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ENZYMATIC SYNTHESIS, RECOVERY, AND PURIFICATION OF ETHYL β-D-GLUCOPYRANOSIDE

Examiners: Prof. Tuomo Sainio
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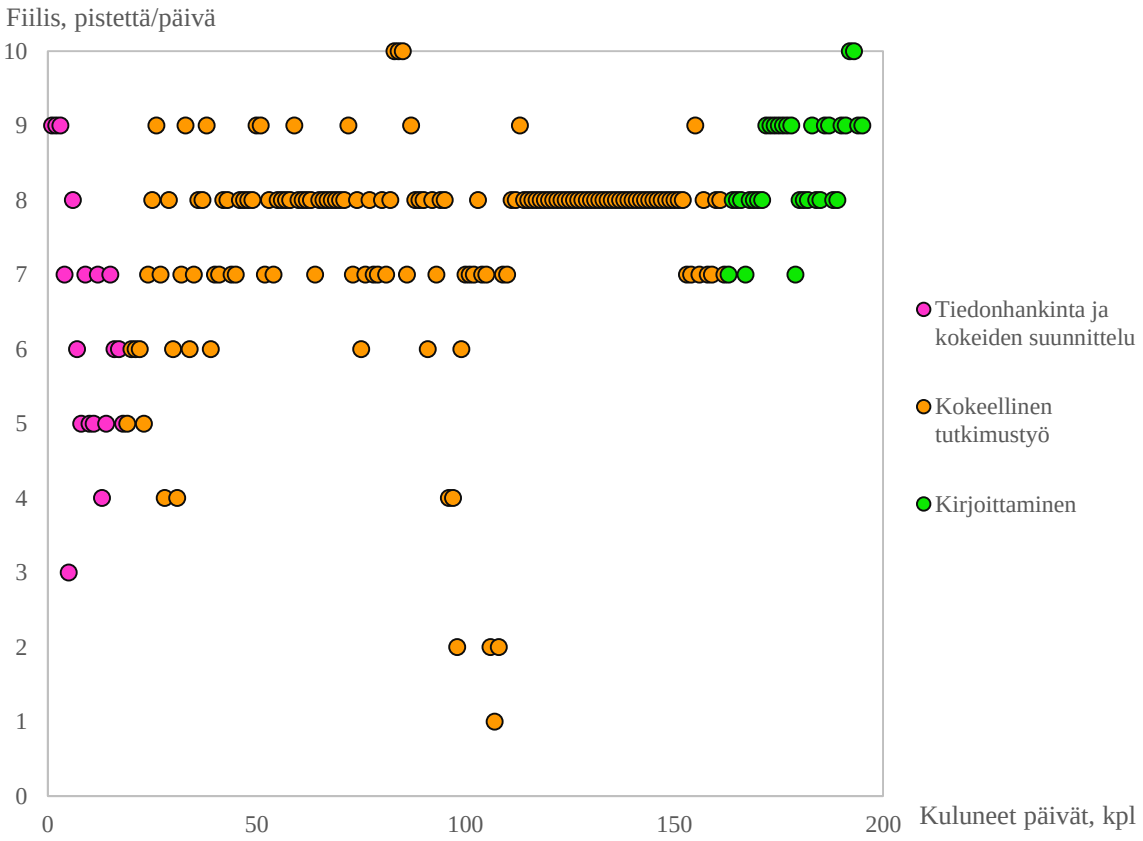
ALKUSANAT

Haluan kiittää professori Tuomo Sainiota ja TkT Jari Heinosta työn ohjauksesta ja tarkistamisesta. Erityiskiitos Jarille myös siitä että pystyin jakamaan kanssasi kaikki työssä kohtaamani ongelmat ja onnistumiset, minkä vuoksi projekti eteni jouhevasti. Kiitän myös laboratorioiden muuta henkilö- ja opiskelijakuntaa, jotka auttoivat minua tarvittaessa. Turun yliopistoa kiitän diplomityöni aiheen mahdollistamisesta.

Iso kiitos kuuluu myös Lappeenrannan Ylioppilasteatteri ActIn jäsenille, jotka pitivät opiskelun ja vapaa-ajan tasapainossa koko opintojeni ajan. Kiitän myös 0990 tiimiä kannustuksesta ja siitä, että kanssanne pystyi jakamaan kaikki ne opintoihin liittyvät asiat, joihin muut eivät olisi pystyneet samaistumaan. Tuesta ja kannustuksesta kiitän myös kaikkia perheenjäseniäni.

Tämä työ oli erittäin kiinnostava, haastava ja opettavainen projekti ylä- ja alamäkineen.



Fiilisasteikko: 0 = luovutan, 2 = voi ***, 4 = ihan kujalla, 6 = ok, 8 = aika jees :), 10 = täähän menee ihan nappiin!

Lappeenranta, 18.8.2015

Henna Niskakoski

TIIVISTELMÄ

Lappeenrannan teknillinen yliopisto
School of Engineering Science
Kemiantekniikan koulutusohjelma

Henna Niskakoski

Etyyli β-D-glukopyranosidin entsymaattinen synteesi, talteenotto ja puhdistus

Diplomityö

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93 sivua, 43 kuvaa, 11 taulukkoa

Tarkastajat: Prof. Tuomo Sainio
TkT Jari Heinonen

Hakusanat: etyyli β-D-glukopyranosidi, etyyliglukoosi, kierrätyskromatografia, nestekromatografia

Työssä etsittiin menetelmä etyyli β-D-glukopyranosidin (BEG) syntetisoimiseksi ja tutkittiin eri ioninvaihtohartsien soveltuvuutta aineen puhdistamiseksi kromatografisesti synteettisestä, binäärisestä liuoksesta, jossa pääasiallisena epäpuhtautena on glukoosi. 99 % puhdasta etyyli β-D-glukopyranosidia haluttiin tuottaa vähintään 50 grammaa suurempaan mittakaavaan skaalatulla panosprosessilla. Tavoitteena oli myös siirtää tuotantoprosessi panostoimisesta prosessista kierrätyskromatografiseen SSR prosessiin.

BEG:iä syntetisoitiin entsymaattisesti käänteishydrolyysillä käyttäen β-glukosidaasi -entsyymiä. Synteesissä 65 % glukoosista reagoi etanolin kanssa BEG:ksi. BEG:n erottamiseksi glukoosista tutkittiin erilaisia ioninvaihtohartsipohjaisia erotusmateriaaleja. Erotusmateriaalivalinta tehtiin styreenipohjaisten vahvojen kationinvaihtohartsien (SAC) ja akryylipohjaisten heikkojen kationinvaihtohartsien (WAC) väliltä perustuen panostoimisiin kolonnikokeisiin.

Erotusmateriaaliksi valikoitui Na^+ muotoinen WAC CA10GC hartsi. Komponenttien erottuminen hartsissa on lineaarinen ja tavoitepuhtauden saavuttaminen on mahdollista jo panosprosessissa ilman kierrätystä, kun virtausnopeus ja pulssikoko ovat tarpeeksi pienet. Panostoiminen erotusprosessi skaalattiin suurempaan mittakaavaan suuremman tuotemäärän tuottamiseksi ja 99 % tuotepuhtaus saavutettiin.

Panostoimisen systeemin läpäisykäyrien dataa ja Matlab simulointeja apuna käyttäen erotusprosessi siirrettiin SSR systeemiin, jonka optimaaliset toimintaolosuhteet määritettiin. SSR systeemillä saavutettuja tuloksia verrattiin vastaaviin panostoimisen prosessin tuloksiin. Valituissa olosuhteissa SSR:llä saatiin valmistettua 98 % puhtaita tuotteita 40 % korkeammalla tuottavuudella ja 40 % pienemmällä eluentin kulutuksella kuin panostoimisella prosessilla vastaavissa tuotepuhtauksissa.

ABSTRACT

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Enzymatic synthesis, recovery, and purification of ethyl β-D-glucopyranoside

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Keywords: ethyl β-D-glucopyranoside, ethyl glucose, recycle chromatography, liquid chromatography

A method to synthesize ethyl β-D-glucopyranoside (BEG) was searched. Feasibility of different ion exchange resins was examined to purify the product from the synthetic binary solution of BEG and glucose. The target was to produce at least 50 grams of 99 % pure BEG with a scaled up process. Another target was to transfer the batch process into steady-state recycle chromatography process (SSR).

BEG was synthesized enzymatically with reverse hydrolysis utilizing β-glucosidase as a catalyst. 65 % of glucose reacted with ethanol into BEG during the synthesis. Different ion exchanger based resins were examined to separate BEG from glucose. Based on batch chromatography experiments the best adsorbent was chosen between styrene based strong acid cation exchange resins (SAC) and acryl based weak acid cation exchange resins (WAC).

CA10GC WAC resin in Na^+ form was chosen for the further separation studies. To produce greater amounts of the product the batch process was scaled up. The adsorption isotherms for the components were linear. The target purity was possible to reach already in batch without recycle with flowrate and injection size small enough. 99 % pure product was produced with scaled-up batch process.

Batch process was transferred to SSR process utilizing the data from design pulse chromatograms and Matlab simulations. The optimal operating conditions for the system were determined. Batch and SSR separation results were compared and by using SSR 98 % pure products were gained with 40 % higher productivity and 40 % lower eluent consumption compared to batch process producing as pure products.

NOMENCLATURE

a_w	water thermodynamic activity	, -
c_i	concentration of component i	, g/L
dq/dc	adsorption isotherm	, -
E_a	activation energy	, kJ/mol
EC	eluent consumption	, L/g
G	Gibbs free energy	, kJ/mol
H_i	Henry constant for component i	, -
n_i	mass of component I	, g
PR	productivity	, g/BV/h
PU	purity	, %
q	loading of the adsorbent bed	, g/L
Q	flow rate	, mL/min BV/h
t_{A1}	beginning of the fraction A collection	, min
t_{A2}	end of fraction A collection, beginning of recycle	, min
t_{B1}	beginning of fraction B collection, end of recycle	, min
t_{B2}	end of fraction B collection	, min
t_c	cycle time	, min
$t_{ret}(c)$	retention time	, min
$t0$	dead time	, min
V_D	dead volume	, mL
V_{ret}	retention volume of the self-sharpening front	, mL
Vs	volume of the adsorbent material	, mL
Y	yield	, %
ε	porosity of the adsorbent bed	, -

Subscripts and superscripts

s.up	scaled-up
exp	experimental scale
i	component i
BV	injection size compared to the column bed volume

Abbreviations

A	the first fraction coming out from the column
B	the second fraction coming out from the column
BEG	ethyl β-D-glucopyranoside (β-ethylglucose)
BV	column bed volume
CLR	closed-loop-recycling chromatography
DVB	divinylbenzene
E	eluent
ECP	elution by characteristic point
ES	enzyme-substrate complex
F	feed
FA	frontal analysis
FACP	frontal analysis by characteristic point
FF	fresh feed
GLU	glucose
MF	mixed feed
R	recycle
SAC	strong acid cation exchanger
SMB	simulated moving bed chromatography
SSR	steady-state recycle chromatography
WAC	weak acid cation exchanger

TABLE OF CONTENTS

1 INTRODUCTION

According to recent studies ethyl β-D-glucopyranoside (BEG) naturally occurs in sea buckthorn berries [1]. Pure BEG is produced in this thesis as it is needed by the University of Turku for further experiments. Effects of BEG to the taste of the berry, and the possible effect of the component on animal sugar metabolism and insulin production will be studied by the researchers in Turku.

The target is to synthesize BEG and purify it to 99 % purity from the synthetic solution. BEG is very similar to glucose and their separation is barely studied. Similar solutes may be separated from each other utilizing liquid chromatography with suitable separation materials. Separation materials to be tested here are styrene based strong acid cation (SAC) exchange resins and acryl based weak acid cation (WAC) exchange resins. The stationary phase material suitability for the separation is studied utilizing batch chromatography.

Batch chromatography is the most traditional way to perform a chromatographic separation. Yet, the problem with batch chromatography is its great eluent consumption and in the case of difficult separation, poor yield. A difficult separation may be improved by recycling the impure mid-fraction back to the system turning the batch process into recycle chromatography process.

As the project proceeded new objectives were set. Due to successful separation the chromatographic process is scaled up and eventually transferred to steady-state recycling chromatography (SSR) process. The design procedure for SSR is studied. The optimal operating conditions for SSR are searched to produce 98 % pure BEG and glucose. The results of SSR are compared to the results of corresponding batch chromatography runs and, it is found out if SSR offers any benefit to separation that is already successful in batch process without recycling.

2 GLYCOSIDES

Glycosides are derivatives of any sugar. They are formed when an aliphatic or aromatic fragment (aglycone) is connected to the anomeric carbon of a sugar fragment of any size (glycone) via heteroatom (any atom that is not carbon or hydrogen) [2]. The glycone part of the glycoside may be a mono- or oligosaccharide. Those glycosides that are derived from glucose are called glucosides. In this thesis the focus is on ethyl β-D-glucopyranoside, the glycoside formed when ethyl group from ethanol combines with glucose via glycosidic bond.

In carbohydrates, no matter if they are polysaccharides like cellulose and starch or complex glycoconjugates like glycolipids and glycoproteins, the monosaccharide units are joined via glycosidic bonds. Glycosidic bond is a covalent bond formed by a glycosylation reaction which is often assisted with an activator. Usually the linkage is formed when leaving group of the glycosyl donor (glycone which has a leaving group at the anomeric position, first compound in Figure 2-1) is displaced with the hydroxyl moiety of the glycosyl acceptor (marked as *Nu* in Figure 2-1) [3].

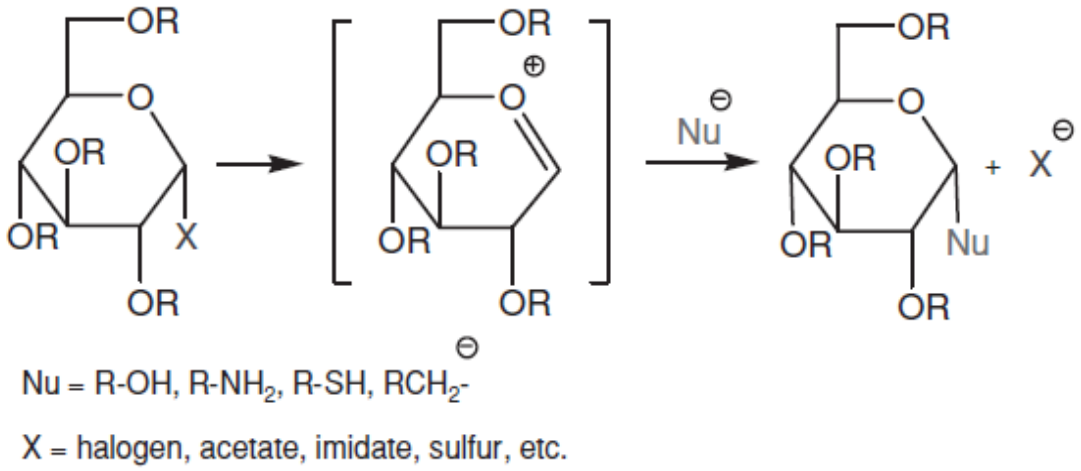


Figure 2-1 Nucleophile displacement on the anomeric carbon [2]. Nu = glycosyl acceptor and X = leaving group.

Leaving group is a component that can replace the anomeric hydroxyl group of a sugar in suitable conditions. Such leaving groups initially were halogens like chlorine and bromine. However, a great amount of other leaving groups have been utilized until now such as sulfur, sulfonates, and acetates. [2].

So, when a nucleophile substitutes the hydroxyl group by replacing the leaving group in the anomeric carbon of the sugar glycosidic bond is formed. Depending on the nucleophile connected to the glucose (alcohol, amine, or carbanion) O-, N-, or C-glycosides are generated [2]. Different types of glycosides are presented in Figure 2-2.

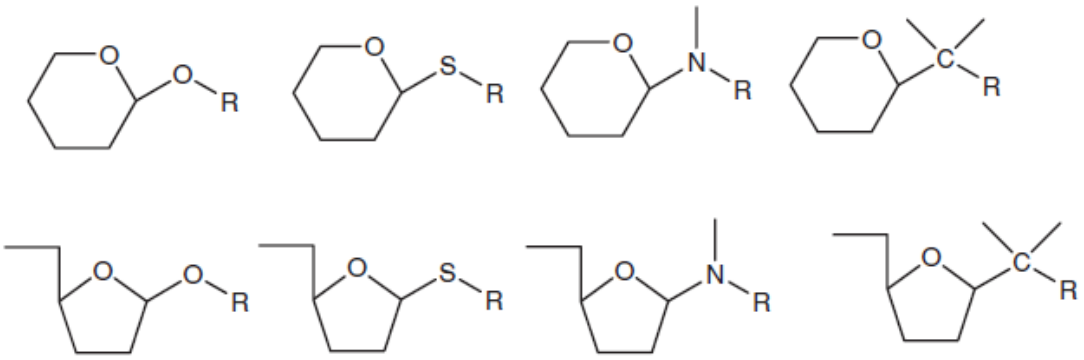


Figure 2-2 General types of glycosides [2].

As it is known for any 6-membered rings (pyranose), there are two conformations: chair and boat. The D enantiomeric form is known as chair and the boat title is used for the L form. In addition to this, the glycosidic bonds have chiralic nature forming α- and β-anomers. [2]. If anomerically pure product is wanted, the formation of different forms becomes a challenge when glycosides are prepared synthetically.

Naturally glycosylation reactions are taking place in plants where a vast variety of glycosides are present. Glycosides are inactive compounds and therefore, the best way for plants to store chemicals. The hydroxyl group of, for example, alcohols, phenols, and mercaptans are protected while they are present as their glycosides that function as precursors for aroma compounds in the plant [4]. When plants are creating glycosides their naturally occurring enzymes take care of the specificity of the stereoselective glycosylation.

Recently the enzymes have become part of the synthetic preparation of glycosides. As they are stereospecific and efficient catalysts under mild conditions they are used in the development of economical and environmentally friendlier manufacturing processes. Yet, their usage has their own drawback especially in high-scale production. For details, see Section 3.2.

2.1 Alkyl Glucosides

Alkyl glucosides contain glucose as a head group and a hydrocarbon tail which usually is a primary alcohol with different chain length and level of saturation. The chemical structure of alkyl glucosides strongly determines their properties in water: the longer the hydrocarbon chain attached to the anomeric carbon, the more hydrophobic the glucoside. Different stereoisomer forms of the compound makes a difference as well [5]. Like every other glycoside, alkyl glucosides also have two resulting stereoisomers called α- and β-anomers.

There is a chance for the aliphatic chain to attach the anomeric carbon of the glucose ring either axially as an α-anomer or equatorically as a β-anomer. The configurations of the anomers are shown in Figure 2-3. The total volume requirement for α-anomer is greater compared to the β-anomer [5] due to their differences in packing.

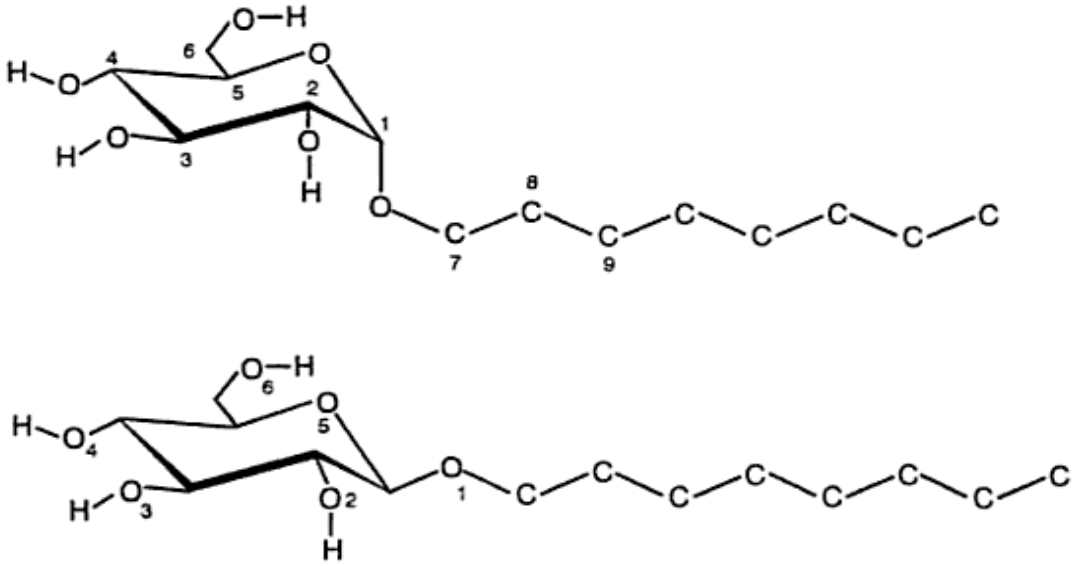


Figure 2-3 Orientation of octyl α-D-glucoside (top) and octyl β-D-glucoside (bottom) **[5]**.

Anomer properties differ no matter if they are in aqueous solution or in solid form. The shape of an α-anomer enables a perfect hydrogen-bonding network of glucopyranoside rings whereas the spacing of for example the n-octyl β-D-glucoside is greater (29 Å) than the spacing of α-anomers (19.7 Å). According to Balzer and Luders [5] this is assumed to be caused by the less perfect packing of β-anomer.

As the anomers show similar melting points but different enthalpy changes it relates to the differences in crystal structures. The anomers also differ in their solubilities. As for example the n-octyl α-D-glucoside can easily be crystallized from water, the β-anomer is higly soluble as shown by Balzer and Luders [5].

2.2 Applications of alkyl glucosides

The most important application area of alkyl polyglucosides is the use as sugar surfactants [6]. Alkyl glycosides are used by surfactant chemistry, in both special and bulk applications because of their favorable properties like biodegradability and chemical stability [5, 7]. They have the molecular structure of surfactants which always consists of hydrophilic head group and a hydrophobic residue. However, when producing alkyl polyglycoside surfactants in industrial scale via different synthesis routes there will be different mixtures of alkyl homologues, oligomers, anomers, and isomers (furanosides and pyranosides) in the product [6]. The purity of the synthesized product will be discussed more under the Section 3.

The surfactants may be used in cosmetics, personal care products, pharmaceuticals, and kitchen detergents. Alkyl glucosides are also useful building blocks in carbohydrate synthesis. In addition, they may be used in membrane protein research. [7, 8]. Alkyl glucosides are relatively easy to tailor to meet the new fields of applications and technical problems [5]. As environmentally friendly compounds, they start to be cost efficient as the manufacturing methods are developing.

According to Yakimchuk et al. [9] alkyl glucosides are stable in alkaline and acidic solutions, they seem to be efficient in the production of detergents and they are characterized as highly biodegradable substances with low toxicity. Short chained alkyl glucosides are assumed to have low foaming and good emulsifying properties. Though, the research has been limited mainly to long-chain alkyl glucosides with 10 to 18 carbons in the alkyl chain by far. Glucosides with short alkyl chain do not work well as surfactants as far as it is known [10]. Moreover, alkyl glucosides are used as building blocks in carbohydrate synthesis, but short chained alkyl glucosides does not have too many applications reported in chemical technology.

3 SYNTHETICAL METHODS TO PREPARE ALKYL GLUCOSIDES

The O-, N-, and C-glycosides discussed in Section 2 have different synthesizing methods [2]. Here, only the methods for O-glycosides are discussed as ethyl-β-D-glucopyranoside is an O-glycoside among all the other alkyl glucosides.

Depending on which alkyl glucoside is the desired product there are some different ways to prepare them. Especially alkyl polyglucosides have generally been interesting among researchers because of their green surfactant properties. They may be synthesized directly from glucose and fatty alcohol having a large excess of the alcohol in the solution which minimizes the sugar oligomerization [11]. In transacetylation short chained alkyl glycosides are used as an intermediate for alkyl polyglucosides. β-glucosidase utilizing enzymatic synthesis is also possible. Research of glucosides with short alkyl chain has not been as extensive as in the case of alkyl polyglucosides but they may be prepared via similar methods.

When synthesizing glycosides it is often important to produce anomerically pure product. The unreacted starting sugar and unwanted sugar derivatives need to be removed as well. Preparation of a certain anomer is usually complicated especially in the case of chemical synthesis methods like Fischer glycosylation and König-Knorr synthesis. [12]. High temperature and pressure, toxic catalysts, multiple steps of protection, deprotection and activation is needed to produce anomerically pure product [7].

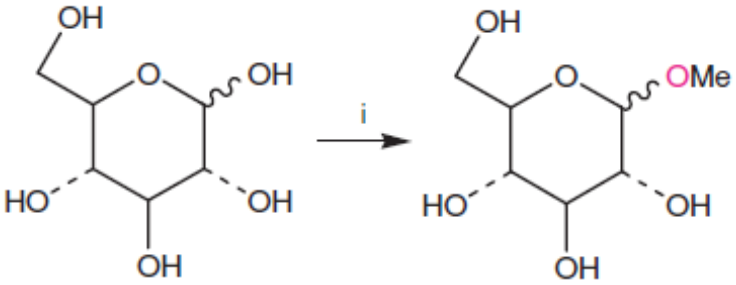
Effective post synthesis anomer separation methods have been neither found nor examined among glucosides. The effort is put to find an efficient way to prepare anomerically pure product at once within the synthesis. Different protecting groups are used to hold the reacting compounds in right position or to prevent unwanted reactions so that anomerically pure product could be synthesized. In enzymatic synthesis the enzymes take care of the production of a certain anomer.

3.1 Chemical synthesis

Summarizing shortly, a glycoside bond is formed when a sugar fragment of any size is condensed with either an aliphatic or aromatic alcohol, or another sugar moiety through an oxygen. The two most common ways to prepare alkyl glucosides via chemical synthesis are Fischer glycosylation and Königs-Knorr synthesis. It is possible to produce anomerically pure product with these methods, but it requires several reaction steps, extreme temperatures, and sometimes toxic catalysts [7]. To name other general methods for the formation of O-glycosides there are for example The Michael reaction, The Helferich reaction, The Sulfur reaction, and The Glycal Reaction presented in more detail for example in the book of Brito-Arias [2].

3.1.1 Fischer glycosylation

Commercial production of glycoside surfactants are mostly carried out through Fischer synthesis [7]. It is considered as the best choice to prepare simple alkyl glycosides from sugars [13] and found to be satisfying method only with small aliphatic alcohols [2]. The advantage is that it does not need protecting groups and is simply combining the free sugar with an alcohol under acidic conditions. An example is presented in Figure 3-1.



i) MeOH-HCl(g).

Figure 3-1 The Fischer Reaction having HCl gas as promoter [2].

Generally, the Fischer reaction is a one-step approach which produces a complex mixture of alkyl glycosides. An alcohol is simply reacted with glucose and mineral acid is used as promoter (e.g. H_2SO_4 or HCl). In addition, Lewis acid, ion exchange resin, and trifluoromethanesulfonic have been also reported providing good yields as promoters [2].

Table I Reacting saccharides with methanol in the presence of 1 % hydrogen chloride at 35°C the corresponding set of products were obtained [5].

Saccharide	Methyl α-furanoside (%)	Methyl β-furanoside (%)	Methyl α-pyranoside (%)	Methyl β-pyranoside (%)	Ref.
D-Glucose	0.6	0.9	65.8	32.7	5
D-Mannose	0.7	0.0	93.9	5.4	5
D-Galactose	6.2	16.3	57.8	19.7	5
D-Xylose	1.9	3.2	65.1	29.8	4
D-Arabinose	21.5	6.8	24.5	47.2	4

Without protection steps a complex mixture of glucose derivatives is formed because of equally reactive hydroxyl groups in the glucose ring [7]. This is illustrated in Table I. The ratio of α- and β-glycosidic products formed depends on the relative thermodynamic stabilities of the isomers [14]. These glucose derivatives are extremely challenging to separate from each other. In addition to mixture of anomers both isomeric forms, funarosides and pyranosides are noticed to be present in the producing solution [2].

Quite a few ways to improve Fischer glycosylation has been examined. In some newer applications for example sulfuric acid immobilized silica has been successfully used as a catalyst to produce glycosides. According to Roy and Mukhopadhyay [13] it was noted to be an efficient alternative to produce alkyl glucosides. The procedure also requires lower quantity of alcohol and shorter reaction time than normally in Fischer glycosylation. Utilizing microwaves to accelerate Fischer glycosylation has also been found to work in some cases [14].

3.1.2 Königs-Knorr synthesis

Königs-Knorr is a substitution reaction of a glycosyl halide with an alcohol. Both alkyl and aromatic alcohol glycosides as well as coupling between sugars may be done via the reaction. Especially glycosides having complex groups, particularly oligosaccharides, are widely synthesized using this reaction.

In the procedure per-O-acylated glycosyl halide is treated with an alcohol. Silver salts like Ag_2O or Ag_2CO_3 are required as catalyst. Oxide, carbonate, nitrate, and triflate silver salts are the most commonly utilized catalysts [2]. The methodology is described as follows by *Brito-Arias* [2]: "When the protecting group is acetate at C (2), there is an intramolecular nucleophilic displacement of the leaving group, generating orthoester. This intermediate is responsible for the incorporation of the alcohol on the β-position". The methodology is presented in Figure 3-2.

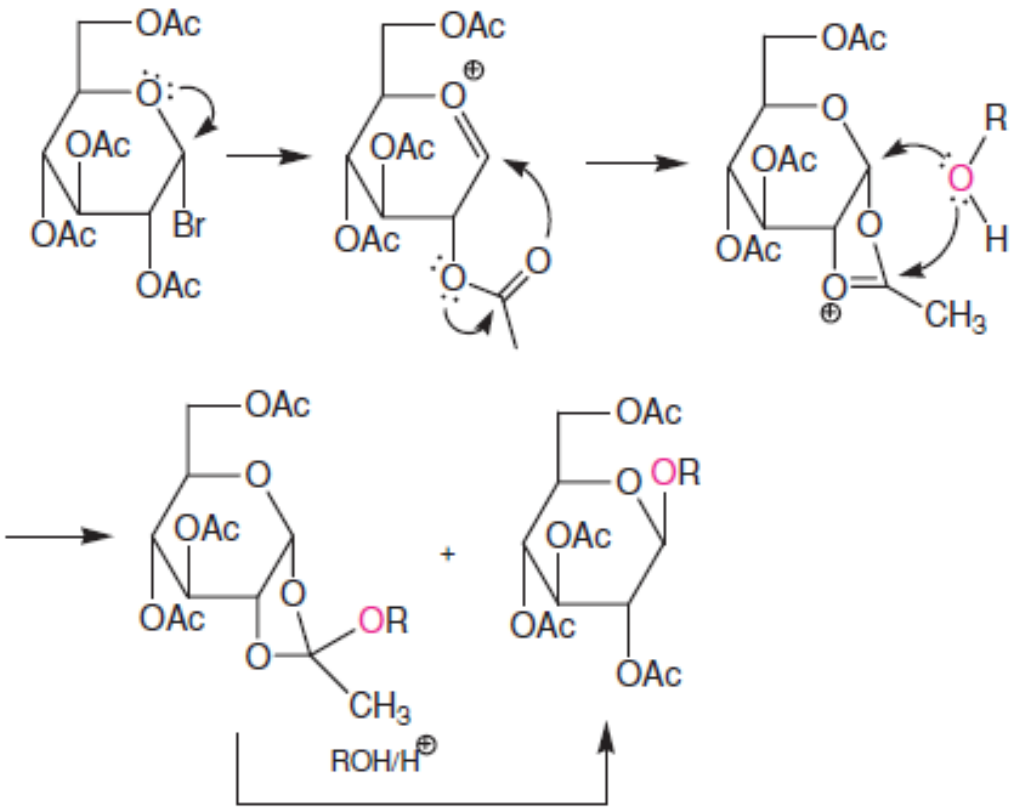


Figure 3-2 Mechanism of Königs-Knorr glycosidic reaction for the synthesis of β O-glycoside formation [2].

The more stable anomer is obtained under the usual reaction conditions and preparation of the less stable anomer occurs only via kinetically controlled reaction. Per-O-acylated glycosyl bromides are preferred for most reactions since they react faster than the corresponding chlorides. Königs-Knorr reaction is the only, but relatively inefficient route to chemically synthesize glucosides only in β-form [4].

When anomerically pure glycosides are of interest chemical syntheses are not the easiest, cheapest, or environmentally friendliest way to produce them. Nowadays there are some effective applications to prepare anomerically pure product more simply and environmentally friendly by using enzymes.

3.2 Enzymatic synthesis

Ecological concern has slowly arose enzyme provoked ways to prepare glycosides on topics because of their environmentally friendly function. Production of anomerically pure alkyl glycosides may be catalyzed by glycosidases under mild conditions without need for a cofactor [4], a molecule that is required by some enzymes in order to function. Glycosidase enzymes catalyze the hydrolysis of glycosidic bond for example in simple glycosides, oligosaccharides, and polysaccharides.

Glycosidases catalyse glycoside hydrolysis *in vivo*, but *in vitro* they can synthesize glycosides via reverse hydrolysis (or glycosylation). In reverse hydrolysis water is the leaving group when a monosaccharide and an alcohol condensates. The reaction is controlled thermodynamically towards an equilibrium. [4]. For example in alkyl glucosidase synthesis, unprotected glucose is required as starting material and alcohol is both reactant and solvent [15]. In its simplicity the equilibrium reaction of reverse hydrolysis is shown in reaction Equation 1.

$$\text{Glycone-OH} + \text{ROH} \leftrightarrows \text{Glycone-OR} + H_2O \quad (1)$$

The approach involving reverse hydrolysis is an equilibrium controlled synthesis. According to Crout and Vic [16] the main parameter controlling the equilibrium is water thermodynamic activity (a_w). Synthesis may be shifted towards equilibrium by reducing the water activity and increasing the substrate concentration so that the substrate itself acts as a solvent media. Successful glycosidic bond formation between glycosyl donor (glycone, for example the sugar fragment having leaving group at the anomeric position) and acceptor (aglycone, for example an alcohol) depends on the reactive intermediate. It should be trapped faster by the glycosyl acceptor than by water. For example alcohols as the glycosyl acceptor tend to bound at the active site of glucose better than water [17].

Simply, glycosyl donor is glycosylated with the enzyme after which the reactive intermediate is released in a certain anomeric position as reacting with a suitable glycosyl acceptor. In addition to stereochemical selectivity at the anomeric center (one enantiomer is preferred over the other) glycosidases show a high degree of chemoselectivity for different hydroxyl groups (selective reactivity, preferring certain substrates over others). The following order of reactivity is noted by Rantwijk et al. [4]: primary alcohols > secondary alcohols > phenols yet, tertiary alcohols are unreactive.

Kosáry et al. have immobilized α- and β-glucosidase enzymes on a modified polyacrylamide-type bead support (Acrylex C-100) resulting in higher yields. With non-immobilized enzymes the yields for β-glucosidase of butanol, pentanol, hexanol, and cyclohexanol changed from 18 – 21 %. Yet, with immobilized enzyme the product yield changed from 36 – 44 % in the same reaction conditions. [15]. Immobilization of the enzymes enables glycosylation via reverse hydrolysis on a larger scale and reuse of the enzyme.

In some applications, native enzymes, especially glucosidases, are used [18] [8] as more inexpensive catalyst for glycosylation reactions of alkyl glycosides. Native enzymes here mean for example powdered almonds that may be used as a source of β-glucosidase in the synthesis. In general, the process seem to work, but when scaling up the reactions yields are sharply reduced due to heterogeneity of reaction mixture and the aggregation of undissolved enzymes in organic media [15]. Kosáry et al. [15] noted that the aggregation of undissolved enzymes could be avoided with immobilized enzymes.

4 ENZYMES

Enzymes are the catalysts of biochemical reactions in living organisms. They are more specific and powerful catalysts than any other chemicals. Nearly all of the reactions in the organisms are catalyzed by enzymes. Most of all known enzymes are protein-based except ribozymes which are made of ribonucleic acids [19].

All enzymes are “true catalysts” which means that they have the following properties. They lower the activation energy barrier, thereby increasing the rate of a reaction as shown in Figure 4-1 which illustrates the conversion of a substrate (reactant in the catalyzed reaction) into a product. Enzymes are not consumed or permanently changed during the catalytic process. They increase the reaction rate but do not change the chemical equilibrium between reactants and products which means that the enzyme equally accelerates both the forward and reverse reactions. They also usually stabilize the transition state by forming a transient complex with the reactant. In addition some enzymes need a cofactor to function properly. A cofactor may be an organic or organometallic molecule (coenzyme) or a metal ion such as Zn^{2+}, Mg^{2+}, or Cu^{2+}. [20].

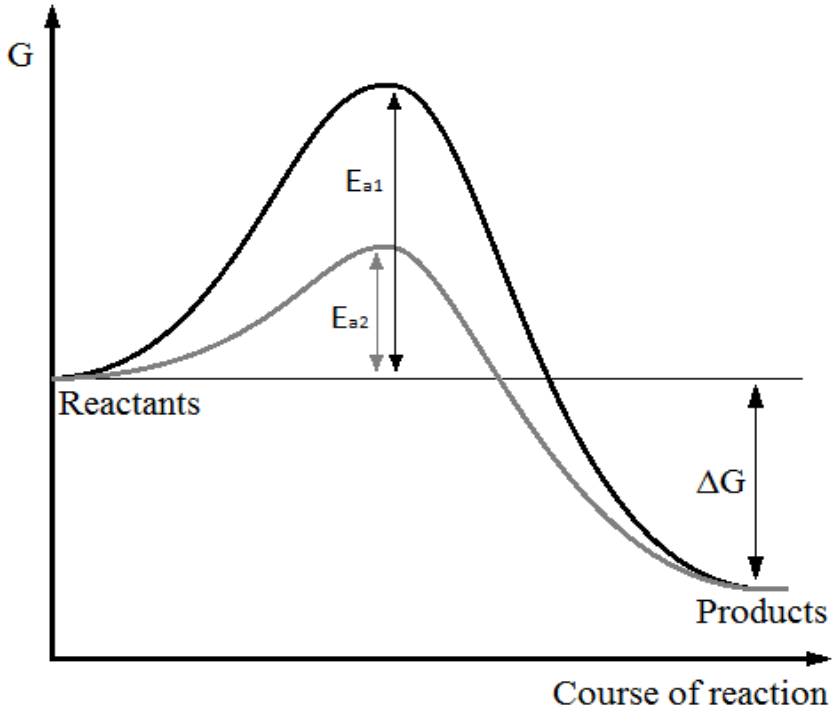


Figure 4-1 Energy diagram for the catalyzed and uncatalyzed reactions. Usage of a catalyst lowers the activation energy (E_a). Uncatalyzed reaction needs higher activation energy (E_{a1}) than catalyzed reaction (E_{a2}). There is a change in free energy (G) between the reactants and the products. The amount of free energy is the highest when the reactants are in transition stage. The reaction proceeds towards the lower energy state.

The experimental factors which affect enzyme activity are substrate concentration, enzyme concentration, pH, and temperature. By adjusting these factors the enzyme reaction rate is changed.

Enzymes are classified into six main types using Enzyme Commission number (E.C.). The E.C. number tells general information about the enzyme, for example its class and the bond it is affecting. The six classes are oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases [21].

When enzymes are used in experiments the exact mass of the enzyme in a sample is rarely known, since the enzyme is generally isolated from micro-organisms, or animal or plant tissues usually containing great amounts of non-catalytic protein [21]. This is why the enzyme concentration may vary from sample to sample and why the enzyme concentrations are often given in “units” instead of mole or mass concentrations.

4.1 Enzyme active sites and substrate binding

An active site is the specific region in the enzyme to which the substrate binds. Generally the active sites are formed by amino acids. Different parts of the polypeptide chain are brought together because of the tertiary structure of the enzyme [22]. The folding of the protein creates a pocket like space in which the catalytic effect occurs. Usually there is only one active site per enzyme subunit [20]. Subunit is a single polypeptide chain assembling with others to form a protein complex, in this case an enzyme. Depending on the protein complex one unit may contain one or more subunits. Since the number of active sites is limited the product may be produced only at a certain rate even if the substrate concentration is much higher than the enzyme concentration, which usually is the case.

The active sites of most enzymes have special characteristics. Enzymes are specific, which means that they recognize the possible substrate molecules due to their "close fit" into the active site. The shape of an active site is closely mirroring the shape of the substrate and some enzymes are even capable to discriminate D and L isomer of a substrate. [22].

The substrate is attached to the active site by weak, non-covalent, and reversible interactions including hydrophobic, ionic, and hydrogen bonding. Only certain interactions are allowed by the functional groups of the enzyme contacting closely with the substrate. When the substrate is bound non-covalently to the active site energy is released to stabilize the unsteady transition state. [20].

Different models of enzymatic catalysis are proposed over time. Lock and key model is probably the simplest. This model introduced by Emil Fischer in 1890 considers the enzyme active site as a lock which is capable to accept only a specific type substrate (key). There is much flexibility in the three-dimensional structure of enzymes but this model implies a very rigid active site. A newer idea by Daniel Koshland was proposed in 1958. Induced-fit model assumes continuous changes in the structure of the active site during substrate binding. The final shape and charge characteristics of the active site are reached not before the substrate is completely bound. [19].

The transition-state analog model is the latest model for enzyme reactivity. According to it the active site not only recognizes the substrate but also position it correctly for the upcoming reaction. When being part of the enzyme-substrate (ES) complex the substrate's bond to be cleaved is already partially broken to enhance the product formation. [20]. All the phases in the formation of ES complex makes the substrate binding to the enzyme the most time consuming phase in the enzyme catalyzed reactions.

In the simple examples there is only a single substrate which binds to an enzyme and reacts into a product. However, usually the biochemical reactions involve interactions between more than one substrate. In such case all the substrates needed in the reaction are considered to bind to the active site of an enzyme in the proper position and orientation to accelerate the production of the product [22]. This is demonstrated in Figure 4-2.

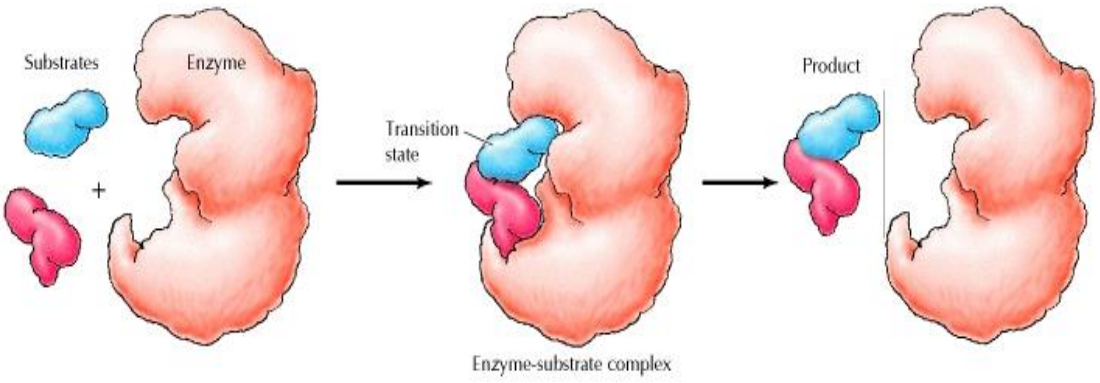


Figure 4-2 Binding of two substrates to an enzyme **[22]**.

Enzyme's other mechanistic features begins after substrate binding and stabilization of its transition state. General acid-base catalysis, metal-ion catalysis and covalent catalysis are the most important and best understood mechanistic processes enzymes perform [20]. Acid-base catalysis is taking place when the enzyme has such functional groups in the active site region that assists in proton transfer reactions and ease bond cleavage. Proton transfer is facilitated due to close locations of donors and acceptors of substrate and proton.

When metal ions are present in enzyme or substrate they are usually taking part in catalysis. There are at least three ways for alkali metal ions and transition metal ions to assist enzyme reactions. Metal ions are capable for holding a substrate properly oriented by coordinate covalent bonds. They can polarize the scissile bond or stabilize a negatively charged intermediate to enhance the reaction. Reversible electron transfer between substrate and metal ions is their way to commiserate in biological oxidation-reduction reactions. [20].

In covalent catalysis a nucleophilic functional group of an enzyme reacts with the substrate to form a covalent bond producing a very reactive temporary intermediate. By combining different mechanisms of catalysis enzymes are capable to produce certain product from a certain substrate.

4.2 β-glucosidase from almonds

β-glucosidase from almonds is a glycoprotein and it is classified as a hydrolase with EC number 3.2.1.21. It releases β -glucose as a reactive intermediate. As a homodimer it consists of two equal subunits of 65 kDa. The molecular weight of the whole enzyme is 135 kDa [23] which apparently contains a 5 kDa space between the subunits. β-glycosidases are easily available, exhibit vast substrate specificity and stereo-selectivity which is why their usage is a good alternative for chemical glycoside synthesis [7] which is circuitous and expensive.

One of the most studied β-glucosidase is the one obtained from sweet almonds since it is noticed to have a good functionality and since almonds are readily available. It has been long known that sweet almond β-glucosidase hydrolyzes glycosides [24] and it obviously follows the standard mechanism shown in Figure 4-3. However, the detailed mechanism of the steps has been remained unclear and researched more by Xie [25].

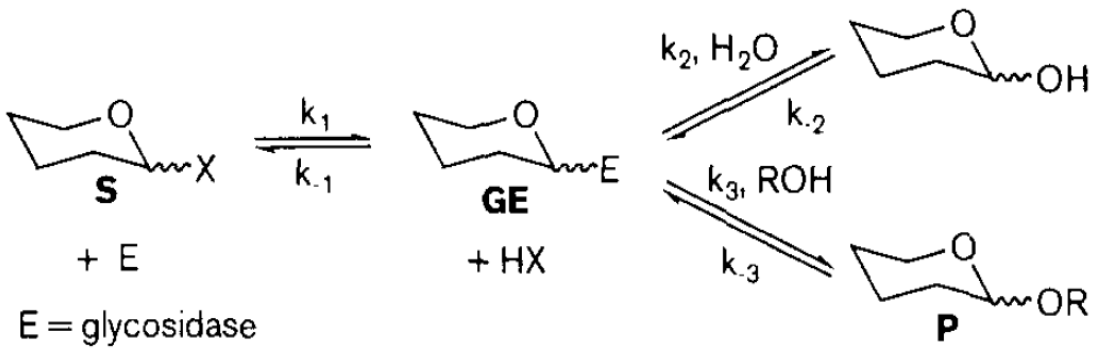


Figure 4-3 Reverse hydrolysis of substrate (S) into product (P) via glycosyl-enzyme intermediate (GE). Hydrolysis is happening via reaction 2. HX is the leaving group (X) combined with hydrogen (H). **[16]**.

When the glycosyl donor (a monosaccharide, oligosaccharide, or activated glycoside) is incubated with the glycosidase enzyme a glycosyl-enzyme intermediate is formed [16]. The intermediate can be intercepted either by water, giving hydrolysis product, or by an acceptor, giving a new glycoside or oligosaccharide.

It is possible to increase the alkyl glucoside yield by increasing the concentration of acceptor alcohol thereby, transferring the reaction equilibrium towards the alkyl glucoside synthesis. Yet, too large amount of alcohol reduces the enzyme stability and sugar solubility. Small amount of water is needed as β-glucosidase is inactive at low a_w [16]. With increased hydration level the enzyme becomes more flexible and active, simultaneously increasing the chance of hydrolysis instead of glycosylation reaction.

5 LIQUID CHROMATOGRAPHY

Liquid chromatography (LC) is a separation method utilized both in analytical and in industrial scale for many purposes; separation of both organic and inorganic compounds like sugars, metal and other ions, pharmaceuticals, different isomers etc. For example the largest facility at an American Crystal Sugar Co. in Hillsboro processes over 545 tons per day of 80 % dry substance molasses [26]. On analytical level, liquid chromatography is commonly used to get individual components analyzed from complex mixtures. Usually, a high performance liquid chromatography (HPLC) equipment is used.

In liquid chromatography, stationary phase particles are packed into a column through which the feed solution containing two or more different solutes is pumped using an inert carrier, eluent. The feed is injected to the eluent stream. Basically, the solute components propagate through the stationary phase with different velocities depending on the differences in affinities towards the stationary phase material (retention). Components with high affinity propagate slower through the column than components with low affinity.

Example of a complete separation of binary solution (solutes A and B) in column is demonstrated in Figure 5-1. Corresponding Gaussian profiles are shown in Figure 5-2.

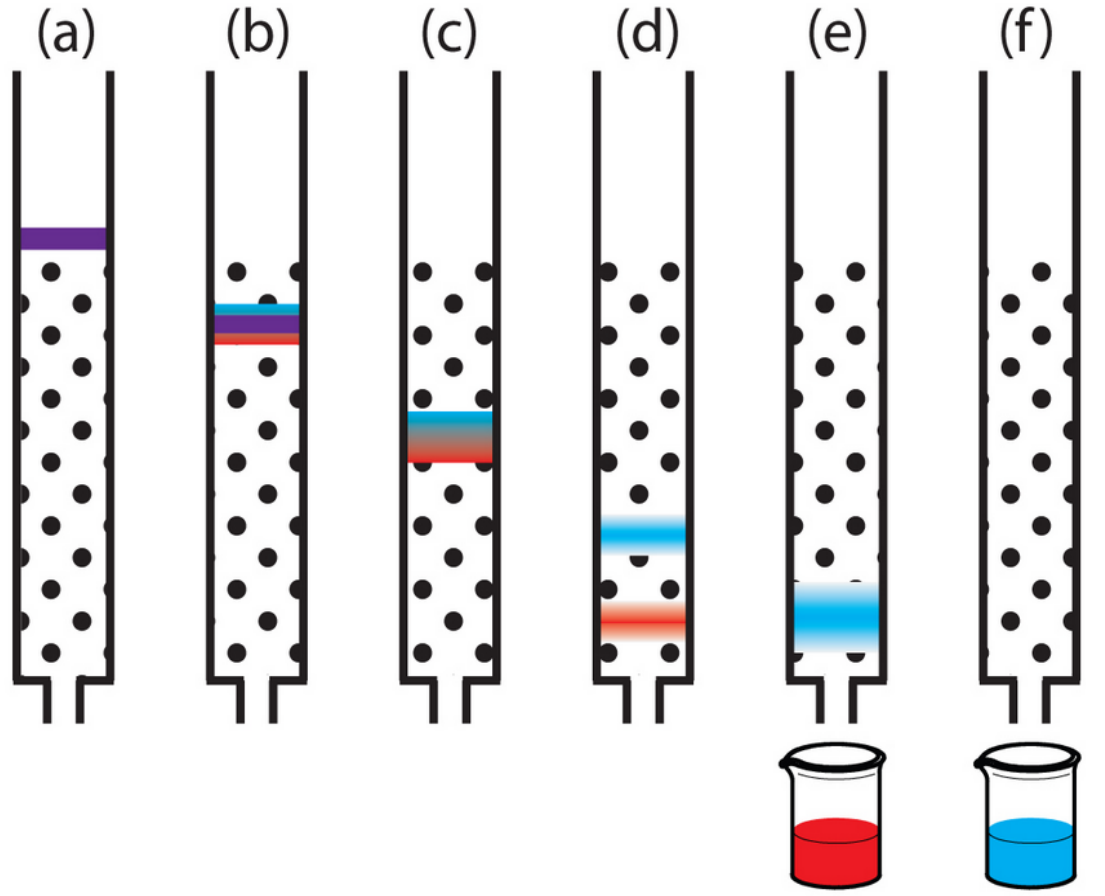


Figure 5-1 (a) Binary sample is just injected and layered on top of the stationary phase. (b-d) Separation of the solutes into two bands as the mobile phase passes through the column. (e-f) Collection of each solute to their own fractions. [27].

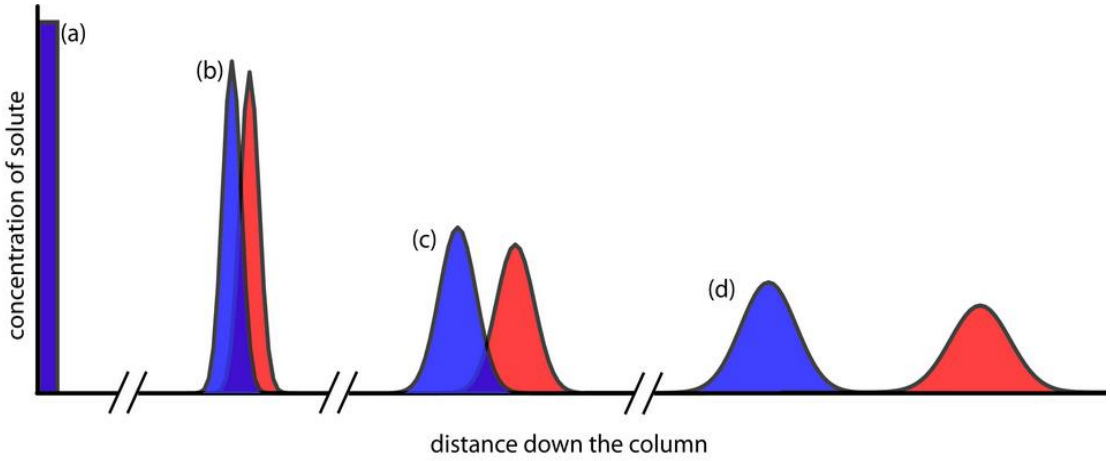


Figure 5-2 Separation above (Figure 5-1) viewed as Gaussian profiles. [27].

However, the compound separation is cause of several factors. Naturally, the temperature, pH, and pressure are physical properties affecting on to the separation yet, there is more. For example, differences in components adsorption isotherms (the strength of the adsorption) enables the separation. The sizes of the stationary phase particles together with the column size determines how great the dispersion effect is. And, greater sized column makes the separation more efficient by increasing the number of theoretical plates. In addition to these separation factors, in every separation case, there are one or more main mechanism enabling the separation to occur depending on the solute and separation material properties. The common mechanisms in sugar separation are discussed further down.

5.1 Adsorption isotherms

Adsorption isotherms are quantitative parameters describing the equilibrium distribution of a solute between mobile and stationary phase involved in chromatographic process [28]. Interactions between solvent, solute, and adsorbent are demonstrated using adsorption isotherms and they are needed when designing and optimizing preparative chromatography.

If the feed solution contains high concentrations of several adsorbing components, they may mutually influence on each other's adsorption. The isotherms obtained under such conditions might be 'competitive isotherms'. On the other hand, 'co-operative sorption' may occur. Individual components perform noncompetitive adsorption. [28].

Usually, the resin is considered to contain water in different states. Water is partly bound around the ionic moieties (hydration water) and partly considered to occur as hygroscopic water (free water). And, only the free water is available for dissolving the solutes. The division of water into these states depends on the ionic form and cross-linkage of the resins. [29]. In turn, Helfferich [30] states that "The fixed ionic groups and counter ions in the ion exchanger form solvation shells and thus tie up solvent molecules". So, in the presence of for two or more components the hygroscopic water is shared by the solutes. Hence, as the amount of free water in the column is limited, the solvated sizes of the solutes may decrease [30].

Decrease in solvated sizes might cause changes in size exclusion and change the adsorption isotherms. Therefore, the shape of an adsorbent isotherm is characteristic to every solute-solvent-adsorbent combination and is determined over a range of solute concentration.

There are five direct chromatographic methods to determine the singe-component isotherms: frontal analysis (FA), frontal analysis by characteristic point (FACP), elution by characteristic point (ECP), pulse methods, and the retention time method. The competitive isotherms are nowadays mainly measured by using column chromatography. Both, frontal analysis and pulse techniques can be easily extended to competitive binary isotherm [31]. Isotherms may also be determined by conventional static method in small batches into which certain amount of resin and changing concentrations of the solutes are added. The mixture is then allowed to reach the equilibrium. The static methods are slower and more prone to inaccuracy than direct chromatographic methods yet, their sample consumption is smaller [31].

In frontal analysis the column with known amount of studied resin is thoroughly saturated with a solution containing a known, constant concentration of the studied compound(s). The adsorption isotherm is calculated from the adsorbed amount of a solute (q) as a function of the liquid phase concentration (c). In order to solve the relation of dq/dc (adsorption isotherm) several breakthrough curves need to be done with different solute concentrations. Figure 5-3 shows an illustrating picture of one step in the frontal analysis. A staircase chromatogram is formed (Figure 8-1) when several different solute concentrations are successively fed to the column.

In the beginning of frontal analysis and after every break through step the stationary phase in the column is in adsorption equilibrium with the current mobile phase at the concentration c^I and loading q^I. Solution having different solute concentration (c^{II}) than previous solution is then started to feed until the platform of the following breakthrough curve reaches the level of c^{II} and loading of q^{II}. The response signal $c(t)$ is recorded. [32]. When several injections with different concentrations are injected sequentially to the column letting the equilibrium to form, the output gives a stair like profile. Depending on the solute concentrations the loading of the column changes.

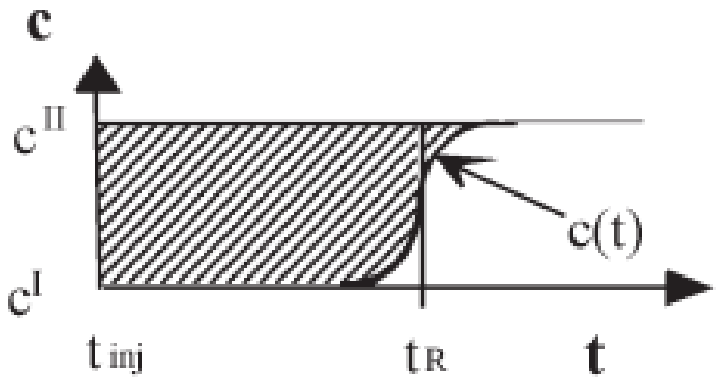


Figure 5-3 Frontal analysis **[32]**. t_R refers to retention time which is labelled as t_{ret} in the text.

Generally, the amount of solute adsorbed into the stationary phase (the equilibrium loading q^{II}) can be calculated from Equation 2

$$q^{II} = q^{I} + \frac{(c^{II} - c^{II})(V_{ret} - V_D)}{V_s} \tag{2}$$

where V_{ret} is the retention volume of the self-sharpening front (volume of the beginning of the breakthrough curve until the retention time t_{ret} is reached), V_D is the dead volume of the system (void volume from the point of injection to the fraction collector caused by bed porosity, tubing, valves, etc.), and V_s is the volume of the adsorbent material in the column.

The retention time, t_{ret}, may be calculated from the Equation 3 when the adsorption isotherm (dq/dc) is known

$$t_{ret} = t0 \cdot \left(1 + F \cdot \left(\frac{dq}{dc}\right)\right) = t0 \cdot \left(1 + \frac{1-\varepsilon}{\varepsilon} \cdot \left(\frac{dq}{dc}\right)\right) \tag{3}$$

where *t0* stand for the ‘dead time’, the time in which the particles without any affinity towards the adsorption material passes the column, and ε is the porosity of the bed.

5.2 Dispersion

Dispersion is the spread of the chromatogram. It is caused by the solutes that take different and random diffusive ways to travel across the solid phase material. Dispersion processes taking place in an LC column are multipath dispersion, dispersion from longitudinal diffusion, dispersion from the resistance to mass transfer in the mobile phase, and dispersion from the resistance to mass transfer in the stationary phase [33].

All of the solute do not go through the column via the same route (multipath dispersion). Mobile phase carrying the solute always splits while hitting a particle. The split stream joins with other split streams and some part of the solute end up going through a longer path than the other part. [34]. Longitudinal diffusion is caused due to tendency of the solutes to find the equilibrium throughout the column. Diffusion also takes place while the solute traverse through the sphere surrounding film from the mobile phase to the surface of the stationary phase particle. The situation is the same while the solute traverse from the particle to the mobile phase. [33].

If there were no dispersion in the column the solute interacting with the stationary phase would show, depending on the pulse size, a bar like chromatogram without taking the form of Gaussian profile shown in Figure 5-2. The smaller the particle size of the solid phase the smaller the gaps between the spheres. Meaning, there is less space for the solutes to twirl around, the propagation through the column is faster, and the profile of the chromatogram is less dispersed.

The efficiency of the separation is measured from the dispersion of the chromatogram. If the column is unevenly packed, all the particles are not soaked, or channeling occurs, the dispersion might increase as the column efficiency is disrupted by lack of uneven radial equilibrium [33].

5.3 Number of theoretical plates

As the mobile phase is continuously flowing through the column the solute do not reach the equilibrium at any point in practice. Yet, as a mathematical concept, the column is considered to be divided into a number of theoretical plates (NTP) each of which has a finite height where equilibrium is considered to be able to take place. The faster the equilibrium, the smaller the theoretical plate. Hence, the more theoretical plates, the more efficient separation. [33]. NTP is increasing as the column size increases which is why longer columns offer better separation. NTP is an indirect way to express chromatogram width (dispersion) at a specific retention time and it reflects the column performance.

5.4 Separation mechanisms

Depending on the mixture and the components to be separated for example ligand exchange, size exclusion, or affinity based chromatography may be applied. Of course, these are just the principal separation mechanisms. Naturally, the mechanisms may happen simultaneously depending on the separation material.

5.4.1 Ligand exchange chromatography

Molecules or anions which have the potential to act as ligands may be separated from each other utilizing highly selective ligand exchange sorbents. Ligand exchangers are ion exchangers containing complex-forming cations such as Cu^{2+}, Ni^{2+}, and Ag^{+} as counter ions. No ion exchange is taking place in the separation as the ion exchanger only carries the complexing metal ions (counter ions) enabling the ligand exchange. [30].

Ligands are ions or neutral molecules binding covalently with a central metal atom or ion. Together they form a complex. In the reaction, ligands are electron pair donors (Lewis base) and the central atom is an electron pair acceptor (Lewis acid). Ligands are mono-, bi-, or polydentate ligands depending on how many donor atoms they have i.e., with how many Lewis acids they are able to bind simultaneously. [27].

With ligand exchange very high selectivity can be achieved. The ligand which has the strongest dependency to form a complex with the metal ion is the most preferred ligand by the exchanger. [30]. Selectivity of the exchanger and differences in the complex strengths causes the separation of the solutes.

Strong ion exchangers are normally used as ligand exchangers to separate weakly binding molecules. The bound molecules may be displaced from the stationary phase with mobile phase containing molecules that are more strongly attracted to the ion exchanger sites [35]. Yet, when neutral compounds like mono- or disaccharides are weakly bound to ion exchange resin, no desorbents are needed as sugars are very soluble to water. Water is used as both the solvent for the sugar in the feed solution, and the mobile phase over the whole chromatographic cycle. The situation is examined over the years among others by Tiihonen et al. [36], and in most of the cases sugars like glucose, fructose, and sucrose are successfully separated with strong acid cation exchange (SAC) resins which have a metal counter ion.

Sugars are bound to divalent metal ions more strongly than to monovalent metal ions. In sugar separation Ca^{2+} is the most used counter ion though, also K^{+}, Na^{+}, Ba^{2+} and Pb^{2+} ions may be functional. [37]. In some cases the sugar complex formed with monovalent metal ions are thought not to be strong enough to gain proper separation. Commonly used polymer base of ion exchange resins is sulfonated polystyrene-divinylbenzene (PS-DVB). In SAC resins the functional group attached to the resin matrix is the sulphonic acid group (SO_3H). The ligand exchange chromatography is also applied in the analysis of sugars using HPLC.

The retention of sugars in SAC resins is primary based on the complex formation between the hydroxyl groups of sugars and metal counter ions attached to the functional group of the resin (ligand conversion) [38]. Different monosaccharides have different molecular structures which affects to the strength of the complexes formed [37]. It is also noticed that increased organic solvent content increases the complex stability between the complexing component and the counter-ion. The stronger the complex formatted in water the more an organic co-solvent strengthens the complex. [39].

The retention varies depending on both the stability of the complexes and the size exclusion effect. Size exclusion is dominant when Na type gel is used whereas, with Ca and Pb type gels it is possible to get sugar alcohols and some monosaccharides separated [38]. The separation of larger molecules, such as disaccharides, from smaller, such as monosaccharides is based on size exclusion as the larger molecules cannot fit into the pores of the stationary phase materials.

5.4.2 Size exclusion chromatography

Size exclusion simply separates components according to their molecular size: the biggest particles elute first since they do not fit into the small pores of the filtration gel through which smaller particles are travelling. Smaller particles are taking the 'longer path' through all the small pores which is why the smaller compounds elute out last from the column. There are two conditions to choose the mobile phase for size exclusion separation: the elute needs to dissolve the components and prevent interactions based on polarity or charge between the solutes and the surface of the stationary phase [35].

In size exclusion chromatography weak and strong acid cation exchangers (WAC and SAC) may be utilized in Na^+ and K^+ form. Depending on the size and the valence of the counter ion the pore size of the resin changes. When using monovalent metal cations as counter ions the resin is allowed to swell more and size exclusive separation of carbohydrates is possible. If di- or trivalent counter ions are used the pores of the resin become too tight for carbohydrates and size exclusion is not occurring. Tiihonen [29] has measured water contents of several strong and weak cation-exchange (SCE and WCE) resins. For example, in water CS16G resin has water content of 46 w-% in Na^+ form and 44 w-% in Ca^{2+} form [29].

Other feature that changes the pore size of the resin is crosslinking level which is expressed as weight percentage of the crosslinking agent in the resin. In the case of the resins used in this thesis (see Section 7.3.1), the crosslinking agent is DVB. The more highly cross-linked the resin the smaller the pores as the cross linkages tightens the resin body.

5.4.3 Affinity based chromatography

In affinity based chromatography the compounds are adsorbed to the active sites of the solid phase material. Adsorption usually depends on the polarities of the compound and the stationary phase, as it is known “polar like polar”. If the compound is similar in polarity with the stationary phase it will be delayed in the chromatographic process since it is attracted by the adsorbent. Compounds having similar polarity with the mobile phase will travel through the solid phase faster as they are attracted more by the eluent which passes the adsorbent bead. [35]. Often used polar adsorbents are silica and alumina.

When non-functionalized polymeric resins are used as adsorbent materials there are three parameters affecting to the binding capacity of a resin for a particular material: the dipole moment, the pore size, and the surface area [40]. The component to be adsorbed needs to reach the adsorbing surface of the pores.

WAC resins are recently found to be effective materials for separating hydrophobic saccharide derivatives from more hydrophilic saccharides [41]. In WAC resins the polymer base of acrylic acid is co-polymerized with DVB as a cross linking agent. The functional group attached to the resin matrix is the carboxylic acid group (COOH). Due to WAC resins hydrophilic nature the most hydrophobic saccharides elute first and the most hydrophilic last from the column. The resin may be used for example in H^+, K^+, Na^+, Mg^{2+}, or Ca^{2+} form. In addition to hydrophobicity, ion and size exclusion are taking place in the separation [41]. By changing the counter ion in the carboxylic group for example the tightness of the resin changes as well as its moisture content.

6 STEADY-STATE RECYCLING CHROMATOGRAPHY

The performance of a batch chromatography process may be enhanced by collecting the impure fraction in between the two separated product fractions, and recycling it back to the column. This allows the usage of greater injection sizes and flowrates without decreasing the yields or purities of the products as the impure fraction of the chromatogram is cut off and recycled. Closed-loop-recycling (CLR) chromatography is the simplest recycling based chromatographic process.

In the CLR procedure, one sample or injection is circulated in the separation system until the wanted purities of the components are reached. Recycled fraction contains the unresolved section of the chromatogram which does not meet the purity demands set for the products. The resolution between the components increase after every cycle. So, the process simulates the use of a longer column as the recycling increases the number of theoretical plates available for the separation. New injection is introduced into the system after completely separating the previous pulse. [42].

Anyhow, the concentration of the solutes in the recycled fraction decreases after every cycle until there is nothing to recycle, and new injection is done. If the feed concentration remained somehow the same, the productivity and eluent consumption would improve as more concentrated feed could be processed within one cycle.

Like CLR, steady-state recycling (SSR) chromatography is an advanced batch process with a single column. SSR works similarly as CLR yet, in the SSR separation process fresh feed is mixed with recycled fraction from the previous pulse. Mixing fresh feed with the recycled fraction never allows the feed concentration to decrease below a certain point (determined by the amount and concentration of recycle in the mixed feed). By doing so, the productivities increases and the eluent consumption decreases compared to batch and CLR processes.

In SSR (as in CLR), the first injection to the column is pure fresh feed. Next injection is made from mixed feed container where recycled fraction and fresh feed is mixed. The size of the recycled fraction determines the share of fresh feed in the following injections as Equation 4 is valid during the whole run. The mixed feed concentration decreases with successive SSR cycles until the steady-state is reached. In steady-state the product purities and concentrations remain constant.

$$V_F = V_{FF} + V_R \tag{4}$$

where V_F is the volume of feed, V_{FF} is the volume of fresh feed, and V_R is the volume of the recycled fraction.

In order to run the SSR the four cut times are needed to know: t_{A1}, t_{A2}, t_{B1}, and t_{B2}. The times are placed into an example chromatogram in Figure 6-1. The first product fraction (A) is collected between t_{A1} and t_{A2}. The recycle fraction (R) is collected between t_{A2} and t_{B1}, and the second product fraction (B) is collected from t_{B1} to t_{B2}.

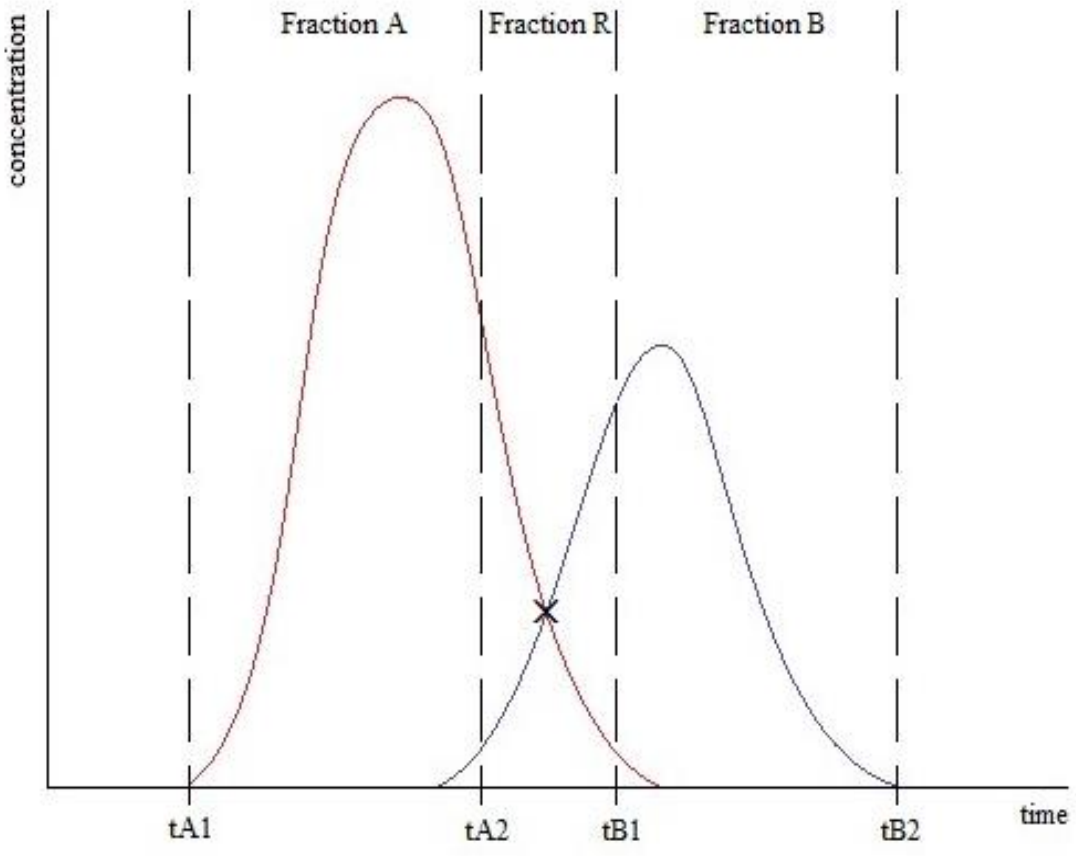


Figure 6-1 An example design pulse for setting the SSR fraction times depending on the purity wanted for each product. t_{A1} and t_{A2} are the starting and finishing times for the collection of the fraction A. t_{B1} and t_{B2} are the starting and finishing times for the collection of the fraction B. Fraction collected between t_{A2} and t_{B1} is recycled fraction R. X indicates the cut time for a normal batch process when comparing its productivity, purity, and eluent consumption to SSR.

Determining the cut times for recycle chromatography is challenging yet, they may be predicted based on batch experiments having similar separation conditions. It is quite easy to find somehow suitable cut times for the recycle (t_{A2} and t_{B1}), but finding the optimal ones with the best productivity, yield, and eluent consumption becomes a challenge. In addition, finding the optimal injection volume and flow rate for a certain separation causes a lot of work without simulation models. Therefore, complex numerical simulation models are often used in the SSR process design. More detailed SSR designing procedure used in the present study is presented in Section 7.4.2.

There are also some designing methods like ‘unified design’ [43] and ‘shortcut design’ [44] to make it easier to transfer the separation scheme from process to process without need of complex simulation models. These designing methods are based on four unchangeable operating m-parameters (one for every cut time) which are determined based on batch processes. The parameters are used to study the separation in a specific separation region while changing the cut times and thereby the volume of the recycle fraction. The topic is discussed more elsewhere [43, 44].

Simulated moving bed (SMB) is a continuous process simulating the use of infinitely long column. Feed is injected, fresh mobile phase is added, and two product streams are collected continuously into and from the SMB system containing several columns in a circulating profile. [42]. Compared to SMB, both SSR and CLR systems have relatively simple setups. They have only one column and some SMB processes have up to 12 columns.

The advantages offered by the continuous SBM process are even better separation and smaller eluent consumption than with SSR. Yet, the high investment cost is one of the main drawbacks of the system. In addition, long time is needed until steady-state operation is achieved and the switching system between columns is complex [31].

Especially when high yields are required, batch processes have limitations with high eluent consumption and low process performance. Compared to batch processes recycling chromatography offers solution to these problems with relatively low investment costs and even when the product chromatograms are greatly overlapping.

7 EXPERIMENTAL

One of the main objective in this thesis was to look for an efficient and easy way to synthesize ethyl β-D-glucopyranoside (BEG), purify the component from the resulting mixture and produce at least fifty grams of BEG in ≥ 99 % purity with a scaled-up process. The other main objective of the thesis was to transfer the batch process into steady state recycling (SSR) process. The designing procedure and efficiency of SSR process were examined. The productivity and eluent consumption of SSR process was compared to corresponding batch process and, the best separation conditions were searched by simulations.

7.1 Materials

Materials used in the experiments of this thesis are presented in Table II.

Table II Material used in the experiments.

Purpose	Substance	Properties	Supplier
Synthesis	Water	Purified water	-
	Ethanol	Purity of 99.9%, analysis quality	VWR
	D-(+)-glucose	Purity of 99.5 %	Sigma-Aldrich
	β-glucosidase	Lyophilized powder, premium, ≥2 units/mg solid	Sigma-Aldrich
Resin	Water	Purified water	-
pretreatment	NaCl	Assay min. 98 %	VWR
	HCl	37 %	Merck KGaA
	$CaCl_2 \cdot 2H_2O$	Assay ≥99 %	Sigma-Aldrich
Resins	CS16GC	More specific information in Table III	Finex
	CS11GC		Finex
	CA16GC		Finex
	CA10GC		Finex
Porosity determination	Blue Dextran 2000	-	GE Healthcare

7.2 Enzymatic synthesis of ethyl β-D-glucopyranoside using β-glucosidase

β-glucosidase enzyme was chosen to enzymatically synthetize ethyl β-D-glucopyranoside. The enzymatic synthesis is a slow process taking over five days to reach equilibrium between the product and the starting material. Yet, it is better choice than chemical synthesis especially when the feed solution to be purified is wanted to be as simple as possible. Chemically synthesized product solution would have contained for example several sugar derivatives and/or toxic metals. Product solution from enzymatic synthesis contains only water, ethanol, glucose, and BEG. It also seems to be the simplest and environmentally friendliest way to implement the synthesis in laboratory scale.

Glucose, ethanol, water, and β-glucosidase enzyme were the starting substances in the ethyl β-D-glucopyranoside synthesis. Ethanol-water ratio was kept in 8:2. Lyophilized sweet almond β-glucosidase powder (≥2 units/mg solid) from Sigma-Aldrich was used in the experiments. Ethanol used had analytical purity of 99.9 % and glucose had purity of 99.5 %. Water used was Millipore grade water. According to the enzyme supplier optimal conditions for the enzyme to function are pH of 5 and temperature of 38^{o}C, and these conditions were implemented over the synthesis.

Equilibrium tests for the synthesis were done by changing the glucose and enzyme concentrations in the test series. In the first test series the glucose concentration was 55 g/L and enzyme concentration was 0.3 g/L. In the second test series the glucose concentration was 28 g/L and enzyme concentration was 0.2 g/L. For further studies the water-ethanol ratio was kept the same (8:2). Glucose concentration was adjusted to be 40 g/L and the enzyme concentration 0.2g/L. According to the enzyme supplier the enzyme solubility in water is 1 mg/mL which is why no more than 0.2 g/L of enzyme was eventually added to the synthesis solution containing 20 % water when preparing feed solution for the separation experiments.

A larger batch of feed solution was prepared in 250 mL bottles. The initial glucose, enzyme, water and ethanol concentrations were the same as above. Synthesis was carried out for over 5 days under 38ºC and 300 rpm shaking after which the glucose concentration was approximately 14-17 g/L and the ethyl glucose concentration was 38-41 g/L (final concentrations varied a little bit from batch to batch). The solution was filtered with VWR's 25 mm Syringe filters (w/ 0.45 µm polypropylene membrane) to remove the enzyme. Subsequently the solution was stored in freezer.

First single-column batch separation experiments were done having ethanol in the feed solution. Yet, large amounts of ethanol was noticed to disturb the separation of glucose and BEG so, its amount was radically decreased for further studies. Ethanol was evaporated off from the solution with rotavapor (120 rpm and 35ºC) when testing the separation materials, and by heating in a fluoropolymer -coated vessel (60 - 75ºC) when producing over 50 grams of pure BEG for the clinical trials conducted by the University of Turku (UTU). The solution with trace amounts of ethanol was used as a feed solution in the search for the best separation material and eventually in the production of pure BEG.

7.3 Purification of ethyl β-D-glucopyranoside

Since ethyl β-D-glucopyranoside is a sugar derivative the development of its purification method was done by using similar methods as used in sugar purification. After finding an appropriate separation material to produce pure BEG the batch process was scaled up and eventually transferred to continuous SSR separation system. The target purity of the final product (BEG) in batch was 99 % and in SSR experiments the purity limit for both BEG and glucose was 98 %.

7.3.1 Stationary phase materials

The materials used in experiments were styrene based strong acid cation exchange resins (SAC) CS16GC and CS11GC, and acryl based weak acid cation exchange resins (WAC) CA16GC and CA10GC. The fundamental properties of the studied stationary phases are listed in Table III. SAC resins were used in both Ca^{2+} and Na^{+} form. WAC resins were used only in Na^{+} form as their size exclusion effect is wanted to be adduced. Due to the smaller size and valence of Na^{+} when compared to Ca^{2+} it keeps the resin body tighter enabling more efficient size exclusion effect. The ion exchange materials were obtained from Finex and Millipore grade water was used as an eluent. Figure 7-1 shows the structures of SAC and WAC resins.

Table III Properties of the resins studied in the thesis. Materials are manufactured by Finex.

Resin	Type	Structure	Functionality	DVB, %	Forms used
CS16GC	SAC	Styrenic	Sulphonic	8	Na+ or Ca2+
CS11GC	SAC	Styrenic	Sulphonic	5.5	Na+ or Ca2+
CA16GC	WAC	Acrylic	Carboxylic	8	Na+
CS10GC	WAC	Acrylic	Carboxylic	5	Na+

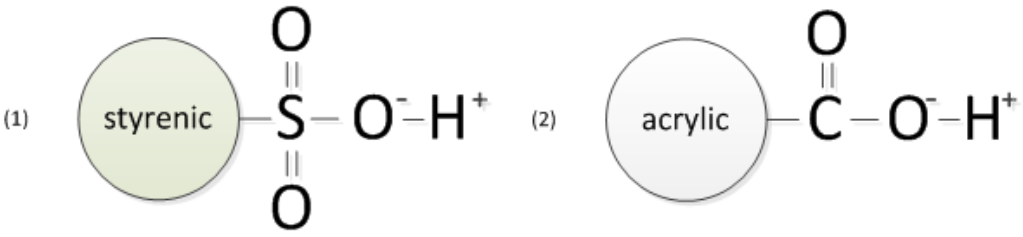


Figure 7-1 (1) SAC resin and (2) WAC resins in their initial form.

Crosslinking level for SAC resins examined were 8 % and 6 %. Crosslinking level for WAC resins examined were 8 % and 5.5 %. Within these crosslinking levels it is still possible for glucose and ethyl glucose to access the pores of the resin yet, some assisting size exclusion is still occurring. With too high crosslinking level the pores become too narrow preventing sugar components to reach the metal counter ions in the pores. With smaller crosslinking the pores become too large from the size exclusion point of view. Nevertheless, amount of water in the resin is an essential characteristic permitting the interaction with the solutes and metal counter ions. The lower the crosslinking level the higher the moisture of the resin.

Styrene based CS16GC resin was tested in Na^+ and Ca^{2+} forms. Since the product should be edible Ca^{2+} was chosen out of divalent metals instead of for example Pb^{2+} which would probably be more effective in current separation, but toxic. WAC exchangers (CA16GC and CA10GC) were tested in Na^+ form.

When testing the separation materials the flow rate was 0.9 BV/h and purified water was used as an eluent. The feed solution injected to the column contained slightly fluctuating concentrations of ethanol, glucose (12-17 g/L), and BEG (30-35 g/L) from run to run. Injection volume was 4.4 $\%_{BV}$ ($\%_{BV}$ is the size of the injection compared to the bed volume). Bed volume was 115 mL. From each run, samples were collected every one, two, or three minutes and analyzed with HPLC. The separation was followed from online RI data during the run.

7.3.2 Resin pretreatment

The resins were prepared for the experiments as follows. WAC resins were used only in Na^+ form. Resins initially in Na^+ form were first washed with water and two bed volumes of 1M NaCl was ran through the bed ensuring the efficiency of the resin. Finally the bed was washed with water.

To transfer SAC resins from their initial form to Ca^{2+} or Na^+ form the resin was first washed with deionized water in open column. Then 5 bed volumes of 1 M HCl was drained through the bed to change the resin into H^+ form. The bed was washed again with water to make sure to remove the free acid. 10 bed volumes of 1 M $CaCl_2$ or NaCl solution was drained through the bed to change H^+ ions to Ca^{2+} or Na^+ ions causing HCl to elute out from the column. The bed was washed again with several bed volumes of water.

CS11GC was initially in Ca^{2+} form and CS16GC was initially in Na^+ form. As a strong acid HCl regenerates the functional group of the SAC resin back to its original SO_3H form after which it is more efficient to change the resin into desired form. Ca^{2+} has higher selectivity towards the sulphonic group than Na^+ which is why Na^+ ions cannot directly replace Ca^{2+} ions of the functional group. In the case of CS16GC the Na^+ ions could be replaced directly with Ca^{2+} ions yet, the ion exchange is more efficient and faster via H^+ form.

7.3.3 Single-column batch chromatography

While testing the separation materials 115 mL bed of pretreated resin was packed into a column having 1.5 cm diameter (bed height of 65 cm). The column temperature was kept in 50°C with a water circulation thermostat. In the process set-up there were online detectors in line. Refractive index, UV, and conductivity detectors gave online data about the separation. The installation is shown in Figure 7-2 and it was controlled with Labview software.

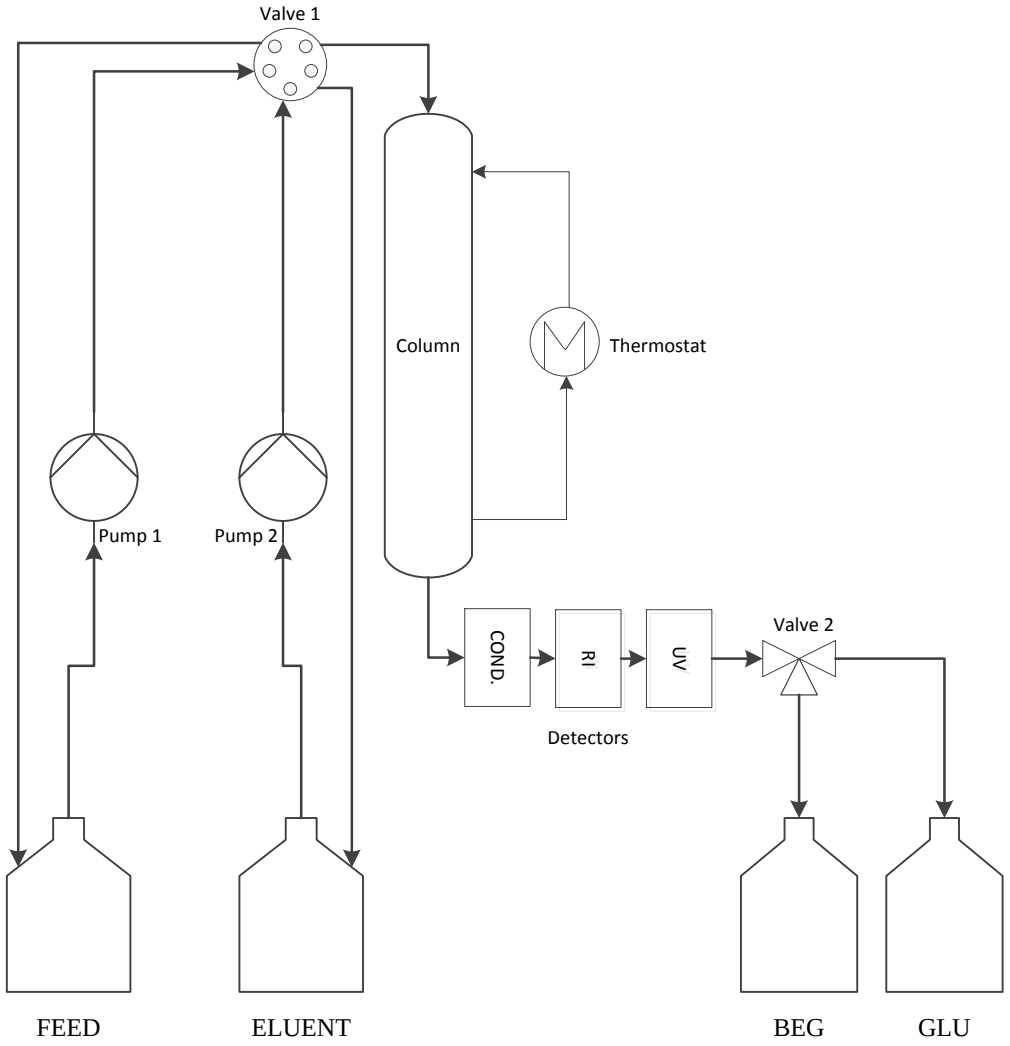


Figure 7-2 Installation for experimental sized batch runs. The scaled-up batch runs were made with similar installation yet, the two pumps were replaced with more efficient one that pumped both F and E to the column. Valve 1 is a motor valve and Valve 2 is a solenoid valve. Pumps are piston pumps for which the eluent degassing was needed.

The single-column experiments done in the thesis may be divided into four parts: experiments to find out the best separation material, scaled-up runs to produce greater amount of 99 % pure BEG, design pulse runs to collect data for SSR design, and eventually SSR runs to find out its efficiency compared to a regular batch separation. Separation runs made with different resins are presented in Table IV. Design pulse runs made are presented in Table V.

Table IV Singe-column batch separation runs done in 115 mL column for separation material testing. Injection volume was 4.4%$_{BV}$, flowrate 0.9 BV/h, and T = 50°C.

Material	c_F(BEG), g/L	c_F(GLU), g/L	c_F(EtOH), g/L
CS16GC, Ca^{2+}	33	12	excess
CS16GC, Na^+	33	12	excess
CS11GC, Ca^{2+}	33	12	excess
CS11GC, Na^+	33	12	excess
CA16GC, Na^+	33	12	excess
CA16GC, Na^+	35	17	4
CA10GC, Na^+	33	12	excess
CA10GC, Na^+	32	16	4

Table V Singe-column design pulses made in batch with 113 mL bed of CA10GC resin in Na^+ form in temperature of 50°C.

Pulse size, %$_{BV}$	c_F(BEG), g/L	c_F(GLU), g/L	c_F(EtOH), g/L
3.7	41	16	< 1
5	38	14	< 1
7	41	16	< 1
10	38	14	< 1
20	38	14	< 1

Wanted amount of pretreated resin is packed into a column letting the packing solution (water in this case) to drain through while the resin is added. The packed column is then placed to the system and purified water is ran through the whole process while the column was heated up to 50°C. All the separations were made in 50°C since high temperature increases the mass transfer kinetics of the sugars.

Depending on the experiment, certain pulse size is injected into the column with a certain flow rate (see Tables IV and V). The detector signals (especially RI) were followed during the separation. Samples from the outcome were collected on regular basis, analyzed with high-performance liquid chromatography (HPLC), and chromatogram of the separation was plotted based on the sample concentrations.

7.3.4 Process scale-up

To produce at least 50 grams of 99 % pure BEG the batch process was scaled up. In the scaled-up process the separation conditions were kept the same as in the experimental process compared to the column bed volume. The *BV* in the scaled-up separation was 1885 mL, over 16 times larger than the experimental column. The flow rate in the larger column ($Q_{s.up}$) was calculated using the flow rate and *BV* of the smaller column, as shown in Equation 5.

$$Q_{s.up} = \frac{Q_{exp}}{BV_{exp}} \cdot BV_{s.up} = \frac{1.77\,\frac{mL}{min}}{114.86mL} \cdot 1884.96mL = 29.05\,\frac{mL}{min} \quad (5)$$

where Q_{exp} and BV_{exp} are the flow rate and bed volume used in experimental scale.

In the same conditions, the breakthrough times for the components will remain the same in smaller and larger scale processes as the relation of the flowrate and the bed volume remains the same. Therefore, fraction collection times for scaled-up process may be determined from the elution profile of the corresponding experimental scale separation. The feed concentrations in the scaled-up run were c(BEG) = 40 g/L, c(GLU) = 17 g/L, and c(EtOH) < 1 g/L.

The breakthrough point of BEG was set as the start point of the BEG fraction collection. The collection was finished at the breakthrough point of glucose. After the first feed pulse, there is no components coming out from the column before BEG breaks through. Let us assume that this happens for example after elution of 0.40 BV. Therefore, the next pulse is fed to the column 0.40 BV before the column is empty. So, if the column was empty after elution of for example 1 BV, new injection should be done after 0.6 BV at the earliest.

Ten successive injections of 10%$_{BV}$ were done. Generally BEG was collected in three fractions. From two injections, samples were collected every two to three minutes to see the whole elution profiles of both components at column outlet hence, the repeatability of the cycles was ensured.

In the production of pure BEG new and clean glassware, containers, tubes, et cetera were used since the product will be tested internally by food scientists at UTU. The flow chart for the scaled-up process was presented earlier in Figure 7-2 of Section 7.3.3.

7.4 Steady state recycling chromatography

BEG and GLU are linearly separated in CA10GC from the binary solution. They are quite easily separated to 99 % pure fractions with one cut in batch process with as low flow rate ($\leq$ 0.9 BV/h) and small pulse size ($\leq$ 10 %$_{BV}$) as used in the former section of this study. Therefore, several design pulses in batch together with simulations in Matlab were done in order to find optimal conditions in which SSR process would offer clearly better results compared to the batch process with purity demand of 98 %. SSR runs made are presented in Table VI.

Table VI An advanced batch process, SSR, ran to steady state with flowrate of 2.64 BV/h, BV = 113 mL, and T = 50^{o}C. c_F(BEG) = 41 g/L, c_F(GLU) = 16 g/L, c_F(EtOH) < 1 g/L.

Pulse size, %$_{BV}$	V_{FF}, %$_{BV}$	V_R, %$_{BV}$	cycles done
7	3.7	3.3	11
10	4.7	5.3	12
20	6.7	13.3	13

7.4.1 SSR set-up

The design pulses and SSR runs were done in a column containing 113 mL BV of CA10GC resin in Na^+ form. The process installation used is shown in Figure 7-3 and flow chart in Figure 7-4. Labview software was used to control the valves, follow the flow rates and read the online data. The Labview control panel view during one SSR run is shown in Figure 7-5.

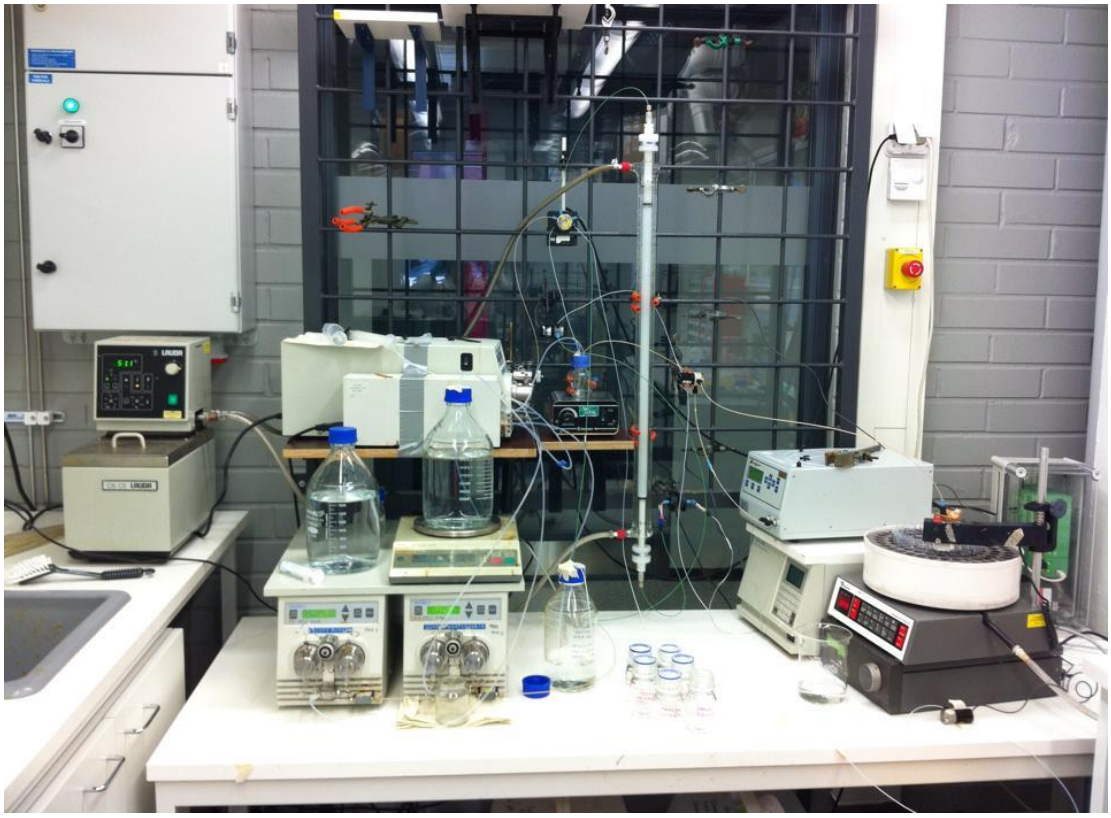


Figure 7-3 SSR installation containing eluent and feed pumps, degassing unit, heating mantles, column, and RI, UV, and conductivity detectors.

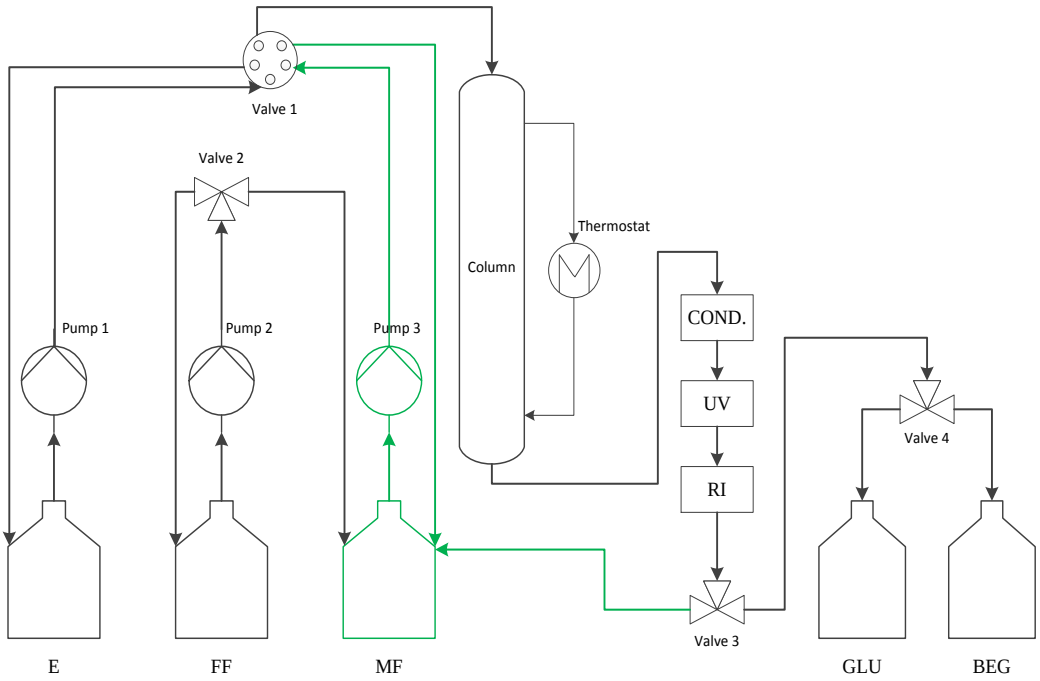


Figure 7-4 SSR flow chart. Green lines are added compared to batch process.

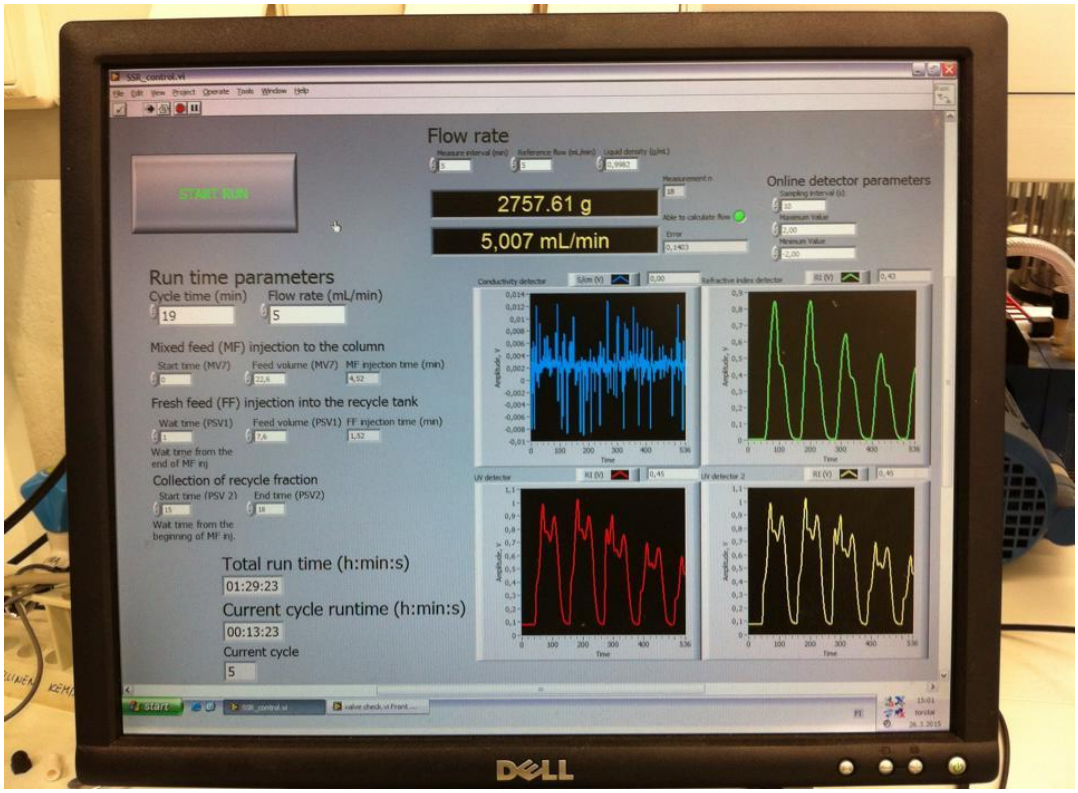


Figure 7-5 Lab view control panel during SSR run.

7.4.2 SSR design

The pathway for designing SSR based on design pulse data is presented in this section. Choosing the fractionation times is the main task in SSR process design. Fractionation times are chosen based on the design pulse data so that the desired purities of the products are reached. The design pulses used in the transfer must have the same flow rates and injection volumes which are intended to be used in SSR.

The optimum conditions (flow rate and pulse size) to run the SSR may be searched via simulations (Section 8). There is an optimum pulse size that may be injected into SSR system with a certain flow rate. With this pulse size the productivity of the SSR system is the best it can get with the chosen flow rate. Factors affecting to the size of the optimum pulse are for example the solutes to be separated, separation material, and purity requirements for the products.

In turn, for every pulse size there is an optimum flowrate which gives the best productivity. As the flowrate increases the dispersion increases yet, the cycle time decreases. Respectively, productivity increases as the cycle time decreases (Equation 7). So, the optimal flowrate with a certain pulse size is eventually determined by the product purity requirement. With fixed pulse size the flowrate may be increased until the purity requirement is reached. With low flowrate greater pulse may be injected into the column as, due to small dispersion effect, the yield of the products on a certain purity level is high. When the flowrate is high the yield of the products on the same purity level is lower than with smaller flowrate due to greater dispersion.

For every pulse size used in the SSR a design pulse exists. After running the design pulse with desired flow rate and pulse size the resulting chromatogram is plotted and the product purities are followed. The cut time of t_{A2} is set to the point in which the product A is still in the limits of wanted purity. The recycle fraction is an impure fraction containing both A and B. From t_{B1} to t_{B2} the product B is collected and again t_{B1} is set to a point from which forward the second product reaches the purity of demand. A new injection is done t_{A1} time units before the end of the cycle (t_{B2}) to reach touching bands; to minimize the eluent consumption and maximize the productivity. An example chromatogram with the fractionation times was presented in Figure 6-1.

If the separation material is effective, there might be a chance where the solutes are possible to be separated in wanted purities in a normal batch process if the flow rate and the injection volume are small enough. In such case, it is important to notice that the lower limit for the amount of fresh feed needed in the mixed feed is the amount that may be purified at once in a batch process. Otherwise, transferring the batch process into SSR process do not offer any advantage in productivities.

Naturally, when the injection volume increases, the product chromatograms faces greater overlapping which leads to a larger recycle fraction. The size of a recycled fraction (or fresh feed) in the mixed feed is linearly dependent on the total injection volume. The injection pulse size needs to be so big that there will be something to recycle yet, there is no reason to feed such a big pulse that causes the solutes to saturate the column. If the column is saturated by the solutes, part of the recycle is practically as concentrated as fresh feed. The design method is summarized in Table VII.

Table VII Summary of the designing method for SSR process when having product purity requirements of almost 100 %. If the purity requirements are lower or, especially, when they are not equal for both of the products, the dilution and dispersion needs more attention. Note that advantage of Matlab has been taken in some parts of the designing.

1	Choose a pulse size (simulations)
2	If possible, find out the pulse size with what the purity demands of the products are fulfilled in batch with one cut time → The amount of fresh feed in mixed feed must be greater than this amount to gain any profit by using SSR
3	Choose flow rate in which you want to operate (simulations)
4	Make a design pulse in a batch in the same conditions you want to use SSR
5	Follow the product purities using the chromatogram of the design pulse and choose *t*A1, *t*A2, *t*B1 and *t*B2 for SSR. Calculate the amount of FF needed in MF based on the size of the recycle fraction
6	Based on the *t*A1 and *t*B2 calculate the cycle time for SSR and set the suitable time suitable time between successive injections → Check that there is enough time for fresh feed and recycled fraction to mix in the MF tank before new injection
7	Keep the successive injections going until steady state is reached
8	Steady state tells the conditions in which man can continuously run SSR to produce uniform product

When running SSR the flow rate was 2.64 BV/h and Millipore grade water was used as an eluent. The feed solution contained glucose (16 g/L) and BEG (41 g/L), and practically no ethanol (< 1 g/L). Bed volume was 113 mL. From each run, samples were collected every 1.75 minutes and analyzed in HPLC in addition to online RI data.

Twelve to fourteen cycles were done per each SSR run. Runs were done with 7 $\%_{BV}$, 10 $\%_{BV}$, and 20 $\%_{BV}$ injection volumes. As it is known, in SSR, part of the feed is recycled from the previous cycle. Therefore, eventually the amount of fresh feed in every injection is 3.7 $\%_{BV}$, 4.7 $\%_{BV}$, and 6.7 $\%_{BV}$, comparable to design pulses done with injection volumes of 3.7 $\%_{BV}$, 5 $\%_{BV}$, and 7 $\%_{BV}$. Generally, BEG and glucose were collected to their own fraction containers. Yet, over some injections the samples were collected every 1.75 minutes to see the whole elution profiles of both components at column outlet and, analyzed in HPLC. Online RI data was followed and the chromatograms of consecutive injections in SSR looked similar to the ones shown Figure 7-6.

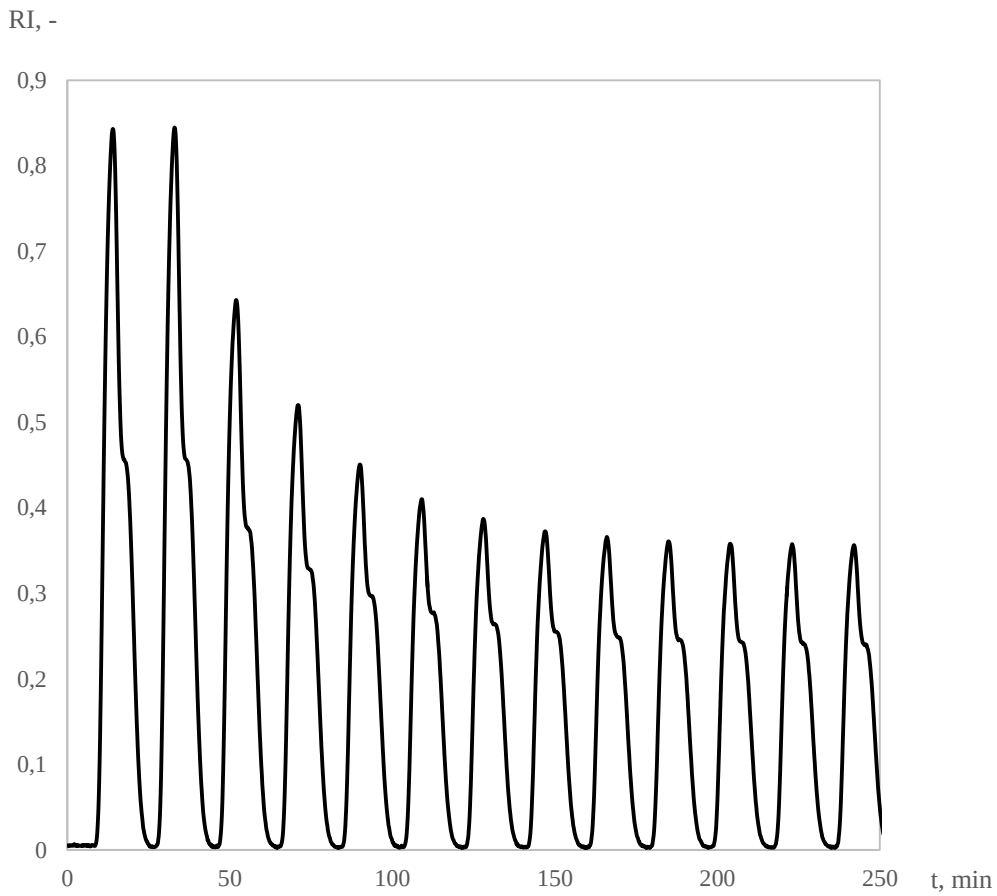


Figure 7-6 RI online data from a SSR run with injection size of 20 $\%_{BV}$ and Q = 2.64 BV/h.

7.4.3 Design pulses

Design pulse is a pulse made in batch with a specific injection volume and flow rate corresponding to those planned to be used in the SSR process under design. The flow rate used in this study was 5 mL/min, equaling to 2.64 BV/h when comparing to the size of the column. Set of five design pulses were done. Three of them were used in designing the SSR runs and to look for the fraction times. The three smallest pulses were done mainly to compare the differences between the batch and the SSR processes.

The design pulses had sizes of 3.7, 5, 7, 10, and 20 $\%_{BV}$. The smallest design pulse represents the greatest pulse size with what 98 % pure products are gained in a normal batch process with one cut time. The smallest SSR pulse (7 $\%_{BV}$) contained the same amount of fresh feed in the mixed feed as the smallest design pulse had in feed. So, the information got from the smallest design pulse is used as comparison values between batch and SSR processes. Greater pulse volumes in batch do not give the desired purity levels.

The fractionation times for SSR were chosen based on the design pulses having the same pulse size (7, 10, and 20 $\%_{BV}$). But, it needs to be remembered that in SSR only part of the injection is fresh feed and the other part is recycled. The smaller design pulse sizes were chosen based on this fact. Therefore, the SSR pulse sizes used contained as large amount of fresh feed as the smaller injection sizes. Table VIII opens up the situation.

Table VIII Choosing the pulse sizes for design pulses. Amounts are percentages compared to BV of the column.

pulse size (batch), %	FF(batch), %	pulse size (ssr), %	FF(ssr), %	R(ssr), %
3.7	3.7	-	-	-
5	5	-	-	-
7	7	7	3.7	3.3
10	10	10	4.7	5.3
20	20	20	6.7	13.3

7.5 Analyses

The analysis was performed utilizing ligand exchange chromatography in Agilent 1100 Series HPLC Value System. The column used contains sulfonated polystyrene gel having lead as a metal counter ion, Shodex SP-0810. Shodex SP-G, containing also Pb^{2+} as counter ion, was used as a guard column. Millipore grade water was used as an eluent with flowrate of 0.5 mL/min. Analysis temperature was 80^{o}C and injection volume 10 µL.

8 MATHEMATICAL MODELLING AND CALCULATION

Matlab was used during the designing phase of SSR. The simulation model used for estimating the behavior of the separation and fractionation times employed the solid film linear driving force approximation. The model is described in more detail for example by Hellstén et al. [45].

Information was collected by changing the parameters and compiling the data from simulations. The suitability of the model was initially tested by fitting it to data of batch chromatography experiments. After adjusting the constants it was possible to predict the practical experiments via simulations, and unnecessary runs were avoided. Changes in the model were mainly done by changing the flowrate and pulse size while planning the following experiments. The behavior of both batch and SSR processes were able to be predicted.

In addition to predict the batch chromatography and SSR separation, simulations were also done to look for the best separation conditions for SSR. Simulation runs were done with several combinations of flow rates and injection volumes to survey the optimal flow rate and pulse size combination. Experiments were done to see that the simulated results are valid in practice as well.

8.1 Simulation parameters

The main simulation parameters determined are the adsorption isotherms and bed porosity. Dispersion was adjusted based on the batch chromatography experiments.

8.1.1 Porosity

The extra column dead volume (tubes, valves, pumps) and the porosity of the bed (ε) were determined with 0.5 M solution of Blue Dextran 2000. Blue Dextran does not adsorb nor fit to the pores of the resin, and it is visible in UV detector with wavelength of 195nm. The elution time of Blue Dextran 2000 equals to the time that is at least needed before elution of any component entering the column. The void of the extra column system was determined without the column to correct the retention volumes obtained in the measurements.

Three consecutive pulses were done in 50^{o}C with flowrate of 0.54 BV/h and an average of consumed eluent volume (V_E) was determined. The porosity was calculated from Equation 6 when the volume of the adsorbent material (V_s) is known

$$\varepsilon = \frac{V_E}{V_s} \tag{6}$$

For CA10GC in Na^+ form the porosity was 0.33.

8.1.2 Adsorption isotherms

Adsorption isotherms were needed to simulate the batch and SSR separations of glucose and BEG with Matlab. They were determined for both glucose and BEG using frontal analysis method. The glucose concentration range for frontal analysis was 0.1 - 30.0 g/L and the BEG concentration range was 1.0 - 40.0 g/L. The adsorption isotherm determinations were done in 50ºC, with flowrate of 0.9 BV/h, and in 115 mL column.

Loading (q) of the adsorbent was determined with every concentration step using Equation 2 of the Section 5.1. An example of the resulting staircase chromatogram is presented in Figure 8-1. Loadings were plotted in the function of concentration and the adsorption isotherm was determined from the slope of the graph as the isotherms were linear. Due to linear isotherms, the adsorption isotherms of BEG and glucose equals the Henry constant (H_i) meaning, their retention time is not influenced by concentration. Yet, the dispersive effect may shape the chromatograms.

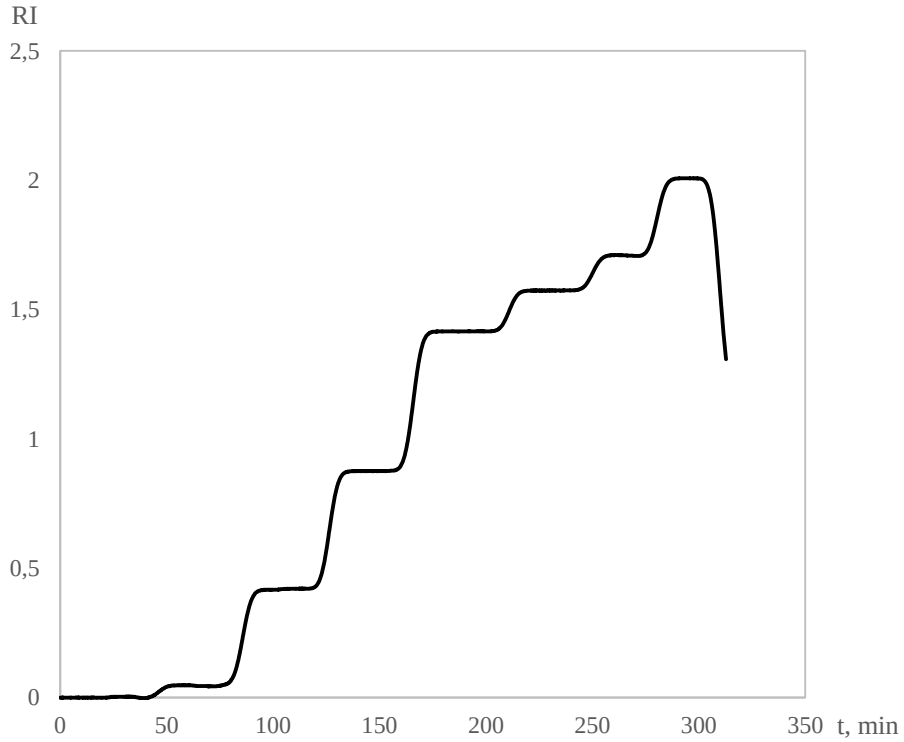


Figure 8-1 Frontal analysis of glucose in water having CA10GC in Na^+ form as adsorbent material. BV = 115 mL, Q = 0.9 BV/h, and T = 50ºC.

8.2 Process performance evaluation

The efficiency of the chromatographic separations was evaluated on the basis of productivity (*PR*), yield (*Y*), purity (*PU*), and eluent consumption (*EC*) (Equations 7 - 10). These values were compared between SSR and corresponding batch chromatography separations.

$$PR = \frac{n_i}{t_c \cdot (1-\varepsilon) \cdot V_s} \left[\frac{g}{BV \cdot h}\right] \tag{7}$$

where n_i is the mass of the produced component i (in grams or moles), t_c is the cycle time, and V_s is the volume of the solid phase in the column (m^3).

$$Y = \frac{n_i}{n_{i,tot}} \cdot 100\ [\%] \tag{8}$$

where $n_{i,\,tot}$ is the total mass of component i fed into the process.

$$PU = \frac{c_A}{c_A + c_B} \cdot 100\ [\%] \tag{9}$$

where c_A is the concentration of the first solute in a binary solution and c_B is the second solute of a binary solution.

$$EC = \frac{t_c \cdot Q}{n_i} \left[\frac{L}{g}\right] \tag{10}$$

where Q is the flowrate.

9 RESULTS AND DISCUSSION

This section contains results of enzymatic synthesis, separation material selection, scale-up, SSR design and runs, and modeling. The results are introduced and the behavior of the examined phenomena is discussed.

9.1 Synthesis

The formation kinetics of BEG from glucose with β-glucosidase was examined superficially. The kinetic test series done are shown in Figure 9-1. In both series the glucose conversion reached 60 % in four days yet, the equilibrium was not reached. From the Figure 9-1 it can be estimated that within these conditions the maximum glucose conversion would be about 65 - 70 %. 65 % glucose conversion was eventually reached when preparing feed solution for further studies over five days incubation having conditions discussed in Section 7.2.

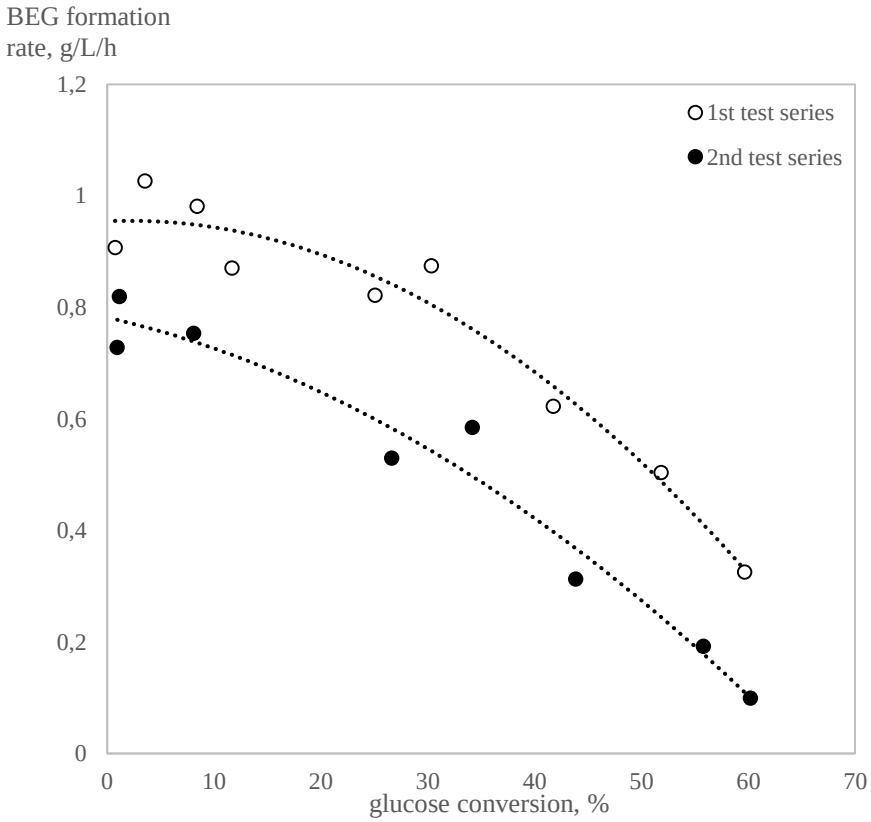


Figure 9-1 The reverse hydrolysis of glucose into ethyl glucose having β-glucosidase as catalyst over four days in 38ºC incubation-shaker (300 rpm). 1st test series: $c(GLU)_i$ = 55 g/L and c(enz) = 0.3 g/L. 2nd test series: c(GLU) = 28 g/L and c(enz) = 0.2 g/L.

The synthesis solution turned cloudy when ethanol was added to glucose-enzyme solution before incubation. Apparently, the enzyme might have partially functioned as a solid catalyst as the opacity of the solution was not preventing the synthesis to occur. In the end of the incubation time white and water-insoluble precipitate had been created on the edges of the container. It was assumed to be aggregate of undissolved enzymes in organic media as the enzymes are supported by proteins [15].

With water-ethanol ratio of 1:4, 0.3 g/L of enzyme in the synthesis reached higher formation rates of BEG than the solution with 0.2 g/L of enzyme. Naturally, higher amount of enzyme work faster than smaller amount as there are more sites for glucose to attach. Yet, change in initial concentration has an effect on the reaction rate as well. The more there is solute the higher is the change the enzyme meets the solute. Still, the state of equilibrium did not change as the water to ethanol ratio remained the same.

Enzymatic synthesis of BEG is not reported before, except briefly by Tiitinen et al. [1]. Alkyl glucosides are more often synthesized using alcohols of longer hydrocarbon chain as long chained alkyl glucosides have better surfactant properties than short chained alkyl glucosides. Though, Kosáry et al. [15] have synthesized alkyl glucosides with changing water content utilizing immobilized glucosidases. They reached the best yield with water content of 15 % as the reaction was run in a mixture containing 0.1 M glucose at 25 ^{o}C during 72 h. For example, the yield of formed butyl- β-glucoside was almost 40 % [15]. In turn, Papanikolaou [46] compared the yields when hexyl-glucosidase was synthesized with free and immobilized enzyme. And, immobilized enzyme resulted in a slightly better conversion yield, 10 % compared to 8 % [46]. Hence, as it is known, the enzymatic synthesis of short chained alkyl glucosides utilizing β-glucosidase is more productive than synthesis of long chained alkyl glucosides.

9.2 Purification

The purification section includes all the chromatographic separation processes and related experiments done in this thesis.

9.2.1 Resin comparison

It was assumed that the complex forming ligand exchange effect of SAC might get glucose and BEG separated. Obviously, the BEG and glucose complexes formed between the hydroxyl groups of the compounds and metal counter ions of the resin had similar stabilities since they closely broke through the column. In the case of CS16GC the breakthrough of BEG occurred after 0.45 BV and glucose after 0.5 BV elution no matter if the counter ion was Ca^{2+} or Na^{+} (Figures 9-2 and 9-3).

In BEG one hydroxyl group is replaced by an ethyl group when comparing to glucose. Because of that the stationary phase (SAC) seemed to be slightly less selective towards BEG causing it to break through the column first. Yet, ethanol seemed to have greater retention in Ca^{2+} formed resin as in Ca^{2+} form the SAC resins had greater density of positive charge and smaller pore size compared to Na^{+} formed resin. It was noticed that excess amounts of ethanol disturbed the elution of the other two compounds as the stationary phase was the most selective towards ethanol, and ethanol seemed to pull BEG and glucose towards it. In addition, ethanol was assumed to decrease the solvated sizes of BEG and glucose.

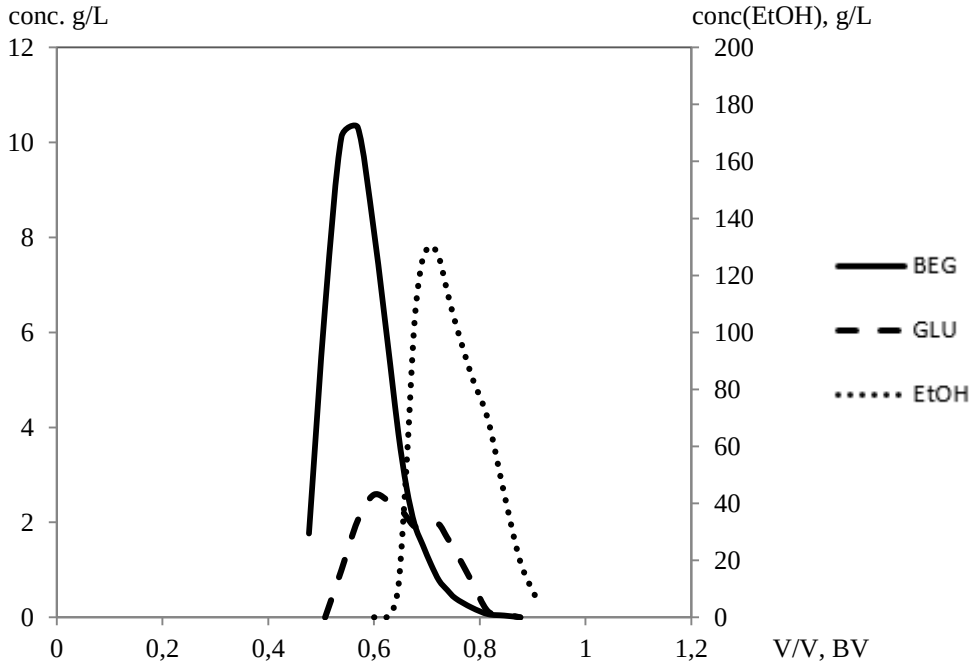


Figure 9-2 Elution profile for CS16GC resin in Ca^{2+} form. In the feed (4.4 $\%_{BV}$) there were c(BEG) = 33 g/L, c(GLU) = 12 g/L, and excess amounts of EtOH.

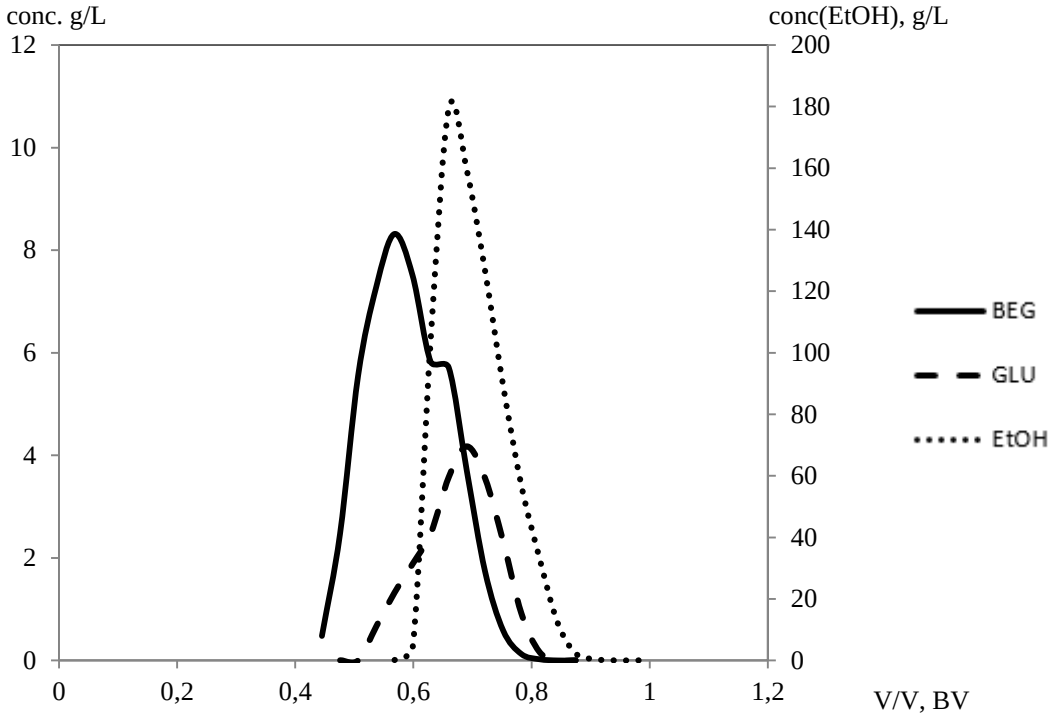


Figure 9-3 Elution profile for CS16GC resin in Na^+ form. In the feed (4.4 $\%_{BV}$) there were c(BEG) = 33 g/L, c(GLU) = 12 g/L, and excess amounts of EtOH.

Less crosslinked CS11GC (Figures 9-4 and 9-5) did not perform any better than the more highly crosslinked CS16GC. Actually, the results were even worse as every component was more retained in the CS11GC column due to larger pores of the less crosslinked resin (BEG and glucose fit better to the larger pores). Glucose and BEG profiles still overlapped strongly and ethanol was disturbing the separation. Even without ethanol, the BEG and glucose profiles would still overlap since ethanol's existence did not presumably change the breakthrough point of the components.

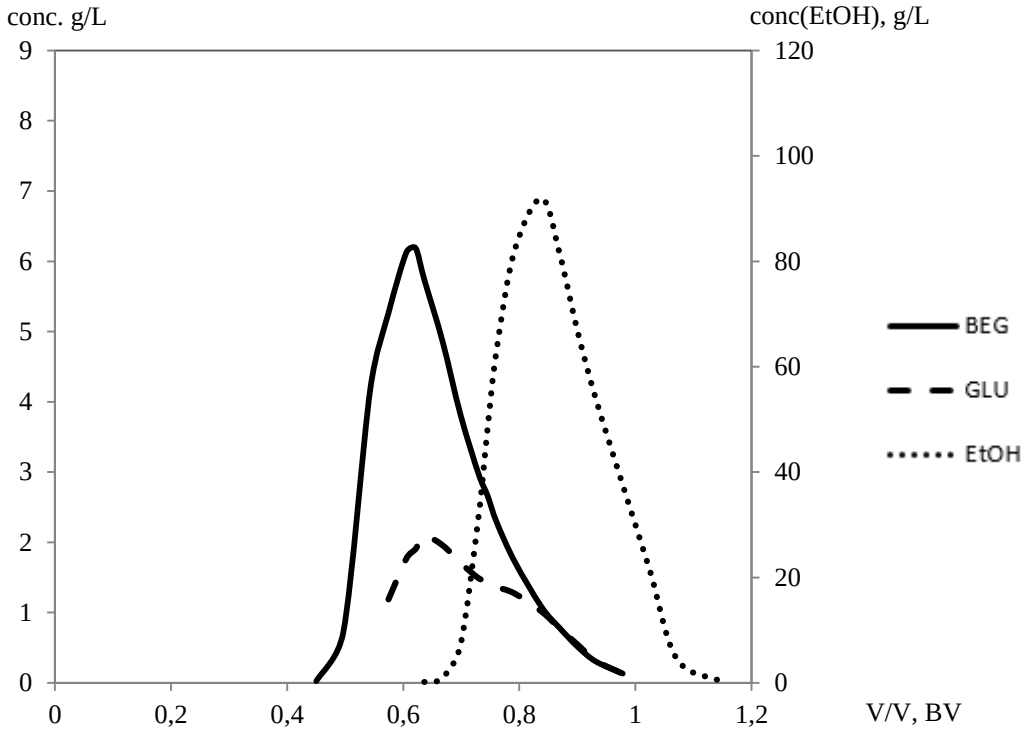


Figure 9-4 Elution profile for CS11GC resin in Ca^{2+} form. In the feed (4.4 $\%_{BV}$) there were c(BEG) = 33 g/L, c(GLU) = 12 g/L, and excess amounts of EtOH.

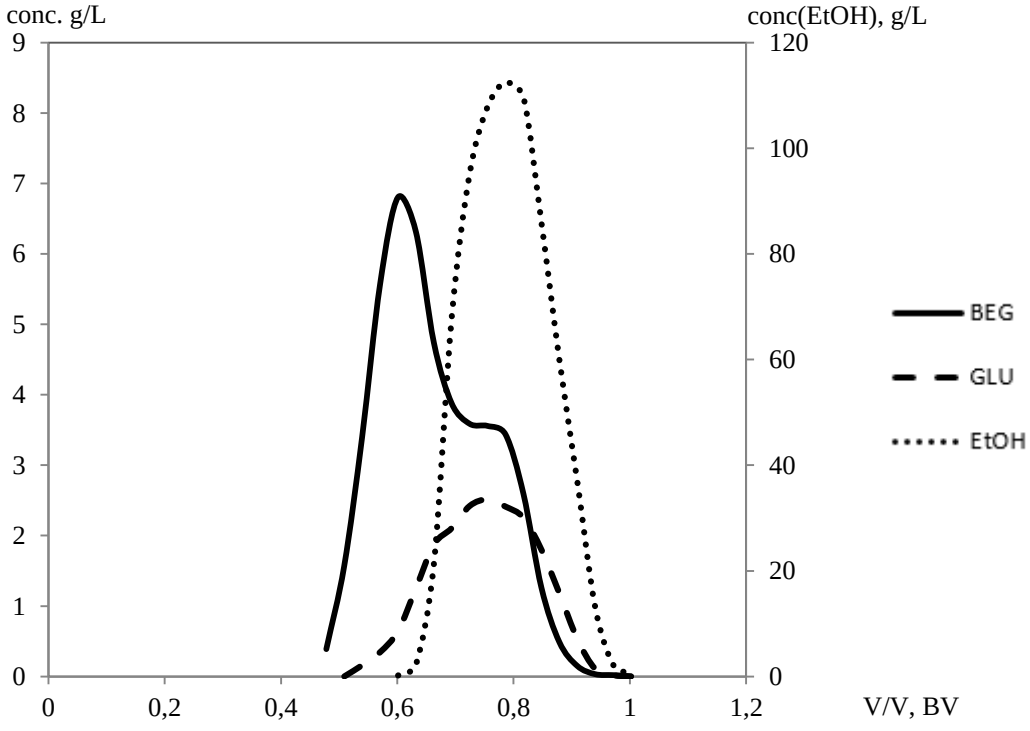


Figure 9-5 Elution profile for CS11GC resin in Na^{+} form. In the feed (4.4 $\%_{BV}$) there were c(BEG) = 33 g/L, c(GLU) = 12 g/L, and excess amounts of EtOH.

It can be seen from Figures 9-2 – 9-5 that both the crosslinking level and the counter ion used changes the elution profiles of BEG and glucose. With less crosslinked resin (CS11GC) the dispersion of all the components is stronger and chromatograms are wider due to larger pore size of the stationary phase compared to CS16GC resin.

In the case of SAC resins the hydroxyl group of ethanol created stronger complex with the separation material than the ones in BEG or glucose being the last component eluting through the column. Size exclusion effect was probably occurring as well as ethanol is smaller molecule than BEG or glucose, and fitted better into the pores of gel like SAC resin. In addition, at least in the presence of ethanol neither BEG nor glucose had symmetrical chromatograms. So, it seems that ethanol increased the retention of both BEG and glucose by preventing them to propagate through the bed as fast as they would without the presence of ethanol.

Tiihonen et al. [39] have studied the complex formation between metal ions and carbohydrates in solvent mixtures by chromatographic measurements. They noticed that the retention times of carbohydrates and sugar alcohols increased while increasing the organic solvent content (e.g. ethanol). This is explained by the increased complex stability between the complexing solute and the counter-ion. [39].

Depending on the form of the resin (Na^+ or Ca^{2+}), the shape of the BEG and glucose chromatograms were changed (Figures 9-2 – 9-5). Though, BEG and glucose chromatograms breakthrough and ending points did not change within changing counter ion. In Na^+ formed SAC resins the peak of the glucose chromatogram had moved under the ethanol chromatogram as in the Ca^{2+} form it was under BEG chromatogram. In addition, BEG had a bump in its chromatogram profile in Na^+ formed SAC resins (Figures 9-3 and 9-5).

On the other hand, it looks like BEG and glucose complexes formed with Ca^{2+} formed resin are stronger compared to those formed in Na^{+} formed resin (Heinonen and Sainio [37]). And, as Tiihonen et al. [39] have noticed, in the presence of organic solvent the complex strengthening effect of for example ethanol is greater in Ca^{2+} than in Na^{+} formed resin. Yet, on the other hand, it looks like the size exclusion effect of SAC resins dominates in the Na^{+} form for BEG and glucose and, in Ca^{2+} form for ethanol. In addition, ethanol probably has an attractive effect on the other two solutes. The separation of BEG and glucose in the SAC resins is result of all the three phenomena. Therefore, the behavior of the chromatograms is really hard to explain.

The experiments with SAC resins were not done without ethanol in the feed solution as the breakthrough points of BEG and glucose are not assumed to change even if the ethanol was removed. With the used SAC resins (CS16GC and CS11GC), BEG and glucose formed complexes of too similar strengths which is why reasonable separation would not be gained.

Acryl based WAC resins (CA16GC and CA10GC) were examined only in Na^{+} form to bring out their size exclusion effect. Overlapping of BEG and ethanol in WAC resins was greater than in SAC resins yet, the separation of BEG and glucose was better. BEG broke through after elution of 0.4 BV and glucose after 0.53 BV from the CA16GC resin when ethanol was present (Figure 9-6).

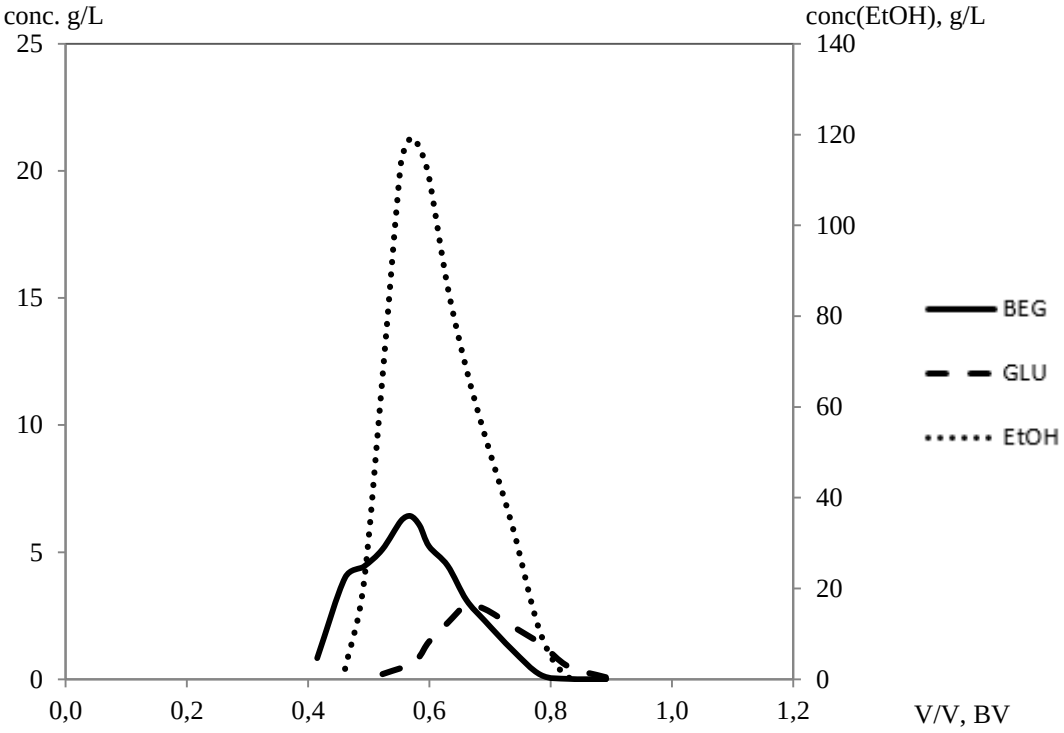


Figure 9-6 Elution profile for CA16GC resin in Na^+ form. In the feed (4.4 $\%_{BV}$) there were c(BEG) = 33 g/L, c(GLU) = 12 g/L, and excess amounts of EtOH.

Due to WAC resins better separation efficiency compared to SAC resins runs were also done with feed from which the amount of ethanol was radically decreased using rotavapor. When the ethanol concentration in the feed solution was decreased the amount of dispersion decreased. Presence of ethanol in the feed greatly disturbed the chromatographic separation. Hence, sugar chromatograms got narrower and more symmetrical, and the separation of BEG and glucose was much more successful (Figure 9-7).

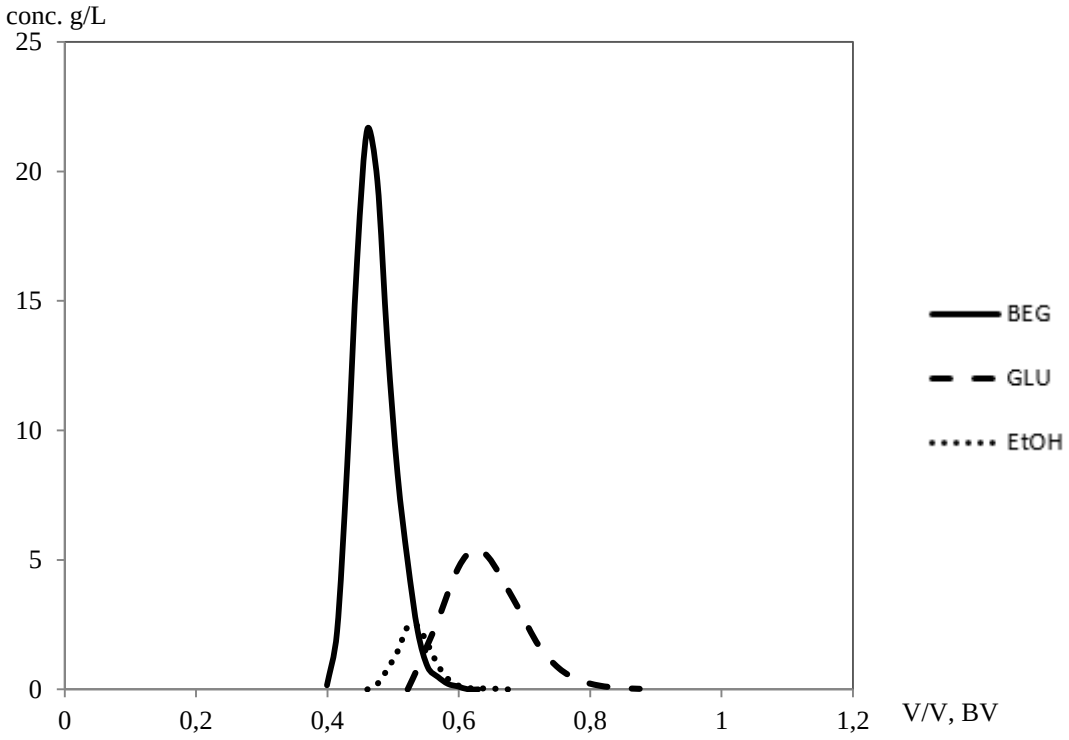


Figure 9-7 Elution profile for CA16GC resin in Na^+ form. Amount of ethanol decreased in the feed. In the feed (4.4 $\%_{BV}$) there were c(BEG) = 35 g/L, c(GLU) = 17 g/L, and c(EtOH) = 4 g/L.

The break through points of the components remained the same as with ethanol in the feed (Figure 9-6) but with smaller amount of ethanol they elute through the column with less dispersion. With WAC CA10GC resin in Na^+ form (cross linking level of 5%) the separation of BEG and glucose was even better than with more highly cross linked (8%) CA16GC resin. The difference between glucose and BEG break through points was about 0.2 BV (Figures 9-8 and 9-9).

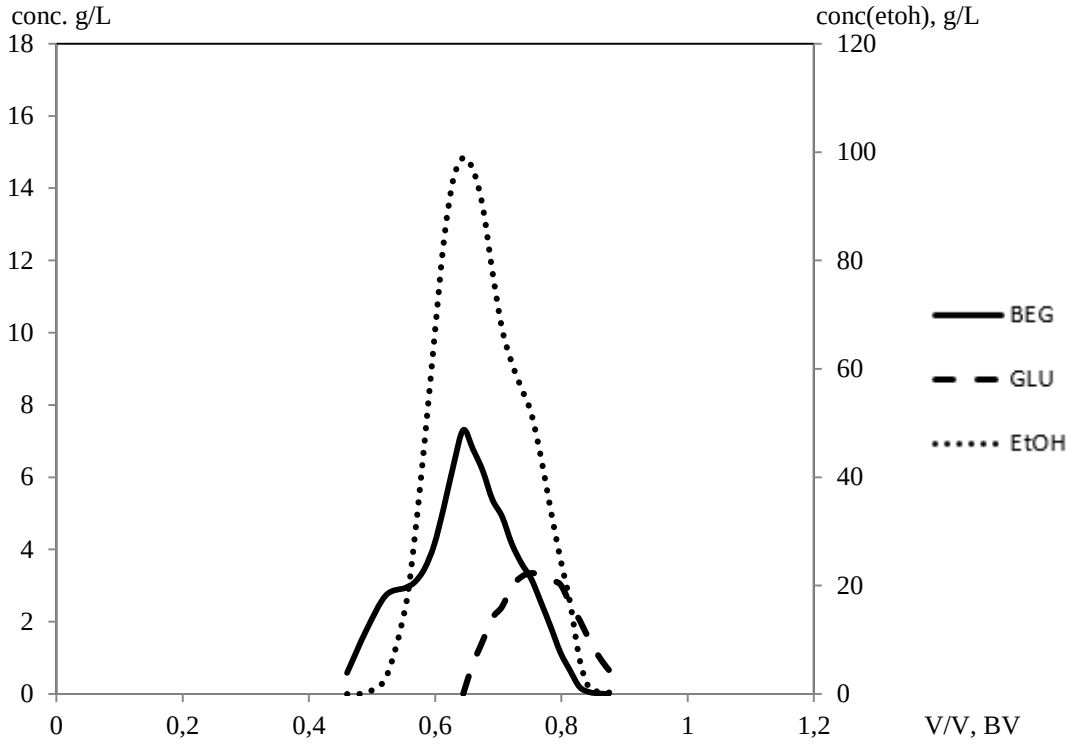


Figure 9-8 Elution profile for CA10GC resin in Na^+ form. In the feed (4.4 $\%_{BV}$) there were c(BEG) = 33 g/L, c(GLU) = 12 g/L, and excess amounts of EtOH.

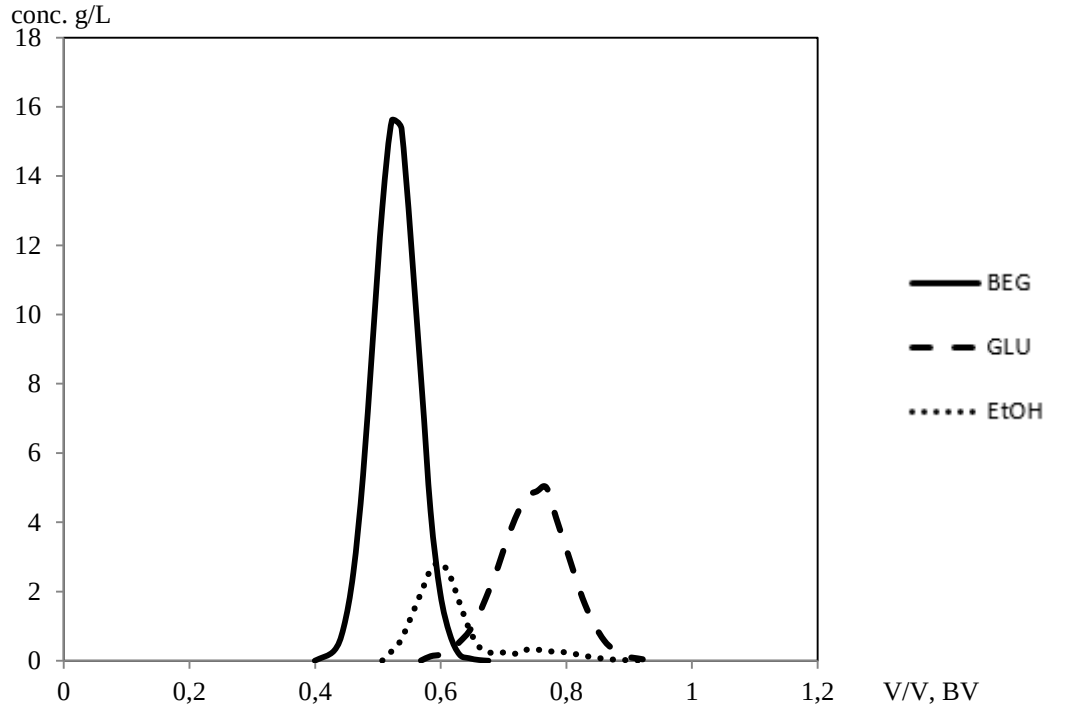


Figure 9-9 Elution profile for CA10GC resin in Na^+ form. Amount of ethanol decreased in the feed. In the feed (4.4 $\%_{BV}$) there were c(BEG) = 32 g/L, c(GLU) = 16 g/L, and c(EtOH) = 4 g/L.

As in the case of SAC resins, ethanol caused greater retention of BEG and glucose. With smaller crosslinking level and thereby with higher pore size, excess amount of ethanol even changed the breakthrough times. BEG seemed to be more disturbed by the ethanol than glucose as glucose chromatogram is just more dispersed when ethanol is present (Figure 9-8).

Separation of glucose and BEG in WAC resins based on their differences in hydrophobicity, implementing affinity based chromatography. As a hydrophilic materials CA16GC and CA10GC retained glucose the most as it is the most hydrophilic compound out of the three components. BEG was the most hydrophobic component which is why it eluted out from the column first with the WAC resins.

As probably in the case of SAC resins, high amount of ethanol also disturbed the separation of sugars with the WAC resins by flattening and broadening the elution profiles. As ethanol was more hydrophobic (relative polarity of 0.654 [47]) than the resin hydrophobic BEG tends to hold back and 'follow' the slower ethanol pulse instead of travelling faster through the column with more hydrophilic eluent (water polarity is 1.000 [47]). The effect reminds locally the situation in hydrophobic interaction chromatography where polar solute travels through the column with polar eluent while non-polar solvent refrains in non-polar adsorbent. With decreased amount of ethanol, the separation was more efficient since there was not enough ethanol to hold BEG back. Since hydrophobic BEG hardly interacted with the hydrophilic resin it could freely travel through the column if there were no ethanol.

When comparing the chromatograms of CA16GC and CA10GC (Figures 9-7 and 9-9) it can be seen that in CA10GC the retention times of BEG and glucose had greater difference. With less crosslinked material the more hydrophilic components (ethanol and glucose) had better access to interact with the separation material as the pores were larger. This is why they eluted out later from the bed packed with CA10GC resin. In addition, with less crosslinked CA10GC, the BEG and glucose profiles seemed to be more symmetrical.

As shown in Figure 9-10, the highest purities were reached using CA10GC and CA16GC, and absence of ethanol radically improved the separation efficiency. Even though CA16GC had higher productivity (4.1 g_{BEG}/BV/h) than CA10GC (3.8 g_{BEG}/BV/h), CA10GC was chosen for further studies. This is because the distance between the break through points of glucose and BEG was longer with CA10GC (0.230 BV) than with CA16GC (0.185 BV) enabling higher injection volumes with higher product purities. This may be seen comparing their elution profiles showing in Figures 9-7 and 9-9.

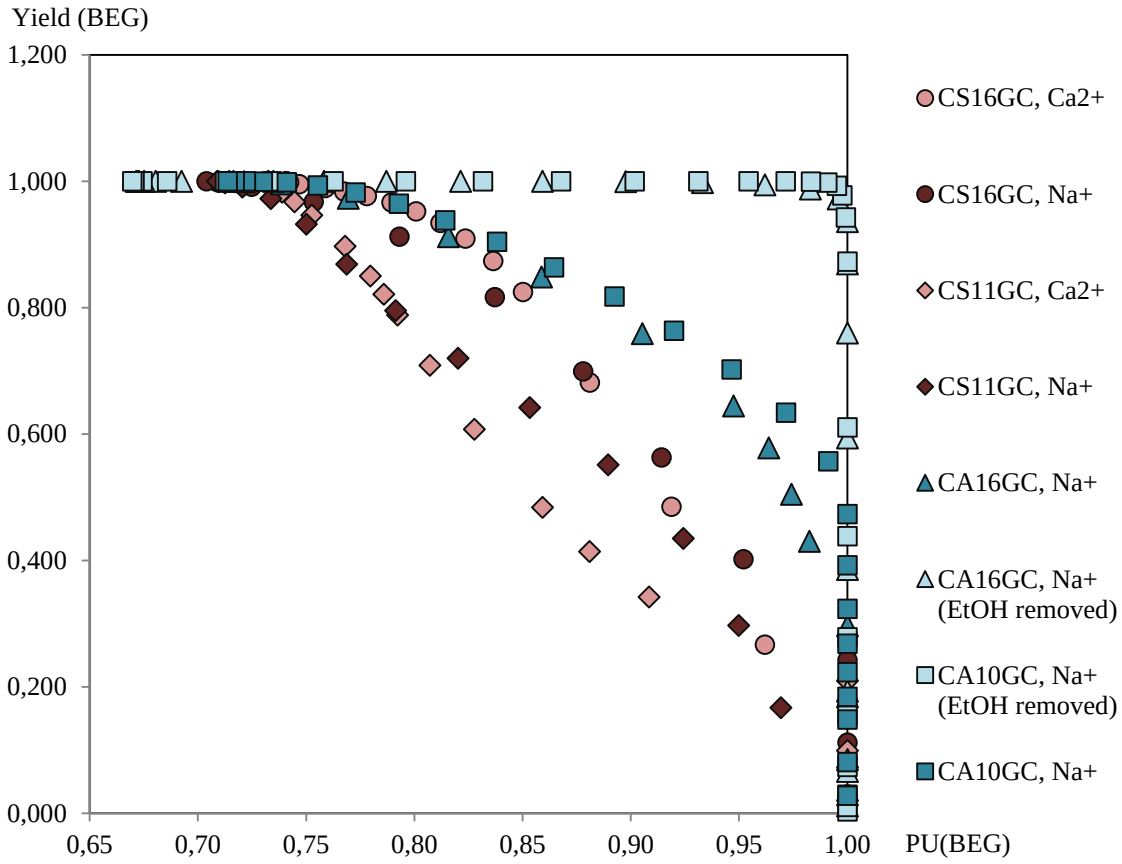


Figure 9-10 Separation efficiency of different separation materials when having BV of 115 mL, injection volume of 4.4 $\%_{BV}$, flow rate of 1.77 mL/min, and temperature of 50°C.

According to Figure 9-10, out from the styrene based resins the Na^+ formed resins performed better results than Ca^{2+} formed resins. Probably because of the larger pore size enabling BEG and glucose to enter the pores and, attractive effect of ethanol which pulled most of the glucose chromatogram under the ethanol phase. If the ethanol was removed the separation might be even worse as BEG and glucose were closely breaking through the column (Figure 9-5 and 9-3). The highly crosslinked CS16GC resin with smaller pore size resulted in better separation as the dispersion of the chromatograms was smaller than in CS11GC resins.

As the separation material was chosen to be CA10GC in Na^+ form the highest reasonable pulse size was looked for with the flow rate of 0.9 BV/h. Dependence of productivity, yield, and eluent consumption on injection size is presented in Figure 9-11.

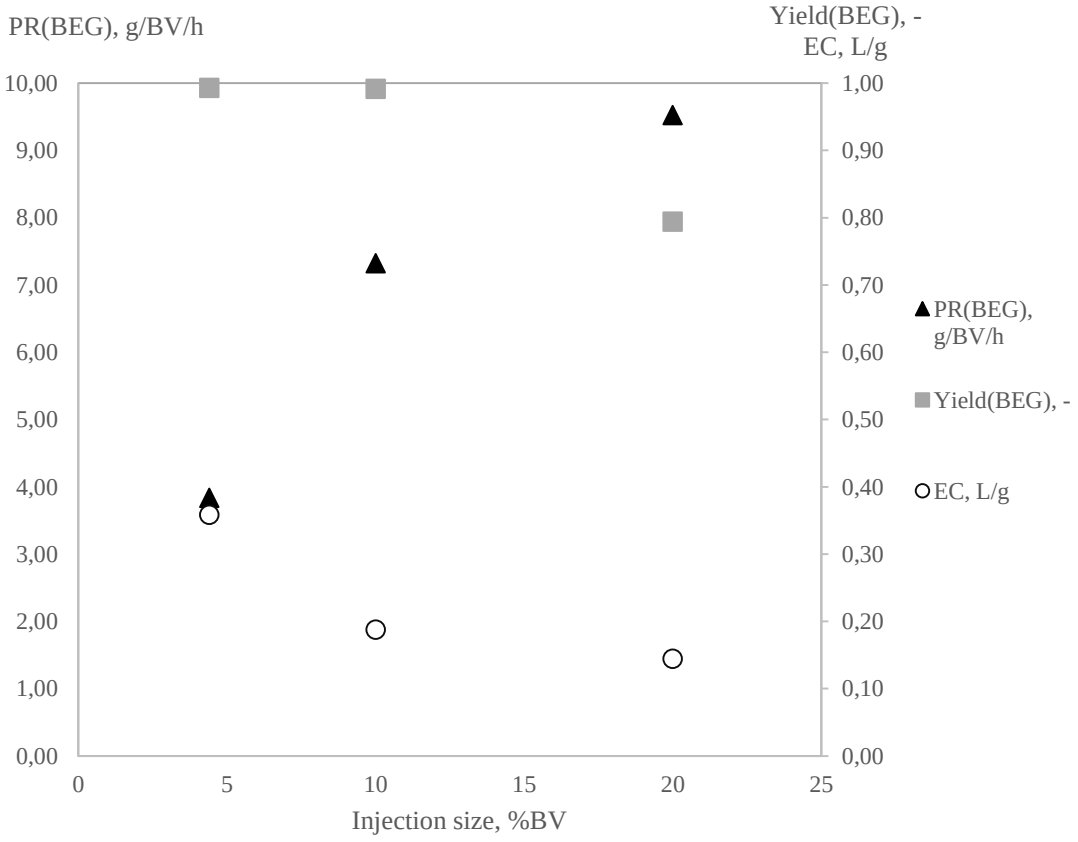


Figure 9-11 **I**njection size experiments in a 115 mL column packed with CA10GC in Na^+ form. In the feed the approximate concentration values of the components were c(BEG) = 31 g/L, c(GLU) = 15 g/L and c(EtOH) = 13 g/L. Flow rate and the column temperature were 0.9 BV/h and 50°C.

9.2.2 Scale-up

Acryl based WAC CA10GC resin in Na^+ form was observed to be the best material to separate glucose from BEG, and the material was used in the scaled-up process. The removal of ethanol from the feed solution clearly enhanced the separation hence, the separation was done with feed solution from which the amount of ethanol was reduced. Amount of ethanol was decreased by heating the feed solution in a fluoropolymer - coated vessel as the product goes to internal testing. Since the target was to produce BEG with the scaled-up batch process the purity of glucose fraction was not under investigation in this part of the experiments.

Injection volume of 10 $\%_{BV}$ was chosen for the scaled up separation. Up to this injection size 99 % pure product with 99 % yield was able to be collected when ethanol is not assumed to be an impurity. Comparison of the different injection sizes of 4.4 $\%_{BV}$, 10 $\%_{BV}$, and 20 $\%_{BV}$ may be seen from the Figure 9-11.

With 4.4 $\%_{BV}$ injection 99.3 % yield with 99 % purity of BEG was gained. Yet, with so small injection volume more than twice the amount of injections and eluent would have needed compared to 10 $\%_{BV}$ injection. In addition, the productivity of 10 $\%_{BV}$ injection was two times higher.

Even though the productivity of 10 $\%_{BV}$ injection was 2.2 units lower than with 20 $\%_{BV}$ injection 10 $\%_{BV}$ was chosen for the scaled up process. If greater injection size than 10 $\%_{BV}$ is separated in a normal batch process with flow rate of 0.9 BV/h the overlapping of BEG and glucose peaks start to be greater and the yield of 99 % pure product decreases. The yield of 99 % pure BEG decreased by 20 % when the injection size was 20 $\%_{BV}$ instead of 10 $\%_{BV}$.

As the column was empty after elution of 1 BV, and it took 0.40 BV before the break through of BEG, a new injection was done when 0.7 BV (to be sure the column was empty) had ran through after every injection. Therefore, the second collection was starting after elution of 1.1 BV. This was continued through all the cycles. Hence, the separation cycle was as effective as possible reaching ‘touching bands’.

When comparing the chromatograms of scaled-up and experimental scale runs the retention times of BEG and glucose varied a little. In addition, from the larger column BEG broke through earlier than from the smaller column as shown in Figure 9-12. The small amount of ethanol in the small scale run might have affected a little bit on the separation. There was practically no ethanol in the feed of scaled-up process. Fraction collection times were set as t_{A1} = 0.4 BV and t_{A2} = 0.64 BV based on the breakthrough points of the design pulse (so that 99 % pure product was possible to be collected).

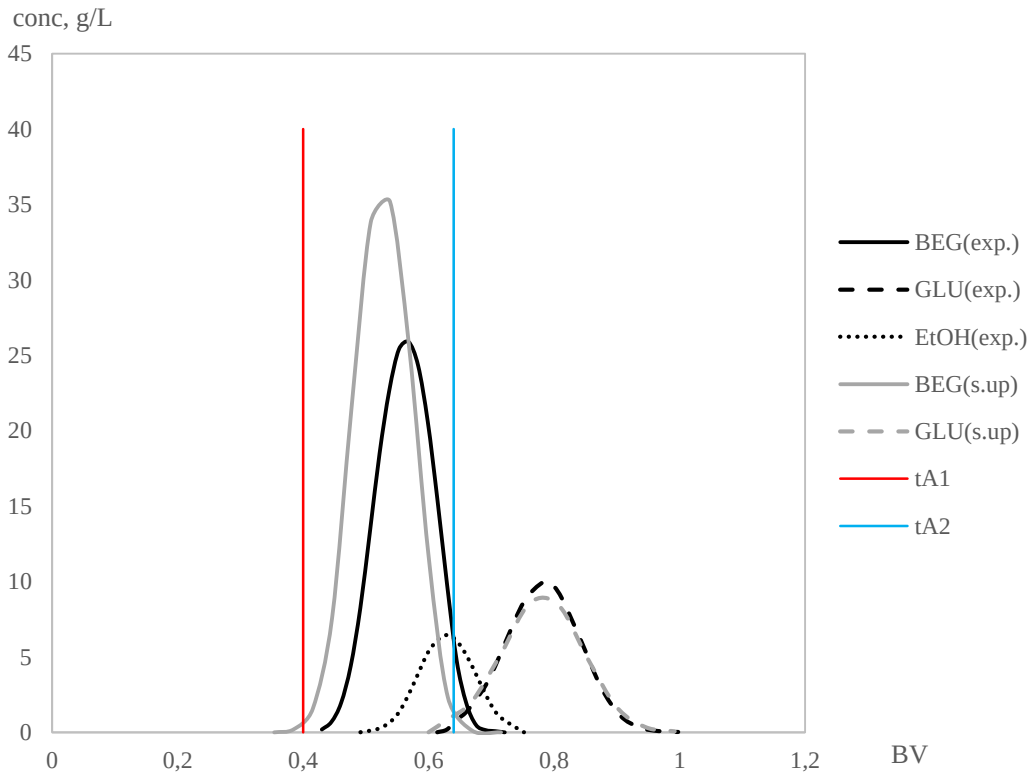


Figure 9-12 Elution profiles with 10 $\%_{BV}$ injection. T = 50°C, $Q_{exp.}$ = 1.76 mL/min and $Q_{s.up}$ = 29 mL/min. $c(BEG)_{s.up}$ = 40 g/L and $c(GLU)_{s.up}$ = 17 g/L. $c(BEG)_{exp}$ = 32 g/L, $c(GLU)_{exp}$ = 16 g/L, and $c(EtOH)_{exp}$ = 5.6 g/L.

For the small scale run the results were $PR(BEG)_{exp}$ = 7.3 g/BV/h and $EC(BEG)_{exp}$ = 0.19 L/g. Productivity for BEG in the scaled-up process was $PR(BEG)_{s.up}$ = 9.3 g/BV/h, and the eluent consumption $EC(BEG)_{s.up}$ = 0.14 L/g. As the feed concentrations differed between the scaled-up and small scale separations, the results were not comparable. However, as the relation of flowrate to the bed volume remained the same in the scale-up, the productivity and eluent consumption should not improve too much. The productivity might be slightly better for scaled-up process as the separation seemed to be more successful in larger column.

It is well known that the higher the plate number the more efficient the column. It is also considered that when using a column with a high number of theoretical plates the peaks are narrower than with a column with less theoretical plates [48]. In the larger column the separation of BEG and glucose seemed to be better which is due to more effective separation compared to experimental sized column, which was over 16 times smaller.

Repeatability of each elution was complete from pulse to pulse during the 10 injections in the scaled-up process. Eventually, 99 % pure BEG was collected within the set fraction collection times if only glucose was considered as an impurity. Ethanol may be completely evaporated off from the product. BEG was produced with yield of 99 %.

9.2.3 Design pulses of SSR

In design pulses the injection size equal to the amount of fresh feed injected to the column in one pulse. As the pulse size increases, productivity increases and eluent consumption decreases. Yet, increase in pulse size affects to the product purities. The purity reached by both components (BEG and glucose) with one cut time decreased as the pulse size increased due to greater overlapping of the chromatograms.

With design pulse size of 3.7 $\%_{BV}$ the products were almost in purity of demand (98 %) which is why this pulse was used as a comparison pulse for SSR runs. Actually, the productivities should be smaller and eluent consumption bigger as the purity of 98 % is truly reached with even smaller pulse size than 3.7 $\%_{BV}$. The design pulses made had purities, productivities, and eluent consumptions shown in Table IX.

Table IX Design pulses made in 113 mL column with CA10GC resin in Na^+ form. Q = 2.64 BV/h and T = 50°C.

$\%_{FF}$	V_{FF}, mL	PU_i, -	PR(BEG)	PR(GLU)	EC(BEG)	EC(GLU)
3.7	4.16	0.978	8.64	3.3	0.46	1.21
5	5.65	0.977	11.72	3.78	0.34	1.05
7	7.91	0.975	16.98	6.61	0.23	0.6
10	11.3	0.956	22.48	9.03	0.18	0.44
20	22.6	0.932	37.74	12.09	0.11	0.33

9.2.4 SSR

The results of SSR are summarized in Table X. The suitable fraction times were chosen based on the design pulses. BEG did not reach the purity of 98 % in 20 $\%_{BV}$ injection as the collection of BEG fraction should have finished earlier.

Table X Results of SSR runs in steady state.

pulse size, $\%_{BV}$	t_{A2}, min	t_{B1}, min	PU(BEG), %	PU(GLU), %	PR(BEG), g/BV/h	PR(GLU), g/BV/h	EC(BEG), L/g	EC(GLU), L/g
7	14	14.75	98.4	98.1	9.6	3.6	0.44	1.1
10	14.25	15.45	98.5	98.3	10.3	4.3	0.39	0.88
20	14.75	17.75	97.0	98.8	15.2	5.2	0.26	0.72

The performance of the SSR process increased with increasing column loading (Table X). However, according to numerical simulations the productivity begins to decline with even higher pulse sizes. As seen from the Figure 9-13, injection size of 20 $\%_{BV}$ is very close to the optimum pulse size as maximum BEG and glucose concentrations in the first cycle are close to the feed concentrations. With too great injection size the column starts to saturate and the productivity starts to decrease. The situation is discussed in more detail with simulations in section 9.4.

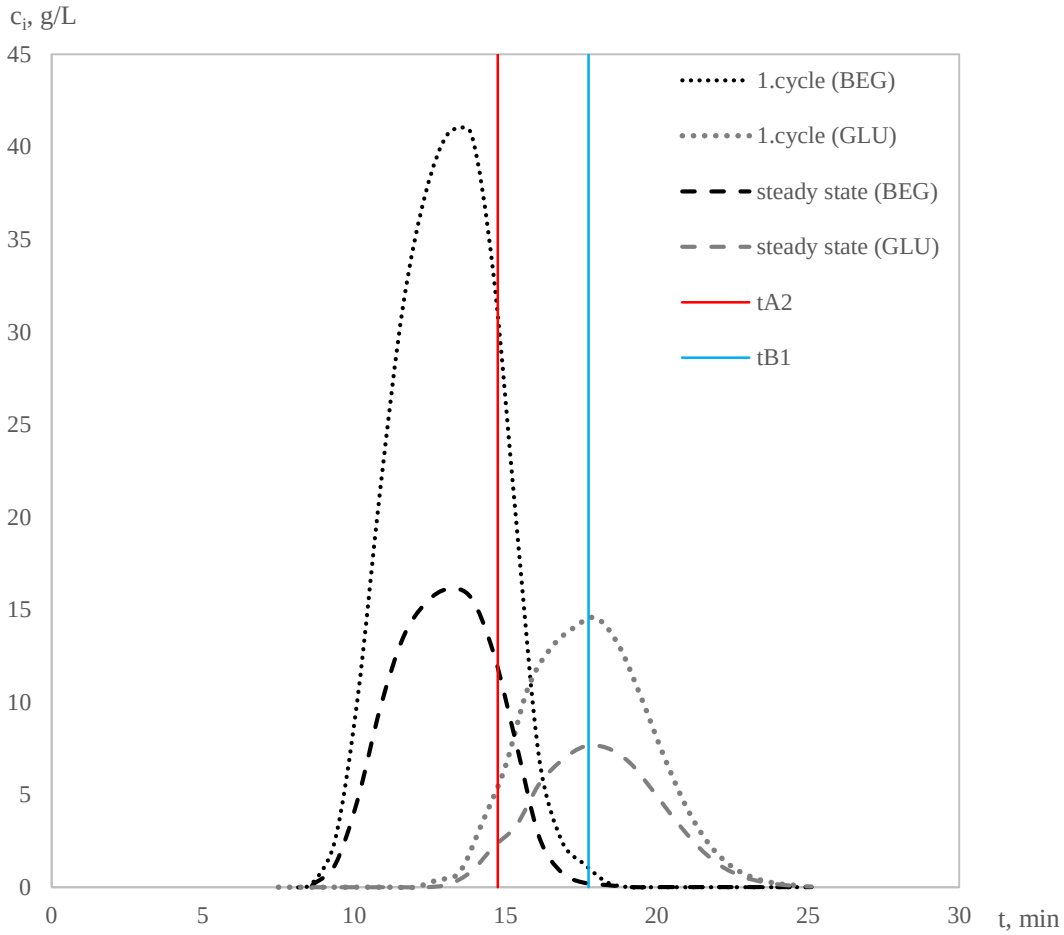


Figure 9-13 SSR chromatogram with 20 $\%_{BV}$ injection size. Recycle fraction delimited by *t*A2 and *t*B1. Initial concentration in feed: c(BEG) = 41 g/L and c(GLU) = 16 g/L.

Selection of the exact fractionation times for the SSR process on the basis of the design pulses is challenging as small differences in the fractionation times affect significantly on the product purities. For example in 20 $\%_{BV}$ run by starting and finishing the recycle fraction 15 second earlier both products would have reached 98 % purity. The slightly too late position of recycle fraction may be seen from Figure 9-13. It can also be seen that the retention times of the components do not change with changing concentration due to the linear isotherms. From 1st injection to steady state the concentrations of the feed have decreased approximately 60 % for BEG and 50 % for glucose.

Figure 9-14 shows how the productivity and eluent consumption change before the steady state is reached. At the steady state the same quality products are produced with constant productivity and eluent consumption.

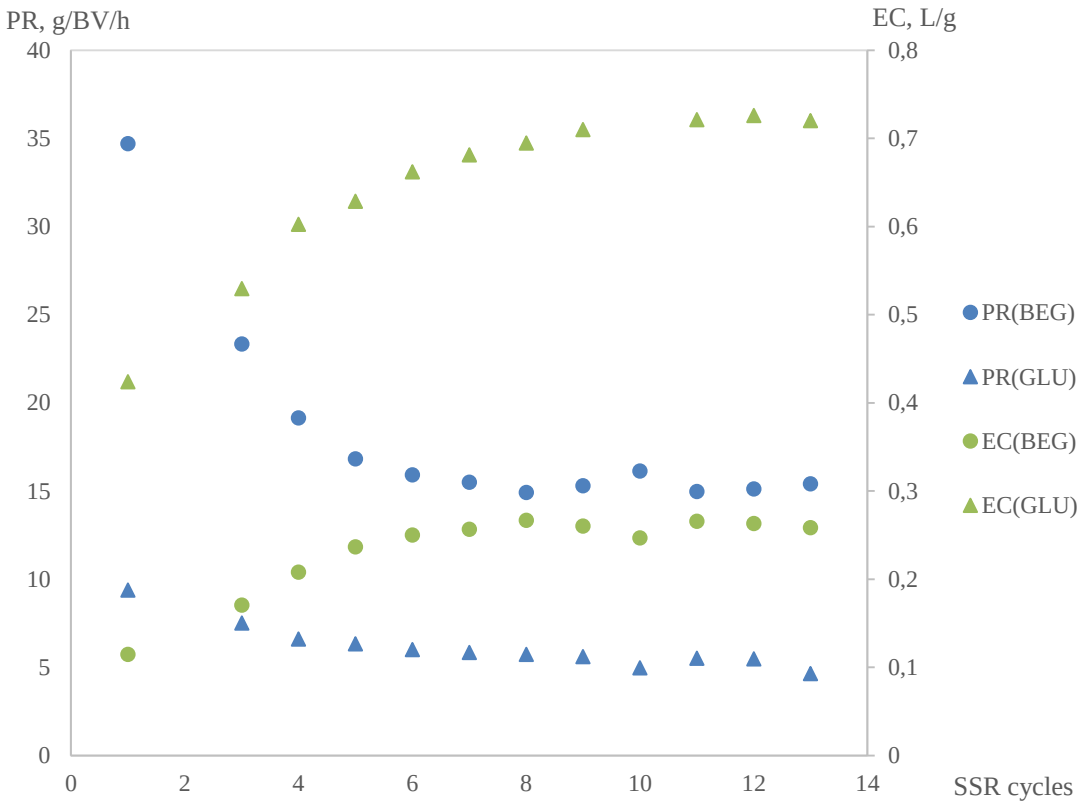


Figure 9-14 Running SSR to its steady state with pulse size of 20%$_{BV}$. Initial concentration in feed: c(BEG) = 41 g/L and c(GLU) = 16 g/L.

As the retention times of the solutes did not change with changing concentration, the purity of the products remained pretty much the same from cycle to cycle. This can be seen from Figures 9-15 – 9-17 for every injection volume used in SSR runs. Correct selection of the fraction times, especially t_{A2} and t_{B1} is very important as even slightly inaccurate cut times cause an inappropriate purities of the products. As in the case of 20 %$_{BV}$ SSR run (Table X). The purity of glucose fraction was close to purity of 99 % whereas, the BEG fraction purity was 97 %.

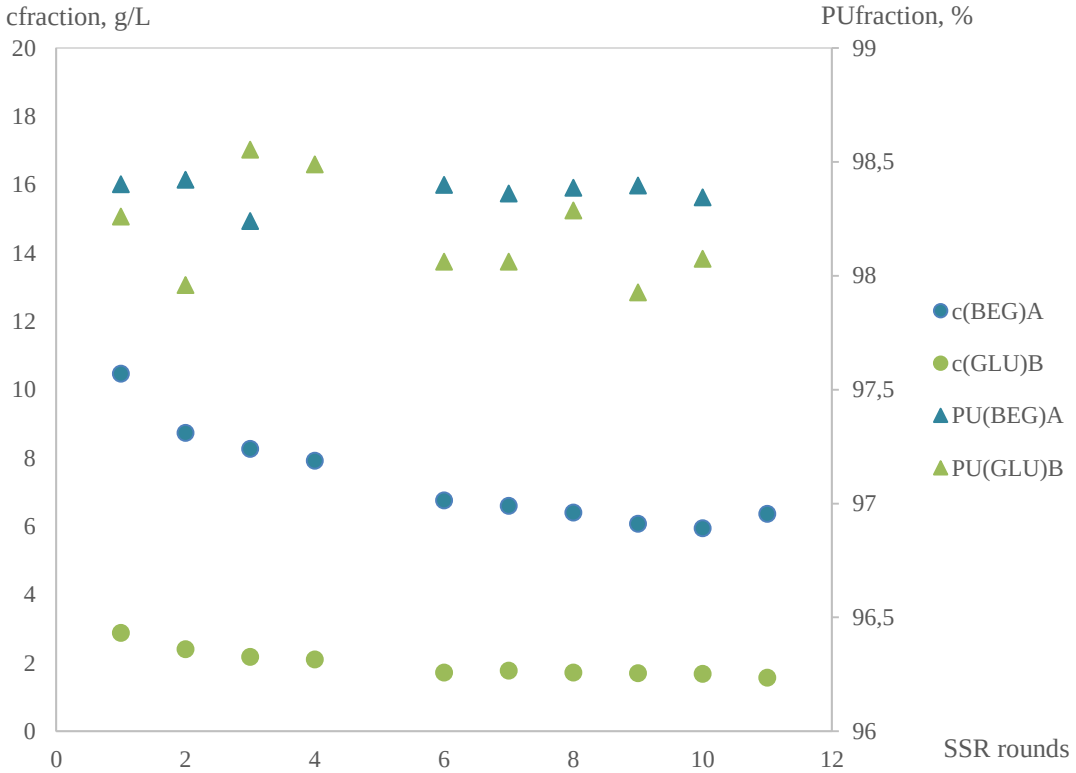


Figure 9-15 Running SSR to its steady state with pulse size of 7 $\%_{BV}$. Initial concentration in feed: c(BEG) = 41 g/L and c(GLU) = 16 g/L.

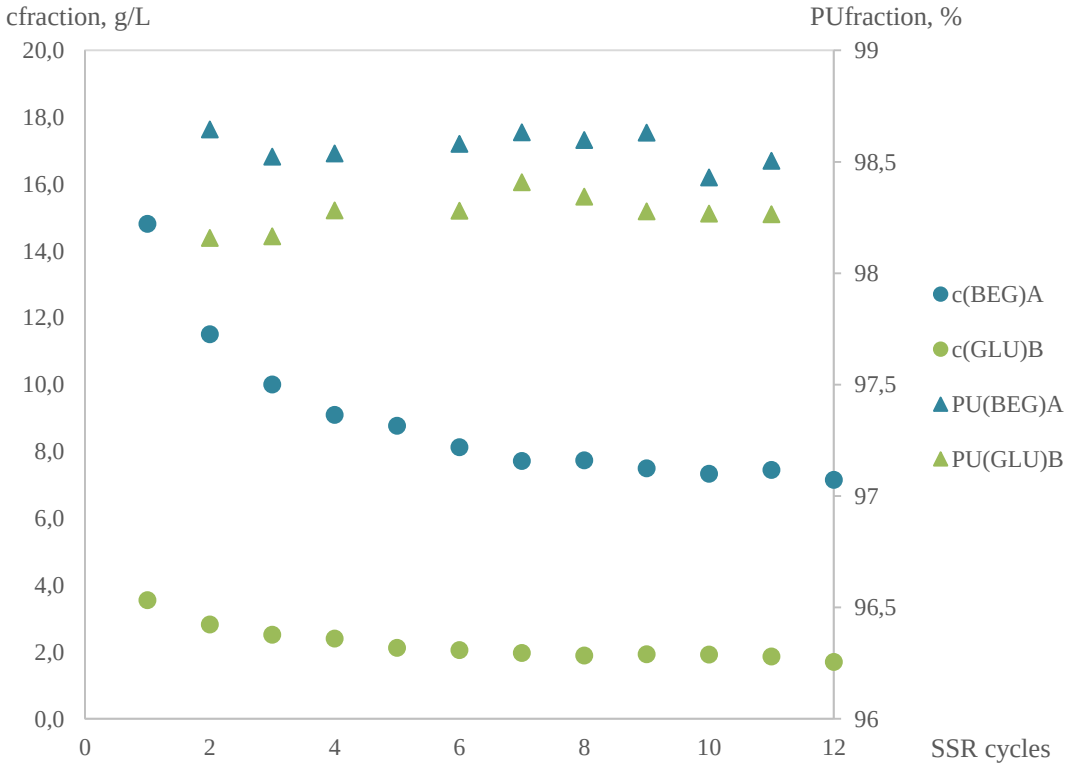


Figure 9-16 Running SSR to its steady state with pulse size of 10 $\%_{BV}$. Initial concentration in feed: c(BEG) = 41 g/L and c(GLU) = 16 g/L.

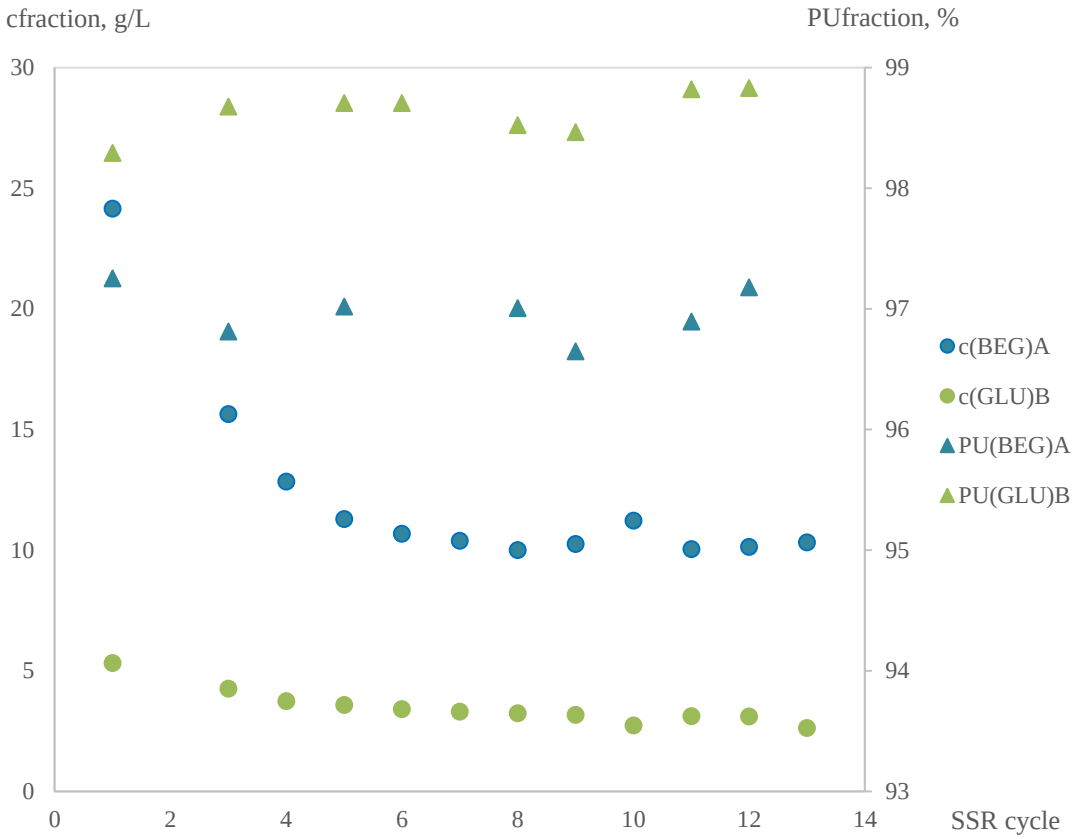


Figure 9-17 Running SSR to its steady state with pulse size of 20 $\%_{BV}$. Initial concentration in feed: c(BEG) = 41 g/L and c(GLU) = 16 g/L.

Figure 9-18 compares the results of SSR to the batch chromatography separation that reached 98 % purity with on cut time. The comparable batch separation run had fresh feed injection volume of 3.7 $\%_{BV}$.

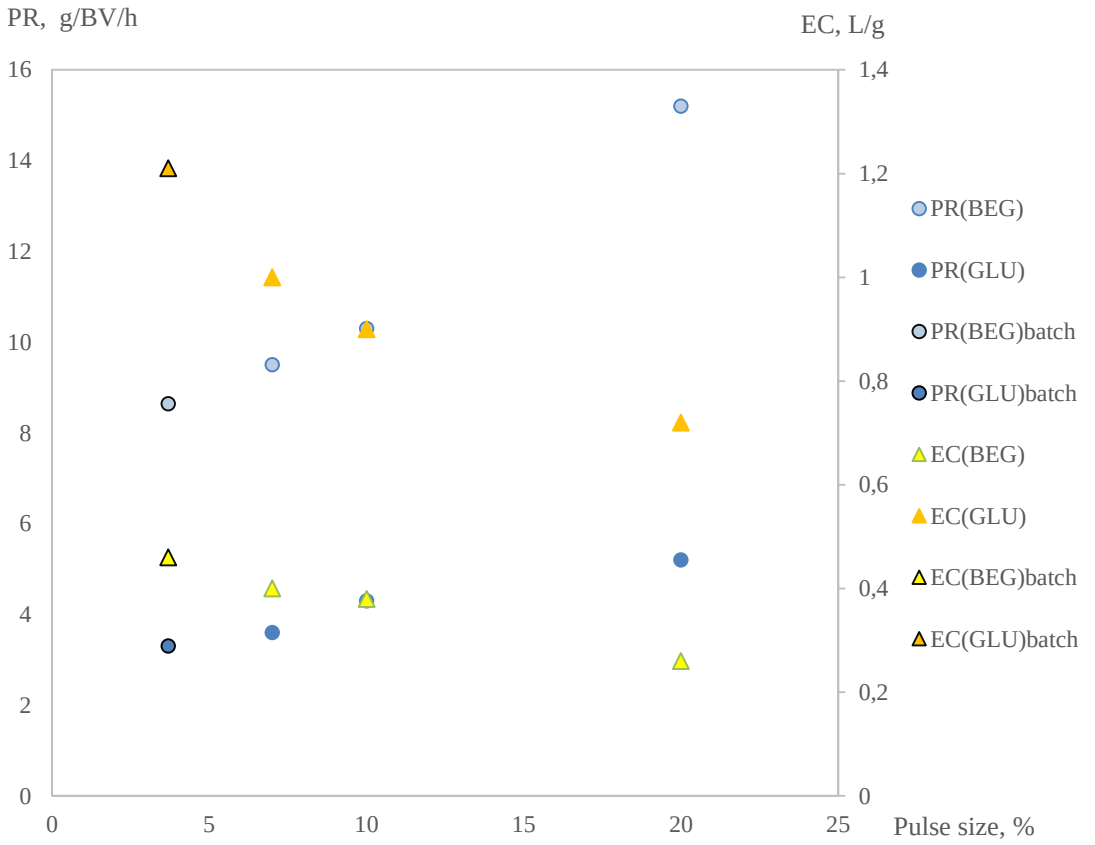


Figure 9-18 Comparing SSR to the design pulse (3.7 $\%_{BV}$) reaching 98 % product purities with one cut.

The SSR run with pulse size of 7 $\%_{BV}$ had 3.7 $\%_{BV}$ of fresh feed in the injection. When utilizing SSR with the same amount of fresh feed the productivity and eluent consumption were already enhanced due to recycle when compared with the batch process. 9.5 g/BV/h compared to 8.64 g/BV/h of BEG, and 3.6 g/BV/h compared to 3.3 g/BV/h of glucose as seen from the Figure 9-18 (or more accurately from the Tables IX and X). The eluent consumption enhanced as well.

With the optimum SSR pulse size (20 $\%_{BV}$) the productivity of 98 % pure BEG was approximately 15.2 g/BV/h and 98 % pure glucose 5.2 g/BV/h. Therefore, the productivities were improved by 43 % and 37 %. Respectively, eluent consumption had decreased by 43 % and 40 %. Yet, it should be remembered that the cut times were not perfectly matched in the experiments so, the results could be even better.

SSR has not been applied too often in the case of separation where the solutes to be separated have linear isotherms and relatively good separation already in batch with low injection volumes and flow rates. Anyhow, in the case of more complicated separations where the solutes are greatly overlapping, different components have been successfully separated with SSR in previous studies. For example, Hellstén and Sainio [49] gained 60% higher productivity and 45% lower eluent consumption with SSR than with batch when separating sulphuric acid and glucose. In turn, Yan and Orihuela [50] have also managed to enhance the productivity (by a factor of 4.5) and decrease the eluent consumption (by a factor 4.1) of a batch HPLC by utilizing SSR. They used the SSR technique for the enantiomeric resolution of three pharmaceutical intermediates at various sample scales.

As stated in the present study, the general trend is, compared to a batch separation, that the use of SSR is greatly increasing the productivity and decreasing the eluent consumption with different separation problems. Within racemic pharmaceutical intermediates Grill et al. [51] have found SSR to be comparable even to the lab-scale SMB process in productivity and solvent consumption. Generally, the harder the batch separation the better the results offered by SSR (if there is a change for recycle). Yet, now it has been presented that even an easy batch separation may be greatly enhanced utilizing SSR and, by increasing the pulse size and flow rate.

9.3 Adsorption isotherms

The adsorption isotherms of BEG and glucose on CA16GC resin in Na^+ form were linear in the concentration ranges of interest (Figure 9-19). The values of the isotherm slopes are given in Table XI. The values of the slopes of the adsorption isotherms (*dq*/*dc*) were different for single components than for mixture of BEG and glucose (Figure 9-19).

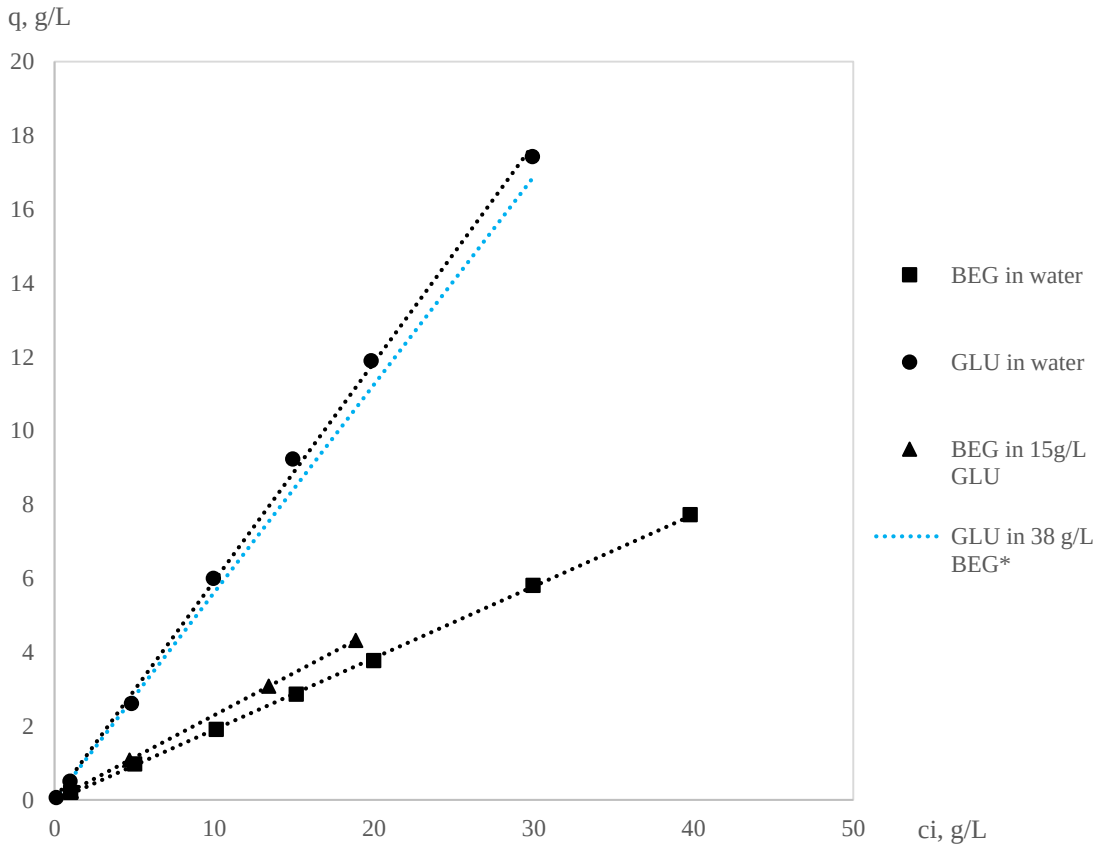


Figure 9-19 Adsorption isotherms determined for glucose and BEG in water and for BEG in 15 g/L glucose. * Estimation according to simulation.

It was found out that the sorption of BEG increased when glucose was present: the slope of the BEG isotherm was found to increase by 0.16 % when the eluent was changed from purified water to 15 g/L glucose solution. However, the linear shape of the isotherm remained. This co-operative interaction was caused by the changes in the solvation of the components in the presence of each other [30, 39]. In pure water, BEG was completely solvated by water molecules and the sorption was lower as the solvated molecules could not fit into the pores of the stationary phase material. Addition of glucose to the solution decreased the solvation of BEG as glucose molecules took part of the water in the solvation shells of BEG. The BEG molecules became less solvated and, thus, smaller. This led to higher sorption of BEG in the presence of glucose, as the less solvated BEG molecules were able to fit to the pores of the stationary phase. The situation would have been the same if pure glucose sorption and glucose sorption in the presence of BEG was compared.

Table XI Adsorption isotherms for components in water and in binary solution.

Component	Eluent	dq/dc
BEG	water	0.193
BEG	15 g/L GLU	0.229
GLU	water	0.593
GLU	38 g/L BEG	0.563*

* Estimation according to simulation.

For the chromatographic runs in which both the components were present, the slopes of the adsorption isotherms had to be adjusted to get a good correlation between the experimental and calculated results. The model did not fit to the data if adsorption isotherms determined in water were used. If their single component adsorption isotherms were valid in binary solution the separation would have been even more efficient.

9.4 Modelling of SSR

The linear isotherm models were validated and the diffusion coefficients ($0.12*10^{-9}$ for BEG and $0.09*10^{-9}$ for glucose) determined by fitting the model predictions with the experimental results obtained from batch elution experiments. The dispersion coefficients were calculated using the correlation of Chung and Wen [31]. The effect of flow rate and column loading on the performance of the SSR process was evaluated using numerical simulations. An example of the correlation between the calculated results and experimentally obtained results with the SSR process is presented in Figure 9-20. A good correlation was obtained between the results with the SSR process and also with the batch process.

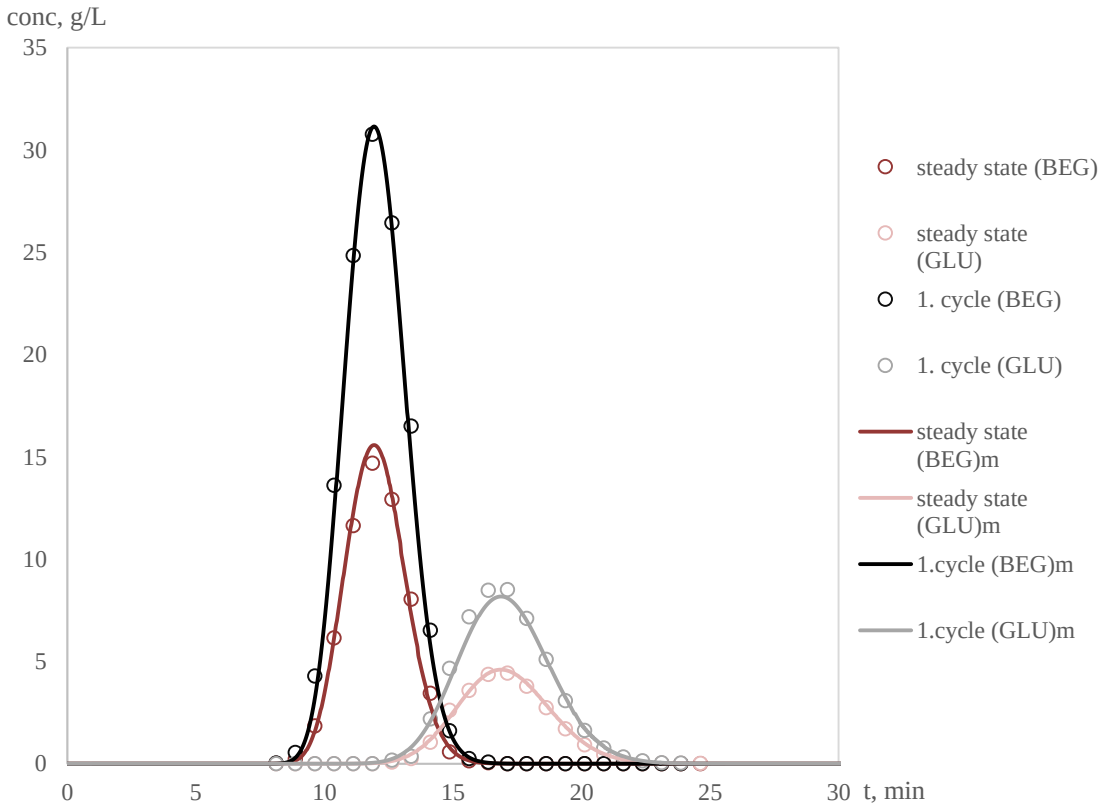


Figure 9-20 Fitting Matlab model to 10 $\%_{BV}$ SSR data. Q = 5 mL/min (2.64 BV/h). Simulation parameters: ε = 0.33, dq/dc(BEG) = 0.229, and dq/dc(GLU) = 0.563. The solute concentrations are c(BEG) = 41 g/L and c(GLU) = 16 g/L.

The effect of the pulse size on the performance of the SSR process is shown in Figure 9-21. With a fixed flowrate, here 2.64 BV/h, it may be seen how the productivity started to decrease with larger pulse sizes due to the increasing cycle time and column saturation. If the injected feed pulse is so large that the column is close to saturate with the solute the shape of the dispersed tail of the chromatogram remain the same even if the pulse size is increased more. This is why the amount of fresh feed in the mixed feed cannot be increased anymore after a certain point. Otherwise, the productivity start to decrease as the recycle fraction keep increasing while the amount of fresh feed remain the same. According to the numerical calculations, the highest productivity was reached with flowrate of 2.64 BV/h at the pulse size of 20 $\%_{BV}$. Eluent consumption decreased with increasing pulse size, but became constant with larger than 20$\%_{BV}$ pulse sizes.

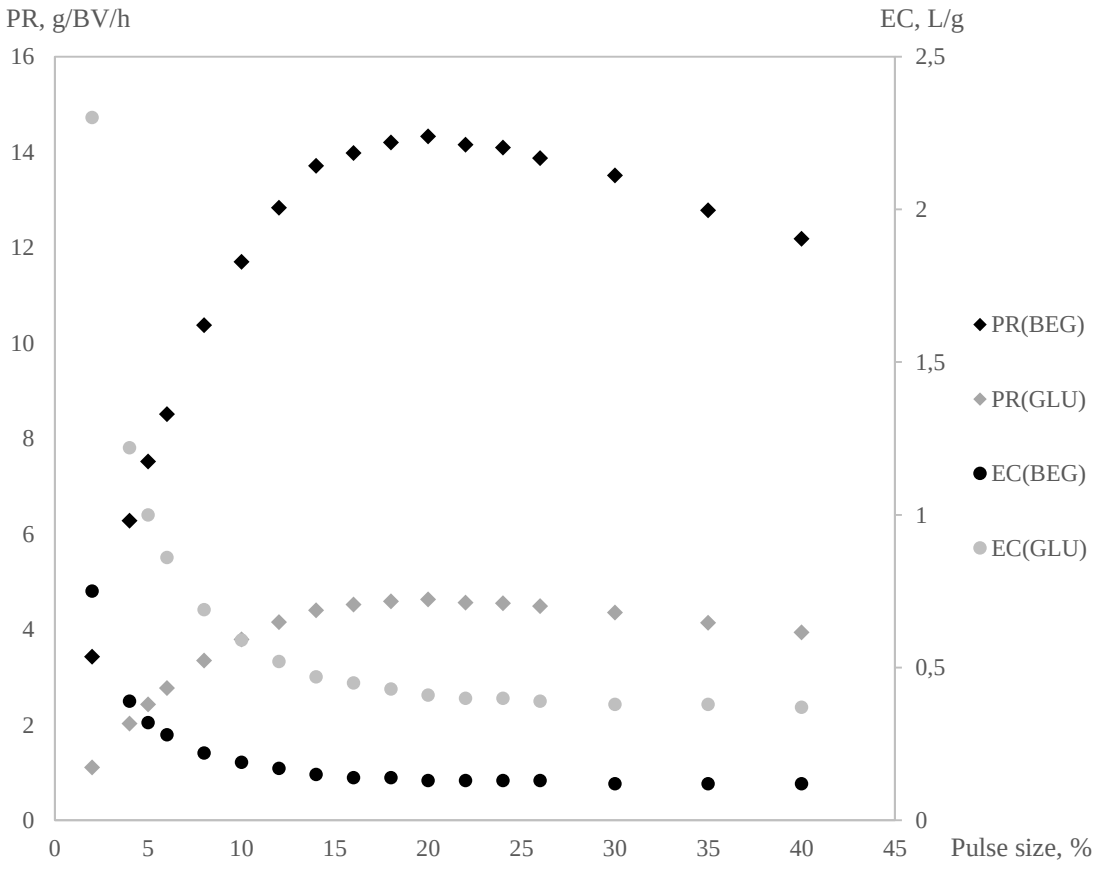


Figure 9-21 Simulations done in Matlab using flowrate of 2.64 BV/h and simulation parameters determined. ε = 0.33, dq/dc(BEG) = 0.229, and dq/dc(GLU) = 0.563. The solute concentrations are c(BEG) = 38 g/L and c(GLU) = 14 g/L.

For a chromatographic process in fixed conditions there is one combination of pulse size and flow rate that gives the best productivity for the SSR system. It is known that the higher the flow rate and the greater the pulse size can be, the better for the process productivity. Anyhow, at some point the productivity start to decrease as stated above. Both of the parameters cannot be increased indefinitely due to dispersion effect, overlapping of the chromatograms, and column saturation. The ratio of fresh feed and recycle in mixed feed becomes unprofitable after a certain point as the amount of fresh feed cannot be increased anymore if the column starts to saturate and the shape of the dispersed tail remains similar.

If the pulse size was kept constant and the flowrate was increased, the size of the recycle fraction would increase due to the dispersion effect. If the flowrate was kept constant and the pulse size was increased, the size of the recycle fraction would increase due to overlapping of the more concentrated chromatograms (if the two components break closely through the column). So, actually, the amount of recycle in the feed indirectly determines the productivity of the process which is why productivity and flowrate are plotted against amount of recycle in the Figure 9-22. Size of the recycle fraction is the link between flowrate and pulse size with what the optimum combination of flowrate and pulse size is possible to find.

To find out the optimum flow rate – pulse size combination three different pulse sizes (15, 20, and 20 $\%_{BV}$) were observed against the range of flow rates (0.9 to 3.72 BV/h) via simulations. The estimated recycle fractionation times were simulated with different flowrates holding pulse size constant, and the amount of recycle compared to the amount of feed in total was calculated. In every pulse size - flowrate combination also the productivity was recorded via simulation.

Through SSR simulations, it was noticed that the optimum flowrate was reached with the optimum pulse size, when the relation of recycle and fresh feed (R/F) in the mixed feed is 50:50 (Figure 9-22). If the pulse size is smaller than the optimum pulse size, here 15 $\%_{BV}$ as an example, the highest productivity with respect to BEG is reached when the R/F is about 35:65. And, if the pulse size is greater than the optimum pulse size, here 25 $\%_{BV}$, the relation is about 58:42 (Figure 9-22). The highest productivity with respect to BEG (15.2 g/BV/h) is obtained with 20 $\%_{BV}$ injection size (Figure 9-22). This is well predicted when compared to the experimental results (see Table X). The highest productivity with respect to BEG of each pulse size was obtained when the flowrate was approximately 2.52 BV/h. Although, 2.52 BV/h flowrate was used in the experiments as it corresponded to 5 mL/min flow rate with the bed volume used.

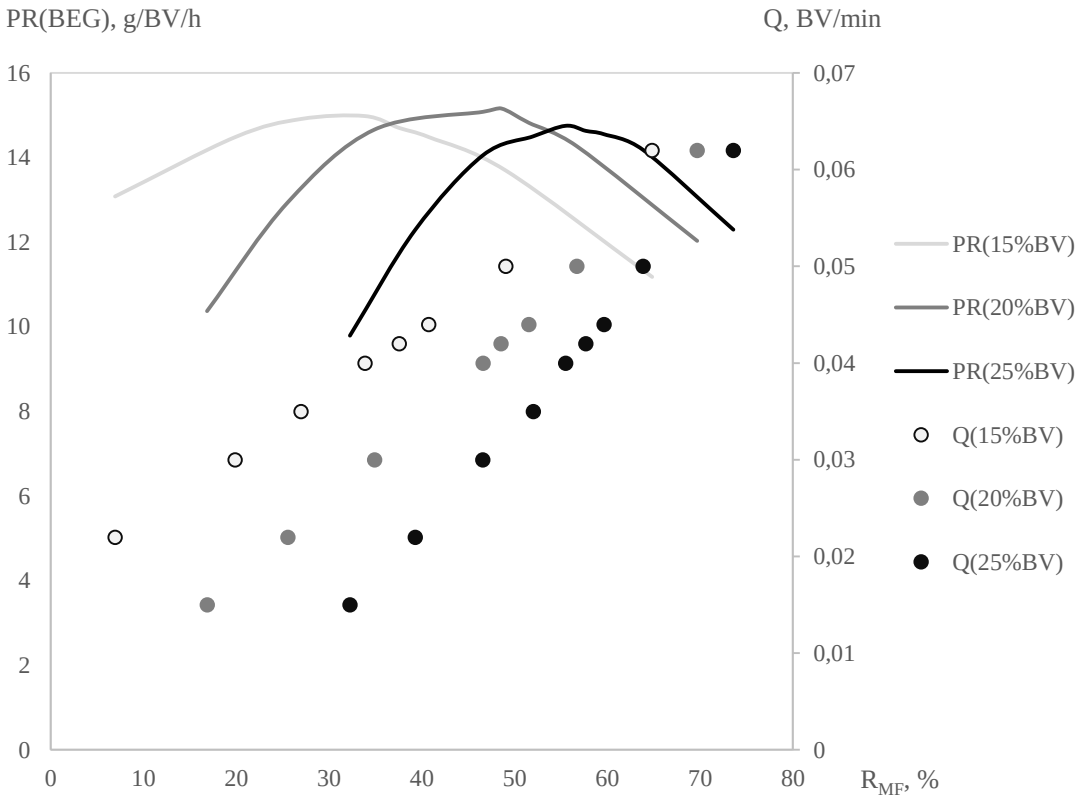


Figure 9-22 Screening optimal pulse size and flowrate with Matlab simulations. R_{MF} (= R/F) is the amount of R in mixed feed (the rest is FF). Simulation parameters: ε = 0.33, dq/dc(BEG) = 0.229, and dq/dc(GLU) = 0.563. The solute concentrations are c(BEG) = 41 g/L and c(GLU) = 16 g/L.

Shortly, according to these simulations, the optimal pulse size is the pulse size which greatest productivity is found at the point where the amount of recycle is 50 % from the total amount of feed through changing the flowrate. Although, with 20 $\%_{BV}$ injection size 33:66 ratio of FF and R was reached instead of 50:50. Yet, the obtained results were still very good. With further adjustment of the fractionation times, even better productivities could be reached.

It was possible to predict the cut times for the SSR as seen from Figure 9-23. In this thesis the short-cut method was used. Yet, taking advantage of the design pulses was more reliable way to set the fractionation times as the simulated times were slightly different to those obtained from the experiments. For example, in the case of 20 $\%_{BV}$ injection the simulated fractionation times were 14.8 min for *tA2* and 17 min for *tB1* (Figure 9-23: cycle 30) as the times determined based on the design pulse were 14.5 min and 17.5 min. Simulation gave shorter recycle period than practical experiments.

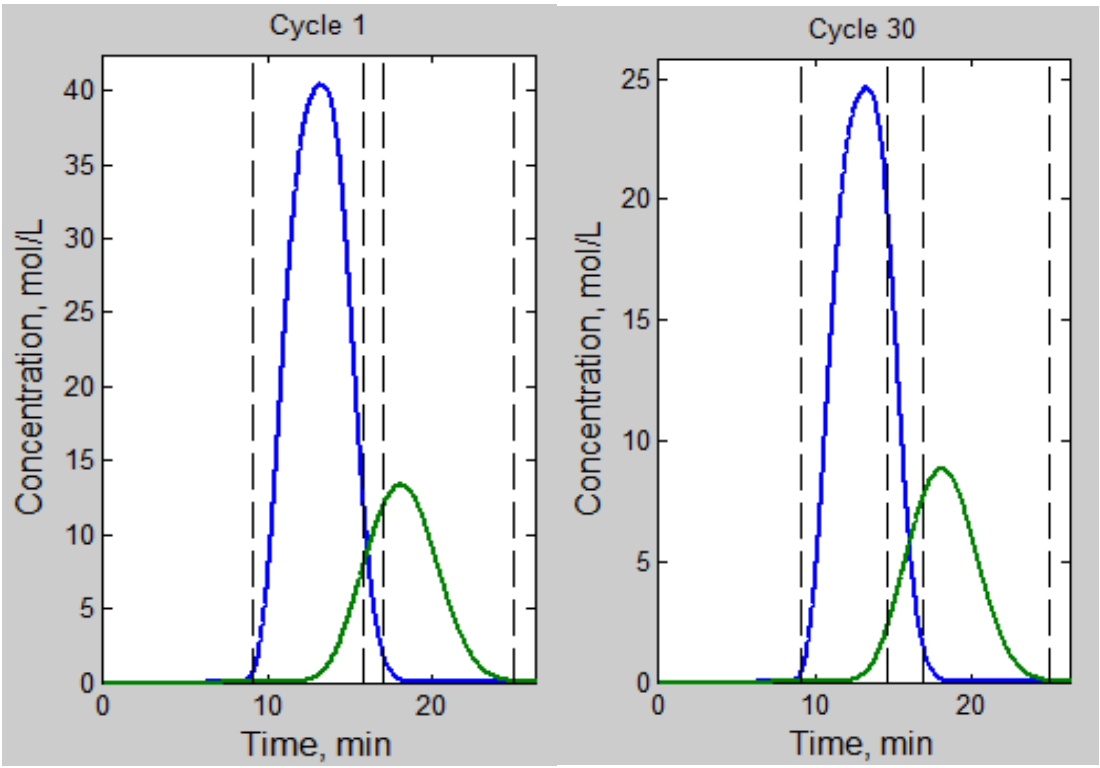


Figure 9-23 Predicting cut times with Matlab model (short-cut design on every cycle) to produce 98 % pure products with flow rate of 5 mL/min and injection size of 20 $\%_{BV}$. Initial concentration for BEG was 41 g/L and for GLU 16 g/L. The dotted lines in figure from left to right are t_{A1}, t_{A2}, t_{B1}, and t_{B2}.

In Figure 9-24 the result of a simulation for 20 $\%_{BV}$ SSR run is showing with flow rate of 2.64 BV/h. It can be seen that the steady state and the set target purity of 98 % were reached in simulation. In practice, changes from cycle to cycle were caused by the concentration changes in mixed feed tank due to recycle fraction collected there until the steady state was reached. The simulation cycles were changing until the model found out the most suitable fraction times for the recycle fraction on the basis of fixed purity. The simulation was done utilizing a short-cut design on every cycle based on the tail of the last eluting component (glucose).

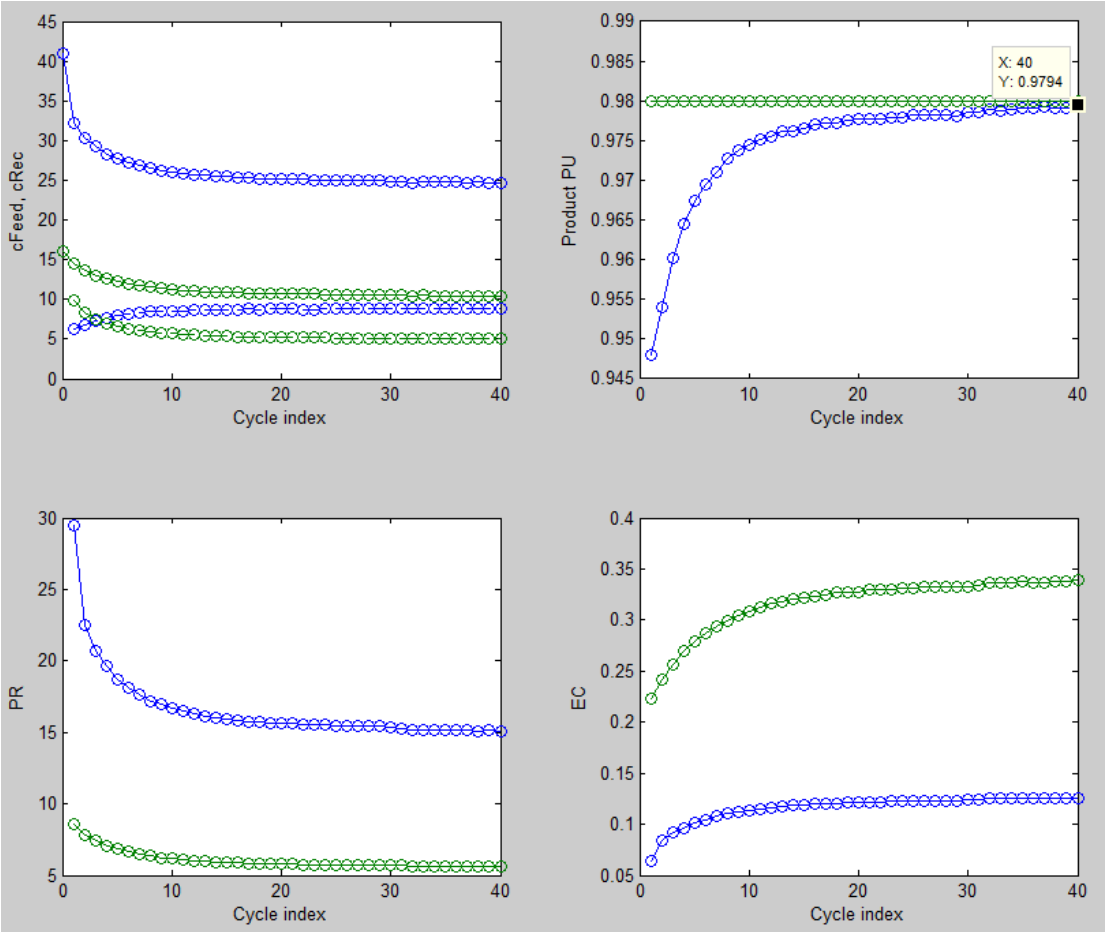


Figure 9-24 Predicting productivities and eluent consumption via Matlab model to produce 98 % pure products with flow rate of 2.64 BV/h and injection size of 20 $\%_{BV}$. Initial concentration for BEG was 41 g/L and for GLU 16 g/L.

CONCLUSION

Enzymatic synthesis of ethyl β-D-glucopyranoside was performed with β-glucosidase as a catalyst. 60 - 65 % glucose conversion was reached. The best separation material was chosen from SAC and WAC resins based on chromatographic experiments. BEG was recovered most efficiently by CA10GC resin in Na^+ form from the synthetic solution.

Scale-up of the batch chromatography separation process was implemented successfully from column size of 115 mL to column size of 1885 mL, and 99 % pure BEG was produced over 50 grams. Successful transfer of the batch process into SSR was implemented utilizing design pulses. Designing of the SSR needed high care and precision in addition to complicated simulation to find out the optimal separation conditions to save time and resource. Even a small inaccuracy in the design changes the product purities. Compared to batch chromatography, with SSR the productivities of the products were improved by approximately 40 %. Respectively the eluent consumption decreased by approximately 40 %.

More thorough experiments among the enzymatic synthesis of BEG could be done by for example changing the ethanol-water ratio in addition to reactant concentration. More experiments about how the amount of ethanol affect to the BEG and GLU separation (also in styrene based SAC resins) could be done as well. What about when the BEG to GLU concentration ratio in feed changes more? And, how are the WAC resins working in Ca^{2+} form? Does the productivity decrease in practice if the injection volume is greater than 20 $\%_{BV}$?

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