AREA
      U=USUP/ETA

      VMAX=K3*EZERO
      VMAX1=VMAX*(1-(VOL/VOID))
      KI=(VMAX1*KM**0.5)/KM

      P=INT(H/DZ)
      TT=XSUP
      INJT=VOL/EFR2
      BC=NINT(INJT/DT)
      BCF=NINT(TT*60/DT)

      DO 10 T=1,BC
      S(0,T)=SCONC
   10 CONTINUE

      DO 20 T=BC+1,BCF
      S(0,T)=0
      G(0,T)=0
      F(0,T)=0
   20 CONTINUE

      DO 30 M=1,P,5
      R1=0

      DO 50 Z=1,5
      R1=R1+1

      S(Z,0)=0
      G(Z,0)=0
      F(Z,0)=0

      DO 60 T=1,BCF

      RR=(VMAX1*S(Z,T))/(KM+S(Z,T)+(S(Z,T)**2)/KI)

C RMK: KDS, KDG AND KDF refer to distribution coefficient with background concentration

      KDS=0.117*(S(Z,T))+kdS0

      ALPHAS=(ETA+(1-ETA)*KDS)/ETA
      PHIS=DT*U/(DZ*ALPHAS)
      BETAS=DT/ALPHAS

      S(Z,T+1)=S(Z,T)-(PHIS*(S(Z,T)-S(Z-1,T)))-(BETAS*RR)
```

```
     ++(DT*9.847E-4*USUP/ALPHAS)*(S(Z+1,T)-(2*S(Z,T))+S(Z-1,T))
      KDG=0.117*(G(Z,T))+KDG0

      ALPHAG=(ETA+(1-ETA)*KDG)/ETA
      PHIG=DT*U/(DZ*ALPHAG)
      BETAG=DT/ALPHAG

      G(Z,T+1)=G(Z,T)-(PHIg*(G(Z,T)-G(Z-1,T)))+(0.526*BETAG*RR)
     ++(DT*9.847E-4*USUP/ALPHAG)*(G(Z+1,T)-(2*G(Z,T))+G(Z-1,T))

      KDF=0.125*(F(Z,T))+KDF0
      ALPHAF=(ETA+(1-ETA)*KDF)/ETA
      PHIF=DT*U/(DZ*ALPHAF)
      BETAF=DT/ALPHAF
      F(Z,T+1)=F(Z,T)-(PHIf*(F(Z,T)-F(Z-1,T)))+(0.526*BETAF*RR)
     ++(DT*9.847E-4*USUP/ALPHAF)*(F(Z+1,T)-(2*F(Z,T))+F(Z-1,T))
      IF (S(Z,T+1).LT.SMIN)THEN
      S(Z,T+1)=0
      RR=0
      ENDIF

 60   CONTINUE

      IF(R1.EQ.5)THEN
      DO 40 T=1,BCF
      S(0,T)=S(Z,T+1)
      G(0,T)=G(Z,T+1)
      F(0,T)=F(Z,T+1)
 40   CONTINUE
      ENDIF

 50   CONTINUE

 30   CONTINUE

      DT1=30/DT

      Z=5

C Program writes time x concentration (sucrose, glucose and fructose) on file

      OPEN(UNIT=9,FILE='RES_1',STATUS='UNKNOWN')

      DO t=1,BCF,DT1
      WRITE(9,*) T*DT/60,',',S(Z,T+1),',',G(Z,T+1),',',F(Z,T+1)
      ENDDO
      CLOSE (9)



C ---------- 2nd part of Salford routines starts ----------
C If the Salford compiler it is not intended to be used then the folowing lines shoud be omitted

C Program reads experimental profiles data from files 'GX1.TXT' AND 'FX1.TXT'
```

```
C The profiles will be plotted on the video together with the profiles from the simulation
      OPEN(UNIT=9,FILE='GX09.TXT',STATUS='OLD')

      READ(9,*) NPOINTS
      DO J=1,NPOINTS
      READ (9,*) Y1(J)
      ENDDO

      CLOSE (9)

      OPEN(UNIT=9,FILE='FX09.TXT',STATUS='OLD')

      READ(9,*) NPOINTS
      DO J=1,NPOINTS
      READ (9,*) Y2(J)
      ENDDO
      CLOSE (9)
      J=0

      YM=0.0001

C GLUCOSE ARRAY

      DO T=1,BCF,DT1
      J=J+1
      YG(J)=G(Z,T+1)
      WHILE (YG(J).GT.YM) DO
      YM=YG(J)
      Y1M=YM
      YMG=YM
      ENDWHILE
      X(J)=T*DT/60
      ENDDO

      NPTS=j

C FRUCTOSE ARRAY

      J=0
      YM=0.0001

      DO T=1,BCF,DT1
      J=J+1
      YF(J)=F(Z,T+1)
      WHILE (YF(J).GT.YM) DO
      YM=YF(J)
      Y1M=YM
      YMF=YM
      ENDWHILE
      X(J)=T*DT/60
      ENDDO

      NPTS=J
```

```
C GLUCOSE EXPERIMENTAL ARRAY
    YM=0.0001

    DO J=1, npoints
    yEXPg(j) = y1(j)
    WHILE (YEXPg(J).GT.YM) DO
    YM=YEXPG(J)
    Y1M=YM
    YMGX=YM
    ENDWHILE
    xexpg(j)=65 +(j-1)*5.0
    endDO

C FRUCTOSE EXPERIMENTAL ARRAY

    YM=0.0001
    DO J=1, npoints
    yEXPF(j) = y2(j)
    WHILE (YEXPF(J).GT.YM) DO
    YM=YEXPF(J)
    Y1M=YM
    YMFX=YM
    ENDWHILE
    xexpF(j)=65 +(j-1)*5.0
    endDO

C COMPARING YM'S

    ymax=ymg

    if(ymf.gt.ymax)then
    ymax=ymf
    endif

    if(ymgx.gt.ymax)then
    ymax=ymgx
    endif

    if(ymfx.gt.ymax)then
    ymax=ymfx
    endif

    CALL HIGH_RESOLUTION_GRAPHICS_MODE@
    CALL VGA@

C sub-routine for y axis legend
    str='Solute concentration (g/cm3)'
    ih=8
    iv=350
    icol=7
    fo=101
    si=1
    ro=90
    call set_text_attribute@(fo,si,ro)
```

```
      call draw_text@(str,ih,iv,icol)
C ROUTINE FOR CAPTIONS

      OPEN(UNIT=6,FILE='graf_3',STATUS='OLD')
      CALL GET_GRAPHICS_RESOLUTION@(HSIZE,VSIZE,ncolours)

C TEXT FOR THE GRAPHICS
C "X" AXIS

      read (6,*) txt1
      call coup@(txt1,7,32,29)

C DRAW BOX
c with the following coordinates

      x0=12
      y0=45
      x1=639
      y11=449
      ICOLOR=CLRBX
      call rectangle@(x0,y0,x1,y11,ICOLOR)

C DRAW INTERNAL BOX (CAPTION)
C with the following coordinates
      xc0=435
      yc0=45
      xc1=639
      yc1=118
      icolOR=CLRBX
      call rectangle@(xc0,yc0,xc1,yc1,icolOR)

C text for caption
      read (6,*) txt2
      call coup@(txt2,7,56,3)
      read (6,*) txt3
      call coup@(txt3,7,56,5)

      close(6)

C GLUCOSE GRAPHIC PROFILES, TIME MARKS, ETC

C NORMALIZE IF INORM ZERO

      IF (INORM.EQ. 0 ) GOTO 100
      DO K =1 ,NPTS
      IF (ABS(YG(K)).GT.YMAX) YMAX =ABS( YG(K))
      ENDDO

100  DO K=1 ,NPTS
      YG(K)= YG(K)/YMAX
      ENDDO
      CONTINUE
      CALL GET_GRAPHICS_RESOLUTION@(HSIZE,VSIZE,NCOLOURS)
      vsize=vsize-32
```

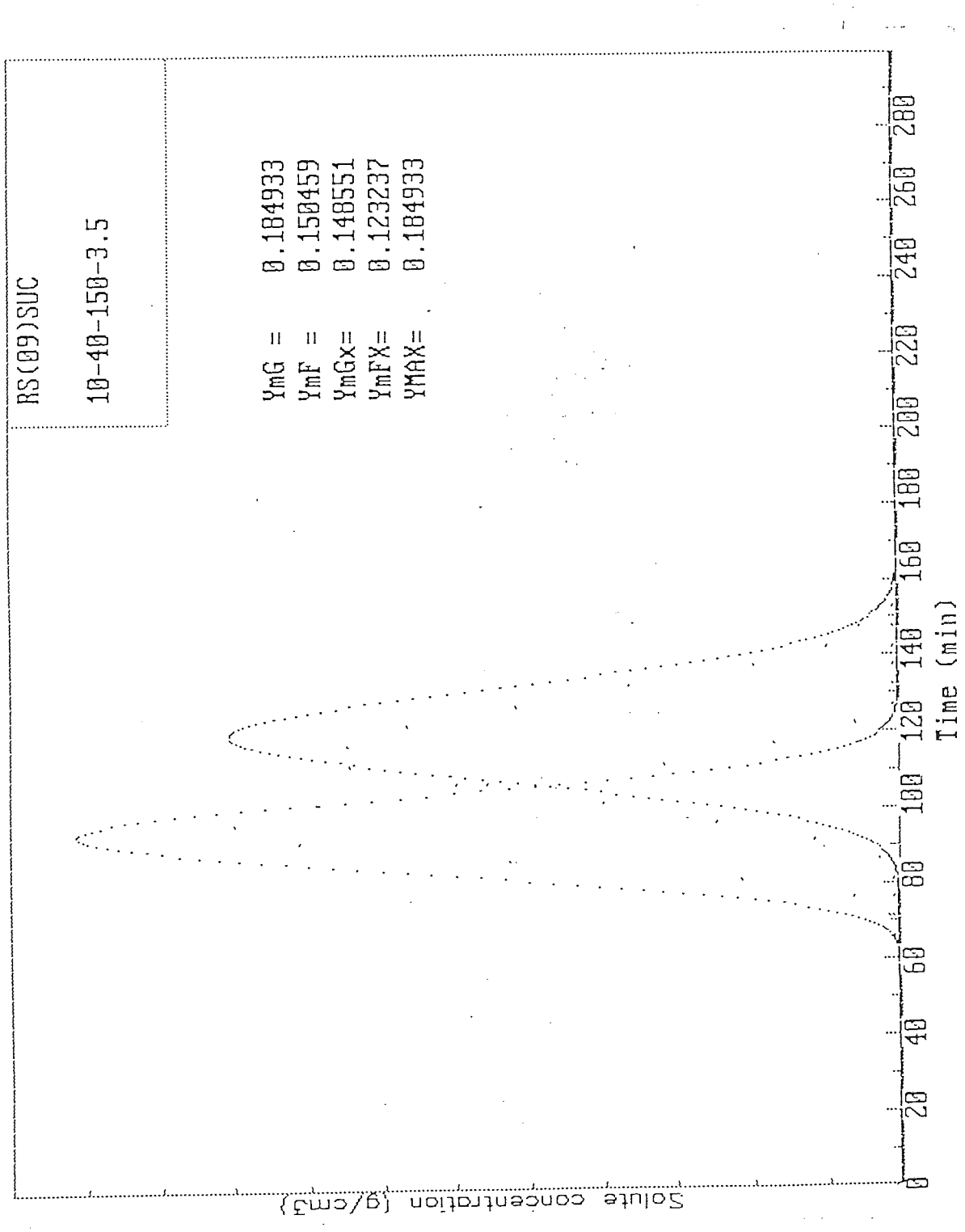
```
C TIME MARKS FOR 20 min INTERVALS
      IX0=12
      IX=IX0
      L=HSIZE-IX0
      NCOL0=INT(80*IX0/HSIZE)
      NCOL=NCOL0

      ICOLOR=CLRMKS

      DO J=1,15
      CALL DRAW_LINE@(IX,VSIZE,IX,VSIZE-6,ICOLOR)
      IX=IX0+INT(L*J/15)
      ENDDO

C TIME MARKS FOR 10 min INTERVALS

      DO J=1,30
      CALL DRAW_LINE@(IX,VSIZE,IX,VSIZE-3,ICOLOR)
      IX=IX0+INT(L*J/30)
      enddo

C WRITE TIME 20 min MARKS

      TP0=0
      TP=TP0

      DO J=1,15
      call set_cursor_pos@(NCOL,28)
      call print_i2@(TP)
      NCOL=NCOL0+INT( 80*L*J*0.067/HSIZE )
      TP=TP+TT/15
      enddo

C CONCENTRATION MARKS

      VSIZE=VSIZE+32

      ix0=449
      IX=IX0
      L=VSIZE-76

      ICOLOR=CLRMKS

      DO J=1,13
      CALL DRAW_LINE@(12,IX,15,IX,ICOLOR)
      IX=IX0-INT(L*J/12)
      enddo

      VSIZE=VSIZE-32

C GLUCOSE PROFILES

      ICOLOR=CLRPROF
```

```
      do j =1 ,NPTS
      XJ=INT((X(J)-XINF)*hsize/(XSUP -XINF)+12 )
      YJ=VSIZE -INT((Yg(J)-YINF)*VSIZE/(YSUP -YINF))
      call set_pixel@(XJ,YJ,ICOLOR)
      ENDDO

C FRUCTOSE PROFILES

C NORMALIZE IF INORM ZERO

      IF (INORM.EQ. 0 ) GOTO 110
      DO K =1 ,NPTS
      IF (ABS(YF(K)).GT.YMAX) YMAX =ABS( YF(K))
      ENDDO

110   DO K=1 ,NPTS
      YF(K)= YF(K)/YMAX
      ENDDO
      CONTINUE

      CALL GET_GRAPHICS_RESOLUTION@(HSIZE,VSIZE,NUCOL)
      vsize=vsize-32

      do j =1 ,NPTS
      XJ=INT((X(J)-XINF)*hsize/(XSUP -XINF)+12 )
      YJ=VSIZE -INT((YF(J)-YINF)*VSIZE/(YSUP -YINF))
      call set_pixel@(XJ,YJ,icolor)
      ENDDO

C EXPERIMENTAL GLUCOSE PROFILE

C NORMALIZE IF INORM ZERO

      IF (INORM.EQ. 0 ) GOTO 120
      DO K =1 ,NPTS
      IF (ABS(YEXPg(K)).GT.YMAX) YMAX =ABS( YEXPg(K))
      ENDDO

120   DO K=1 ,NPTS
      YEXPg(K)= YEXPg(K)/YMAX
      ENDDO
      CONTINUE

      CALL GET_GRAPHICS_RESOLUTION@(HSIZE,VSIZE,NUCOL)
      vsize=vsize -32

      ICOLOR=CLRPROFX

      DO J=1,NPTS
      XJ=INT((Xexpg(J)-XINF)*hsize/(XSUP -XINF)+12)
      YJ=VSIZE -INT((YEXPg(J)-YINF)*VSIZE/(YSUP -YINF))
      CALL DRAW_TEXT@(TP1,XJ,YJ,ICOLOR)
      enddo
```

```
C EXPERIMENTAL FRUCTOSE PROFILE

C NORMALIZE IF INORM ZERO

    IF (INORM.EQ. 0 ) GOTO 150
    DO K =1 ,NPTS
    IF (ABS(YEXPF(K)).GT.YMAX) YMAX =ABS( YEXPF(K))
    ENDDO

 150  DO K=1 ,NPTS
    YEXPF(K)= YEXPF(K)/YMAX
    ENDDO
    CONTINUE

    CALL GET_GRAPHICS_RESOLUTION@(HSIZE,VSIZE,NUCOL)

    vsize=vsize -32

    ICOLOR=CLRPROFX

    DO J=1,NPTS

    XJ=INT((XexpF(J)-XINF)*hsize/(XSUP -XINF)+12)
    YJ=VSIZE -INT((YEXPF(J)-YINF)*VSIZE/(YSUP -YINF))
    CALL DRAW_TEXT@(TP1,XJ,YJ,ICOLOR)
    enddo

    call set_cursor_pos@(55,10)
    WRITE(*,*) 'YmG =',YMG

    call set_cursor_pos@(55,11)
    WRITE (*,*) 'YmF =',YMF

    call set_cursor_pos@(55,12)
    WRITE (*,*) 'YmGx=',YMGX

    call set_cursor_pos@(55,13)
    WRITE (*,*) 'YmFX=',YMFX

    call set_cursor_pos@(55,14)
    WRITE(*,*) 'YMAX=',YMAX

    call GET_KEY@(K)
    call text_mode@
C ---------- 2nd part of Salford routines ends ----------

    stop
    end
```

APPENDIX A-12

Typical Profile from the Simulation Displayed in the Computer Monitor



PUBLICATIONS

1. Taddei, L. E. M. and Barker, P. E. (1993), "Bioreaction-Separation in Chromatographic Columns", in Proceedings of The 1993 IChemE Research Event - Vol 1, Rugby, UK, Institution of Chemical Engineers, pp. 22-24.

2. Taddei, L. E. M. and Barker, P. E. (1994), "The Chromatographic Column as a Bioreactor-Separator", in Proceedings of The 1994 IChemE Research Event - Vol 1, Rugby, UK, Institution of Chemical Engineers, pp. 214-216.