Universidade do Minho
Escola de Engenharia

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Fructo-oligosaccharides recovery from fermentation processes

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Trabalho efectuado sob a orientação do
Professor Doutor José António Couto Teixeira
e da
Professora Doutora Lígia Raquel Marona Rodrigues

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Fructo-oligosaccharides recovery from fermentation processes

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ABSTRACT

FRUCTO-OLIGOSACCHARIDES RECOVERY FROM FERMENTATION PROCESSES

The main purpose of this thesis was to recover the fructo-oligosaccharides (FOS) from the sugar mixtures obtained by fermentation. Among the current downstream techniques that are available, the separation method chosen was the liquid chromatography. Two different stationary phases were studied, namely the activated charcoal and several ion-exchange resins.

A single activated charcoal column was used to study the adsorption/desorption of FOS contained in a fermentative broth mixture through a gradient of water/ethanol. After optimizing the separation method, 74.5% (w/w) of FOS were recovered as well as fractions with purity up to 97% (w/w). This method was also efficient in the desalting of the fermentative broth. The process using a single activated charcoal column was a good starting point to obtain large amounts of pure FOS in a simple and economic way. Nevertheless, the main goal of this thesis was to develop a separation process to be used at an industrial level, thus economically attractive. Therefore, the Simulated Moving Bed (SMB) chromatography was selected as the most adequate separation method.

The SMB chromatography has the advantage of working in a continuous mode, using water as eluent and homogeneous stationary phases, leading to very reproducible results. Hence, several potential ion-exchange resins were studied in order to be used in a SMB chromatography pilot plant.

The suitability of the resins in the separation of mono- and disaccharides was evaluated by adsorption equilibrium, kinetic and mechanical resistance studies. A Dowex Monosphere 99K/320 resin, gel-type with 320 μm of particle diameter, in potassium form, was selected. The fermentative broth chromatographic elution on a single column was modelled and kinetic and adsorption parameters were identified. FOS were successfully purified from 37.1 to 62.9% (w/w) using the SMB pilot plant. Furthermore,

the FOS yield and productivity obtained in the raffinate was 69.4% (w/w) and 82.1 $g.L^{-1}.h^{-1}$, respectively.

Until now, no scientific work has reported the recovery of FOS from fermentative broths using SMB chromatography. With the vast expansion of the FOS market in the last decade, due to the increase of interest by consumers in health foods, the results gathered in this thesis are very promising for the food industry, in particular for the development of new processes for the recovery of sugars produced by fermentative processes.

KEYWORDS: Fructo-oligosaccharides, separation, chromatography, activated charcoal, ion-exchange resins, simulated moving bed.

SUMÁRIO

RECUPERAÇÃO DE FRUCTO-OLIGOSSACARÍDEOS DE PROCESSOS DE FERMENTAÇÃO

A presente tese teve como objectivo principal a recuperação de fructo-oligossacarídeos (FOS) de misturas de açúcares obtidas por fermentação. Entre as técnicas de separação actualmente disponíveis, a cromatografia líquida foi o método escolhido. Duas fases estacionárias foram estudadas, nomeadamente o carvão activado e várias resinas de troca iónica.

A adsorção/dessorção de FOS contidos numa mistura de caldo de fermentação, usando um gradiente de água/etanol, foi estudada numa coluna de carvão activado. Após optimização do método foi possível recuperar 74.5% (p/p) de FOS em relação aos FOS inicialmente contidos no meio de fermentação. Adicionalmente, obtiveram-se fracções com graus de pureza até 97% (p/p). Este método mostrou ser também foi eficiente na dessalinização do caldo fermentativo. O método de separação usando a coluna de carvão activado foi um bom ponto de partida para a obtenção de grandes quantidades de FOS puros de uma forma simples e pouco dispendiosa. No entanto, o principal objectivo desta tese consistia no desenvolvimento de um processo de separação para ser usado numa escala industrial, portanto, economicamente atractivo. Assim, a técnica de cromatografia de leito móvel simulado (SMB) foi seleccionada como sendo o método de separação mais adequado.

A cromatografia de SMB tem a vantagem de trabalhar de um modo contínuo, usando água como eluente e fases estacionárias homogéneas, levando a resultados muito reprodutíveis. Como tal, estudaram-se várias resinas de troca iónica com potencial suficiente para serem utilizadas numa planta piloto de SMB.

A adequação das resinas na separação de mono- e dissacarídeos foi avaliada através dos estudos de equilíbrio de adsorção, cinética e de resistência mecânica. A resina seleccionada foi uma Dowex Monosphere 99K/320, tipo gel, com 320 μm de diámetro das partículas, em forma de potássio. Estabeleceu-se um modelo matemático para

descrever a eluição cromatográfica do caldo fermentativo em coluna e identificaram-se os parâmetros cinéticos e de adsorção. Os FOS foram purificados com sucesso de 37.1 a 62.9% (w/w), usando o SMB. Além disso, o rendimento e a produtividade obtidos para FOS no refinado atingiu 69.4% (w/w) e 82.1 $g.L^{-1}.h^{-1}$, respectivamente.

Até agora, nenhum trabalho científico reportou a recuperação de FOS de caldos fermentativos utilizando o SMB. Com a grande expansão do mercado de FOS na última década, devido ao crescente interesse dos consumidores por alimentos saudáveis, os resultados obtidos nesta tese mostram-se muito promissores para a indústria alimentar, nomeadamente no desenvolvimento de novos processos para a recuperação de açúcares produzidos por processos fermentativos.

PALAVRAS-CHAVE: Fructo-oligossacarídeos, separação, cromatografia, carvão activado, resinas de troca iónica, leito móvel simulado.

RÉSUMÉ

RÉCUPÉRATION DE FRUCTO-OLIGOSACCHARIDES DE PROCESSUS DE FERMENTATION

L'objectif principal de la présente thèse est la récupération des fructo-oligosaccharides (FOS) de mélanges de sucres obtenus par fermentation. Parmi les techniques actuellement disponibles, la méthode de séparation sélectionnée a été la chromatographie liquide. Deux phases stationnaires différentes ont été étudiées, le charbon actif et plusieurs résines échangeuses d'ions.

Une colonne remplie de charbon actif a été utilisée pour étudier l'adsorption/désorption des FOS contenus dans le milieu de fermentation avec un gradient d'eau/éthanol. Après optimisation de la méthode de séparation, une récupération de 74.5% (w/w) de FOS et des fractions avec une pureté jusqu'à 97% (w/w) ont été obtenus. Cette méthode a aussi été efficace pour dessaler le milieu de fermentation. Le procédé utilisant une colonne de charbon actif a été un bon point de départ pour obtenir de grandes quantités de FOS purs d'une manière simple et peu coûteuse. Néanmoins, le but principal de cette thèse est de développer un procédé de séparation utilisable à l'échelle industriel, donc économiquement attractif. C'est pourquoi, la chromatographie sur lit mobile simulé (SMB) a été considérée comme la méthode de séparation la plus adéquate.

La chromatographie SMB a l'avantage de travailler en continu, en utilisant de l'eau comme éluant et des phases stationnaires homogènes, permettant d'obtenir des résultats très reproductibles. Par conséquent, plusieurs résines échangeuses d'ions potentiellement adéquates pour une utilisation sur un équipement de chromatographie SMB ont été étudiées.

L'adéquation des résines pour la séparation des mono- et disaccharides a été évaluée à travers des études de résistance mécanique, de cinétique et d'équilibre d'adsorption. Une résine Dowex Monosphère 99K/320, de type gel avec un diamètre de particules de 320 µm, dans sa forme potassium, a été sélectionnée. L'élution chromatographique sur une seule colonne du milieu de fermentation a été modélisée et les paramètres d'adsorption et

cinétiques ont été identifiés. Les FOS ont été purifiés de 37.1 à 62.9% (w/w) en utilisant l'équipement SMB. De plus le rendement et la productivité obtenus dans le raffinat ont été 69.4% (w/w) et 82.1 $g.L^{-1}.h^{-1}$, respectivement.

Jusqu'à présent, aucun travail scientifique n'a reporté une récupération de FOS des milieux de fermentation en utilisant la chromatographie SMB. Avec la vaste expansion du marché des FOS dans les dernières décennies, et grâce à l'intérêt grandissant des consommateurs pour les aliments sains, les résultats présentés dans cette thèse sont très prometteurs pour l'industrie alimentaire, en particulier pour le développement de nouveaux procédés de récupération des sucres produits par des processus de fermentation.

MOTS-CLÉS : fructo-oligosaccharides, séparation, chromatographie, charbon actif, résines échangeuses d'ions, lit mobile simulé.

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LIST OF SYMBOLS

UPPERCASE LATIN LETTERS

SYMBOL	DESCRIPTION
C	Concentration
C_0	Initial concentration
C_{ads}	Adsorbed sugar concentration
C_E	Concentration of sugar in the extract
C_F	Concentration of sugar in the feed
C_R	Concentration of sugar in the raffinate
C_{Exp}	Concentration obtained in the experiments
C_{Mod}	Concentration calculated by the model
C_{Sim}	Concentration calculated by the simulation
D_{app}	Apparent diffusion coefficient
$Di_{i,j}$	Dilution of component *i* in *j:* extract (E) or raffinate (R)
$D_{L,i}$	Diffusion coefficient
F value	Fisher parameter
H_i	Henry constant of the component *i*
J	Cost function
K	Distribution coefficient
K_L	Langmuir adsorption equilibrium constant
K_F	Freundlich isotherm constant
K_R	Redlich & Peterson isotherm constant
K_T	Toth isotherm constant

L	Column length
M	Langmuir isotherm parameter
M_i	Mass of component *i* inside the column after the equilibrium
N	Number of experimental points
N_i	Number of theoretical plates for the component i
N_L	Efficiency per meter
P value	Statistical parameter
$Pr_{i,j}$	Productivity of component *i* in *j:* extract (E) or raffinate (R)
$Pu_{i,j}$	Purity of component *i* in *j:* extract (E) or raffinate (R)
Q	Flow-rate
Q_s	Flow-rate of the solid phase
Q_j^{SMB}	Flow-rate of the SMB unit
Q_j^{TMB}	Flow-rate of the TMB unit
Q_I, Q_{II}, Q_{III}, Q_{IV}	Flow-rate of the zone I, II, III or IV, respectively
Q_D	Flow-rate of the desorbent
Q_E	Flow-rate of the extract
Q_F	Flow-rate of the feed
Q_R	Flow-rate of the raffinate
R	Coefficient of determination
R_s	Resolution
S_j^{TMB}	Number of subsections in the *j*th section of the SMB unit
V	Solution volume
V_c	Column volume
V_d	Void volume
V_j^{TMB}	Volume of the *j*th section of the TMB unit
V_{Loop}	Loop volume

V_{SMB}	Volume of the fixed bed of the SMB unit
$Y_{i,j}$	Yield/recovery of component *i* in *j:* extract (E) or raffinate (R)

LOWER LATIN LETTERS

SYMBOL	DESCRIPTION
a	Langmuir isotherm parameter
a_R	Redlich & Peterson isotherm parameter
a_T	Toth isotherm parameter
k_1	Pseudo-first order rate constant
k_2	Pseudo-second order rate constant
k_i^{rel}	Relative mass transfer coefficient of component *i*(kinetic model)
$k_{F,i}$	Mass transfer coefficient of component *i*
k_s	Retention factor
m	Dried adsorbent mass
m_i	Dimensionless flow-rate ratios
n	Freundlich isotherm parameter
p	Number of parameters estimated
q	Equilibrium loading
q_i^{eq}	Adsorbed equilibrium concentration
q_{exp}	Adsorbed concentration obtained in the experiments
q_{mod}	Adsorbed concentration calculated by the model
q_1	Amount of sugar adsorbed at equilibrium (pseudo-first order model)
q_2	Amount of sugar adsorbed at equilibrium (pseudo-second order model)
q_t	Amount of sugar adsorbed at time *t*
r	Redlich & Peterson isotherm parameter

t	Toth isotherm parameter
t^*	Switching time
t_0	Column dead time
t_d	Time delay
t_i	Retention time of the sugar *i*
t_p	Injection duration
u	Fluid velocity
$w_{(1/2)}$	Width at half height
z	Axial coordinate

GREEK LETTERS

SYMBOL	DESCRIPTION
α	Separation factor
β	Security factor
ε	Void fraction
ε_p	Porosity of the resin
ε_T	Total bed porosity
$\Phi(t)$	Injection profile
γ	Distribution coefficient
τ	Time constant of the valve

ABBREVIATIONS

ABBREVIATION	DESCRIPTION
ANOVA	Analysis of variance
ARE	Average relative error
COS	Chito-oligosaccharides
CVD	Constant volume of diafiltration
CA	Cellulose acetate
DP	Degree of polymerization
DP_{av}	Average of the degree of polymerization
DVB	Divinylbenzene
EABS	Sum of absolute errors
ED	Equilibrium dispersive model
ERRSQ	Sum square errors
F	Fructose
FD	Finite differences
FOS	Fructo-oligosacharides
FOS-Na^+	FOS contained in the cell-free fermentative broth
FOS-Ca^{2+}	FOS contained in a fermentative broth saturated with calcium ions
G	Glucose
GAs	Genetic algorithms
GF	Sucrose
GF_2	Kestose
GF_3	Nystose
GF_4	Fructofuranosylnystose
GI	Gastrointestinal

GOS	Galacto-oligosaccharides
HDL	High-density lipoprotein
HETP	Height of an equivalent theoretical plate
HPLC	High-Performance Liquid Chromatography
HYBRID	Hybrid fractional error function
IMO	Isomalto-oligosaccharides
LDF	Linear Driving Force
LF	Low frequency
LS	Lactosucrose
LDL	Low-density lipoprotein
MPSD	Marquardt's percent standard deviation
MOS	Malto-oligosaccharides
ODE	Ordinary Differential Equations
PDE	Partial Differential Equations
PI	Prebiotic index
PlzP	Plasma polymerization
PS-DVB	Sulfonated poly(styrene-*co*-divinylbenzene)
RI	Refractive index
RTM	Retention time method
S	Sucrose
SBA	Strong base anion
SCA	Strong acid cation
SCFA	Short-chain fatty acids
SE	Sum of the errors
SL	Slope limiter
SMB	Simulated Moving Bed

SOS	Soybean oligosaccharides
TMB	True Moving Bed
TOS	Transgalactooligosaccharide n
UV	Ultraviolet
VVD	Variable volume of diafiltration
WAC	Weak acid cation
WBA	Weak base anion
XOS	Xylo-oligosaccharides

GENERAL INTRODUCTION

CONTEXT AND MOTIVATION

The daily consumption of fructo-oligosaccharides (FOS) is proven to promote the growth of beneficial bacteria in the human gut improving host's health and well-being (Gibson *et al.*, 2004). Through modulation of the gut microflora it is possible to prevent and treat overall number of gastrointestinal disorders ranging from discomfort or colitis to cancer (Kelly, 2009). With the increased consumers interest in food providing additional health benefices, FOS have gained an important place in the functional food market.

Moreover, besides the nutritional aspects, FOS have also great technological properties resulting in improved taste, mouthfeel, texture and shelf-life of the products and enabling also fibre incorporation in liquid foods. Since FOS are less caloric than common sugars and have great organoleptic properties, they have been used as sweeteners substitutes or in combination with other sweeteners in many food products (Franck, 2002; Crittenden and Playne, 2002).

FOS are present in many edible plants and vegetables, however, they are season-limited and their content is very low and probably not enough to produce the prebiotic effect. Therefore, at large scale, FOS are produced from sucrose by microbial enzymes (Sangeetha *et al.*, 2005). FOS produced by fermentation are obtained together in a complex mixture containing biomass and salts, that must be removed, and other small saccharides namely, fructose, glucose and sucrose. Small saccharides are metabolized by humans. Therefore, in order to achieve a product less caloric and cariogenic, with increased dietary fibre content, allowing its use in health, dietetic and diabetic foods, mono- and disaccharides must be also removed.

Currently, isolated FOS are only available for analytical purposes and even pure FOS commercialized mixtures are still very expensive. Since few studies reported the separation of mono- and disaccharides from FOS contained in fermentative broth mixtures, it is important to investigate and develop new and economically viable separation processes to obtain pure FOS mixtures at large scales. The purification of FOS is a challenge due to the physicochemical similarities between the different oligosaccharides and with smaller saccharides. Until date no scientific work reported the

separation of FOS from fermentative broths at large scales, and therefore further research in this field is needed.

RESEARCH AIMS

The main purpose of this thesis was the recovery of FOS from sugar mixtures obtained by fermentation. Isolated FOS are only available for analytical purposes and even pure FOS commercialized mixtures are still very expensive. The development of new separation processes that enable the large scale recovery of pure FOS fractions would be extremely important for the food industry. Therefore the specific aims of the current thesis were:

- to select an adequate and inexpensive separation process for the recovery of high percentages of FOS from fermentative mixtures;
- to select the best liquid and stationary phase to be used, based on adsorption/desorption equilibrium parameters, namely capacity and selectivity.
- to optimize the recovery of FOS from fermentative sugar mixtures based on the selected and previously defined separation process.

The separation process developed, optimized and characterized in this thesis is intended to be applicable at an industrial scale and holds a great promise for the food industry.

OUTLINE OF THE THESIS

This thesis comprises seven chapters that cover the research aims stated above. The thesis subjects are introduced in this chapter, while Chapter 7 regards to the main conclusions and recommendations drawn from the current work. A brief outline of the other chapters is given below.

IN CHAPTER 1, an overview on FOS, namely kestose (GF_2), nystose (GF_3) and fructo-furanosilnystose (GF_4), its occurrence, physicochemical properties, technological functionality, commercial products, functional properties and production, is given. Also, the most recent and currently available methods for the FOS purification are discussed.

IN CHAPTER 2, a process to purify FOS from a fermentative broth using a single activated charcoal column is proposed. The adsorption kinetics onto activated charcoal of the sugars contained in the fermentative broth are studied and modelled. Adsorption time, flow-rate, volume of treated fermentative broth and volume and ratio of water/ethanol used in the desorption step are optimized.

IN CHAPTER 3, adorption equilibrium of FOS from a purified mixture and a fermentative broth on a Dowex Monosphere Calcium resin is studied. Experimental isotherms data are analysed using linear, Langmuir/anti-Langmuir, Freundlich, Redlich & Peterson, and Toth type models. Estimation of the isotherm parameters, using linear and non linear correlations for the minimization of several error functions, is provided.

IN CHAPTER 4, adorption equilibrium of FOS from a pure mixture and a fermentative broth on three different ion-exchange resins, Lewatit (S2568) (macroporous resin in sodium form), Amberlite (CR1320Ca) (gel-type resin in calcium form) and Diaion (UBK530) (gel-type resin in sodium form), is studied. The effect of the counter-ion, resin structure and the liquid phase containing the FOS mixture in the adsorption behavior is evaluated. Multi-component anti-Langmuir and linear isotherm models to fit

equilibrium data are analyzed and compared. Resins capacity and selectivity are compared.

IN CHAPTER 5, the adorption equilibrium of fructose, glucose and sucrose on a gel-type resin in potassium form, and on a macroporous resin in sodium form, is studied. The influence of the cation, effect of the resin structure and temperature for mono- and multi-component mixtures is evaluated.

IN CHAPTER 6, the separation of FOS from fructose, glucose and sucrose contained in a fermentative broth mixture by Simulated Moving Bed (SMB) chromatography is evaluated. The selection of the stationary phase between two ion-exchange resins, a Dowex Monosphere 99K/320 and Dowex 50WX2, both in the potassium form, is addressed according to the separation performance and stability when operating in the SMB pilot plant. Equilibrium and kinetic studies are conducted with the selected resin. Also, a model of the chromatographic elution on a single column is developed and kinetic and adsorption parameters are identified.

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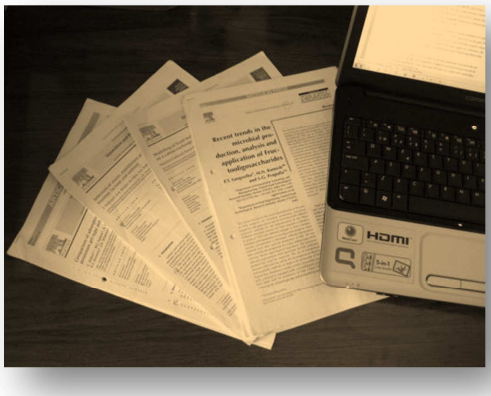
CHAPTER 1



INTRODUCTION

1.1 THE GASTROINTESTINAL TRACT

The gastrointestinal (GI) tract contains a substantial number of diverse bacteria that plays an extremely important role in human health. More than 400 different species of bacteria colonizing the human large bowel have been identified (Crittenden and Playne, 1996; Gibson, 1999). Therefore, the colon is considered the most metabolically active organ in the human body (Gibson, 2004).

Among the colon microflora there are a certain number of pathogenic bacteria, as well as predominant content of beneficial bacteria that contribute to the global well-being of the host by reducing the risk of disease. The modulation of the large bowel microflora by promoting the specific growth of the beneficial bacteria, named probiotic, and keeping a low level of pathogenic species is therefore essential to improve human health through the prevention and treatment of GI disorders ranging from discomfort or colitis to cancer.

Carbohydrates are the main substrate for growing gut bacteria (Gibson, 2004). These sugars are metabolized by the bacteria via fermentation to several end products such as gases (hydrogen, carbon dioxide and methane), organic acids (lactate, succinate, and pyruvate), ethanol and short-chain fatty acids (SCFA) (acetate, propionate and butyrate), which further contribute to generate energy for the host (Gibson, 1998; Gibson, 2004).

1.2 PREBIOTICS

The concept of prebiotic was initially introduced by Gibson and Roberfroid (1995) as “a non digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health". This concept was updated in 2004 (Gibson *et al.*, 2004) and a series of new criteria was established in order to consider any food ingredient as a prebiotic. Therefore, a prebiotic should fulfill the following criteria:

- “resistance to gastric acidity, to hydrolysis by mammalian enzymes, and to gastrointestinal absorption;

- fermentation by intestinal microflora;
- selective stimulation of the growth and/or activity of those intestinal bacteria that contribute to health and well-being".

Most recently, Roberfroid (2007a) suggested a new definition for a prebiotic as "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the GI microflora that confers benefits upon host well-being and health". It is expected that in the near future, a wide variety of food will be characterized as functional food due to its scientifically proven beneficial effects (Roberfroid, 2007b).

Prebiotics, probiotics and synbiotics are considered functional food compounds (Roberfroid, 2007b). Synbiotics are foods that combine both prebiotics and probiotics (Crittenden and Playne, 1996).

A broad range of carbohydrates with potential prebiotic activity has been studied in the recent years, such as fructans (inulin and fructo-oligosaccharides (FOS)); galacto-oligosaccharides/transgalactooligosacchariden (GOS/TOS); gluco-oligosaccharides; isomalto-oligosaccharides (IMO); malto-oligosaccharides (MOS); xylo-oligosaccharides (XOS); soybean oligosaccharides (SOS); lactosucrose (LS) and lactulose; raffinose and stachyose (Manning and Gibson, 2004; Gibson *et al.*, 2004; Torres *et al.*, 2010; Aachary and Prapulla, 2011). These oligosaccharides are classified according to the types of monosaccharide units that compose them and to the degree of polymerization (DP). The monosaccharides include glucose, fructose, galactose and xylose. Additionally, other carbohydrates have also been investigated based on their prebiotic potential. However, evidences of their activity are still scarce. Examples of such carbohydrates include germinated barley foodstuffs; gentio-oligosaccharides; mannan oligosaccharides; chito-oligosaccharides (COS); oligosaccharides from melibiose; pectic oligosaccharides; oligodextrans; palatinose; polydextrose; gluconic acid; glutamine and hemicelluloses-rich subtracts; lactose; resistant starch and its derivatives; and sugar alcohols (Manning and Gibson, 2004; Gibson *et al.*, 2004).

Among all the carbohydrates reported only fructans and GOS/TOS fulfill all the above mentioned criteria required to be considered prebiotics (Roberfroid, 2007a):

- Fructans cannot be hydrolysed nor adsorbed by either pancreatic or by brush border digestive enzymes in the upper intestinal tract of humans (Pool-Zobel et al., 2002).
- Fructans are specifically fermented by probiotic bacteria, as *Bifidobacteria*, promoting the growth and activity of this particular population and inhibiting the growth of pathogenic bacteria (Gibson, 2004; Ferreira et al., 2008). The effects on the growth of other colon microorganisms are less consistent (Kelly, 2008).
- FOS are able to change the microflora composition of the colon inducing beneficial effects in the host (Kolida and Gibson, 2007).

1.3 FRUCTANS

Fructans are a category of carbohydrates that include inulin and oligofructose. They consist in fructose molecules linked to each other that can or cannot have glucose residue in their initial configuration. Therefore, fructans are mixtures of both GF_n and FF_n molecules, with n > 2; where G and F stand for glucose and fructose, respectively, and *n* represents the DP.

Inulin is naturally found in plants and industrially is extracted mainly from the chicory root. The inulin DP varies between 2 and 60 units with an average (DP_{av}) of 12 units. Oligofructose are short chain molecules, including FOS from hydrolysed inulin, that can be a mixture of GF_n and FF_n molecules ($2 < DP < 7$, $DP_{av} = 4$), and FOS enzymatically synthesised from sucrose, that are all GF_n type ($2 < DP < 4$, $DP_{av} = 3.6$) (Roberfroid, 2007b).

Independently of the type of fructans in a given mixture, it is important to take note that all have the same nutritional effects. However, the technological properties can vary accordingly to their DP (Roberfroid and Delzenne, 1998).

1.3.1 OCCURRENCE

FOS are naturally present in more than 36000 plants and vegetables (Havenaar *et al.*, 1999). Most of the species belong to the *Compositae* or *Liliales* families (Peters, 2007). In industrial processes, chicory is the most commonly used plant from which inulin is extracted. Chicory contains about 15 to 18% of inulin (Peters, 2007). Other examples of plants, fruits and vegetables that contain FOS include: onions, honey, wheat, bananas, rye, garlic, leeks, Jerusalem artichoke, dahlia and blue agave (Manning and Gibson, 2004; Peters, 2007). In 2007 the World production of inulin was estimated to be about 350 000 tons (Peters, 2007).

However, the FOS content in these foods is very low and probably not enough to produce a prebiotic effect in the host. Furthermore, these FOS-containing foods are season-limited. Therefore, for a large scale FOS productions, microbial enzymes with transfructosylation activities are used (Roberfroid, 2000).

1.3.2 PHYSICOCHEMICAL PROPERTIES

FOS obtained by transfructosylation consist mainly in β(1←2)-D- fructosyl–fructose linkages starting with an α-D-glucose. As previously mentioned, FOS are GF_n compounds namely α-D-glucopyranosyl-(β-D-fructofuransoyl)$_{n-1}$-β-fructofuranoside characterized by a DP between 2 and 4 (Gibson *et al.*, 2004).

Humans are only able to hydrolyze alpha glycosidic linkages. Therefore, due to the beta configuration of the anomeric C_2 in the fructose monomers of fructans, that form the β-(2-1) glycosidic linkages of the molecules, fructans cannot be hydrolysed by humans (Roberfroid, 2002). Consequently, FOS are non-digestible oligosaccharides.

FOS compounds are named kestose (GF_2), nystose (GF_3) and fructofuranosylnystose (GF_4). The chemical structures of the FOS compounds are presented in Fig. 1.1.

FOS are very hygroscopic and their viscosity is relatively higher than that of sucrose at the same concentration level. The thermal stability of FOS is also superior to sucrose.

They are extremely stable in the normal pH range found in food (4.0 – 7.0) and at refrigerated temperatures over the course of a year. The solubility, freezing and boiling points, and crystal data of FOS seem to be very similar to sucrose (Yun, 1996).

Some physicochemical properties of the sugars involved in FOS production are summarized in Table 1.1.

Table 1.1 Physicochemical properties of sugars involved in FOS production.

FOS	Molecular formula	Molecular weight	Melting point (ºC)	Solubility*		
				Water	Alcohol	Ether
F	$C_6H_{12}O_6$	180	95-105	Very soluble	8 (at 18ºC)	
G	$C_6H_{12}O_6$	180	146 (α-D-Glucose) 150 (β-D-Glucose)	82 (at 17.5ºC)	Slightly soluble	Insoluble
GF	$C_{12}H_{22}O_{11}$	342	170-186	179 (at 0ºC)	0.9	Insoluble
GF_2	$C_{18}H_{32}O_{16}$	504	199-200			
GF_3	$C_{24}H_{42}O_{21}$	666				
GF_4	$C_{30}H_{52}O_{26}$	828				

Data from (Liley *et al.*, 1997) and (Crittenden and Playne, 1996).
(*Solubility is given in parts by weight of the sugars per 100 parts by weight of the solvent).

The caloric content of a digested carbohydrate, such as glucose and fructose, is 3.75 $kcal.g^{-1}$ (Cummings *et al.*, 1997). On the other hand, the caloric content of the fermented byproducts (SCFA, lactate and gases) derived from FOS vary between 1.0 and 2.0 $kcal.g^{-1}$ depending on the chain length (Roberfroid *et al.*, 1993; Cummings *et al.*, 1997). Therefore, FOS have only 25 to 50% of the caloric value of a digested sugar molecule (Roberfroid *et al.*, 1993; Gibson and Roberfroid, 1995). The relative sweetness of FOS, estimated on the basis of 10% (w/v) sucrose solution, corresponding to 100%, is 31, 22, and 16%, for GF_2, GF_3 and GF_4, respectively (Yun, 1996).

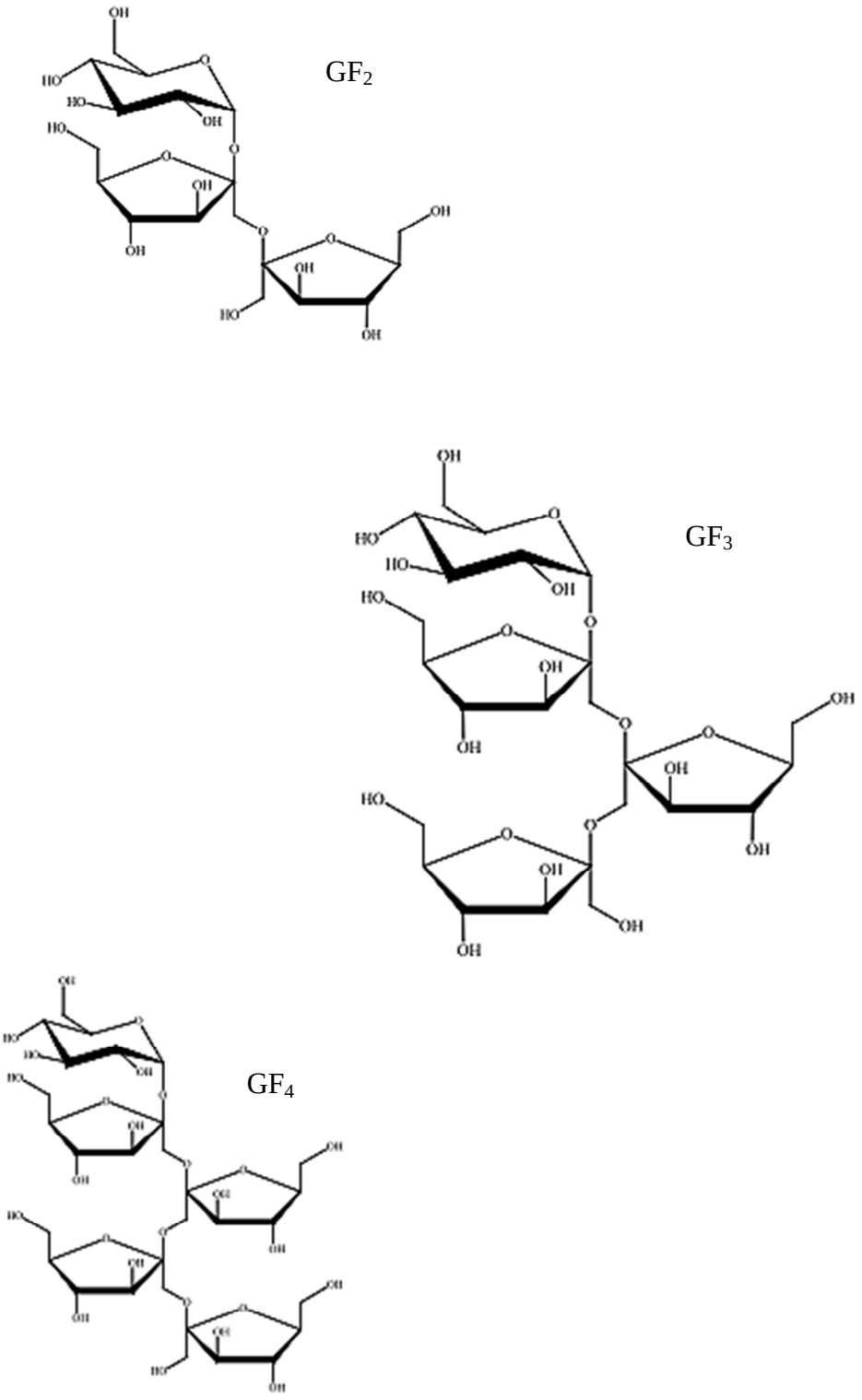


Fig. 1.1 Chemical structures of fructo-oligosaccharides: kestose (GF_2), nystose (GF_3) and fructofuranosylnystose (GF_4).

Monosaccharides are the elementary units of all carbohydrates. Typically their molecules contain a linear saturated chain of five or six carbon atoms, each of them carrying a hydroxyl substituent. One of these is oxidized to a group of aldehyde or ketone. Fructose and glucose are hexoses containing the same molecular formula but each as a different structure. Fructose is a ketose while glucose is an aldose.

As a liquid solution glucose and fructose can be present in at least five different tautomeric forms: α- and β-pyranose, α- and β-furanose and open chain (Fig. 1.2 and Fig. 1.3). However in glucose the pyranose form is dominant, while in fructose the dominant form is the furanose form. Fructose has a higher solubility than other sugars and therefore it is difficult to crystallize from an aqueous solution.

Sucrose is a disaccharide and results from the combination of a unit of α-D-glucose and a unit of β-D-fructose, linked by a glycosidic bond between C1 on the glucosyl subunit and C2 on the fructosyl unit (Fig. 1.4). Sucrose is also named β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside. Since sucrose contains no anomeric OH groups it is classified as a non-reducing sugar compound.

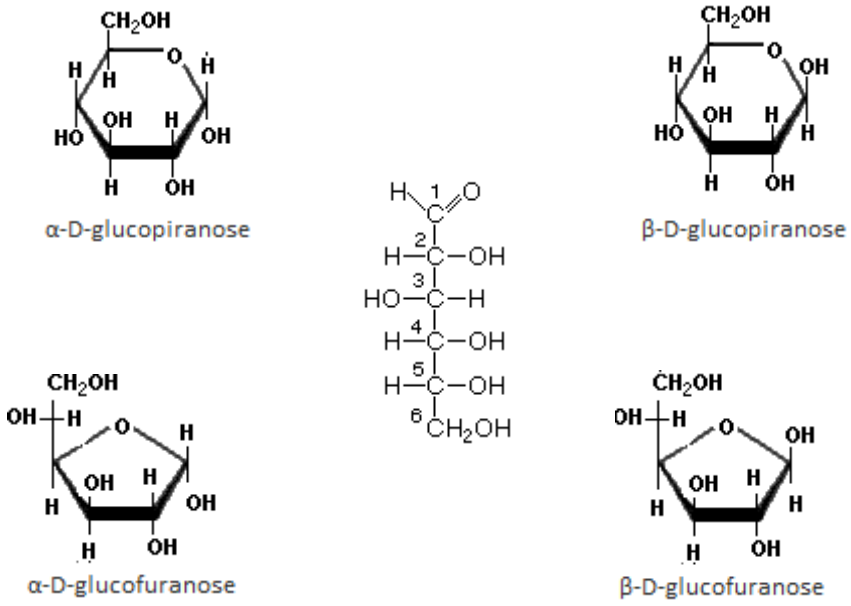


Fig. 1.2 Haworth's structure of glucose.

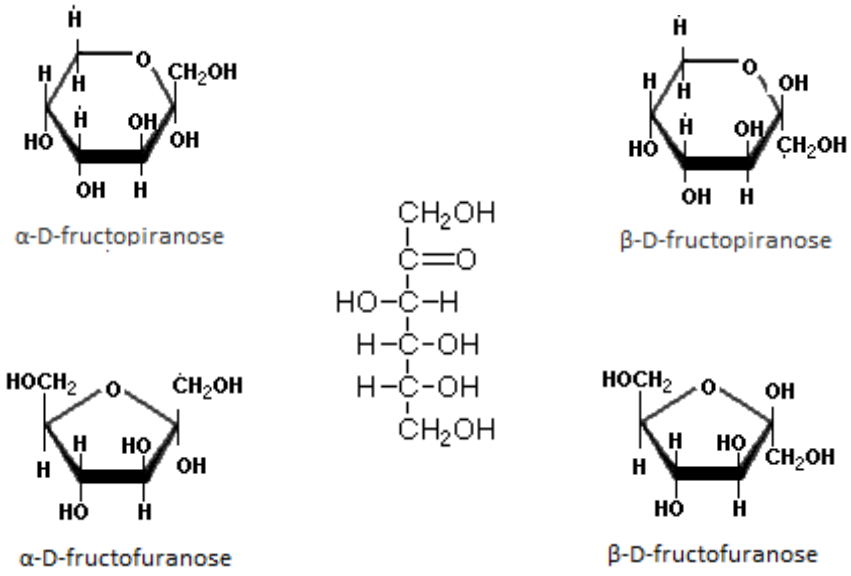


Fig. 1.3 Haworth's structure of fructose.

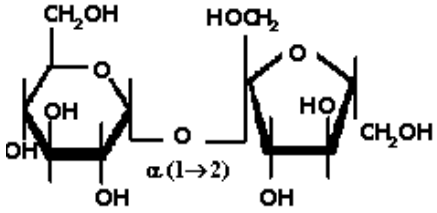


Fig. 1.4 Haworth's structure of sucrose.

The OH sugar groups are polar and form hydrogen bonds with water. The steric orientation of the OH group determines the ability of sugars to form hydrogen bounds and complexes with other substances. The OH groups are axial-equatorial (eq-ax) oriented in *cis*-diol groups. The stability of the complex formed by the sugars depends on the eq-ax sequences. The number of pairs of the eq-ax oriented OH groups and its relative composition in D-fructose and D-glucose are presented in Table 1.2. Sucrose has no pairs of eq-ax oriented OH groups.

Table 1.2 Equilibrium composition of D-fructose and D-glucose in water (mole percentage) (Angyal, 1991).

Sugar	T (°C)	Pyranose				Furanose			
		α	#Pairs	β	#Pairs	α	#Pairs	β	#Pairs
D-Fructose	30	2	1	70	2	5	0	23	1
D-Glucose	27	38.8	1	60.9	0	0.14	1	0.15	0

Sugars are highly polar due to the presence of many OH groups and non soluble in organic solvents. However, when certain conformations are adopted, oligosaccharides can generate apolar surfaces which are capable of interacting with non polar solutions and adsorbents. The hydrophobicity of sugars is determined by several factors such as CH-surface area, hydration effect of the OH groups and molecular planarity and rigidity (Yano *et al.*, 1988). As the hydrophobicity is related with the extent of the CH and -CH_2 moieties, it increases with the extension of the chain length, provided that the configuration and the glycosidic link stereochemistry are favorable (Sundari and Balasubramanian, 1997). Therefore, the hydrophobicity of di- and higher saccharides increase with the number of monosaccharide units that constitute the oligosaccharide (Yano *et al.*, 1988).

1.3.3 TECHNOLOGICAL FUNCTIONALITY

FOS are the most studied oligosaccharides and the best implemented in the European market. These compounds are used as food ingredients not only for their nutritional

value, but also for their great technological properties, such as the improvement of the organoleptic quality of the products (Franck, 2002). Generally FOS are added to food in a dose that varies between 2 and 50% (Franck, 2002). A detailed description of the technological functionality of FOS has been previously described by several authors (Crittenden and Playne, 1996; Franck, 2002).

FOS improve the taste, mouthfeel and texture (crispness and expansion) of the products. Since they provide a high moisture-retaining capacity, preventing excessive drying, and reducing the water activity content, the microbial development decreases, and thus an increase of the products shelf life is achievable (Crittenden and Playne, 1996).

In the food industry, FOS have been used as low-calorie substitutes for sugar in dietetic and diabetic food (Cummings *et al.*, 1997). However, as the pure FOS sweetening power is lower than sucrose they have only partially been used as a sugar substitute. Typically, FOS are combined with other sugars or sweeteners with higher sweetener intensity, without a significant increase to the final product's caloric content (Coussement, 1999).

Furthermore, FOS can be used to modify the freezing temperature of frozen foods, and to control the amount of browning due to Maillard reactions in heat-processed foods (Crittenden and Playne, 1996). Since FOS are very soluble, they enable fibre incorporation within liquid foods.

The DP of a given fructan determines its water solubility, viscosity, water retention capacity as well as the capacity to form a cream-like texture (Roberfroid and Delzenne, 1998). For example, due to its long chain, only inulin (fructan) can be used as a fat replacer. Typically, 0.25 g of inulin replaces 1g of fat (Coussement, 1999). On the other hand, FOS are much more soluble than inulin (Niness, 1999). Consequently, FOS are mainly used as sugar substitutes, due to the similar technical properties compared to glucose, fructose and sucrose.

FOS have been commonly used as ingredients in functional food such as biscuits; baking products; fillings, drinks; yoghurts; dairy products; breakfast cereals; tablets; frozen desserts; infant formulae; fruit preparations; dietetic products; meal replacers; and sweeteners (Crittenden and Playne, 1996; Franck, 2002; Manning and Gibson, 2004).

1.3.4 COMMERCIAL OLIGOSACCHARIDES

Nowadays, consumers have the tendency to seek food that can provide some additional heath benefits. Therefore, food products are increasingly supplemented with prebiotics, resulting in a large commercial interest in FOS.

In Japan, synthesized FOS using enzymes have been in the market since 1984 (Crittenden and Playne, 1996). The first FOS industrial producer was the Meiji Seika Kaisha, Ltd. with the product named "Meioligo" (Oku *et al.*, 1984; Crittenden and Playne, 1996). FOS produced by Meiji were named "Neosugar" (Oku *et al.*, 1984). Meioligo products are commercialized as several product types with different purity level. Currently in Japan, FOS can be found in more than 500 food products (Macfarlane *et al.*, 2008). In 2007, the retail FOS market was estimated to be about US$200/kg (Macfarlane *et al.*, 2008).

Other companies also commercializing FOS synthesized using enzymes are GT Nutrition in the United States with NutraFlora®; Cheil Foods and Chemicals in Korea with Oligo-Sugar; and Victory in China with Beneshine®. FOS are commercialized with a purity level above 95% (*data obtained from the suppliers*).

In Europe, FOS synthesized using enzymes are only commercialized by Beghin-Meiji Industries. These FOS are produced from sucrose by the company TEREOS sugar group in France. Beghin-Meiji commercializes FOS for two industry segments: the Actilight® for food industry, and the Profeed® for animal feed industry. Actilight® is available in powder and liquid form, with a fibre content ranging from 55% to 95%.

Inulin and oligofructose extracted from the chicory root are produced in Europe by Beneo-Orafti, Cosucra and Sensus. Cosucra commercializes it by the name of Fibruline® and Fibrulose®; Beneo-Orafti as Orafti® ingredients; and Sensus as Frutafit® inulin and Frutalose® oligofructose. By varying the contents in dietary fibre and sugar, these companies can offer more than one type of product.

In Mexico, Nutriagaves de Mexico S.A. de C.V. (Namex) produces and commercializes inulin and oligofructose extracted from the desert succulent blue agave hearts of the

Tequiliana Weber variety under the brand name OLIFRUCTINE. A summary of the fructans currently produced and commercially available is presented in Table 1.3.

Table 1.3 Fructans currently produced and available in the market*.

Company	Country	Product name	Type of Fructan
Meiji Seika Kaisha	Tokyo, Japan	Meioligo	FOS
GTC Nutrition	Golden, Colorado, US	NutraFlora®	FOS
Cheil Foods and Chemicals	Seoul, Korea	Oligo-Sugar	FOS
Victory Biology Engineering	Shangai, China	Beneshine™ P-type	FOS
Beghin-Meiji Industries	Paris, France	Actilight® Profeed®	FOS
BENEO-Orafti	Brussels, Belgium	Orafti®	Inulin and oligofructose
Cosucra	Warcoing, Belgium	Fibruline® Fibrulose®	Inulin and oligofructose
Sensus	Roosendaal, Netherlands	Frutafit® inulin Frutalose® oligo-fructose	Inulin and oligofructose
Nutriagaves de Mexico S.A. de C.V.	Ayotlan, Jalisco, Mexico	OLIFRUCTINE-SP®	Inulin and oligofructose

*data obtained from a survey of the major worldwide FOS manufacturers

Individual FOS molecules obtained from the purification of commercial FOS mixtures are only available for analytical purposes. A compilation of the principal companies supplying GF_2, GF_3 and GF_4 and their respective prices is summarized in Table 1.4.

Table 1.4 Prices and purities of individual FOS molecules available for analytical purposes.

Company	FOS	Purity	Quantity (mg)	Price (€)
Sigma Aldrich	GF_2	$\geq$ 98%	25	27.7
			100	76.8
	GF_3	$\geq$ 98%	25	35.4
			100	98.7
Megazyme	GF_2	≈ 95%	100	141.0
	GF_3	≈ 95%	100	141.0
	GF_4	≈ 95%	40	141.0
Wako Chemicals GmbH	GF_2	99%	500	1 Set containing GF_2, GF_3 and GF_4: 307.0
	GF_3	99%	500	
	GF_4	80%	500	

*data obtained from the suppliers at April of 2011

1.3.5 SAFETY

As mentioned previously, fructans naturally occur in many edible fruits and vegetables; consequently they are regularly ingested by humans as a part of a normal diet. Hence, these sugars do not pose any safety concerns to consumers. Furthermore, many *in vivo* and *in vitro* studies have proved along the years the prebiotic characteristics of FOS (Gibson *et al.*, 1995; Gibson *et al.*, 2004; Yen *et al.*, 2011).

In most European countries natural occurring FOS are recognized as food ingredients and in United States, Australia, Canada and Japan they have a recognized GRAS (generally regarded as safe) status (Roberfroid and Delzenne, 1998; Franck, 2002).

Synthetic FOS have been designated as a “novel food” (EU Regulation on Novel Foods and Novel Food Ingredients 258/97) by the ad hoc authorities of the European Commission (Roberfroid and Delzenne, 1998). Although safe, when consumed in high levels, FOS can cause some digestive problems due to the osmotic effect. This increases the water content in the bowel, leading to a high level of flatulence and bloating causing abdominal discomfort (Coussement, 1999). The recommended daily intake of FOS seems to vary. The tolerance towards these carbohydrates differs from person to person. It is associated with the type of oligosaccharide that is being consumed, the type of food

in which it is incorporated as well as the number of times that it is consumed per day (Coussement, 1999; Bruhwyler *et al.*, 2009).

The long-chain of oligosaccharides present a slower digestion compared to the short-chain ones, hence they appear to be more tolerated than the smaller oligosaccharides (Roberfroid *et al.*, 1998). In addition to this, fructans incorporated in liquid foods are always more likely to not be tolerated than in solid foods (Roberfroid and Delzenne, 1998).

Many studies have been conducted to set the adequate dose of the daily intake of fructans. The values that have been suggested vary from 3 to 40 g/day (Yamashita *et al.*, 1984; Gibson, 1999; Pereira and Gibson, 2002; Bruhwyler *et al.*, 2009; Gibson and Shepherd, 2010).

According to Roberfroid and Delzenne (1998), a single 10 g daily dose of FOS in a liquid product is well tolerated, while a single daily dose of 20 g may cause some discomfort in most people. Nevertheless, very resistant consumers may tolerate up to 30 g per day in a single dose. Besides, if the dose is split along the day and consumed with meals, daily doses of 20–30 g are well tolerated (Roberfroid and Delzenne, 1998).

A recent study with three types of inulin-fructans, with different chain lengths, suggested that the intake of 20 g/day distributed in three doses was well tolerated by healthy young people regardless of the fructan consumed (Bruhwyler *et al.*, 2009).

1.3.6 DOSE AND THE PREBIOTIC ACTIVITY

The average daily intake of natural FOS has been estimated to be between 1 and 4 g in the United States (Moshfegh *et al.*, 1999), and between 3 to 11 g in Europe (Quemener *et al.*, 1994).

The effect of a prebiotic depends on the number of *Bifidobacteria* that is colonizing the colon before initiating the prebiotic supplement in the diet (Roberfroid, 2007a). Therefore, a recommended daily intake of a prebiotic can be senseless. Instead, a

prebiotic index (PI) should be used. The PI is defined as "the increase in the absolute number of *Bifidobacteria* expressed divided by the daily dose of prebiotic ingested" (Roberfroid, 2007a).

Moreover, the effective dose to induce a bifidogenic effect also seems to vary depending on the type of oligosaccharide ingested (Crittenden and Playne, 1996). The minimum daily dose that has been suggested to produce a bifidogenic effect in adults varies between 2.5 to 5 g (Gibson *et al.*, 1995; Bouhnik *et al.*, 2006). It was also reported that an intake up to 10 g per day does not translate into any significant improvement of the therapeutic effect (Kelly, 2008).

1.3.7 HEALTH CLAIMS

Several health claims have been made on behalf of the prebiotic activity of fructans. Present claims concerning the risk of diseases diminishing are very promising. Many studies have been done *in vitro* with successful results; however more clinical trials are needed to prove the benefits in humans. Several reviews have been written about the health benefits of fructans (Roberfroid and Delzenne, 1998; Roberfroid, 2007b; Kelly, 2009; Qiang *et al.*, 2009; Delgado *et al.*, 2010; De Preter *et al.*, 2011).

Bifidobacteria constitutes up to 25% of the total population in the gut of an adult and 95% in newborns (Gibson and Roberfroid, 1995). Fructans are selectively fermented in the colon by the *Bifidobacteria* sp. promoting the growth of this specific population. Most of the health benefits associated with the daily intake of fructans are linked to the end products formed during their fermentation. *Bifidobacteria* produce strong acids as SCFA, acetate and lactate that lower the pH of the colon producing an antibacterial effect (Gibson, 1999). The selective fermentation of FOS by *Bifidobacteria* with consequent inhibition of pathogenic growth has been extensively confirmed in many human studies (Gibson and Roberfroid, 1995).

Conducted Clinical investigations concerning the health claims related with fructans intake may be divided in four main categories:

- Blood sugar regulation;
- Blood lipids;
- Mineral absorption and biomarkers of bone health;
- GI health.

The effect of fructans has been reported to be beneficial for glucose levels in the bloodstream both in animal models and humans (Yamashita *et al.*, 1984; Causey *et al.*, 2000). However, the recent reviews suggest that fructans do not appear to lower significantly the serum glucose in normo- glycemic humans (Kelly, 2009).

For individuals possessing high levels of lipids, it has been suggested that inulin reduces triglyceride, serum/plasma total cholesterol, low-density lipoprotein **(**LDL)-cholesterol and increases high-density lipoprotein **(**HDL)-cholesterol (Yamashita *et al.*, 1984; Davidson *et al.*, 1998; Causey *et al.*, 2000; Williams and Jackson, 2002; Pereira and Gibson, 2002; Russo *et al.*, 2008; Ooi and Liong, 2010; Bonsu *et al.*, 2011).

The low pH caused by the SCFA production is responsible for the increase of the minerals solubility. As a result, calcium absorption, as well as bone calcium accretion and bone mineral density, also appears to increase due to the daily intake of fructans, which consequently reduces the risk of osteoporosis (Scholz-Ahrens and Schrezenmeir, 2002; Coxam, 2005; Franck, 2005; Abrams *et al.*, 2005). In a recent study conducted with adolescent girls, the daily consumption of fructans did not benefit the calcium absorption or retention. Nevertheless, the authors concluded that this result can be related to their highly efficient calcium absorption (Martin *et al.*, 2010).

Concerning other minerals, several studies suggest that there is no impact on the absorption of iron and zinc due to the intake of fructans, but it might induce a positive effect on copper and magnesium absorption in some populations (Scholz-Ahrens and Schrezenmeir, 2002; Azorin-Ortuno *et al.*, 2009).

The daily intake of fructans has been shown to decrease obesity by promoting satiety and reducing food intake, consequently lowering the body weight gain (Cani *et al.*, 2006; Abrams *et al.*, 2007; DiBaise *et al.*, 2008).

The increased production of SCFA and butyrate in the colon by the *Bifidobacteria* has also been linked to the potential anti-carcinogenic properties of fructans (Pool-Zobel *et al.*, 2002; Taper and Roberfroid, 2002; Pool-Zobel, 2005; Fotiadis *et al.*, 2008; Munjal *et al.*, 2009). Munjal and co-workers (2009) state that "these fermentation products are shown to be protective in different stages of cancer onset since they regulate the colonic epithelial turnover and inducing apoptosis in colon adenoma and cancer cell lines".

Potential health benefits of the daily intake of fructans may also be related with the decrease of the risk of intestinal infectious diseases (Gibson, 1998; Gibson *et al.*, 2005; Guarner, 2007), constipation (Kleessen *et al.*, 1997; Sabater-Molina *et al.*, 2009), diarrhoea (Cummings *et al.*, 2001; Duggan *et al.*, 2003) and inflammatory bowel disease (Welters *et al.*, 2002; Lindsay *et al.*, 2006; Casellas *et al.*, 2007).

Furthermore, *Bifidobacteria* is also thought to stimulate the immune system, produce vitamin B, reduce blood ammonia and help the renewal of the normal gut microbiota after antibiotic therapy (Gibson *et al.*, 1995; Crittenden and Playne, 1996; Jenkins *et al.*, 1999; Bouhnik *et al.*, 2006).

1.4 PRODUCTION

Guio *et al.* (2009) have recently reviewed the patents related with FOS production and applications. The different production processes of FOS have been extensively reviewed by several authors (Yun, 1996; Prapulla *et al.*, 2000; Sangeetha *et al.*, 2005). Additionally, the microbial production of fructosyltransferase was reviewed by Maiorano and collaborators (2008).

FOS can be produced by chemical glycosylation and *de novo* synthesis using glycosidase and glycosyltransferase activities (Barreteau *et al.*, 2006). The chemical FOS synthesis using this method is a laborious multi-step endeavour, uses hazardous/expensive chemicals and provides low yields (Palcic, 1999; Prapulla *et al.*, 2000). Therefore, its implementation on an industrial scale does not seem to be economically feasible (Barreteau *et al.*, 2006).

Industrially, FOS are produced by transfructosylation of sucrose. Fructosyl Transferases (FTase) are the enzymes responsible for the FOS production from a consecutive set of disproportionation reactions (Jung *et al.*, 1989; Kim *et al.*, 1998):

$$GF_n + GF_n \rightarrow GF_{n-1} + GF_{n+1} \text{ (with n = 1, 2, 3)} \quad (1.1)$$

and in part from,

$$GF_n + GF \rightarrow GF_{n+1} + G \quad (1.2)$$

By transferring between one to three molecules of fructose to the fructose residue in sucrose, FTase produces firstly GF_2, then GF_3 and finally GF_4. The FOS production mechanism is schematically represented in Fig. 1.2.

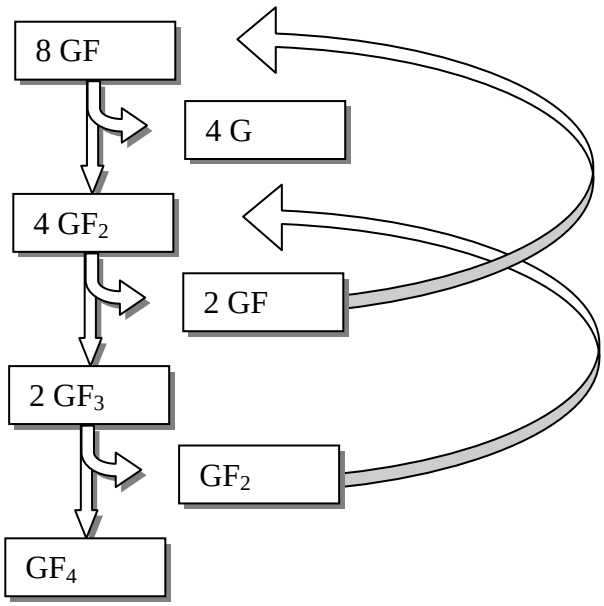


Fig. 1.5 Schematic representation of the reaction mechanism for FOS production with a fructosyl transferase derived from *Aureobasidium pullulans* (adapted from (Jung *et al.*, 1989)).

Many microorganisms have been reported to produce FTase. Mostly are fungi strains, such as *Aspergillus* sp., *Aureobasidium* sp., *Fusarium* sp., *Penicillium* sp., among others (Yun, 1996; Chen and Liu, 1996; Sangeetha *et al.*, 2004; Park *et al.*, 2005; Prata *et al.*, 2010). Additionally, there are some bacteria that have also been reported as FTase producers, such as *Arthrobacter* sp., *Zymomonas mobilis* and *Bacilus macerans* and yeasts such as *Kluyveromyces*, *Candida* and *Saccharomyces cerevisiae* (Yun, 1996; Sangeetha *et al.*, 2005; Caicedo *et al.*, 2009).

The enzymes used for the industrial FOS synthesis are produced by the fungi *A. pullulans* and *Aspergillus niger* (Maiorano *et al.*, 2008).

There are two main reasons for which FOS are synthesised by microorganisms (Yun, 1996):

- it allows the elimination of free fructose that has an unfavorable effect in the microorganism growth when present in high concentrations;
- it is a way to store glucose in an oligosaccharide form, that can be used when there is no more free glucose available.

FOS can be produced using the whole cells of a given microorganism, suspended or immobilized (Sangeetha *et al.*, 2004; Caicedo *et al.*, 2009; Mussatto *et al.*, 2009), or, more frequently, using the extracted enzymes in free or immobilized conditions (Sangeetha *et al.*, 2005).The enzymes involved in FOS production can be either intra or extra cellular.

The maximum theoretical yield obtained for FOS, produced by microbial FTases, is around 55-60% based on the initial sucrose concentration (Yun and Song, 1993; Sangeetha *et al.*, 2005). This yield cannot be increased due to the high amounts of glucose co-produced during the fermentation which inhibits the transfructosylating reactions (Yun and Song, 1993). Therefore, at the end of the fermentation, FOS are obtained with high levels of other contaminant sugars, mostly glucose, but also fructose and non consumed sucrose.

1.5 PURIFICATION

Most of the commercialized FOS products are not pure due to the low yields of the industrial production process. Additionally, FOS products also have small chain saccharides in their composition. Hence companies supply several types of products with different FOS contents. Typically commercial products contain about 5% of other sugars, although products with 55 to 99% of purity can be found in the market.

The presence of other sugars in the mixture decreases the prebiotic, caloric and cariogenic values of the final product, preventing the inclusion of these mixtures in health, dietetic and diabetic foods. Moreover, pure FOS are essential when pharmaceutical applications are envisaged. In addition, pure FOS are also needed to evaluate their individual functional properties (e.g. *in vitro* prebiotic activity) and to determine their physicochemical properties. As previously mentioned, apart from their nutritional value, FOS also have great physicochemical properties that allow improvement of the organoleptic quality of food (Franck, 2002; Crittenden and Playne, 2002).

Currently, the isolated FOS are only available for analytical purposes and their prices are prohibited. Indeed, even pure FOS mixtures are still very expensive. Besides, relatively few reports are available regarding the FOS purification. Therefore, the isolation and purification of FOS is nowadays a challenging and important task for the scientific community.

Several downstream techniques that are currently available could be used for the isolation and purification of FOS from an industrial fermentative process. Some of these techniques will be discussed further below.

1.5.1 INCREASE OF THE FERMENTATION YIELDS

The presence of glucose in the final fermentative broth of a FOS production process inhibits the transfructosylating activity of the FOS-producing enzymes (Yun and Song, 1993). Therefore, in order to achieve higher fermentation yields, many authors have

studied the impact of the continuous removal of glucose and residual sucrose from the fermentative broth during the FOS conversion. Consequently, by increasing the fermentation yields a purer final product could be obtained. The use of enzymes and membrane reactors has been proposed for the glucose and sucrose removal. Purification of FOS from commercial mixtures could also be achieved using the same approach.

Several authors used mixed enzyme systems with β-fructofuranosidase and glucose oxidase to increase the FOS production yields in fermentative processes (Yun and Song, 1993; Yun *et al.*, 1994; Sheu *et al.*, 2002). The fermentation can be conducted with enzymes or microorganisms containing β-fructofuranosidase and/or glucose oxidase enzymes. Lin and Lee (2008) used calcium alginate-immobilized mycelia of *Aspergillus japonicus* (β-d-fructofuranosidase producer) and *A. niger* (glucose oxidase producer). The enzyme β-fructofuranosidase has the ability to synthesize FOS from sucrose, while glucose oxydase enzymes are able to convert glucose into gluconic acid that can be afterwards removed from the broth by adsorption onto ion-exchange resins or precipitation with calcium carbonate solutions (Sheu *et al.*, 2002). Therefore, the combination of the two enzymes has been successfully used and contents in FOS up to 98% have been achieved (Yun *et al.*, 1994). Nevertheless, Yun *et al.* (1994) found a significant difference with respect to the composition in the FOS produced by the combined enzymes as compared to that produced by Ftase/furanosidases. They found that a higher content of GF_3 was accumulated and only a trace amount of GF_4 was detected when using the combination of the two enzymes.

Reactor systems using nanofiltration membranes, through which glucose permeates but not sucrose and FOS, can also be used to remove glucose continuously during FOS production. Studies showed an increase of FOS production up to 90% (Nishizawa *et al.*, 2000; Nishizawa *et al.*, 2001).

Another way to increase the fermentation yields is by preventing the occurrence of oligosaccharides hydrolysis during the enzymatic synthesis. Based on that, an activated charcoal column connected to the fermenter has been used to remove oligosaccharides from the fermentative broth during the enzymatic synthesis (Ajisaka *et al.*, 1987; Nunoura *et al.*, 1997; Boon *et al.*, 2000). Depending on their molecular weight, sugars

can be adsorbed differently onto activated charcoal. Sugars with high molecular weight are more adsorbed than small saccharides.

1.5.2 MICROBIAL TREATMENT

S. cerevisiae and *Z. mobilis* are able to ferment some common mono- and disaccharides but do not possess carbohydrases able to hydrolyze most of the oligosaccharides. Therefore, the use of these microorganisms to eliminate small saccharides from broth mixtures with oligosaccharides has been studied by several authors (Crittenden and Playne, 2002). The two microorganisms mentioned above convert glucose, fructose and sucrose to ethanol and carbon dioxide. During sucrose fermentation by *Z. mobilis* a minimal amount of by-products, such as sorbitol and FOS can also be formed. In a study conducted with a commercial FOS mixture (Meioligo G), containing 57% of FOS in total sugars, no degradation of FOS was observed and glucose, fructose and sucrose were completely fermented by *Z. mobilis* without the formation of by-products (Crittenden and Playne, 2002). Studies conducted with *S. cerevisiae* showed that the yeast is able to completely remove fructose, glucose and sucrose from a mixture of sugars. Some authors achieved also a complete removal of galactose during the fermentation (Yoon *et al.*, 2003; Hernandez *et al.*, 2009). However, for high concentrated mixtures, the ethanol produced during the fermentation may inhibit its assimilation, thus only a slight reduction of galactose is observed (Goulas *et al.*, 2007). On the other hand, *S. cerevisiae* do not ferment disaccharides with D-galactose at the non reducing-end and/or those with β-glycosidic linkages. Furthermore, oligosaccharides with four or more monosaccharide units were not fermented by the yeast (Yoon *et al.*, 2003; Goulas *et al.*, 2007; Hernandez *et al.*, 2009).

Microbial treatment seems to be a good alternative to increase the percentage of FOS in a mixture by removing mono- and disaccharides. Moreover, the process can even be used during the enzymatic synthesis of FOS. However, the use of microbial treatments implies a further step of purification for removal of biomass and the metabolic products formed during the fermentation, in order to obtain a FOS product with few contaminants. Also,

the use of yeast treatment was found to modify the oligosaccharides composition of honey (Sanz *et al.*, 2005).

1.5.3 ULTRA AND NANOFILTRATION

Membrane technology, mainly ultrafiltration and nanofiltration, has been used by several authors to fractionate and purify oligosaccharides (Pinelo *et al.*, 2009). In the area of food applications, the membrane technology was widely extended with the development of nanofiltration membranes that are more selective (Pontalier *et al.*, 1999). The membrane technology for purification of oligosaccharides produced with enzymes was recently reviewed (Pinelo *et al.*, 2009).

The separation by membrane filtration relates to the structural and physicochemical properties of sugars with the theory of membrane separation, taking into consideration the operational modes and operational conditions on the separation efficiency.

Physicochemical properties of sugars that influence membrane separation processes include the solubility, viscosity, substitution and branching (defined as the existence of monomers chains attached to a monosaccharide in the main chain). The solubility is directly determined by the chemical structure, hydrophilicity and hydrophobicity ratio, glycosidic linkages and special orientation of the functional groups. The concept of molecular weight cut-off loses significance when the solubility of the sugar is taken into account, because the cut-off values of sugars can change depending on the solubility/hydrophobicity of the solute (Pinelo *et al.*, 2009). The physicochemical properties of FOS, fructose, glucose and sucrose were previously discussed in section 1.3.2.

Different operational modes can be used in the membrane separation processes. The most commonly used for the oligosaccharides purification are the cross-flow filtration, dead-end membrane filtration and free/immobilized enzymes in a membrane reactor (Fig. 1.6). The operational conditions affecting membrane separation performance are the membrane type, temperature, feed concentration and pressure.

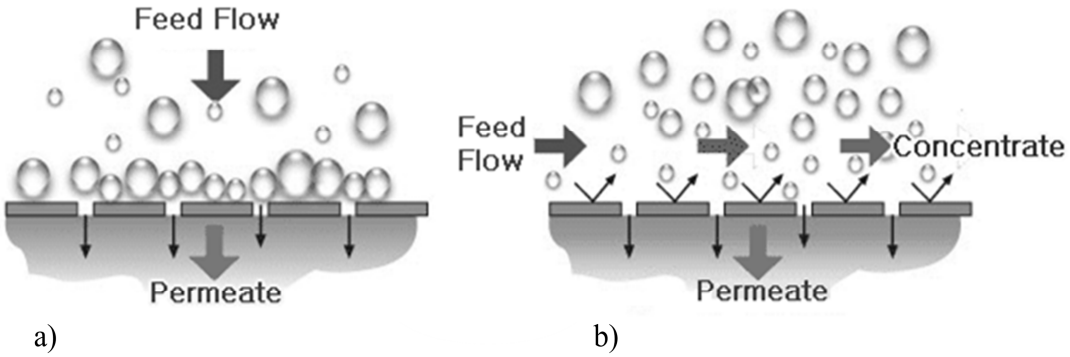


Fig. 1.6 Schematic representation of operational modes used in membrane separation processes a) cross-flow filtration b) dead-end filtration (Roplant, 2002).

Leiva and Guzman (1995) used cross-flow filtration to concentrate GOS produced from the enzymatic hydrolisys of lactose. The retention factors obtained were 15.4% for glucose and galactose, 37% for lactose and 74.7% for GOS. Sarney *et al.* (2000) also used cross-flow filtration for the recovery of oligosaccharides from human milk. Goulas *et al.* (2002) used cross-flow filtration, in a continuous diafiltration module, for the separation of a model sugar solution containing raffinose, sucrose and fructose and a commercial GOS mixture. Higher yields were obtained for raffinose and GOS, 81 and 98% respectively. Monosaccharides and disaccharides presented lower yields, 14-18% and 59-89%, respectively (Goulas *et al.*, 2002). Later, the same authors studied the fractionation of several commercial oligosaccharide mixtures using diafiltration in a 'dead-end' filtration cell and the same yields were obtained after four filtration steps (Goulas *et al.*, 2003).

On both works performed by Goulas *et al*. (2002; 2003), a constant volume of diafiltration (CVD) was used. One characteristic of the CVD operation mode is the excessive consumption of the dilution water solution (Barba *et al.*, 1998). In order to reduce the dilution water, a variable volume of diafiltration (VVD), during which dilution water flux is different to that of permeate, was introduced by Li *et al.* (2004; 2005). In these works, the purification of FOS by nanofiltration under different operation modes was studied. Results showed that the relationship between purity and yield was

independent of the dilution ratio. Consequently, the same purity of FOS was obtained for both operational modes. However, the water consumed under VVD was found to be almost half as much as that of CVD. Hence, the use of VVD enabled the saving of dilution water (Li *et al.*, 2004; Li *et al.*, 2005).

The dead-end filtration can be used to purify commercial FOS mixtures or FOS from a fermentative broth. In this system, the sugar mixture contained in a tank is pumped to the separation module where small saccharides are excluded while oligosaccharides are concentrated and recycled again to the feed tank. An ultrafiltration step, operating in the same way, can be previously performed to eliminate larger molecules from the broth (e.g. proteins and insoluble substances), and followed by the nanofiltration step. Kamada *et al.* (2002b) reduced from 9.0 to 2.6% the content of fructose, glucose and sucrose from the commercial product Raftiline (oligosaccharides from chicory) using nanofiltration in a dead-end module. The collected permeate contained only 6.3% of oligo-fructose in total sugars (Kamada *et al.*, 2002b).

Several types of membranes have been used to separate oligosaccharides by nanofiltration. Recently, a low-pressure plasma modified cellulose acetate (CA) membrane was used by Gulec *et al.* (2010) to separate highly concentrated sugar mixtures (40%, w/v) of lactose, glucose and galactose. CA membranes were modified by means of plasma polymerization (PlzP) using low-frequency (LF) excitation. Very high retention (>94%) was obtained with the LF/PlzP-modified CA membrane for lactose, while the retention for monosaccharide was rather low (<73%) at the same reaction time (Gulec *et al.*, 2010). Botelho-Cunha *et al.* (2010) *s*tudied the nanofiltration potential for fractionation of GOS mixtures by CA membranes submitted to annealing treatments. The study researched the effect of temperature, transmembrane pressure and total concentration of solutes in the feed. From a 150 $g.L^{-1}$ sugar feed solution, trisaccharides were totally retained. Using a more concentrated feed solution (300 $g.L^{-1}$), trisaccharides retention decreased for all the studied pressures, reaching 90% (Botelho-Cunha *et al.*, 2010).

Other related works reported the use of membrane technology for the recovery of non-digestible saccharides from yacon rootstock (mainly FOS and inulo-poysaccharide)

(Kamada *et al.*, 2002a), and the concentration of oligosaccharides from soybean extracts (Matsubara *et al.*, 1996), caprine milk (Martinez-Ferez *et al.*, 2006), rice husks xylan (Vegas *et al.*, 2008) and unwanted compounds coming from lactic acid recovered from fermentative broths derived from apple pomace (Gullón *et al.*, 2011).

1.5.4 ACTIVATED CHARCOAL

Activated charcoal, also called activated carbon or activated coal, is a form of carbon that was submitted to a sequence of chemical and terminal processes in order to develop a large surface area (between 500 and 2000 m^2g^{-1}), conducting to a good sorption capacity (Unger *et al.*, 2005). The surface area developed in the pores of the carbon is actually much higher than the external surface area of the particles, and most of the sugars adsorption occurs on the pore's surface.

Sugars are physically adsorbed onto the activated charcoal. The adsorption is due to Van der Walls' forces and is a reversible process (Hung *et al.*, 2005). The major surface area of the activated charcoal is non polar or hydrophobic. Therefore, when the solute is more hydrophobic more adsorbed it will be (Abe et al., 1983). Since the hydrophobic character of the sugar is related with the extent of the CH groups, sugars will be adsorbed accordingly to their molecular weight, provided the configuration and the glycosidic link stereochemistry are favorable (Sundari and Balasubramanian, 1997). Hence, FOS are more adsorbed onto the activated charcoal than the other small saccharides as sucrose, fructose and glucose enabling their separation.

After the adsorption step, sugars can be selectively desorbed using different percentages of water/ethanol. Monosaccharides have been recovered using water or very small percentages of ethanol. For the recovery of disaccharides, a percentage between 5 and 10 % of ethanol in water is used. Finally, oligosaccharides have been recovered using 15 to 50% of ethanol in water (Whistler and Durso, 1950; Hidaka *et al.*, 1988; Swallow and Low, 1990; Kaplan and Hutkins, 2000; Sanz *et al.*, 2005; Morales *et al.*, 2006).

The most common process used to purify oligosaccharides with activated charcoal is divided in three steps. Firstly the activated charcoal is loaded with the oligosaccharide

mixture. Afterwards, the non retained compounds are washed with pure water. Finally, the adsorbed sugars are selectively recovered using an ethanol gradient.

In addition to the good characteristics of adsorption, the activated charcoal is cheap and easily regenerated (Chinn and King, 1999). Therefore, it has been largely used by many authors to purify oligosaccharides. The activated charcoal has been used in slurry or in packed columns as a single adsorbent or in combination with Celite.

Whistler and Durso (1950) were the first to propose the use of an equal part by weight mixture of charcoal:Celite to separate sugars. Different mixtures of sugars including glucose, maltose, raffinose, sucrose and melibiose were successfully separated using a single column packed with the charcoal:Celite. The procedure used consisted in successive elutions of sugars with water, 5% and 15% of ethanol (Whistler and Durso, 1950). Uchiyama *et al.* (1985) extracted FOS from *L. radiate* bulbs and used a charcoal-Celite column to separate GF_2, GF_3, pentaose and hexaose in different fractions. Sugars were successively eluted with water, 5%, 7.5% and 10% of ethanol. However, each fraction was found to be contaminated with other sugars (Uchiyama *et al.*, 1985). Samain *et al.* (1997) recovered COS from a culture medium using vegetable powder activated charcoal and Celite. Sugars were recovered by a stepwise elution with ethanol, and a 62% recovery of COS could be obtained in the 30% ethanol faction (Samain *et al.*, 1997).

Activated charcoal has been widely used to characterize the composition of honey (Swallow and Low, 1990; Weston and Brocklebank, 1999; Sanz *et al.*, 2005; Morales *et al.*, 2006; Morales *et al.*, 2008). The identification of oligosaccharides in honey by chromatographic techniques is very difficult due to the lack of commercial standards, the low and large contents in oligo- and monosacharides, respectively, and the similarity of the sugar structures (Morales *et al.*, 2006). Therefore, the separation of monosaccharides from honey before the oligosaccharides identification process is essential. Swallow *et al.* (1990) and later Weston *et al.* (1999) used the procedure of Whistler and Durso (1950) with few modifications to purify and concentrate oligosaccharides from honeys. In this procedure, 99% of monosaccharides were removed with 0.1% of ethanol solutions and honey oligosaccharides were recovered with 50% of ethanol (Swallow and Low, 1990;

Weston and Brocklebank, 1999). Later, Morales *et al.* (2006) replaced the use of charcoal:Celite columns by a stirred system containing the activated charcoal and the water/ethanol solutions mixed with honey samples. The optimized process consisted in stirring 0.5 g of honey with 3 g of activated charcoal and 100 mL of the water/ethanol solution, for 30 min, at ambient temperature. After the adsorption/desorption process, sugars were recovered by filtration. An ethanol concentration of 10% was found to be optimal in order to recover mono- and disaccharides from the mixture. On the other hand, oligosaccharides were recovered with 50% ethanol. This process proved to be simpler and less time-consuming than the charcoal:Celite columns used in the official method (Morales *et al.*, 2006). This method was later used to detect the adulterations of honey with added sugar syrups preparations (Morales *et al.*, 2008). Hernandez *et al.* (2009) also used this same procedure, with few modifications, to remove mono- and disaccharides from a commercial GOS product. In order to find the optimal ethanol concentration to recover GOS with maximum purity and minimum losses, the authors dissolved the samples, mixed them with charcoal, and used different ethanol solutions (1, 5, 8, 10 or 15%). GOS were recovered from the charcoal with a 50% ethanol solution. In this work, it was found that using up to 1% of ethanol solution the non monosaccharides are adsorbed. However, it was not possible to completely remove the disaccharides using any of the ethanol concentrations studied. Despite the residual recovery of disaccharides using the 15% ethanol solution, only 20% of trisaccharides GOS were recovered. Therefore, the 10% ethanol solution was chosen as the best compromise between purity and the amount of GOS recovered. With this ethanol solution it was possible to recover 50% of trisaccharides, 90% of tetrasaccharides, and 10% of disaccharides (Hernandez *et al.*, 2009).

Activated charcoal columns have been also used to purify FOS mixtures. FOS produced by *A. niger* were purified and fractioned by Hidaka *et al.* (1987) using a preparative activated charcoal column (8.0 x 43 cm). A volume of 160 mL of fermentative broth was purified by successive elutions with water and ethanol. Results showed that monosaccharides were totally eluted with the washing water; GF_2, GF_3 and GF_4 were collected with ethanol elutions at 5, 10 and 20%, respectively. Amounts of 9, 16 and 8 g of GF_2, GF_3 and GF_4 were recovered with purities of 70, 69 and 44%, respectively. Pure

FOS solutions were recovered with fractions of GF_3 and GF_4. However, with the GF_2 fraction, 22% of the collected sugars were sucrose (Hidaka *et al.*, 1988). Kaplan and Hutkins (2000; 2003) purified FOS from a commercial mixture by removing monosaccharides and disaccharides with water and 5% ethanol, respectively. FOS were further recovered with a 15% ethanol solution (Kaplan and Hutkins, 2000; Kaplan and Hutkins, 2003).

Sanz and co-workers (2005) compared nanofiltration, yeast treatment and activated charcoal regarding their potential to separate monosaccharides from oligosaccharides in honey, having concluded that activated charcoal was the most efficient method. In another study, Hernandez et al. (2009) compared diafiltration, yeast treatment, activated charcoal and size exclusion chromatography for GOS fractionation. Although size exclusion was the best technique to fractionate GOS at an analytical scale, activated charcoal was also the most appropriated method to remove mono- and disaccharides.

Kuhn and Maugeri (2010) recently studied the purification of FOS from a fermentative broth using an activated charcoal fixed bed column with ethanol as eluent. Operating conditions such as temperature and ethanol concentration were evaluated using a 2^2 central composite design. Results showed that with 15% ethanol at 50 ºC, 80% recovery of FOS with about 97.8% purity could be obtained. Nevertheless, a poor efficiency for separation of FOS from sucrose was found. Although the process seemed promising, the authors also found that column efficiency varies deeply with the type of charcoal used. Thus, a different pore distribution and pore volume size may be obtained as a function of the initial pyrolysis and activated procedures used. Consequently, the method did not demonstrate a very good reproduction between the different batches conducted (Kuhn and Maugeri, 2010).

Other related works also reported the use of activated charcoal for the recovery of GOS from fermentative broths (Sai Prakash *et al.*, 1989); horse milk oligosaccharides (Urashima *et al.*, 1991) and goat colostrums (Urashima *et al.*, 1994); oligosaccharides from human milk (Priem et al., 2002); XOS produced by the auto-hydrolysis of almond shells, in this case, lignin-related products were the highest adsorbed compounds (Montane *et al.*, 2006); XOS from fermentative broths (Zhu *et al.*, 2006; Yang *et al.*,

2007); and two new oligosaccharides obtained by fermentation of fruits and vegetables in a fermented beverage (Kawazoe *et al.*, 2008).

Synthesised FOS by microorganisms are obtained in mixtures containing not only small saccharides, but also large amounts of salts that must be removed to guarantee certain quality parameters in the final product. One advantage of using activated charcoal to purify FOS from fermentative broths, rather than other separation techniques, is the potential that charcoal has to simultaneously desalt solutions. Whistler and Durso (1950) concluded that the presence of sodium chloride, sodium bicorbonate and sodium acetate, in various concentrations, do not affect neither the adsorption nor the desoprtion of sugars onto activated charcoal. Salt present in the sugar mixtures were completly eliminated with the water effluent while washing the loaded column (Whistler and Durso, 1950). Samain *et al.* (1997) desalted a culture medium using vegetal powder activated charcoal and Celite. The slurry containing the adsorbent and the culture medium was filtered and washed with distillated water, and the salt found was completely removed during the washing stage. Packer *et al.* (1998) demonstrated that graphitized carbon can be used for desalting oligosaccharides mixtures released from glycoproteins. Results showed that during the washing stage, neutral monosaccharides and salts were completlty eluted with the water (Packer *et al.*, 1998).

1.5.5 ION EXCHANGE CHROMATOGRAPHY

Several authors have studied the potential of using ion exchange resins to separate carbohydrates. Chromatography separation is based in the molecular differences of carbohydrates instead of macroscopic properties. Therefore, this method allows the separation of very similar carbohydrates, such as isomers, and shows high potential to separate FOS.

At an industrial scale, ion exchange resins have been applied as adsorbents in Simulated Moving Bed (SMB) chromatography. The adsorbents used in chromatography must be non-toxic and must have a good mechanical, chemical and biological stability to allow a long lifetime. Ion exchange resins of sulfonated poly(styrene-*co*-divinylbenzene) (PS-

DVB) have been largely used due to their chemical inertness, higher capacity and selectivity (Okada, 1995; Stefansson and Westerlund, 1996; Tiihonen *et al.*, 2002; Luz *et al.*, 2008). PS-DVB resins are obtained by cross-linking the linear chain of the styrene polymer with DVB (Fig. 1.7). This cross-linking increases the molecular weight of the average polymer chain length, which decreases the polymer solubility and increases its mechanical stability (Fritz and Gjerde, 2009). Gel-type resins normally contain less than 12% of DVB, while macroporous resins have DVB contents greater than 20%. Macroporous resins are characterized by a permanent well-developed porous structure and do not shrink. Since these resins have a high content in DVB, they are resistant to degradation caused by osmotic shock and oxidation. On the other hand, gel-type resins are soft, compressible and are able to swell with the appropriated solvent. However, these properties may restrict their use in chromatographic applications in which the back pressure may reach high values (Abrams and Millar, 1997; Sherrington, 1998).

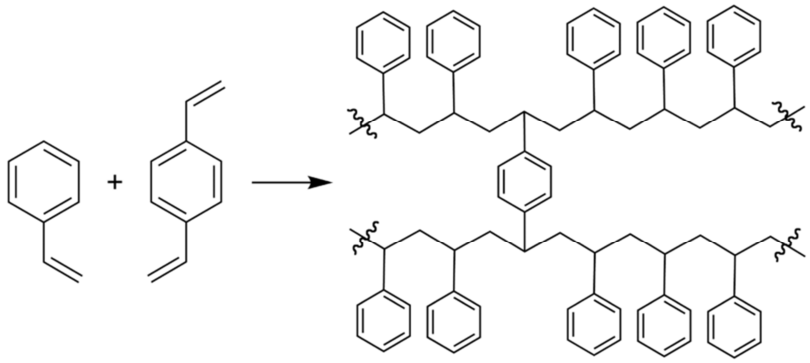


Fig. 1.7 Schematic representation of styrene-*co*-divinylbenzene copolymer.

PS-DVB resins can be functionalized with counter-ions which are attached to the polymer. Functionalized resins become considerably more polar, consequently, for the softer resins (such as the gel-type ones) are expected to have different behaviors in the presence of polar or non-polar solvents. In the presence of polar solvents these resins tend to swell, while with non-polar solvents tend to dehydrate and shrink (Fritz and Gjerde, 2009).

Counter-ions are able to form complexes with the OH groups of sugars. The strength of the complex formed will depend on the hydration and ionic radius of the counter-ion, and the number and orientation of the OH group (Angyal, 1989; Tiihonen *et al.*, 2002). Therefore, the sugar conformation and the counter-ion involved determine the cation-sugar affinity, and consequently, the degree of adsorption. The stability of the complex formed depends on the number of pairs of eq-ax oriented OH groups. Since fructose has more pairs than glucose (Table 1.2), it is expected to be more adsorbed. Sucrose and FOS have no eq-ax oriented group, so they are excluded from the resin. As a result, the separation of the high molecular size sugars occurs mainly by size exclusion.

The separation mechanism involved in sugar purification with ion exchange-resins is mainly based in three phenomena: size exclusion, partition and ligand exchange based on complex formation (Churms, 1996; Stefansson and Westerlund, 1996).

The water contained in the resin is present in two forms: as hydration water (water contained in the hydration shell of the counter-ion available for complex formation) and as hygroscopic water (free water inside the resin available for partition). The total amount of water contained in the resin is therefore dependent on the resin type, cross-link density and ionic form.

As discussed above, the gel-type resins are able to swell. Hence, their water retention capacity is greater compared to the macroporous resins, resulting in faster kinetic diffusions (Abrams and Millar, 1997; Sherrington, 1998). The cross-linking of the resin decreases its elasticity. Therefore, the ability to swell and retain water for partition also decreases. On the other hand, the amount of ions per volume of unit water increases with the DVB content. Hence, the adsorption by complex formation may increase. The hydration number of the counter-ion increases with the ionic valence (Tiihonen *et al.*, 2002). Consequently, univalent cations only form weak complexes with sugars. Therefore, for these resins the separation mechanism is mainly based on the combination of size exclusion and restricted diffusion effects. In contrast, ions with increasing ionic valence may form strongest complexes with sugars. Nevertheless, the water uptake decreases with the increase of ionic valence (Tiihonen *et al.*, 1999).

Several studies have been carried out to evaluate the performance of ion-exchange resins in the separation of sugars. The characteristics of resins as stability, resistance, selectivity and adsorption capacity have been investigated. Studies were conducted to evaluate the influence on the adsorption of the resin type, ionic form and cross-link density. The influence of temperature and additional sugars in the mixture has been also studied. A summary of the most relevant and recent studies in adsorption equilibrium of sugars on ion-exchange resins is presented in Table 1.5.

RESIN TYPE

The strong acid cation (SAC) resins are the most commonly used for the industrial sugar separations. Therefore, SAC are the best well studied type of resins (Luz *et al.*, 2008; Pedruzzi *et al.*, 2008; Nobre *et al.*, 2009; Lei *et al.*, 2010). Recently, Saari *et al*. (2010) evaluated the use of weak acid cation (WAC), weak base anion (WBA), and strong base anion (SBA) ion-exchange resins for the separation of sugars from lignocellulosic biomass hydrolysates. Results showed that SBA resins in SO_4^{2-} form could be an alternative to separate xylose and rhamnose from biomass hydrolysates (Saari *et al.*, 2010). WBA resins in Cl^- form have also been used to separate lactic acid from oligosaccharides found in fermentative broths derived from apple pomace (Gullón *et al.*, 2010) .

Among the SAC resins, gel-type resins have been more applied in sugar separations than macroporous resins, therefore just few works investigate the two different resin types. Gramblicka et *al.* (2007) studied the adsorption equilibrium of FOS, fructose, glucose and sucrose on two Na^+ resins with different structures. In this work, based on the GF_2/sucrose selectivity, the gel-type resin was more effective in FOS purification than the macroporous one (Gramblicka and Polakovic, 2007). Recently, Nobre *et al.* (2009) compared the adsorption equilibrium of fructose, glucose and sucrose onto a gel-type (K^+) and macroporous (Na^+) resins. Based on selectivity results, the gel-type (K^+) resin was found to be the best choice for the separation of the three sugars.

Table 1.5 Studies on the adsorption equilibrium of sugars on ion-exchange resins.

Sugars	Resin	Process conditions	Adsorption isotherms/Method	Main observations	Reference
Fructose (F) Glucose (G)	Purolite PCR642 Ca^{2+}	▪ C: [10 – 70] $g.L^{-1}$; ▪ T: 30 and 60 ºC; ▪ Single-component mixtures; and data syrup.	▪ Linear isotherms ▪ Adsorption-desorption in batch and in column.	▪ Adsorption capacity decrease with increasing T. ▪ Results suggest that F and G may be successfully separated from data syrup.	(Mostafazadeh *et al.*, 2011)
Fructose (F) Glucose (G) Sucrose (S) FOS	Amberlite CR 1320 Ca^{2+}	▪ C: [0 – 400] $g.L^{-1}$; ▪ T: 60 ºC; ▪ Single-component and binary mixtures.	▪ Linear isotherms ▪ Frontal analysis.	▪ The binary adsorption equilibrium showed that the distribution coefficients of all saccharides besides F were slightly lower in the presence of another saccharide.	(Vankova *et al.*, 2010)
Glucose (G) Xylose (X) Arabinose (A)	Resin 001*4 Ca^{2+} Resin 001*6 Ca^{2+} Resin 001*8 Ca^{2+} Resin 001*6 K^{+} Resin 001*6 Fe^{3+}	▪ C: [10 – 100] $g.L^{-1}$; ▪ T: 25 ºC ▪ Single-component mixtures;	▪ Linear isotherms ▪ Adsorption-desorption in batch.	▪ Order of sugars adsorption: $K^{+} > Ca^{2+} > Fe^{3+}$. ▪ Resin in Ca^{2+} form was more suited to separate the mixture. ▪ A 6% DVB was more suitable to separate A/X; while 8% DVB was more suitable for X/G.	(Lei *et al.*, 2010)
Arabinose (A) Fructose (F) Galactose (Ga) Glucose (G) Mannose (M) Rhamnose (R) Sucrose (S) Xylose (X)	Finex CS 11 GC Na^{+} (SAC) Finex CA 12 GC Na^{+} (WAC) Finex AS 510 GC SO_4^{2-} (SBA) Finex AA 12 GC FB SO_4^{2-} (WBA)	▪ C: [0 – 350] $g.L^{-1}$; ▪ T: 65 ºC ▪ Single-component mixtures;	▪ Linear isotherms ▪ Adsorption-desorption in batch.	▪ The use of the WAC exchange resin was especially restricted due to the decomposition of sugars. ▪ The WBA exchange resin showed high selectivity for X and A. ▪ The SBA exchange resin showed good selectivity for X and R, which implies that these could be separated from biomass hydrolysates.	(Saari *et al.*, 2010)

Table 1.5 (Cont.) Studies on the adsorption equilibrium of sugars on ion-exchange resins.

Fructose (F) Glucose (G) Sucrose (S)	Dowex Monosphere 88 Na^+ Dowex Monosphere 99 Ca^{2+}/320	▪ C: [5 – 250] $g.L^{-1}$; ▪ T: 25 and 40 ºC; ▪ Single- and multi-component mixtures;	▪ Linear isotherms ▪ Adsorption-desorption in batch.	▪ Competitive effect on the adsorption at 25ºC; Synergetic effect on the adsorption at 40ºC. ▪ Adsorption capacity decreased on mono-component mixtures; and increased on multi-component mixtures with increasing T ▪ Dowex Monosphere 99Ca/320 was more suited for F, G, S separation.	(Nobre *et al.*, 2009)
Lactobionic acid (La) Fructose (F) Lactose (L) Sorbitol (So)	Dowex Monosphere 99 Ca^{2+}/320 Dowex 50WX8-400 (H^+ and K^+ form) Dowex 50WX4-400 H^+ and K^+ form)	▪ C: [5 – 130] $g.L^{-1}$; ▪ T: 20, 40 and 60 ºC; ▪ Single-component mixtures;	▪ Linear isotherms: F, L and So; Anti-Langmuir model: La. ▪ Frontal Analysis; Adsorption-desorption in column.	▪ The best results for the retention factor and separation factor were obtained with K^+ resins. ▪ Dowex 50WX4-400 in K^+ form showed better resolution to separate La. ▪ Adsorption capacity decreased, and mass transfer coefficient increased with increasing T.	(Pedruzzi *et al.*, 2008)
Fructose (F) Glucose (G) Sucrose (S)	Lewatit MDS 1368 Ca^{2+}	▪ C: [0– 600] $g.L^{-1}$; ▪ T: 60 and 80 ºC; ▪ Single- and multi-component mixtures;	▪ Anti-Langmuir model. ▪ Frontal Analysis; Adsorption-desorption in column.	▪ Synergetic effect on the competitive adsorption. ▪ Adsorption capacity decreased for F with increasing T. G and S were not affected.	(Nowak *et al.*, 2007)
Fructose (F) Glucose (G) Sucrose (S) FOS	Diaion UBK 530 Na^+ Dowex Monosphere 99 Ca^{2+}/320 Lewatit S 2568 Na^+ Amberlite CR 1320 Ca^{2+}	▪ C: [0 – 450] $g.L^{-1}$; ▪ T: 60 ºC; ▪ Single-component: F, G, S; and multi-component: FOS.	▪ Linear isotherms: F, G and FOS; Convave isotherm model: S. ▪ Adsorption-desorption in batch	▪ Amberlite CR 1320 Ca^{2+} resin was more suited to purify FOS.	(Gramblicka and Polakovic, 2007)
Fructose (F) Glucose (G) Sucrose (S) Galactose (Ga) Lactose (L)	Dowex 50WX4-400 (K^+, Na^+ and Ca^{2+} forms)	▪ C: [0 – 400] $g.L^{-1}$; ▪ T: 60 ºC; Single-component: F, G, S; and multi-component: FOS.	▪ Linear isotherms: F, G, Ga and L; Convave isotherm model: S. ▪ Frontal Analysis; Adsorption-desorption in batch.	▪ Order of sugars adsorption: $K^+ > Na^+ > Ca^{2+}$. ▪ Resin in K^+ form was more suited to separate G from oligosaccharides. ▪ Resin in Ca^{2+} form was more suited to separate F from oligosaccharides.	(Vente *et al.*, 2005)

IONIC FORM

The separation of glucose from fructose is the most well-known sugar separation process. As Ca^{2+} cations are able to form strong complexes with fructose enabling the isomers separation, Ca^{2+} resins are the most reported (Howard *et al.*, 1988; Saska *et al.*, 1992a; Beste *et al.*, 2000; Luz *et al.*, 2008; Nowak *et al.*, 2009). However, the separation mechanism of monovalent counter-ions provides higher adsorption kinetic rates compared with resins that form strong complexes with sugars. Therefore, counter-ions such as K^+ and Na^+ have been studied for sugar separation processes. Vente *et al.* (2005) studied the influence of K^+, Na^+ and Ca^{2+}, as the counter-ions of a SAC resin, in the adsorption equilibrium of mono- and disaccharides that are relevant for the separation of oligosaccharides. Results showed that Ca^{2+} resin provided the highest selectivity values for fructose/oligosaccharides separation, due to the complexes formed with fructose. While for glucose/oligosaccharides separation, all resins studied presented the same selectivity. However, with the K^+ resin, a higher capacity compared to Na^+ and Ca^{2+} resins could be obtained. Therefore, the K^+ resin seems to be most suitable for the envisaged separation. Accordingly, Nobre *et al.* (2009) and Dendene *et al.* (1995) also found better results for the separation of mono- and disaccharides, with a resin in the K^+ form rather than with a Na^+ one. Furthermore, Pedruzi *et al.* (2008) elected a resin in K^+ form for the separation of lactobionic acid from a mixture of sugars, instead of resins in H^+ and Ca^{2+} form. Nevertheless, the resin in Ca^{2+} form showed higher selectivity to separate sorbitol/lactose and fructose/sucrose. In a study conducted with four commercial resins in Na^+ and Ca^{2+} forms, for FOS purification, Gramblicka *et al.* (2007) found almost no selectivity of sucrose/kestose for Na^+ resins. On the other hand, Ca^{2+} resins presented higher selectivity values. Recently, in a study on the separation of glucose, xylose and arabinose, the adsorbance of sugars to the resins varied in the order $K^+ > Ca^{2+} > Fe^{3+}$, however Ca^{2+} resin was elected due to its higher selectivity (Lei *et al.*, 2010).

CROSS-LINK DENSITY

The effect of the cross-link density was also investigated in the same study. Results showed that the choice of DVB content depends on the sugar molecules involved. A 6% DVB was more suitable to separate arabinose/xylose, while 8% DVB was more suitable

for the xylose/glucose separation (Lei *et al.*, 2010). Pedruzzi *et al.* (2008) found that a 4% DVB resin was better to separate lactobionic acid/lactose instead of an 8% one. However, the resin with lower DVB content presented higher selectivity values for the separation of sorbitol/lactose and fructose/sorbitol. The effects of the DVB content and ionic form of cation-exchange resins on the chromatographic separation of MOS was evaluated by Adachi *et al*. (1989). In this study, the resin with higher DVB content gave the smaller coefficient of distribution. Also, as the radius of the hydrated ion became larger and the distribution coefficient was reduced (Adachi *et al.*, 1989a).

TEMPERATURE

The high concentration of sugar leads to an increased viscosity of the liquid phase, with a consequent increase in pressure drop and a decrease in solubility of sugars. As a result, many authors have used temperatures above 70ºC to promote a decrease of viscosity and an increase of solubility (Azevedo and Rodrigues, 2001; Luz *et al.*, 2008). Besides, the use of high temperatures also avoids microbial growth (Beste *et al.*, 2000).

The temperature effect in sugar adsorption equilibrium onto gel-type resins, using single-component mixture of sugars was investigated by many authors (Dendene *et al.*, 1995; Pedruzzi *et al.*, 2008; Nobre *et al.*, 2009; Mostafazadeh *et al.*, 2011). These works reported a decrease on the adsorption capacity at high temperatures. Studies conducted with multi-component sugar mixtures are scarce. In this case, the temperature effect seems to vary according to the resin, sugar and range of temperatures tested. Nowak *et al.* (2009) studied the influence of temperature on the adsorption of a mixture of sucrose, glucose and fructose on a Lewatit MDS 1368 in Ca^{2+} form. Experiments were conducted at 60ºC and 80ºC. Results showed no temperature dependency of the adsorption of glucose and sucrose. A decreased adsorption of fructose was found for increasing temperatures (Nowak *et al.*, 2007; Mostafazadeh *et al.*, 2011). However, Best *et al.* (2000) studied the temperature effect (25ºC to 80ºC) in the adsorption of a mixture of fructose and glucose onto the same resin, and an increased adsorption behavior of glucose was found increasing the temperature. Regarding the adsorption of fructose, the authors reported a decrease in temperatures up to 60ºC, and an increase to 80ºC. On the other hand, in a study conducted with a gel-type (K^+) and a macroporous (Na^+) resin with

a mixture of fructose, glucose and sucrose, the temperature increases conducted to an increased adsorption, however with decreased selectivity for the K^+ resin (Nobre *et al.*, 2009).

MULTI-COMPONENT MIXTURES

Nowak *et al.* (2007) also studied the competitive loading of fructose, glucose and sucrose onto a gel type resin in Ca^{2+} form, at 60ºC and 80ºC, by adding the individual sugars to a mixture. Results showed that the presence of additional sugars in solution conducted to a synergistic effect in the adsorption, *i.e.* the increased concentration of one sugar conducted to the increased adsorption of the others. Accordingly, Nobre *et al.* (2009) also found a synergistic effect at 40ºC; however, for a lower temperature (25ºC) a reverse effect was found. Nonetheless, Vankova *et al.* (2010b) reported a competitive effect for all sugars except fructose in the adsorption of binary mixtures of fructose, glucose, sucrose and FOS, working at 60ºC, with a different gel-type resin in Ca^{2+} form.

SMB CHROMATOGRAPHY

PS-DVB resins have been largely and successfully used in SMB plants for sugar separations (Beste *et al.*, 2000; Coelho *et al.*, 2002; Luz *et al.*, 2008). SMB has been used for the separation of glucose from fructose (Beste *et al.*, 2000; Azevedo and Rodrigues, 2001; Lee, 2003), sugarcane molasses (Neuzil, 1982; Saska *et al.*, 1992b) and high-fructose corn syrups (Toumi and Engell, 2004). SMB chromatography consists in multiple chromatographic columns connected in series and a complex valve arrangement that allows an appropriated shift of injection and collection points. The system works in continuous counter-current motion of the solid phase relatively to a liquid phase, without the real motion of the adsorbent. Instead, the movement of the adsorbent is simulated by the valves that move the position of two inlet (feed and eluent) and two outlet streams (raffinate and extract) by switching one column in the direction of the liquid phase flow, at a fixed interval of time (Charton and Nicoud, 1995).

The main advantages of this separation method are that it works as a continuous system and enables very difficult separations (even with components presenting low selectivity).

Compared to elution chromatography, higher productivities are obtained with lower solvent consumption and it becomes less expensive for large scale separations (Mazzotti *et al.*, 1997; Gomes *et al.*, 2006). However, the design, operation, optimization and control of the process are complex requiring longer time for the start up.

The number of reports on oligosaccharides adsorption onto ion-exchange resins for SMB applications is very limited. Ion-exchange resins for MOS purification were extensively studied by Adachi *et al.* (Adachi *et al.*, 1989a; Adachi *et al.*, 1989b; Adachi *et al.*, 1995). Kawase *et al.* (2001) studied the simultaneous production and separation of lactosucrose from glucose in a SMB reactor. Gramblicka *et al.* (2007) selected, among four commercial resins, the Amberlite CR1320 resin in Ca^{2+} form for FOS purification, based on the adsorption equilibrium studies of fructose, glucose, sucrose and FOS. Later, the same authors studied the adsorption equilibrium of binary mixtures (Vankova *et al.*, 2010b) and modelled a fixed-bed adsorption of these sugars onto the same resin (Vankova *et al.*, 2010a). In another work, the separation of FOS from mono- and disaccharides using a single-column was optimized (Vankova and Polakovic, 2010). A recovery of 86% of FOS, with purity increasing from 61.7 to 82%, was obtained for a column load of 2% and superficial velocity of $5.0x10^{-5}$ $m.s^{-1}$. This work also demonstrated that the scale up was feasible, and 90% of FOS yields and purity could be obtained using a larger column. Geisser *et al.* (2005) were the only authors reporting the separation of a complex mixture of oligosaccharides using a SMB plant. The separation of lactose from the human milk oligosaccharides mixture was studied. Results showed that an almost complete lactose separation was reached, under stable conditions (Geisser *et al.*, 2005).

1.6 CONCLUSIONS

An increased commercial interest in FOS has emerged in the last decade due to their prebiotic activity. Many authors have investigated their technological properties and health benefits. Also, a great amount of research has been carried out in FOS production by microbial enzymes from sucrose. The FOS obtained by fermentation consist of mixtures of FOS but also fructose, glucose and sucrose, mainly due to the low yields of the fermentation process. The presence of these smaller sugars in the mixture decreases the prebiotic, caloric and cariogenic values of the final product. Therefore, many studies have been conducted to obtain a product with increased FOS purity.

Some authors have proposed the continuous removal of glucose or FOS from the fermentative broth during the production process as a way to increase the production yields. Fermentations carried out with mixed enzymes, reactor systems using nanofiltration membranes and charcoal columns connected to the bioreactors have been successfully used.

The recovery of FOS from mixtures of sugars has been a challenge for the scientific community. Several separation techniques with potential for FOS purification have been evaluated namely, microbial treatment, ultra- and nanofiltration, activated charcoal systems and ion-exchange chromatography.

Microbial treatment with *S. cerevisiae* and *Z. mobilis* has demonstrated high potential to eliminate mono- and some disaccharides. However, the presence of metabolic products, formed during the fermentation, as well as the biomass requires a further purification step.

Membrane technology, mainly nanofiltration, in different operational modes has also been used to purify FOS. However, membranes needed to separate very concentrated mixtures are not stable at elevated temperature and lead to diluted products. Furthermore, the choice of the membrane is very challenging and many authors could not purify oligosaccharides using nanofiltration systems.

On the other hand, activated charcoal systems have proven to be an easy and efficient method used to recover large amounts of oligosaccharides. Also, compared to other downstream processes, the activated charcoal was found to be superior and more versatile for the recovery of different saccharides. Nevertheless, charcoal has a heterogeneous surface and its efficiency varies with the type of charcoal used. Activated charcoal was found to be the only downstream process that promotes the desalting of the mixtures during FOS purification.

Several types of ion-exchange resins have recently been evaluated for further use in SMB chromatography systems. The SMB chromatography works in a continuous mode, does not require organic solvents and resins are very stable. Therefore, although it is a complex system, its application at an industrial scale is advantageous compared to the batch activated charcoal columns. This method has already proved to be suitable to separate binary sugar mixtures and some complex oligosaccharide mixtures efficiently. Hence, it is expected that SMB will play an important role on the large scale purification of FOS.

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CHAPTER 2

PURIFICATION OF FRUCTO-OLIGOSACCHARIDES FROM A FERMENTATIVE BROTH USING AN ACTIVATED CHARCOAL COLUMN

ABSTRACT

In this study, a simple and efficient process to purify fructo-oligosaccharides (FOS) from a fermentative broth was proposed using a single activated charcoal column. The FOS adsorption onto the activated charcoal was modeled by a pseudo-second order model. Several volumes and concentrations of water/ethanol were studied to optimize the selective desorption of sugars from the broth mixture at 25ºC. Mixtures containing 50.6% (w/w) of FOS (FOS content in the fermentative broth) were purified to 92.9% (w/w) with a FOS recovery of 74.5% (w/w). Moreover, with the proposed process, fractions with purity up to 97% (w/w) of FOS were obtained. This purification process was also found to be efficient in the desalting of the fermentative broth.

2.1 INTRODUCTION

Fructo-oligosaccharides (FOS) have gained large commercial interest due to their beneficial properties in the human health as prebiotics. FOS, namely, kestose (GF_2), nystose (GF_3) and fructo-furanosylnystose (GF_4), are non digestible food ingredients that are selectively fermented in the colon, increasing the numbers of beneficial bacteria by modulation of the gut microflora (Kolida and Gibson, 2007).

Although FOS occur naturally in many common foods such as fruit, vegetables, milk and honey, they are present in low concentrations and are season-limited. On a large scale FOS can be produced by fermentation using microorganisms, such as *Aureobasidium* sp. (Gibson, 1998; Sangeetha *et al.*, 2005). These fungi produce enzymes with transfructosylation activities that convert sucrose into FOS (Sangeetha *et al.*, 2004). At the end of the process, the fermentative broth contains FOS together with microorganisms, salts and sugars with low molecular weight, such as fructose (F), glucose (G) and sucrose (S). These small sugars do not meet the requirements to be considered as prebiotics since they are metabolized by humans. Therefore, to obtain a mixture with high prebiotic activity these small sugars must be eliminated or reduced. Additionally, salts and microorganisms must also be removed.

Several methods have been developed to purify oligosaccharides from sugar mixtures (Sanz and Martinez-Castro, 2007), such as the use of ion exchange resins, activated charcoal, size exclusion chromatography, solvent precipitation, nanofiltration and yeast treatments (Goulas *et al.*, 2003; Ku *et al.*, 2003; Li *et al.*, 2004; Sanz and Martinez-Castro, 2007). Sanz and co-workers (2005) compared three of these methods - nanofiltration, yeast treatment and activated charcoal - regarding their potential to separate monosaccharides from oligosaccharides in honey. The authors found that activated charcoal was the most efficient method when a pure mixture in oligosaccharides is envisaged. Therefore, among other methods that have been studied, the adsorption onto activated charcoal persists as one of the most suitable methods. Activated charcoal is cheap and has a large surface area and pore volume, conducting to a good sorption capacity. In addition, this sorbent is easily regenerated (Chinn and King,

1999) and has the potential to desalt solutions (Packer *et al.*, 1998; Whistler and Durso, 1950). Other advantages consist in the simplicity of the system, the low cost of the process, and its capacity to separate large amounts of oligosaccharides using only a single small column (Capek and Stanek Jr, 1975; Whistler and Durso, 1950).

Since Whistler and Durso (1950) reported the use of activated charcoal in combination with Celite to separate sugars by elution with increasing ethanol concentrations, several researchers used this method with different combinations of adsorbent and ethanol concentrations to purify sugars from honey (Morales *et al.*, 2006; Swallow and Low, 1990; Weston and Brocklebank, 1999); oligosaccharides from human milk (Priem *et al.*, 2002); galacto-oligosaccharides from reaction mixtures (Ajisaka *et al.*, 1987; Boon *et al.*, 2000); and FOS from sugar mixtures (Hidaka *et al.*, 1988; Kaplan and Hutkins, 2000; Kuhn and Maugeri, 2010).

The isolated FOS (GF_2, GF_3 and GF_4) are only available for analytical purposes and their prices are prohibitive. Indeed, even pure FOS mixtures are still very expensive. Hence, it is crucial to optimize the FOS purification processes for large scale applications. In this study, a simple and efficient process to purify FOS from a fermentative broth using a single activated charcoal column is proposed and optimized. FOS were produced by fermentation of sucrose using *Aureobasidium* sp. The adsorption kinetics onto activated charcoal of the sugars contained in the fermentative broth was studied and modelled using pseudo-first and pseudo-second order models. The purification process was conducted in three steps: 1) adsorption of sugars onto the activated charcoal column; 2) column washing with water; 3) desorption of sugars with an ethanol gradient. Finally, the effectiveness of the activated charcoal on the mixture desalting was also evaluated.

2.2 MATERIALS AND METHODS

2.2.1 FOS PRODUCTION

A 10 mL pre-culture of *Aureobasidium* sp. stored in glycerol was added to 75 mL of inoculation medium, in a shaken flask (inoculation medium: 100 $g.L^{-1}$ of sucrose, 10 $g.L^{-1}$ of $NaNO_3$, 0.5 $g.L^{-1}$ of KCl, 0.35 $g.L^{-1}$ of K_2SO_4, 0.5 $g.L^{-1}$ of $MgSO_4.7H_2O$, 7.89 $g.L^{-1}$ of KH_2PO_4 and 0.01 $g.L^{-1}$ of $FeSO_4.7H_2O$). The inoculum was grown for 3 days at 28 ºC and 140 rpm and afterwards transferred to a 5L bioreactor - Model micro DCU system, equipped with Twin and MCU-200 controllers (B. Braun Biotech International, Melsungen, Germany) using a working volume of 4L of culture medium (culture medium: 200 $g.L^{-1}$ sucrose, 20 $g.L^{-1}$ $NaNO_3$ and the same salt concentrations as the ones used in the inoculum medium). Fermentations were carried out at 28 ºC, 300 rpm and pH 5.0. Several samples were taken at different points in time in order to evaluate their sugar profile. After detecting the production of the highest FOS molecule (GF_4) by High-Performance Liquid Chromatography (HPLC), the fermentation was stopped and cells were removed by filtration with a paper filter, followed by a microfiltration with a 0.2 µm cut-off cellulose acetate filter. Residual proteins were removed by filtration with a centrifugal filter Amicon Ultra-15 (50 NMWL) from Millipore, at 4000 g for 10 min. Afterwards, the cell free fermentative broth was stored at -18ºC for further purification. All chemicals used were of analytical grade.

2.2.2 ADSORBENT

The adsorbent used in all the purification experiments was extra pure activated charcoal, supplied by Merck (Darmstadt, Germany) in a granular form, with 1.5 mm of mean particle diameter.

2.2.3 ADSORPTION KINETIC STUDIES

According to previous studies (*data not shown*), a volume of 35 mL of fermentative broth was added to 18 g of activated charcoal contained in a shaken flask. Adsorption was held for 5.5 h, at 25 ºC, and 140 rpm of agitation. Samples were collected during the adsorption process and sugars from the liquid phase were analyzed by HPLC. Experiments were carried out in duplicate.

The concentration of sugars adsorbed onto the resin, defined as the loading (q), was determined according to the following equation:

$$q = \frac{(C_o - C) * V}{m} \quad (2.1)$$

where C_o and C are the initial concentration and the concentrations at time t or at the equilibrium, respectively, V is the volume of solution and m the dried activated charcoal mass.

2.2.4 ACTIVATED CHARCOAL COLUMN

Before filling the column, activated charcoal was washed and autoclaved to remove particles and air from the pores. Next, 180 g of adsorbent was loaded into a column glass (33 x 4.5 cm internal diameter), previously filled with Milli-Q pure water. Milli-Q pure water was pumped from the bottom of the column using a peristaltic pump (Watson Marlow) in order to equilibrate the charcoal inside the column. To remove the fines, the pump needs to work at a higher flow-rate than the one which was used to purify the sugars (25 mL/min), although this flow-rate should not be too high to prevent the charcoal elution. To avoid air entrapment in the charcoal bed and reduce the formation of preferential channels, the system was operated in an up-flow regime.

2.2.5 FOS PURIFICATION

Activated charcoal column was loaded with 455 mL of fermentative broth at a flow rate of 25 mL/min. The first 200 mL of the outlet stream was discarded. The broth was re-circulated until the equilibrium between the sorbent and the moving phase was obtained. Samples were collected from the column outlet for sugars quantification by HPLC.

To remove the non adsorbed sugars, 7 L of Milli-Q pure water was passed through the column. Samples were continuously withdrawn from the column outlet and analyzed by HPLC to guarantee that all the non adsorbed sugars were removed. The retained sugars were then recovered by elution with a gradient of ethanol (up to 50% (v/v)). All experiments were performed at 25 ºC.

Fractions collected in the desorption phase were evaporated at 60 ºC, to remove ethanol and concentrate sugars using a rotavapor (B. Braun Biotech International), and further freeze-dried (Heidolph lyophilizer).

The sodium, magnesium and potassium ions concentration was determined by flame atomic absorption spectrophotometry (Varian Spectra AA-400) in the final fractions and compared with the initial fermentative broth.

2.2.6 SUGARS ANALYSIS

Samples were analysed by HPLC using a Jasco device equipped with a refractive index detector and a Prevail Carbohydrate ES (5 μm, 250 × 4.6 mm) (Alltech). The mobile phase consisted of acetonitrile (HPLC grade; Carlo Erba) and water (70:30 v/v), with 0.04% of ammonium hydroxide in the water (HPLC grade; Sigma). Elution was conducted at 1 mL/min flow rate and at room temperature (Dias *et al.*, 2009; Nobre *et al.*, 2009).

FOS standards used to determine the calibration curves were purchased from Wako, Chemicals GmbH, (Germany). Sucrose, fructose and glucose (analytical grade) were obtained from Merck.

2.3 RESULTS & DISCUSSION

2.3.1 FOS PRODUCTION

The sugar profile obtained in the fermentation of sucrose into FOS is illustrated in Fig. 2.1. Operation parameters were previously optimized in order to obtain the maximum production yield of FOS (*unpublished data*). A mass yield of 56% of FOS in total sugars was achieved at 50 h of fermentation, which is in accordance with the results from Sangeetha and co-workers (Sangeetha *et al.*, 2004). These authors reported a yield up to 57% (w/w) of FOS using a culture broth homogenate of *Aureobasidium* sp.

However, it is important to notice that when the maximum yield is reached, the last sugar being produced, GF_4, is usually present in residual amounts. Therefore, as a process for the purification of the whole FOS mixture is envisaged, the fermentative broth was collected after this point and before GF_2 starts to be degraded with consequent production of high contents of glucose. Based on this, the fermentation was stopped at 98 h of operation.

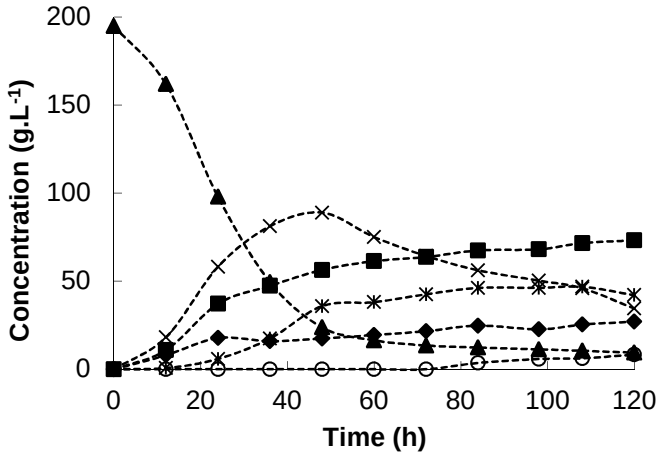

Fig. 2.1 FOS production by *Aureobasidium* sp. Symbols: ◆F, ■G, ▲S, × GF_2, ∗GF_3, and ○GF_4.

2.3.2 ADSORPTION

2.3.2.1 Adsorption assays

In order to evaluate the activated charcoal potential as an adsorbent for the purification of FOS from a fermentative broth mixture, equilibrium and adsorption kinetic studies were conducted in shaken flasks.

Adsorption curves demonstrated that sugars are selectively adsorbed according to their molecular weights (Fig. 2.2). Therefore, the sugar retention increased in the following order: $GF_4 > GF_3 > GF_2 > S > G > F$.

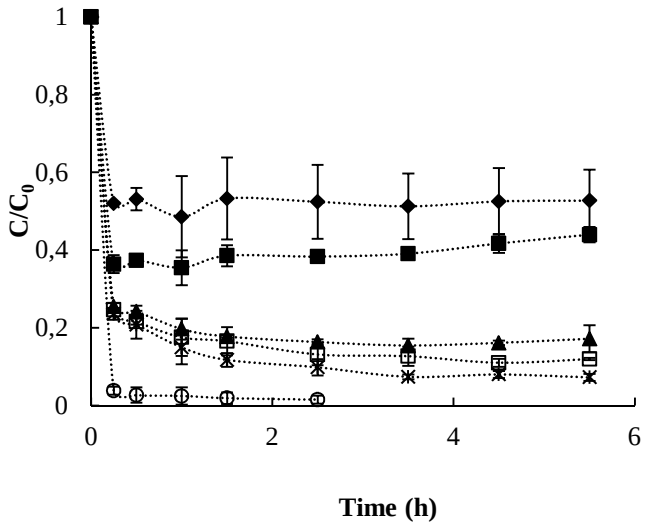

Fig. 2.2 Adsorption profile of ◆F, ■G, ▲S, □GF_2, ✳GF_3, and ○GF_4 from a fermentative broth, adsorbed on activated charcoal, in a shaken flask at 25ºC and 140 rpm.

The adsorption behavior of each adsorbate (in this case each sugar) to a specific activated charcoal depends on the micropores diameter distribution of the granular activated charcoal (Hidaka *et al*., 1981). The strength of the interaction between the adsorbate and activated charcoal increases with increasing molecular weight and size of the adsorbate molecules. According to Hung and co-workers (2005), an adsorption dependence on the molecular weight and size occurs for highly agitated batch reactors,

such as the system used in the current work. Therefore, it is expected that oligosaccharides adsorb more than the smaller saccharides (e.g. glucose or fructose) present in a given mixture.

In addition, a major portion of the carbon surface of the activated charcoal is non polar or hydrophobic (Hung *et al.*, 2005). Accordingly, the more hydrophobic the analyte is, the more retained it will be in the charcoal (Abe *et al*., 1983). Moreover, the hydrophobic character of the sugar is related with the extent of the CH groups surface area present in the saccharide structure (Sundari and Balasubramanian, 1997). Therefore, as oligosaccharides have an increased chain of CH groups, it is likely that they are more adsorbed to the activated charcoal than other small saccharides.

The type, position and orientation of the functional groups that contact with the adsorbent are also relevant for the retention process (Koizumi, 1996). Although glucose and fructose have the same molecular weight, glucose was found to adsorb more onto activated charcoal than fructose, which may be related with the accessibility of the CH groups of glucose (Sundari and Balasubramanian, 1997). Accordingly, other authors also reported a higher adsorption of glucose in comparison to fructose (Abe *et al.*, 1983; Whistler and Durso, 1950). However, it is important to note that glucose is present in higher concentrations in the fermentative broth than fructose, and charcoal is known to retain according to the amount of adsorbent in water, thus glucose is expected to be more adsorbed than fructose.

In the current work, the adsorption process was very fast and the equilibrium was reached for the monosaccharides during the first 15 minutes. The remaining saccharides present in the mixture reached the equilibrium after around 2 h of contact. Therefore, for the following studies conducted in the activated charcoal column, a 3 h contact time was selected. For the shaken flask experiments, the equilibrium loading (q) calculated for FOS and mono- with disaccharides adsorbed onto activated charcoal was 223 and 150 mg, respectively, per g of activated charcoal.

2.3.2.2 Adsorption kinetic models

Different kinetic models such as pseudo-first order (Lagergren, 1898) and pseudo-second order (Ho and Mckay, 1999) have been used to predict the mechanism involved in the adsorption processes. In the present study the application of the pseudo-first and pseudo-second order models were tested for the sorption of FOS from a fermentative broth onto activated charcoal.

The pseudo-first order model is defined according to the following equation:

$$\frac{dq_t}{dt} = k_1(q_1 - q_t) \tag{2.2}$$

where q_t and q_1 are the amount of sugar adsorbed at time *t* and equilibrium, respectively, and k_1 is the pseudo-first order rate constant.

The pseudo-second order equation is defined by the following equation:

$$\frac{\mathrm{d}q_t}{\mathrm{d}t} = k_2(q_2 - q_t)^2 \tag{2.3}$$

where q_2 is the amount of sugar adsorbed at equilibrium and k_2 is the pseudo-second order rate constant.

Plots representing the linear equations of the first- and second-order model (Eqs. 2.2 and 2.3) are shown in Fig. 2.3. Values of the adsorption rates - k_1 and k_2 - and the respective loading equilibrium - q_1 and q_2 - were calculated from the slopes and intercepts of the linearized models (Table 2.1).

The pseudo-second order model seem to better describe the FOS adsorption mechanism since the coefficients of determination (R^2) obtained are higher compared to the ones obtained for the pseudo-first order model. Moreover, the pseudo-second order model predicted the adsorption behavior for the whole range of contact time studied, while the pseudo-first order model was only applicable for the initial contact time. Accordingly, Ho and McKay (1998) reported that in most cases the pseudo-first order model is generally applicable only for the initial contact time.

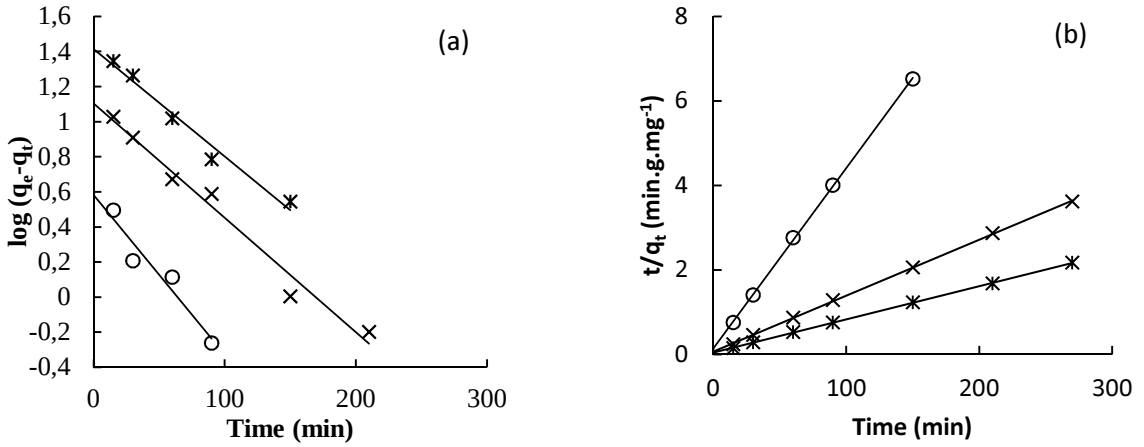

Fig. 2.3 Linearized data of (a) the pseudo-first-order kinetic model and (b) the pseudo-second-order kinetic model for × GF_2, ∗GF_3, and ○GF_4 adsorption onto activated charcoal.

Table 2.1 Coefficients of determination of the linearized pseudo-first and pseudo-second order models and the respective modeled and experimental parameters.

Sugar	**Pseudo-first order**			**Pseudo-second order**			**Experimental**
	q_1 ($mg.g^{-1}$)	k_1 (min^{-1})	R^2	q_2 ($mg.g^{-1}$)	k_2 ($g.mg^{-1}.min^{-1}$)	R^2	q_e ($mg.g^{-1}$)
GF_2	12.7	$1.50x10^{-2}$	0.978	75.8	$2.68x10^{-3}$	1.000	74.0
GF_3	25.9	$1.40x10^{-2}$	0.975	126.6	$1.53x10^{-3}$	0.999	125.6
GF_4	3.8	$2.10x10^{-2}$	0.934	23.4	$1.28x10^{-3}$	1.000	23.0

Finally, the pseudo-second order model was found to describe perfectly the adsorption of FOS onto activated charcoal as the R^2 values for all sugars are very high and the equilibrium capacities, q_2, determined by the model, are in agreement with the experimental q_e values.

2.3.3 FOS PURIFICATION

Several experiments were previously conducted in order to determine the best operating parameters for purifying FOS using a fixed bed activated charcoal column (*data not shown*). Parameters to be used in the process, such as flow rate, recirculation time, water volume, ethanol volume and percentage, were rigorously studied.

Since the adsorption process is exothermic (Inglezakis and Poulopoulos, 2006), the use of high temperatures can reduce the degree of adsorption. Based on that, and envisaging a lower impact on the process costs, a 25ºC room temperature was used in the current work.

The fermentative broth used in the column experiments had the following composition in mono- and disaccharides: 23.74 $g.L^{-1}$ of F, 58.34 $g.L^{-1}$ of G and 9.87 $g.L^{-1}$ of S. Moreover, the FOS composition of the fermentative broth was: 41.88 $g.L^{-1}$ of GF_2, 47.98 $g.L^{-1}$ of GF_3 and 4.16 $g.L^{-1}$ of GF_4. Hence, the initial percentage of FOS in total sugars was 50.6% (w/w).

2.3.3.1 Column adsorption (Step 1)

After conditioning the column, the charcoal was loaded with sugars by recirculation of the fermentative broth. To avoid dilution of the broth, the first 200 mL of the outlet stream containing the water from the dead volume and the first eluted sugars in very low concentrations, were discarded. In this step, 3.9% (w/w) of the FOS present in the initial mixture were lost.

The broth was kept in recirculation until the equilibrium between the concentration of sugars in the liquid and solid phases was reached. As shown in Fig. 2.4, the ratio C/C_0 stabilizes after 1.5 h of contact time. These results are in accordance with the ones previously obtained in the shaken flask experiments (adsorption assays). However, to assure that the maximum amount of sugars were adsorbed during the loading phase, the fermentative broth was kept in recirculation during 3h.

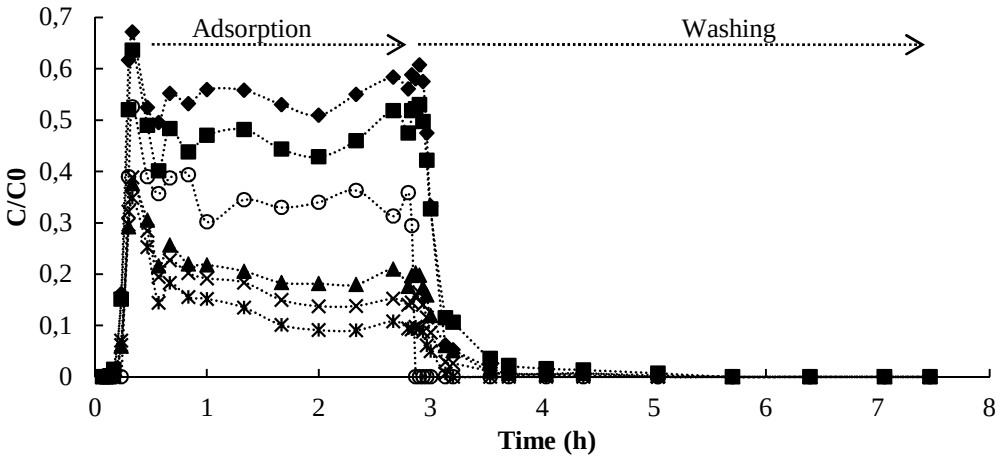

Fig. 2.4 Adsorption and desorption kinetics of ◆F, ■G, ▲S, × GF_2, ✳GF_3, and ○GF_4 from a fermentative broth on activated charcoal in a fixed column during the recirculation and washing phases (Step 1 and 2).

The ratios C/C_0 obtained in the adsorption phase, at the equilibrium, were 0.55 ± 0.02 for F; 0.46 ± 0.03 for G; 0.19 ± 0.01 for S; 0.14 ± 0.01 for GF_2; 0.10 ±0.01 for GF_3; and 0.34 ± 0.02 for GF_4. As expected, the C/C_0 ratio increased as the molecular weight of the sugar decreased, except for GF_4. The amount of GF_4 in the fermentative broth was very low (C_0 = 4.16 $g.L^{-1}$), thus the concentration in the equilibrium was also very low and difficult to measure. Therefore, the C calculated for GF_4 may have been overestimated leading to the high C/C_0 ratios observed.

Results show that the least adsorbed sugars from the fermentative broth were fructose and glucose. Only 53.2% and 62.5% (w/w) of the fructose and glucose contained in the initial broth were adsorbed. Furthermore, 83.9%, 86.9%, 90.4% and 95.1% (w/w) of the S, GF_2, GF_3 and GF_4 from the fermentative broth, respectively, were adsorbed onto the activated charcoal column. In summary, only 24% (w/w) of the total sugars contained in the fermentative broth were not adsorbed and were initially discarded. Also, 77% (w/w) of them constitute the small sugars (mono- and disaccharides) that should be eliminated in a FOS purification process. Losses of 7.0% (w/w) of FOS from the fermentative broth were estimated in the adsorption step, namely in the recirculation.

The equilibrium loading (*q*) determined on the column assay was 222 mg of FOS per g of activated charcoal which is in accordance with the results obtained previously on the shaken flask experiments (adsorption assays).

2.3.3.2 Column washing with water (Step 2)

A washing stage was included in the procedure to guarantee that all the non adsorbed sugars were removed and the adsorbed monosaccharides were eluted. Therefore, the washing stage was carried out until no sugar was detected in the mobile phase. During this step, the outlet concentration was monitored by HPLC (Fig. 2.4). After 2 h of washing, the outlet concentration indicated that the liquid phase was free of sugars. Furthermore, to assure a total cleansing, the washing continued for an extra 2 hours.

In this step, the desorption ratio representing the amount of sugar desorbed per adsorbed sugar was: 96% (F), 93% (G), 25% (S), 13% (GF_2), 7% (GF_3) and 1% (GF_4) (w/w). Almost all the adsorbed monosacharides (step 1) were removed during the washing step. Moreover, FOS losses were estimated to be only 8.4% (w/w).

2.3.3.3 Desorption (Step 3)

As previously demonstrated by the kinetic studies (section 3.2) and the adsorption onto the column (section 3.3.1), sugars were selectively adsorbed onto activated charcoal. FOS were adsorbed more strongly than mono- and disaccharides. Thus, an adequate desorption strength should be used in order to selectively recover the sugars.

Several authors used water or very small percentages of ethanol to recover monosaccharides. As for disaccharides recovery, percentages between 5 and 10 % of ethanol in water have been used. Oligosaccharides have been recovered using 15 to 50% of ethanol in water (Hidaka *et al.*, 1988; Kaplan and Hutkins, 2000; Morales *et al.*, 2006; Sanz *et al.*, 2005; Swallow and Low, 1990; Whistler and Durso, 1950). Therefore, the volume and percentage of ethanol/water used in each step the desorption process was critically evaluated. Taking into account previous (*data not shown*) and current data, the

optimized volume and percentage of ethanol were established for the desorption process (Table 2.2).

Table 2.2 Volume and ethanol concentrations used in step 3 to recover the sugars adsorbed onto the activated charcoal column.

% Ethanol (v/v)	1	2	3	4	5	10	20	30	40	50
Volume of elution (L)	0.5	0.5	0.5	0.5	0.5	1	2	2	1	0.5

Since FOS are only desorbed when ethanol concentrations are between 10 and 40 % (v/v), a greater ethanol volume was used during the elution at these concentrations in order to recover all adsorbed FOS. The ethanol concentration that yielded the highest amount of sugars recovery was 20% (v/v). Non-oligosaccharides need a lower strength to desorb, thus, a lower gradual increase of ethanol concentrations was used, up to 5% (v/v). The gradient allowed the removal of more small saccharides at low ethanol concentrations, thus reducing the loss of FOS at the highest ones. In the end of the desorption step, and to assure that all sugars were desorbed, a 50% (v/v) ethanol concentration was used.

Fig. 2.5 shows the desorption ratio of each sugar, representing the percentage of sugar desorbed in step 3 per sugar adsorbed in step 1, and the corresponding composition of the ethanol solution used to collect each fraction.

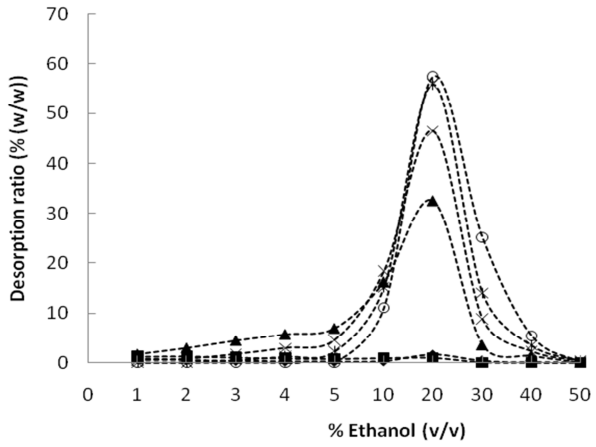

Fig. 2.5 Desorption ratio of sugars (◆F, ■G, ▲S, × GF_2, ∗GF_3, and ○GF_4) collected in each fraction.

Fig. 2.6 illustrates the amount of sugars recovered in each fraction (total mass of sugars per fraction), and the corresponding individual sugar composition percentage (% w/w).

From both figures (Figs. 2.5 and 2.6), a peak can be observed during the desorption step showing that ethanol efficiently desorbs the sugars retained in the column. S and the remaining monosacharides (F and G) were desorbed at ethanol percentages bellow 5% (v/v). The fraction that yielded the highest percentage of FOS (97% (w/w)) was eluted with 30% (v/v) of ethanol. Fractions rich in FOS were obtained with ethanol percentages between 10 and 40% (v/v). The purity of FOS in these fractions was found to be 92.9% (w/w) representing 74.5% (w/w) of FOS from the fed fermentative broth. Furthermore, 32.7 g of FOS were recovered from these rich fractions.

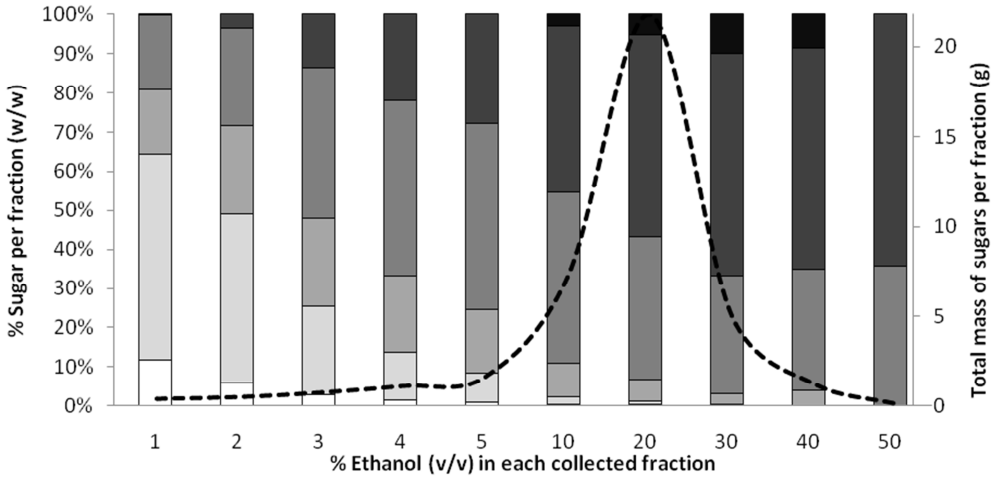

Fig. 2.6 Recovery sugars per fraction desorbed (--) and respective percentage of sugar (□ F ■ G ■ S ■ GF_2 ■ GF_3 ■ GF_4) in each collected fraction.

Through the analysis of the desorption ratios it was also possible observe that S, GF_2, GF_3 and GF_4 were preferentially desorbed with 5, 10, 20 and 30% ethanol, respectively.

2.3.4 DESALTING

As previously discussed, the culture medium used for the microbial production of FOS contains high amounts of salt. Consequently, in the end of the fermentation process many ions are present in the solution. Desalting the fermentative broth constitutes an important step to guarantee certain quality parameters. Therefore as sodium, potassium and magnesium are the main cations present in the fermentation culture medium, their amounts were determined in the recovered pure FOS fractions. Results showed that these pure FOS fractions were free of ions, confirming the effectiveness of activated charcoal in the desalting of the fermentative broth, which is in accordance with several studies that have been reported (Packer *et al.*, 1998; Whistler and Durso, 1950).

2.4 CONCLUSION

Adsorption kinetic experiments show that sugars were selectively adsorbed and that FOS adsorption onto activated charcoal was well described by a pseudo-second order model.

The fermentation of sucrose by *Aureobasidium* sp. to produce FOS resulted in a sugar mixture containing 56% (w/w) of FOS. This fermentative broth was further purified using an activated charcoal column. After the optimization of the ethanol volume and concentration used in the global desorption step, ethanol solutions ranging from 10 to 40% (v/v) were used to recover FOS. These ethanol solutions enabled a FOS recovery of 74.5% (w/w) with a 92.9% (w/w) of purity, thus leading to a FOS product with a higher market value. Moreover, with the proposed process, fractions were recovered with purities up to 97% (w/w) of FOS. Activated charcoal was also found to be very efficient in the desalting of the fermentative broth.

In summary, this work described a simple and efficient process to purify FOS from a fermentative broth using a single column filled with commercial activated charcoal that can be easily applied at a larger scale.

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CHAPTER 3

EQUILIBRIUM STUDIES FOR THE ADSORPTION OF FRUCTO-OLIGOSACCHARIDES ON A DOWEX MONOSPHERE CALCIUM RESIN

ABSTRACT

Fructo-oligosaccharides (FOS), namely kestose, nystose and fructo-furanosylnystose, are prebiotics that can be obtained by fermentation. The resulting fermentative broth is a complex mixture containing also salts and other sugars that must be removed. Adsorption equilibrium studies were conducted using the static method in batch mode. Adsorption of FOS contained in a fermentative broth and purified mixtures onto Dowex Monosphere calcium resin was evaluated at 60ºC. Experimental isotherms data were analyzed using linear, Langmuir/anti-Langmuir, Freundlich, Redlich & Peterson, and Toth type models. Isotherm parameters were estimated using linear and non-linear correlations for the minimization of several error functions. The non-linear correlations were found to provide the best isotherm parameters for the models. The experimental adsorption data of FOS contained in purified mixtures were well fitted with the anti-Langmuir isotherm, while FOS in a fermentative broth were better fitted with Toth and Langmuir isotherms.

3.1 INTRODUCTION

Nowadays, the production of fructo-oligosaccharides (FOS) has gained a large commercial interest due to its beneficial properties in the human health through modulation of the microbiota. Based on these features, FOS such as kestose (GF_2), nystose (GF_3) and fructo-furanosylnystose (GF_4) are named prebiotics (Gibson, 2008).

For large scale production of FOS, fermentative processes appear to be a good alternative due to the possibility of increasing the production yields and obtaining differentiated products. In these processes, FOS are produced from sucrose through fructosyltransferase that is present in several microorganisms (Prapulla et al., 2000). However, the fermentative broth obtained in such processes is a complex mixture of salts and sugars, among other minor components. These sugar mixtures include FOS, but also mono- and disaccharides that must be removed since they have no prebiotic effect.

Among many techniques that have been reported for the fractionation of carbohydrates (Sanz and Martinez-Castro, 2007) Simulated Moving Bed (SMB) seems to be the most efficient at an industrial scale. The main advantage of this continuous process is that it can be used for difficult separations, besides the fact that it presents low selectivity, high productivities and low reagents consumption (Gomes *et al.*, 2006).

In the sugar industry, the SMB chromatography has been mainly used for the separation of glucose from fructose (Azevedo and Rodrigues, 2001); sugarcane molasses (Neuzil, 1982; Saska *et al.*, 1992b); and high-fructose corn syrups (Toumi and Engell, 2004); using sulfonated poly(styrene-*co*-divinylbenzene) (PS-DVB) as adsorbent. The gel-type resins of calcium (Ca^{2+}) are the most popular among the PS-DVB resins used in the sugar industry, especially due to the strong complexation of calcium with fructose (Howard *et al.*, 1988; Beste *et al.*, 2000; Nowak *et al.*, 2007; Luz *et al.*, 2008). The different interaction between calcium and the hydroxyl groups of the sugars enables their separation.

The isotherm parameters of the individual sugars constitute important information for the design of the SMB equipment. Few works addressed the study of the adsorption of FOS

onto ion exchange resins for SMB applications (Kawase *et al.*, 2001; Geisser *et al.*, 2005; Gramblicka and Polakovic, 2007; Vankova *et al.*, 2010a; Vankova *et al.*, 2010b). Therefore, in this work, the adsorption of GF_2, GF_3 and GF_4 onto Dowex Monosphere Ca resin was studied. Adsorption studies were carried out for mixtures of purified FOS and compared with the adsorption of FOS contained in a fermentative broth. This broth was collected at a given time point of a fermentation conducted with *Aureobasidium* sp. (Rocha *et al.*, 2009), and the final mixture also included salts and other small sugars, as mono- and disaccharides, in high concentrations.

Although equilibrium isotherms of sugars onto calcium resins are known to be linear for a range of small sugar concentrations, several authors reported a non-linear adsorption for high sugar concentrations (> 250 g/L) (Ching *et al.*, 1987; Saska *et al.*, 1992a; Beste *et al.*, 2000; Vente *et al.*, 2005; Gramblicka and Polakovic, 2007). In this study, several equilibrium isotherm models were used to fit the experimental data obtained for each FOS, namely linear, Langmuir/anti-Langmuir, Freundlich, Redlich & Peterson, and Toth. The isotherm parameters were determined using linear and non-linear correlations for the minimization of several error functions, enabling the choice of the model that best fitted the experimental results.

3.2 THEORY

3.2.1 ADSORPTION ISOTHERM MODELS

According to Gritti and collaborators (2004), the non-linear isotherms can be divided in two groups depending on their initial curvature. The first group includes the isotherms that have a downward curvature (Group I). This group contains the Langmuirian isotherms, such as Langmuir, Freundlich, Redlich & Peterson, and Toth. The second group includes the isotherms with an upward curvature represented by the anti-Langmuir isotherm (Group II). The limit case of a linear isotherm belongs to both groups.

3.2.1.1 Linear isotherm

In the linear isotherm, the concentration of the solute in the solid phase, *q*, is considered to be proportional to that in the liquid phase, *C*. The linear isotherm is represented by the following equation:

$$q = K.C \tag{3.1}$$

where *K* is the slope of the isotherm representing the distribution coefficient.

Isotherms Group I

3.2.1.2 Langmuir isotherm

The Langmuir isotherm described in Eq. (3.2), takes into account the finite surface occupied by a molecule adsorbed on the surface. It is a model used for homogeneous surfaces, where only a single layer of adsorbed solute molecules is formed, and no lateral interferences between the adsorbed molecules are considered (Guiochon *et al.*, 1994; Schulte and Epping, 2005):

$$q = \frac{MK_L C}{1 + aK_L C} \tag{3.2}$$

being *M* and K_L the isotherm parameters. For the Langmuir isotherm the parameter *a* is set equal to *+1*.

The Langmuir parameters K_L and *M* can be determined by linearization of the previous equation (Eq. (3.2)):

$$\frac{1}{q} = \left(\frac{1}{K_L M}\right)\frac{1}{C} + \frac{a}{M} \tag{3.3}$$

Plotting *1/q versus 1/C* it is possible to calculate the values of *M* and K_L using a linear regression analysis, being (*a/M*) equal to the intersection value with the ordinate axis,

and ($1/K_LM$) equal to the slope value. Furthermore, the equation is thermodynamically consistent and follows the Henry's Law at low concentrations.

3.2.1.3 Freundlich isotherm

The Freundlich isotherm is an empirical model used for heterogeneous surfaces for which there are no significant adsorbate-adsorbate interactions. This model is only applicable in certain concentration limits as this isotherm equation does not follow the Henry's Law behavior at low concentrations, and does not have a finite limit when the concentration is sufficiently high (Duong, 1998). The Freundlich isotherm can be described by the following equation:

$$q = K_F C^{1/n} \tag{3.4}$$

where K_F is the Freundlich isotherm constant and n is a fitting parameter that is greater than 1. Linearization of the Eq. (3.4) using natural logarithms results in a straight line with a slope of ($1/n$) and an intercept of ln (K_F):

$$\ln(q) = \frac{1}{n}\ln(C) + \ln(K_F) \tag{3.5}$$

3.2.1.4 Redlich & Peterson isotherm

The Redlich & Peterson isotherm combines the Langmuir and Freundlich equations. This isotherm includes two parameters related to the adsorption capacity (K_R) and surface energy (a_R), and a third one, r, which varies between 0 and 1. The equation is described as follows (Redlich and Peterson, 1959):

$$q = \frac{K_R C}{1 + a_R C^r} \tag{3.6}$$

It is not possible to determine these parameters using a linear transformation. Therefore, a maximization of the regression coefficient ($R^2 \approx 1$) was conducted to determine those

parameters, which were used afterwards in the linear method. When *r* is equal to 1, the Redlich & Peterson isotherm is simplified into the Langmuir equation. Therefore, the Langmuir parameters with *r* = 1 were used as a first approximation.

3.2.1.5 Toth isotherm

The Toth isotherm is an empirical equation that can be used at low and high concentration ranges. This model is adequate to describe heterogeneous systems, and is represented by the following equation (Duong, 1998):

$$q = \frac{K_T C}{(a_T + C^t)^{1/t}} \tag{3.7}$$

where K_T and a_T are the equation constants and *t* is the Toth isotherm exponent. When *t* is equal to*1* the Toth isotherm is simplified into the Langmuir equation. Thus, the same procedure as the one described for the Redlich & Peterson isotherm was used to find the three parameters.

Isotherms Group II

3.2.1.6 Anti-Langmuir isotherm

The anti-Langmuir isotherm can be also described by Eq. (3.2). This isotherm presents an opposite shape as compared to the Langmuir isotherm. As the curvature of this model is upward, the Langmuir equation term *a* has a value equal to -1. The anti-Langmuir isotherm should be regarded as an empirical equation, because it cannot be derived from a physicochemical background (Kaczmarski *et al.*, 2004).

3.2.2 ERROR FUNCTIONS

Error functions allow the estimation of fitting errors taking into account both the q values calculated by the models (q_{mod}) and the ones obtained in the experiments (q_{exp}). Some functions also consider the number of parameters estimated (p), and the number of experimental points (N). The five error functions used in this work are described in the Table 3.1.

Table 3.1 Error functions.

Error Function	Abbreviation	Expression	Eq.	Reference
Sum square errors	ERRSQ	$\sum_{i=1}^{N}(q_{mod}-q_{exp})_i^2$	(3.8)	(Foo and Hameed, 2010)
Hybrid fractional error function	HYBRID	$\frac{100}{N-p}\sum_{i=1}^{N}\left[\frac{(q_{mod,i}-q_{exp,i})^2}{q_{exp,i}}\right]$	(3.9)	(Ng *et al.*, 2003)
Marquardt's percent standard deviation	MPSD	$100\left[\sqrt{\frac{1}{N-p}\sum_{i=1}^{N}\left[\frac{(q_{mod,i}-q_{exp,i})}{q_{exp,i}}\right]^2}\right]$	(3.10)	(Marquardt, 1963; Ng *et al.*, 2003)
Average relative error	ARE	$\frac{100}{N}\sum_{i=1}^{N}abs\left[\frac{(q_{mod}-q_{exp})}{q_{exp}}\right]_i$	(3.11)	(Kapoor and Yang, 1989)
Sum of absolute errors	EABS	$\sum_{i=1}^{N}abs(q_{mod,i}-q_{exp,i})$	(3.12)	(Foo and Hameed, 2010)

In order to choose the best fitted isotherm model, the sum of the errors calculated for the five above mentioned error functions was considered. Briefly, each error function was minimized and the respective isotherm parameters were determined. Next, these parameters were used to determine the values of the other four error functions. Afterwards, all the error values were summed. Finally, the isotherm parameters were selected based on the lowest sum of the errors (SE).

3.3 EXPERIMENTAL METHODS AND MATERIALS

3.3.1 MATERIALS

A Dowex Monosphere Ca ion-exchange resin (in calcium form), from Dow Chemicals, Midland, MI, was used (Table 3.2).

Table 3.2 Physical and chemical properties of the Dowex Monosphere Ca ion-exchange resin according to the supplier.

Ionic form	Ca^{2+}
Structure	Gel-type
Matrix	Styrene-DVB
Functional group	Sulfonate
Total capacity (eq/L)	>1.5 (H^+ form)
Water content (%)	57-61 (H^+ form)
Volume median diameter (µm)	300-330

The fermentative broth consisted of a complex mixture obtained in our lab from the fermentation of sucrose by *Aureobasidium* sp. (Rocha *et al.*, 2009). Biomass and residual proteins were removed from the broth by centrifugation and filtration with a centrifugal filter Amicon Ultra-15 (50 NMWL) from Millipore, at 4000 g for 10 min. The FOS-rich mixture contained 9, 71 and 72 $g.L^{-1}$ of GF_4, GF_3 and GF_2, respectively, corresponding to 38% (w/w) of FOS in total sugars. This mixture was also rich in fructose, glucose, sucrose and salts. The cations present in the mixture were mainly sodium (3.3 $g.L^{-1}$) and potassium (1.7 $g.L^{-1}$). To avoid any calcium precipitation, the fermentative broth was previously passed through a calcium column. The volume of resin used in this pre-treatment corresponded to half of the volume of fermentative broth treated. Preliminary experiments proved that this ratio is enough to guarantee the saturation of the broth in calcium ions and the elimination of total sodium and potassium ions (*data not shown*).

Mixtures of purified FOS (with residual amounts of other sugars and without salts) were obtained in our lab by purification of the FOS-rich mixture from the fermentative broth using a column with activated charcoal (for details see Chapter 2). These FOS-rich mixtures contained 19, 94 and 77 $g.L^{-1}$ of GF_4, GF_3 and GF_2, respectively; representing 90% (w/w) of FOS in total sugars.

FOS standards used for High Performance Liquid Chromatography analysis (HPLC), containing GF_2, GF_3 and GF_4, were purchased from Wako, Chemicals GmbH, Germany.

3.3.2 DETERMINATION OF ADSORPTION ISOTHERMS

A static method, specifically the adsorption-desorption mode in batch, was used to obtain the adsorption isotherms. Resins were washed several times with double distilled water. Additionally, the water content in excess was removed from the resin by filtration. A volume of 3.5 mL of sugars solution with different initial sugar concentration from purified FOS mixture or fermentative broth were added to 1.50 ± 0.01 g of wet resin. Adsorption was held for 8 h under agitation at 60ºC. This time was chosen based on preliminary experiments (*data not shown*) as it was proved to be enough to reach equilibrium without the occurrence of hydrolysis. The experiments were carried out in duplicate. Five samples of the wet resin were dried at 105ºC to determine the resin water content.

For determining the adsorption isotherms, the solution of purified FOS was diluted several times, in order to have a range of total sugar concentrations between 20 and 220 $g.L^{-1}$. The same procedure was adopted for the fermentative broth, being the final range of concentrations in total sugars between 50 and 360 $g.L^{-1.}$

Before desorption of sugars, the bulk solution was decanted and the resin was washed three times with double distilled water. A 5 mL water volume was added to the resin, and desorption of the sugars was conducted overnight, under agitation, at 25 ºC.

Sugar concentrations were determined by HPLC. The HPLC equipment consisted of a Knauer chromatograph (Berlin, Germany) equipped with a LCP 4020 pump (Ecom,

Prague, Czech Republic) and an on-line degasser model A1050 (Knauer, Berlin, Germany). A Gilson (Middleton, WI) automated injector with a 10 µl loop was used. A differential refractive index detector type 298.00 (Knauer, Berlin, Germany) operating at 30ºC was used. The column used was a REZEX RSO-Oligosaccharide (Ag^+ form, 200x10 mm, Phenomenex, Torrance, CA). Analysis were carried out in isocratic mode at 40 °C using a 0.3 $mL.min^{-1}$ flow rate of water as eluent (Gramblicka and Polakovic, 2007).

3.3.3 DETERMINATION OF ADSORPTION PARAMETERS

Adsorption equilibrium parameters determined for each sugar were obtained from the experiments conducted with a mixture of purified FOS and with the fermentative broth. The equilibrium data were represented by plotting the equilibrium solid phase concentrations of sugar, defined as the equilibrium loading (q), that correspond to the mass of sugar adsorbed per mass of resin, *versus* the equilibrium concentrations of the sugar in the liquid phase (C).

The equilibrium loading (q) required to calculate the parameters in all the models evaluated in this work, was determined according to the following equation:

$$q = \frac{(C_{ads} * V)}{m} \tag{3.13}$$

where C_{ads} is the concentration of the adsorbed sugar, V the volume of solution and m the dried resin mass.

3.3.4 ISOTHERMS DATA PROCESSING

Linearization of isotherms, such as Langmuir and Freundlich, is the most frequently used approach to determine the isotherm parameters. However, some researchers also used non-linear methods to successfully estimate those parameters (Ho *et al.*, 2002; Ng *et al.*, 2003; Ho *et al.*, 2005; Hadi *et al.*, 2010). These non-linear approaches use error functions, as described in the section 3.2.2, which must be minimized to obtain the best

fit between model and experimental data. The sum of the squares is the type of error most commonly used, although others can also be considered. In this work, five types of error functions were assessed, in order to identify the one that predicts the parameters with a minimum error.

Identification of the parameters using the non-linear method requires that initial estimates are established for each of them. For the isotherms for which a linearization is possible (Langmuir/anti-Langmuir and Freundlich), the parameters determined using the respective linearized equation (Eqs. (3.3) and (3.5)) were used as initial estimates. For Toth and Redlich & Peterson isotherms (three parameters isotherms), the initial estimates were set equal to the parameters values obtained from the Langmuir isotherm assuming that β and t were equal to 1. Using the *solver add-in* of Microsoft Excel (Microsoft Corporation, 2007), the parameters were determined by minimizing the error function. Since this approach can lead to local minima, the process was repeated several times using different initial estimates of the parameters to guarantee that a global minimum is obtained. Furthermore, the parameters obtained using the other error functions were also used as initial estimates. Therefore, using this procedure it was virtually assured that the solution converges to a global minimum.

3.4 RESULTS & DISCUSSION

3.4.1 MIXTURE OF PURIFIED FOS

Due to the low availability of single FOS and their prohibitive costs, a mixture of the three purified FOS was used in this work to determine the adsorption behavior of each sugar onto Dowex Monosphere Ca.

Figs. 3.1 to 3.3 show the adsorption isotherms obtained for each one of the three FOS contained in the purified mixture. Plotting the experimental data revealed that the amount of adsorbed sugars onto the resin increased almost linearly with the increasing sugar concentration of the solution. However, the isotherms appear to have an upward

curvature with increasing sugar concentration in the bulk. Thus, due to the upward shape of the isotherms, the experimental data were fitted with the anti-Langmuir model and compared with the linear model (Tables 3.3 to 3.5).

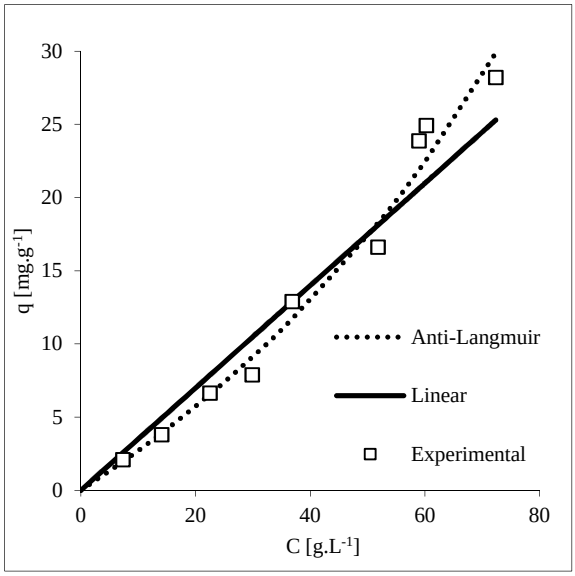

Fig. 3.1 Adsorption isotherms for GF_2 in the purified mixture using the parameters estimated with the HYBRID function.

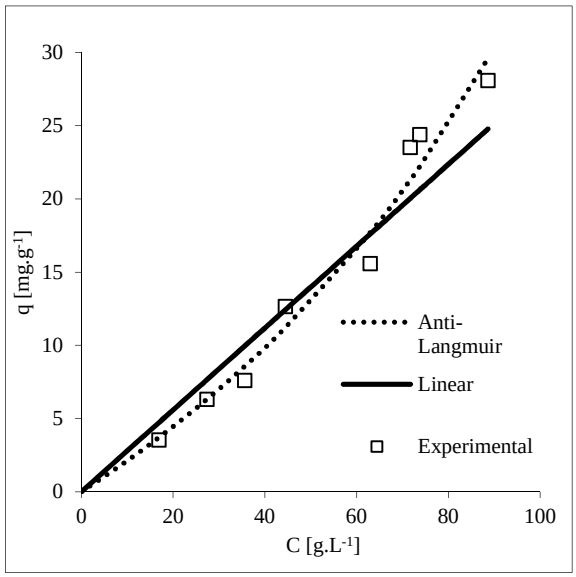

Fig. 3.2 Adsorption isotherms for GF_3 in the purified mixture using the parameters estimated with the HYBRID function.

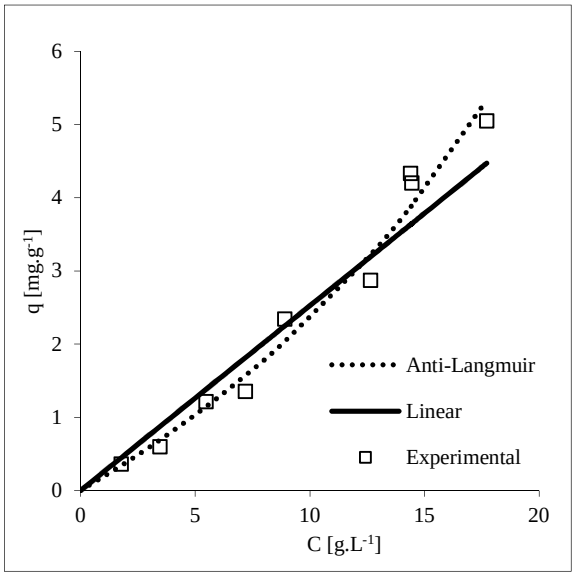

Fig. 3.3 Adsorption isotherms for GF_4 in the purified mixture using the parameters estimated with the HYBRID function.

The upward isotherm shape is related to the increase in the sugar activity coefficient with increasing solute concentrations in the liquid phase (Tiihonen et al., 1999). The adsorption process involves the hygroscopic water available in the resin pores and the diffusion of the sugar molecules into the pores. When the sugar concentration in the liquid phase is very high, the diffusion of sugars into the pores is accelerated due to the shrink of the resin that occurs with the displacing of the hygroscopic water from the micropores to the liquid phase. The increasing sugar concentration in the liquid favours this process, since the sugar clusters are known to have smaller diameters and are less hydrated at high concentrations (Saska et al., 1992a). Consequently, the uptake of the adsorbent increases substantially at high sugar concentrations, thus leading to isotherms with an anti-Langmuir shape.

Table 3.3 Adsorption isotherm parameters obtained for GF_2 in a mixture of purified FOS according to the error function

		Linear	ERRSQ	HYBRID	MPSD	ARE	EABS
Linear isotherm	K ($L.kg^{-1}$)	0.37	0.37	**0.35**	0.31	0.29	0.39
	R^2	0.954	(linear)	0.940			
	ERRSQ	35.48	35.48	45.66	98.80	148.79	40.20
	HYBRID	44.69	44.69	37.54	53.49	76.53	57.74
	MPSD	26.22	26.22	20.92	17.25	18.50	30.49
	ARE	20.51	20.51	17.09	14.64	14.42	22.85
	EABS	16.27	16.27	16.96	21.23	25.36	15.80
	SE	143.16	143.16	**138.16**	205.41	283.59	167.07
Anti-Langmuir	K_L ($L.g^{-1}$)	0.004	0.005	**0.005**	0.005	0.005	0.004
	M ($mg.g^{-1}$)	73.86	59.83	**49.12**	48.05	49.62	77.66
	R^2	0.995	(linear)	0.977			
	ERRSQ	34.74	16.93	17.73	18.21	17.65	19.89
	HYBRID	23.14	14.69	13.53	13.64	13.74	17.26
	MPSD	11.39	10.91	9.64	9.59	9.84	12.13
	ARE	8.28	7.98	7.54	7.60	7.50	8.94
	EABS	12.63	10.23	10.32	10.39	10.28	10.16
	SE	90.17	60.74	**58.77**	59.44	59.01	68.37

(Bold-labeled data represent the minimum sum of the errors and the respective isotherm parameters that best represent the experimental data).

Table 3.4 Adsorption isotherm parameters obtained for GF_3 in a mixture of purified FOS according to the error function.

		Linear	ERRSQ	HYBRID	MPSD	ARE	EABS
Linear isotherm	K ($L.kg^{-1}$)	0.30	0.30	**0.28**	0.25	0.23	0.32
	R^2	0.948	(linear)	0.933			
	ERRSQ	38.91	38.91	50.33	107.47	170.24	46.31
	HYBRID	49.45	49.45	41.23	58.50	88.18	67.99
	MPSD	27.86	27.86	22.01	18.09	19.84	33.46
	ARE	21.85	21.85	18.40	15.24	14.73	24.90
	EABS	16.97	16.97	18.14	21.94	26.57	16.34
	SE	155.03	155.03	**150.12**	221.23	319.55	189.01
Anti-Langmuir	K_L ($L.g^{-1}$)	0.003	0.004	**0.004**	0.004	0.004	0.004
	M ($mg.g^{-1}$)	82.22	54.94	**45.73**	45.16	48.35	48.35
	R^2	0.992	(linear)	0.974			
	ERRSQ	49.38	18.54	19.32	19.86	20.60	20.60
	HYBRID	32.20	16.55	15.42	15.55	16.02	16.02
	MPSD	13.25	11.68	10.51	10.46	10.53	10.53
	ARE	10.30	9.09	8.38	8.36	8.23	8.23
	EABS	15.48	10.80	10.77	10.78	10.56	10.56
	SE	120.62	66.66	**64.40**	65.01	65.93	65.93

(Bold-labeled data represent the minimum sum of the errors and the respective isotherm parameters that best represent the experimental data).

Table 3.5 Adsorption isotherm parameters obtained for GF_4 in a mixture of purified FOS according to the error function.

		Linear	ERRSQ	HYBRID	MPSD	ARE	EABS
Linear isotherm	K ($L.kg^{-1}$)	0.27	0.27	**0.25**	0.22	0.22	0.29
	R^2	0.950 (linear)		0.935			
	ERRSQ	1.20	1.20	1.57	3.70	3.72	1.42
	HYBRID	9.24	9.24	7.77	11.54	11.60	12.34
	MPSD	30.23	30.23	24.39	20.02	20.02	35.45
	ARE	22.23	22.23	19.31	16.17	16.17	25.13
	EABS	2.92	2.92	3.24	4.10	4.11	2.82
	SE	65.83	65.83	**56.28**	55.54	55.62	77.15
Anti-Langmuir	K_L ($L.g^{-1}$)	0.016	0.019	**0.022**	0.023	0.021	0.017
	M ($mg.g^{-1}$)	12.29	10.71	**8.38**	7.58	9.41	11.74
	R^2	0.985 (linear)		0.972	0.970	0.971	0.970
	ERRSQ	1.41	0.63	0.67	0.71	0.71	0.71
	HYBRID	5.56	3.54	3.21	3.29	3.80	3.67
	MPSD	14.33	14.61	12.43	12.16	14.67	14.31
	ARE	11.38	10.13	10.23	10.40	10.06	9.95
	EABS	2.71	1.98	2.11	2.18	2.04	1.94
	SE	35.39	30.89	**28.64**	28.74	31.28	30.59

(Bold-labeled data represent the minimum sum of the errors and the respective isotherm parameters that best represent the experimental data).

The coefficient of determination (R^2) of the linear regression analysis was 0.95 for the three sugars studied, suggesting a poor fitting of the experimental data by this method. However, when the HYBRID function was chosen to minimize the error, the SE value decreased. It should be noted that the ERRSQ error function is the same that is used when the parameters are estimated by linear regression. Thus, both methodologies (linear regression analysis and ERRSQ) lead to the same results for the linear isotherm model.

Among the distribution coefficients calculated, the values that best described the equilibrium data were 0.35, 0.28 and 0.25 $L.kg^{-1}$ for GF_2, GF_3 and GF_4, respectively (Tables 3.3 to 3.5).

The adsorption mechanism onto Dowex Monosphere Ca resin for larger carbohydrates is mainly pore partitioning affected primarily by the size exclusion effect (Howard *et al.*, 1988). The decreasing distribution coefficients found for increasing molecular weights of the sugars, $GF_2 > GF_3 > GF_4$, confirmed the size exclusion effect. Besides, the small values of the distribution coefficients found ($K < 1$ $L.kg^{-1}$) also suggested that adsorption

is mainly driven by partition affected by steric effects. Taking into account the ion-exchange capacity of the resin provided by the supplier, these distribution coefficient values are very low (Gramblicka and Polakovic, 2007). However, as the size of the adsorbent particle pores is similar to the size of the hydrated sugar molecules, the migration of sugars towards the inside of the pores is limited (Saska *et al.*, 1992a). Moreover, small sugar molecules tend to block the pores preventing their adsorption to the active sites that are still free inside the pores. Thus, these phenomena could explain the lower sorption obtained as compared to the supplier guidelines.

In addition, isotherms were also fitted with the anti-Langmuir model. The anti-Langmuir isotherm parameters, M and K_L, determined for the three sugars are given in Tables 3.3 to 3.5.

To determine the anti-Langmuir parameters, the linearization of Eq. (3.2) is generally used. The values of R^2 found for these isotherms, when a linearization approach was used, were greater than 0.98, suggesting that this model has a satisfactory fitting for all the sugars studied. The non-linear analysis, using the error functions to determine the isotherm parameters of the anti-Langmuir isotherm, yielded even smaller SE values compared with the values obtained for the linearization approach. Also, the error function that allowed better fitting results was the HYBRID function.

Comparing the sum of the five errors (SE) obtained for the two model isotherms discussed above, it was found that the values obtained for the anti-Langmuir isotherm were much smaller than the ones obtained for the linear model (Tables 3.3 to 3.5). Regarding the R^2 values, it was found that, both for the linear and non-linear regressions, they were much higher for the anti-Langmuir than for the linear isotherms. Therefore, the results indicate that anti-Langmuir isotherm is the model that best described the adsorption of FOS in purified mixtures onto the Dowex Monosphere Ca resin. Several researchers reported upward isotherms for carbohydrates adsorption onto calcium columns (Ching *et al.*, 1987; Saska *et al.*, 1992a; Beste *et al.*, 2000). Nowak and collaborators (2007) successfully predicted the adsorption isotherms of several sugars using the anti-Langmuir model. Therefore, the results presented in the current work are in good agreement with the ones reported in the literature.

3.4.2 FOS IN A FERMENTATIVE BROTH

In the fermentative broth mixtures, FOS isotherms were found to have a downward curvature as illustrated in Figs. 3.4 to 3.6. Therefore, the experimental data were fitted with isotherm models adequate to describe adsorption data with this shape. The isotherm models chosen belong to the group I, namely Langmuir, Redlich & Peterson, Freundlich and Toth. The results obtained with these models were afterwards compared with the ones obtained using the linear model.

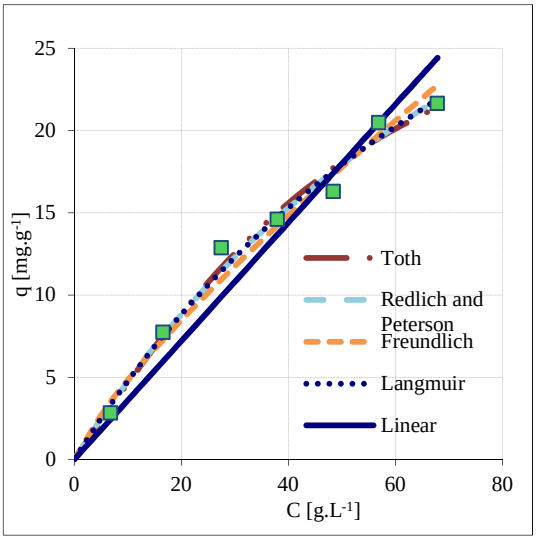

Fig. 3.4 Adsorption isotherms for GF_2 in the FOS-rich broth mixture using the parameters estimated with the best error function.

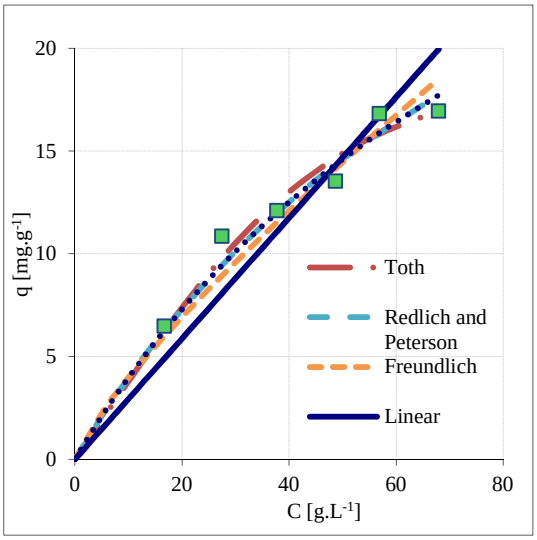

Fig. 3.5 Adsorption isotherms for GF_3 in the FOS-rich broth mixture using the parameters estimated with the HYBRID function.

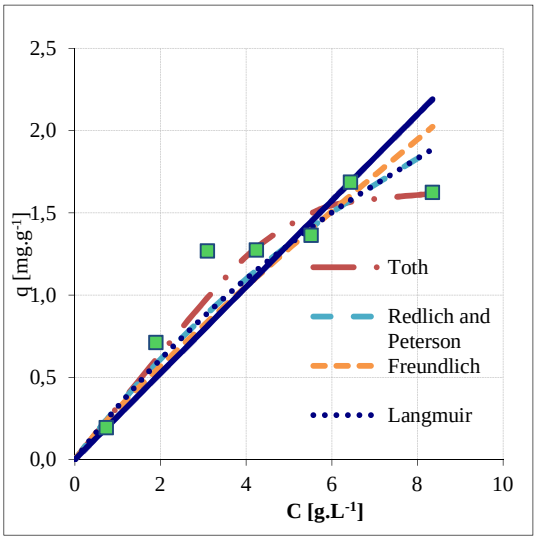

Fig. 3.6 Adsorption isotherms for GF_4 in the FOS-rich broth mixture using the parameters estimated with the best error function.

Regardless of the use of these isotherm models to fit the experimental data, it should be emphasized that those correlations were used in this work just as empirical models and that these results are discussed only qualitatively. The main mechanism of sorption in ion-exchange PS-DVB resins is pore partitioning, affected by the limited accessibility of

the internal surface of the resin to the sugar molecules, due to the pore blocking effect (Saska *et al.*, 1992a). Therefore, the sorption is not dependent on the specific pore surface area and cannot be directly related with the adsorbent active sites.

The R^2 values found for the linear model, when a linear regression approach was used, were low ($R^2 < 0.92$) (Tables 3.6 to 3.8), thus indicating that sugar adsorption, in these conditions, is not well described by the linear isotherms. Even so, values obtained for the distribution coefficients were 0.36, 0.29 and 0.26 $L.kg^{-1}$ for GF_2, GF_3 and GF_4, respectively. The higher values of the smaller sugars confirmed once more the existence of a size exclusion effect.

On the other hand, R^2 values obtained for the linearization of the Langmuir isotherm were greater than 0.97, indicating accurate satisfactory description of the experimental data (Tables 3.6 to 3.8).

The Redlich & Peterson equation, as mentioned above, is a combination of the Freundlich and Langmuir isotherms. When the parameter *r* tends to zero this equation is simplified into the linear isotherm. On the other hand, if *r* value tends to one, this equation is transformed into the Langmuir isotherm. For the three FOS contained in the fermentative broth, the *r* value was found to be *1*. Therefore, the adsorption behavior was confirmed to be well fitted with the Langmuir isotherm model.

Table 3.6 Adsorption isotherm parameters obtained for GF_2 in a FOS-rich broth mixture according to the error function.

		Linear	ERRSQ	HYBRID	MPSD	ARE	EABS
Linear isotherm	K ($L.kg^{-1}$)	0.35	0.35	0.36	0.38	0.38	**0.36**
	R^2	0.922	(linear)				0.919
	ERRSQ	21.22	21.22	22.33	29.30	34.47	21.93
	HYBRID	28.15	28.15	27.10	30.15	33.47	27.15
	MPSD	16.48	16.48	15.52	14.85	14.99	15.68
	ARE	12.80	12.80	12.16	12.07	12.02	12.17
	EABS	10.17	10.17	10.11	11.45	12.05	9.95
	SE	88.83	88.83	87.22	97.81	106.99	**86.88**
Langmuir	K_L ($L.g^{-1}$)	0.002	0.011	**0.009**	0.007	0.010	0.010
	M ($mg.g^{-1}$)	215.14	51.76	**57.66**	72.82	53.25	53.25
	R^2	0.992	(linear)	0.982			
	ERRSQ	28.30	4.51	4.75	6.15	4.61	4.61
	HYBRID	31.51	8.46	7.89	9.22	8.09	8.09
	MPSD	13.79	12.25	10.43	9.29	11.34	11.34
	ARE	10.12	6.95	6.58	6.68	6.59	6.59
	EABS	11.09	4.50	4.66	5.45	4.38	4.38
	SE	94.81	36.68	**34.31**	36.78	35.01	35.01
Freundlich	K_F ($L.g^{-1}$)	0.600	0.989	**0.747**	0.577	0.470	1.080
	n	1.15	1.35	**1.23**	1.14	1.07	1.41
	R^2	0.976	(linear)	0.973			
	ERRSQ	11.59	6.06	7.40	11.26	16.01	6.72
	HYBRID	15.89	17.03	13.05	15.98	21.94	20.10
	MPSD	11.92	20.85	14.01	11.76	13.01	23.15
	ARE	8.61	10.30	9.11	8.64	8.43	11.05
	EABS	7.12	5.55	6.35	7.32	8.12	5.51
	SE	55.13	59.78	**49.92**	54.97	67.50	66.53
Redlich & Peterson	K_R ($L.g^{-1}$)	0.55	0.55	**0.52**	0.48	0.52	0.56
	a_R ($L.mg^{-1}$)	0.011	0.011	**0.009**	0.007	0.010	0.015
	r	1.00	1.00	**1.00**	1.00	0.98	0.94
	R^2	0.983	(linear)	0.982			
	ERRSQ	4.51	4.51	4.75	6.15	4.92	4.59
	HYBRID	10.58	10.58	9.86	11.52	10.05	10.74
	MPSD	13.69	13.69	11.66	10.38	11.49	13.77
	ARE	6.95	6.95	6.58	6.68	6.49	6.78
	EABS	4.50	4.50	4.66	5.45	4.59	4.35
	SE	40.24	40.24	**37.51**	40.19	37.54	40.23
Toth	K_T ($L.g^{-1}$)	36.45	36.54	**31.49**	34.27	38.26	38.26
	a_T ($L.mg^{-1}$)	447.64	440.89	**1637.00**	1637.51	433.86	433.86
	t	1.42	1.42	**1.76**	1.71	1.39	1.39
	R^2	0.983	(linear)	0.982			
	ERRSQ	4.56	4.56	4.79	5.12	4.61	4.61
	HYBRID	9.14	9.14	8.76	9.35	8.99	8.99
	MPSD	11.16	11.16	9.66	9.21	10.55	10.55
	ARE	6.26	6.27	6.07	5.80	6.13	6.13
	EABS	4.52	4.52	4.78	4.66	4.47	4.47
	SE	35.64	35.65	**34.06**	34.14	34.76	34.76

(Bold-labeled data represent the minimum sum of the errors and the respective isotherm parameters that best represent the experimental data).

Table 3.7 Adsorption isotherm parameters obtained for GF_3 in a FOS-rich broth mixture according to the error function.

		Linear	ERRSQ	HYBRID	MPSD	ARE	EABS
Linear isotherm	K ($L.kg^{-1}$)	0.31	0.29	**0.29**	0.31	0.32	0.30
	R^2	0.851	(linear)	0.880			
	ERRSQ	26.06	20.40	21.05	25.16	32.29	21.50
	HYBRID	32.39	30.61	29.74	31.73	37.48	29.77
	MPSD	16.48	17.79	17.00	16.46	16.92	16.83
	ARE	12.59	13.60	12.87	12.60	12.54	12.66
	EABS	10.40	9.66	9.46	10.25	11.20	9.40
	SE	97.92	92.08	**90.12**	96.20	110.43	90.15
Langmuir	K_L ($L.g^{-1}$)	0.0004	0.014	**0.010**	0.006	0.013	0.015
	M ($mg.g^{-1}$)	764.94	36.28	**43.82**	63.95	37.31	33.97
	R^2	0.986	(linear)	0.974			
	ERRSQ	49.05	3.99	4.64	7.57	4.27	4.40
	HYBRID	64.25	12.36	10.36	13.49	11.33	13.13
	MPSD	21.16	19.74	15.05	12.58	17.70	20.19
	ARE	14.88	10.16	8.99	9.36	8.97	9.82
	EABS	13.76	4.66	4.91	6.28	4.29	4.22
	SE	163.09	50.91	**43.94**	49.28	46.56	51.77
Freundlich	K_F ($L.g^{-1}$)	0.46	0.93	**0.62**	0.44	0.36	1.01
	n	1.13	1.43	**1.24**	1.11	1.07	1.49
	R^2	0.962	(linear)	0.953			
	ERRSQ	13.79	6.34	8.30	13.28	16.42	8.43
	HYBRID	22.12	25.10	17.59	22.20	30.05	29.57
	MPSD	15.42	30.16	18.66	15.10	17.35	31.78
	ARE	11.14	14.24	11.93	11.14	11.11	14.52
	EABS	7.57	5.85	6.72	7.82	8.58	5.70
	SE	70.03	81.69	**63.19**	69.53	83.50	90.00
Redlich & Peterson	K_R ($L.g^{-1}$)	0.49	0.49	**0.44**	0.38	0.46	0.50
	a_R ($L.mg^{-1}$)	0.01	0.01	**0.01**	0.01	0.01	0.01
	r	1.00	1.00	**1.00**	1.00	1.00	1.00
	R^2	0.977	(linear)	0.974			
	ERRSQ	3.99	3.99	4.64	7.57	4.18	4.40
	HYBRID	15.45	15.45	12.95	16.86	13.45	16.45
	MPSD	22.07	22.07	16.82	14.07	18.94	22.62
	ARE	10.16	10.16	8.99	9.36	8.93	9.84
	EABS	4.66	4.66	4.91	6.28	4.45	4.22
	SE	56.32	56.32	**48.31**	54.14	49.95	57.53
Toth	K_T ($L.g^{-1}$)	35.04	22.71	**19.81**	21.29	35.21	33.51
	a_T ($L.mg^{-1}$)	78.14	1465.38	**29629.53**	29629.53	78.59	78.91
	t	1.02	1.82	**2.61**	2.52	1.01	1.03
	R^2	0.98	(linear)	0.980			
	ERRSQ	3.96	3.58	3.85	4.58	4.70	4.43
	HYBRID	15.59	10.58	9.23	10.57	15.00	15.51
	MPSD	22.29	16.15	12.49	11.54	20.14	21.42
	ARE	2.30	1.64	1.34	1.35	2.14	9.42
	EABS	1.53	1.11	0.90	0.89	1.42	4.18
	SE	45.67	33.06	**27.81**	28.94	43.40	54.95

(Bold-labeled data represent the minimum sum of the errors and the respective isotherm parameters that best represent the experimental data).

Table 3.8 Adsorption isotherm parameters obtained for GF_4 in a FOS-rich broth mixture according to the error function.

		Linear	ERRSQ	HYBRID	MPSD	ARE	EABS
Linear isotherm	K ($L.kg^{-1}$)	0.25	0.25	0.25	0.26	**0.26**	**0.26**
	R^2	0.665	(linear)			0.640	0.640
	ERRSQ	0.57	0.57	0.58	0.62	0.61	0.61
	HYBRID	7.50	7.50	7.40	7.59	7.54	7.54
	MPSD	25.45	25.45	24.87	24.57	24.58	24.58
	ARE	18.83	18.83	18.14	17.33	17.32	17.32
	EABS	1.53	1.53	1.51	1.50	1.49	1.49
	SE	53.87	53.87	52.50	51.61	**51.54**	**51.54**
Langmuir	K_L ($L.g^{-1}$)	0.0277	0.216	0.130	**0.062**	0.097	0.198
	M ($mg.g^{-1}$)	9.73	2.67	3.44	**5.56**	3.91	2.61
	R^2	0.973	(linear)		0.845		
	ERRSQ	2.20	0.11	0.14	0.26	0.22	0.15
	HYBRID	28.28	4.20	3.05	4.24	3.75	3.83
	MPSD	43.19	40.53	26.81	20.93	22.16	33.48
	ARE	27.44	19.05	15.35	15.37	15.19	16.00
	EABS	2.68	0.76	0.83	1.10	0.98	0.73
	SE	103.79	64.65	46.19	**41.90**	42.30	54.19
Freundlich	K_F ($L.g^{-1}$)	0.33	0.53	0.39	**0.31**	0.26	0.48
	n	1.15	1.73	1.34	**1.13**	1.03	1.65
	R^2	0.909	(linear)		0.757		
	ERRSQ	0.43	0.19	0.26	0.41	0.53	0.20
	HYBRID	6.49	8.10	5.02	6.40	8.23	6.70
	MPSD	25.48	57.99	31.45	24.17	26.23	48.99
	ARE	18.02	26.00	19.46	17.56	16.98	22.59
	EABS	1.29	0.98	1.12	1.33	1.46	0.95
	SE	51.71	93.25	57.30	**49.86**	53.42	79.43
Redlich & Peterson	K_R ($L.g^{-1}$)	0.58	0.58	0.45	**0.34**	0.38	0.52
	a_R ($L.mg^{-1}$)	0.22	0.22	0.13	**0.06**	0.10	0.20
	r	1.00	1.00	1.00	**1.00**	1.00	1.00
	R^2	0.936	(linear)		0.845		
	ERRSQ	0.11	0.11	0.14	0.26	0.22	0.15
	HYBRID	5.25	5.25	3.81	5.30	4.71	4.79
	MPSD	45.31	45.31	29.98	23.40	24.71	37.41
	ARE	19.05	19.05	15.35	15.37	15.19	15.99
	EABS	0.76	0.76	0.83	1.10	0.99	0.73
	SE	70.48	70.48	50.12	**45.43**	45.81	59.07
Toth	K_T ($L.g^{-1}$)	1.70	1.70	1.65	**1.63**	1.69	1.66
	a_T ($L.mg^{-1}$)	67.62	67.65	452.22	**9189.85**	268.00	268.00
	t	2.93	2.93	4.05	**5.61**	3.50	3.79
	R^2	0.96	(linear)		0.93		
	ERRSQ	0.07	0.07	0.08	0.13	0.11	0.08
	HYBRID	2.51	2.51	2.13	2.75	2.40	2.21
	MPSD	27.77	27.77	21.31	18.09	19.34	23.66
	ARE	13.20	13.20	11.40	10.94	10.74	11.54
	EABS	0.63	0.63	0.64	0.71	0.63	0.60
	SE	44.19	44.19	35.55	**32.61**	33.21	38.09

(Bold-labeled data represent the minimum sum of the errors and the respective isotherm parameters that best represent the experimental data).

Among the five error functions used to select the best fitting model, the HYBRID error function gave the lowest sum of the errors in most of the cases (Tables 3.6 to 3.8). Thus, with this function it was possible to obtain the isotherm parameters that were further used in the sorption models, in order to find the best experimental data fittings.

The analysis of the sum of the errors obtained for the best fitting error function for each isotherm model enabled the comparison of all the sorption models studied. The Toth isotherm was the model that presented the lowest SE values, followed by the Langmuir and Redlich & Peterson isotherms. The Freundlich isotherm yielded SE values slightly higher, and together with the linear isotherm were undoubtedly the worst fitting models.

The coefficients of determination evaluated for all isotherm models assessed, also confirmed that the Toth and Langmuir isotherms were the best models to represent the GF_2, GF_3 and GF_4 adsorption onto Dowex Monosphere Ca.

3.4.3 FOS IN PURIFIED MIXTURE VERSUS FOS IN A FERMENTATIVE BROTH

In this work, FOS contained in two types of liquid phases were studied, namely water in the case of the purified mixture, salts and smaller sugars in the fermentative broth. In both mixtures it was found that the adsorption capacity decreases with increasing molecular sizes of the sugars, being this fact related with the size exclusion mechanism. The distribution coefficients determined for GF_2, GF_3 and GF_4 kept almost the same values independently from the liquid phase that contained the sugars. However, slight increase on the adsorption capacity was found for all the sugars contained in the fermentative broth. This phenomenon can be related with the high concentration of ions in the bulk and the presence of other sugars in the liquid phase. Adachi and collaborators (Adachi *et al.*, 1989) concluded that the increase of the ionic strength of a solution could lead to an increase of the distribution coefficient of saccharides for an ion-exchange resin. Other sugars present in the solution may also contribute to increase the global sorption. Nobre *et al.* (2009) and Nowak *et al* (2007) recently reported the existence of synergetic effects on the adsorption of carbohydrates when multi-component mixtures are used. Furthermore, in the current work, it was found that the degree of selectivity

between the sugars was not dependent on the composition of the mixtures. The shape of the isotherm, as well as the sorption mechanism between sugars and Dowex Monosphere Ca resins, are still not fully understood. Significant differences in the type of isotherm used to fit experimental data for the same adsorbent can be found in the literature (Vente, 2005), even for the same range of sugar concentrations. Consequently, the adsorption parameters that have been reported are also different and cannot be compared.

Nowak and collaborators (2009) reported that the isotherm curve shape was mainly due to the non-idealities in the liquid phase. In the current work, FOS in pure water (purified mixture) were found to have an upward curvature, contrary to the downward curvature found for FOS contained in a fermentative broth. The isotherm shape seems to be influenced by the complexity of liquid phase at high sugar concentrations.

3.5 CONCLUSIONS

Adsorption of FOS onto a Dowex Monosphere Ca resin showed different behaviors depending on the complexity of the liquid phase of the FOS mixtures studied. FOS isotherms in purified mixtures showed an upward curvature, and consequently were fitted with a model derived from the anti-Langmuir isotherm with a good correlation. On the other hand, FOS contained in a fermentative broth showed a downward curvature. Thus, the experimental data were best fitted with the Toth and Langmuir isotherms.

For both studied mixtures, the adsorption capacity of individual sugars was primarily determined by their molecular size and was significantly dependent on the liquid phase composition, whereas the selectivity was essentially constant.

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CHAPTER 4

ADSORPTION EQUILIBRIUM OF FRUCTO-OLIGOSACCHARIDES FROM A FERMENTATIVE BROTH ON CATION EXCHANGE RESINS

ABSTRACT

At large-scale, fructooligosaccharides (FOS), considered as prebiotic sugars, can be produced by fermentative processes. The fermentative broth obtained is a complex mixture of FOS, salts and other small sugars that have to be removed. For that purpose, the adsorption isotherms of FOS contained in a fermentative broth and in purified mixtures were determined. Equilibrium studies were conducted at 60 ºC using different cationic adsorption resins, namely Lewatit (S2568) (macroporous resin in sodium form), Amberlite (CR1320Ca) (gel-type resin in calcium form) and Diaion (UBK530) (gel-type resin in sodium form). The effect of the counter-ion, resin structure and the liquid phase containing the FOS mixtures in the adsorption behavior was evaluated. Resins capacity and selectivity were compared. Multi-component anti-Langmuir and linear isotherm models were used to fit the equilibrium data. Results showed that in purified mixtures anti-Langmuir isotherms described better FOS adsorption onto the resins. On the other hand, for FOS contained in a fermentative broth linear isotherms were chosen, except for the Diaion resin. Also, FOS contained in a fermentative broth were found to be more adsorbed than the ones contained in purified mixtures. Lewatit was the resin that showed the greater capacity, however due to its higher selectivity, the Amberlite resin was elected as the one showing the best performance for the separation envisaged.

4.1 INTRODUCTION

Fructooligosaccharides (FOS) are non digestible sugars considered prebiotics due to their ability to selectively promote the growth of beneficial gut microflora (Gibson and Roberfroid, 1995). Several studies have shown that FOS have non-carcinogenic properties, alleviate constipation, enhance calcium and magnesium absorption, stimulate the immune system and the synthesis of vitamins, decrease levels of phospholipids, triglycerides, and cholesterol (Yamashita *et al.*, 1984; Crittenden and Playne, 1996; Morohashi *et al.*, 1998; Fotiadis *et al.*, 2008). Nowadays, consumers tend to seek for food that can provide some additional health benefits; therefore the consumption trend of food products supplemented with prebiotics is increasing, consequently resulting in a large commercial interest on FOS production.

Fermentative large scale production of FOS (kestose (GF_2), nystose (GF_3) and fructo-furanosilnystose (GF_4)) is conducted by microorganisms that contain enzymes with transfructosylation activity (Sangeetha *et al.*, 2005b), namely fructosyl-transferase enzymes that are able to synthesize FOS from sucrose (Fernandez *et al.*, 2004; Tuohy *et al.*, 2005). FOS produced from sucrose using microbial enzymes have low sweetness intensity when compared to commercial sucrose, however their sweet taste is very similar. Therefore, these oligosaccharides have attracted special attention, particularly as ingredients in foods aimed at diabetics or low-calorie foods (Yun, 1996; Sangeetha *et al.*, 2005a).

As a result of the fermentative process, besides FOS, the final mixture contains microorganisms, salts, proteins and other small sugars. Since these “contaminants” do not contribute to the prebiotic activity of FOS, the other sugars including glucose, fructose and sucrose, must be reduced or removed from the mixture. Consequently, the caloric value of the mixture is decreased and the suitability of the syrups for diabetic applications is improved (Gibson and Roberfroid, 1995; Crittenden and Playne, 1996).

Simulated moving bed chromatography (SMB) appears to be an efficient downstream process for sugar separation at an industrial scale (Gomes *et al.*, 2006). However, although separation of fructose/glucose mixtures has been extensively reported and is

well known, very few studies addressed the oligosaccharide recovery by SMB (Geisser *et al.*, 2005). Sulfonated poly(styrene-*co*-divinylbenzene) (PS-DVB) resins have been largely and successfully used in SMB plants for sugars separation (Beste *et al.*, 2000; Vente *et al.*, 2005; Luz *et al.*, 2008). Depending on the resin structure, PS-DVB resins can be classified as gel-type or macroporous resins (Abrams and Millar, 1997; Sherrington, 1998). Gel-type resins are highly prone to swelling, with DVB contents lower than 12%, resulting in a high water retention capacity and fast diffusion kinetics. On the other hand, macroporous resins have a permanent well-developed porous structure with higher contents of DVB. Due to the high degree of cross-linking, macroporous resins do not compress as the gel-type ones, being advantageous for large scale applications in which the back pressure can reach high values and consequently destroy the adsorbent structure (Abrams and Millar, 1997; Sherrington, 1998).

Gel-type and macroporous PS-DVB resins can be functionalized with counter-ions, such as calcium, potassium and sodium. The counter-ions form complexes with sugars and depending on the cation, the number and orientation of the sugar hydroxyl group involved, these complexes present different strengths. The ionic radius of the cation determines also the strength and stability of the complex formed (Angyal, 1989; Tiihonen *et al.*, 2002). The separation mechanisms using those cationic adsorption resins are mainly based on size exclusion, hydrophilic or hydrophobic interactions, ligand exchange and ion exchange (Churms, 1996; Stefansson and Westerlund, 1996).

In this work, three commercial PS-DVB resins potentially interesting for SMB separation of sugars were studied. Considering that an efficient adsorbent must show great capacity and a good selectivity, the adsorption isotherms of FOS contained in a fermentative broth and in purified mixtures were determined for each resin. Also, the possible effects of the fermentative broth non-idealities, the resin structure and the counter-ion form in the adsorption process were evaluated. Furthermore, experimental adsorption data were fitted with linear and multi-component anti-Langmuir isotherm models. The isotherm parameters for each model were determined by minimization of the hybrid fractional error function (HYBRID) (Porter *et al.*, 1999; Ng *et al.*, 2003), which allows the comparison of models having different numbers of adjusted

parameters, and takes into account the size of the different experimental datasets used. The minimization of this function corresponds to the minimization of relative distances, which is preferable to the usually minimization of the Euclidean distances, between the value of the model and the experimental data (Oliveira and Saramago, 2010). Since no physical meaning is attributed to the best-fit parameters, the isotherms used in this study are empirical equations. Finally, the statistical significance of the identified parameters and of each model was evaluated using the Fisher's test (Quinones and Guiochon, 1996a; Quinones and Guiochon, 1996b).

4.2 ADSORPTION THEORY

4.2.1 ISOTHERM MODELS

Several isotherm models have been used to study the retention mechanism in sugar chromatography. The linear isotherm is the most commonly used model, although several authors also reported the use of quadratic and Langmuir equations as good fitting models for sugar isotherms (Ching *et al.*, 1987; Saska *et al.*, 1992; Beste *et al.*, 2000; Vente *et al.*, 2005; Gramblicka and Polakovic, 2007). In this work, the linear and the multi-component Langmuir isotherm models were chosen to fit the adsorption experimental data obtained. Since in this study only multi-component experimental data was used, the competitive binding models proposed herein are non-predictive models (Cano *et al.*, 2007).

4.2.1.1 Linear isotherm

The linear isotherm follows the Henry's law and correlates the concentration of adsorbate into the stationary phase, q, with the concentration in liquid phase, C. The isotherm is represented by the following equation:

$$q_i = \gamma_i.C_i, \text{ i} = 1, \ldots, \text{n} \quad (4.1)$$

where γ_i, the slope of the isotherm, represents the distribution coefficient (Henry's constant).

The selectivity is defined as γ_i / γ_j, with $i \neq j$.

4.2.1.2 Langmuir isotherm

To describe competitive adsorption isotherms in multi-component systems, the Langmuir isotherm (Butler and Ockrent, 1930) can be used:

$$q_i = \frac{MK_{Li}C_i}{1 + \sum_{j=1}^{n} aK_{Lj}C_j}, \text{ i} = 1, \ldots, \text{n} \quad (4.2)$$

where M and K_L are the isotherm parameters. The parameter a assumes a value of +1 for the Langmuir favorable isotherms, and -1 for the anti-Langmuir unfavorable isotherms (Kaczmarski *et al.*, 2004; Mazzotti, 2006a; Mazzotti, 2006b). The parameter M should assume the same value for all components of the fluid mixture, to ensure that the model is thermodynamically consistent with the Gibbs-Duhem adsorption isotherm equation (Levan and Vermeulen, 1981). At an infinite dilution, the slope of the isotherm, defined as $H_i = MK_{Li}$, represents the Henry's constant of the i^{th} component, corresponding to a system characterized by the linear isotherm (Eq. 4.1) with $H_i = \gamma_i$ (Golshanshirazi and Guiochon, 1989; Mazzotti, 2006b).

It is important to notice that the anti-Langmuir model does not derive from a physicochemical background, thus it should be regarded as an empirical equation (Kaczmarski *et al.*, 2004). However, in many situations an empirical description of a real multi-component adsorption phenomenon is useful (Zhang *et al.*, 2001). Since the main objective of the present study was to identify a model that best fits the experimental data, by minimization of the error associated to each estimation, the above-mentioned empirical equation was used has an explicit formalism.

As the parameter *M* is assumed to be the same for all components, the selectivity is defined as H_i/H_j, where $i \neq j$. The selectivity is composition independent, and by definition will always be larger than one (Mazzotti, 2006b).

4.3 EXPERIMENTAL METHODS AND MATERIALS

4.3.1 MATERIALS

4.3.1.1 Resins

Three ion-exchange resins were used; one resin in calcium form (Ca^{2+}): Amberlite (CR1320Ca) (Roehm and Haas, Philadelphia, PA); and two resins in sodium form (Na^{+}): Diaion (UBK530) (Mitsubishi Chemical Corporation, Tokyo, Japan) and Lewatit (S2568) (Bayer, Leverkusen, Germany). Physical and chemical properties of the above mentioned resins are presented in Table 4.1.

Table 4.1 Physicochemical properties of the ion-exchange resins used in the current work.

Physicochemical Properties	Lewatit S 2568	Diaion UBK 530	Amberlite CR1320Ca
Ionic form	Na^{+}	Na^{+}	Ca^{2+}
Structure	Macroporous	Gel-type	Gel-type
Matrix	Styrene-DVB	Styrene-DVB	Styrene-DVB
Functional group	Sulfonate	Sulfonate	Sulfonate
Total capacity (eq/L)	1.75	>1.6	>1.5 (H^{+} form)
Water content[b] (%)	60-63	34-40	39-41
Volume median diameter (μm)	680	220	310-350

[a] Data obtained from the supplier [b]Data obtained by the dry weigh method

4.3.1.2 FOS mixtures

FOS were produced in the laboratory by fermentation of sucrose with *Aureobasidium* sp. (Rocha *et al.*, 2009). The final fermentative broth was a complex mixture containing sugars (fructose, glucose, sucrose and FOS), salts, microorganisms and residual proteins. When the production of the highest FOS molecule (GF_4) was detected by high performance liquid chromatography (HPLC), the fermentation was stopped (~ 50 h), and the broth was collected and filtrated through a 0.2 µm cut-off cellulose acetate filter to remove the microorganisms. Residual proteins were removed by centrifugation and filtration with a centrifugal filter Amicon Ultra-15 (50 NMWL) from Millipore, at 4000 g for 10 min.

Cations present in the broth mixture were determined using a flame atomic absorption spectrophotometer (Varian Spectra AA-400) to check if cation exchange was required before proceeding with adsorption experiments.

The cell-free fermentative broth obtained is referred in this study as FOS-Na^+ mixture, since the main cation present in the mixture is Na^+.

Regarding the assays conducted with the calcium resin, to avoid calcium precipitation due to the exchange with ions from the fermentative broth, the FOS-Na^+ mixture was previously passed through a column containing the calcium resin Amberlite CR1320Ca. The volume of resin used in this pre-treatment corresponded to half the volume of fermentative broth treated. Preliminary experiments proved that this ratio is enough to guarantee the saturation of broth with calcium ions and remove the primary ions from the fermentative broth (*data not shown*). The mixture obtained in this step is referred in the present study as FOS-Ca^{2+} mixture due to the Ca^{2+} ion content.

Adsorption studies were also conducted with a mixture of pre-purified FOS containing only low concentrations of small sugars and free of salts. This mixture was obtained in our lab by purification of the cell-free fermentative broth using a column with activated charcoal (for details see chapter 2) and is referred in the present study as FOS-pure mixture.

FOS standards used in the HPLC analysis (pure GF_2, GF_3 and GF_4) were purchased from Wako, Chemicals GmbH, Germany.

4.3.2 ADSORPTION ISOTHERMS

Resins were washed several times with double distilled water, and afterwards the remaining excess of water was removed from the resin by filtration with a glass filter.

The three mixtures used were successively diluted in double distilled water in order to obtain solutions with a decrease of 10% in the total sugar concentration for FOS-Na^+ and FOS-pure mixtures, and 15% for the FOS-Ca^{2+} mixture. A volume of 3.5 mL of each FOS mixture, with different initial sugar concentration, was added to test tubes containing 1.50 ± 0.01 g of wet resin. FOS-Na^+ mixtures were used in the studies conducted with Lewatit and Diaion resins. FOS-Ca^{2+} mixtures were used in the studies conducted with the Amberlite resin. FOS-pure mixtures were studied with all the resins. Experiments were carried out in duplicate. Five samples of each wet resin were dried at 105ºC to determine the resin water content.

Adsorption equilibrium experiments were conducted in a batch process. Briefly, test tubes containing the resin and sugar solution were incubated 8h under agitation at 60ºC.

Before sugar desorption, the bulk solution was decanted and the resin was washed three times with double distilled water. Five milliliters of water were added to the resin and sugar desorption was conducted overnight, under agitation at 25 ºC.

Sugars concentrations were determined by HPLC using a Knauer chromatograph equipped with a differential refractive index detector type 298.00 (Knauer, Berlin, Germany) operating at 30ºC. A REZEX RSO-Oligosaccharide column (Ag^+ form, 200x10 mm) from Phenomenex was used. Elution was conducted at 0.3 $mL.min^{-1}$ flow rate and 40 ºC using water as eluent.

The equilibrium loading *q*, representing the mass of sugar adsorbed per mass of resin, used to calculate the adsorption parameters was calculated from the following mass balance:

$$q = \frac{C_{ads}.V}{m} \tag{4.3}$$

where C_{ads} is the concentration of sugar adsorbed in the resin, *V* the volume of solution and *m* the dried resin mass.

4.3.3 ISOTHERMS DATA PROCESSING

To empirically fit the whole set of parameters of each model (3 and 4 parameters for linear and Langmuir models, respectively) the hybrid fractional error function was used (Porter *et al.*, 1999; Ng *et al.*, 2003):

$$\frac{100}{N-p}\sum_{i=1}^{N}\left[\frac{(q_{mod,i}-q_{exp,i})^2}{q_{exp,i}}\right] \tag{4.4}$$

The HYBRID function allows the estimation of fitting errors taking into account both the *q* values calculated by the model (q_{mod}), and the ones obtained in the experiments (q_{exp}). This error function was chosen since it takes into account the number of adjustable parameters (*p*), the number of experimental data available for each FOS (in purified mixture or fermentative broth) (*N*) and it minimizes the relative distance instead of the Euclidean distance, which is recommended (Oliveira and Saramago, 2010). The best fitting parameters were determined using a genetic algorithm.

Genetic algorithms (GAs) are usually applied for the optimization of complex non-linear models. They are stochastic algorithms based on the mechanisms of natural selection and genetics. First, an initial population, containing a pre-established number of individuals is created randomly considering the specific lower and upper bounds for each individual, and coded as a vector (chromosome). Then, the goodness of fit of this possible solution of the multidimensional search space is evaluated by using a predefined fitness criterion. Finally, a new generation of individuals is created from the actual population using three

genetic operators (reproduction, crossover and mutation) (Roubos *et al.*, 1999; Veloso *et al.*, 2005).

In the present work, GAs were applied to empirically identify the parameters of the linear adsorption (Eq. 1) or the Langmuir adsorption (Eq. 2) equations by minimizing the HYBRID error function, considering simultaneously the three FOS present in the multi-component mixtures (purified FOS mixture or fermentative broth). For this purpose, a *Genetic and Evolutionary Algorithm Toolbox* (*GEAtbx 3.3*) for MATLAB developed by Pohleim (Pohlheim, 2003) was used. It works with several genetic operators and supports binary, integer and real-valued representations, being the last the one chosen in this work. Also, a globally oriented evolutionary optimization with four subpopulations with the same number of individuals was chosen. In total, 200 individuals were evaluated per iteration. The optimization procedure was interrupted when the value of the objective function did not alter during 500 iterations, being at least 5000 iterations performed.

To infer about the statistical significance of the identified parameters the Fisher's test was applied at a significance level of 5%. Since the conventional use of the Fisher distribution is not possible (Quinones and Guiochon, 1996b) for each model and each set of experimental data, the Fisher parameter (F-value) was calculated according to:

$$F = \frac{N-p}{N-1}\frac{\sum_{i=1}^{N}\left(q_{exp,i}-\overline{q_{exp,i}}\right)^2}{\sum_{i=1}^{N}\left(q_{exp,i}-q_{mod,i}\right)^2} \tag{4.5}$$

Where $\overline{q_{exp,i}}$ represents the mean value of the data, i.e. of the $q_{exp,i}$ obtained experimentally.

Using Eq. 4.5 it is possible to compare models with different number of parameters. Highest values for the Fisher parameter mean greater statistical significance of the model used and of the identified parameters. Therefore, the model performance was evaluated by mean of the magnitude of the F-value associated.

4.4 RESULTS & DISCUSSION

4.4.1 FOS MIXTURES CHARACTERIZATION

The cations that were quantified on the FOS-Na^+ mixture were sodium (3.3 $g.L^{-1}$) and potassium (1.7 $g.L^{-1}$). Since sodium was the main cation present, this mixture was used on the adsorption experiments carried out with the sodium resins without further treatment. The total sugar concentration of the FOS-Na^+ mixture, determined by HPLC, was 400 $g.L^{-1}$; including FOS with 91, 86 and 9 $g.L^{-1}$ of GF_2, GF_3 and GF_4, respectively; and small saccharides (fructose, glucose and sucrose). FOS represented 47% (w/w) of the total sugars in the mixture solution.

In the FOS-Ca^{2+} mixture, calcium was the only cation identified. Therefore, suggesting that the pre-treatment of the FOS-Na^+ mixture was efficient since sodium and potassium were successfully removed. The total sugar concentration of the FOS-Ca^{2+} mixture determined by HPLC was 358 $g.L^{-1}$; including FOS with concentrations of 72, 71 and 9 $g.L^{-1}$ of GF_2, GF_3 and GF_4, respectively; and small saccharides. FOS represented 42% (w/w) of total sugars in the mixture solution.

The FOS-pure mixture contained only 10% of small sugars and 90% (w/w) of FOS. The total sugar concentration determined was 210 $g.L^{-1}$; including 77, 94 and 19 $g.L^{-1}$ of GF_2, GF_3 and GF_4, respectively. This mixture was free of salts.

4.4.2 ADSORPTION ISOTHERMS

Several authors have investigated the sorption of fructose, glucose, sucrose and FOS onto ion-exchange resins in the last years (Gramblicka and Polakovic, 2007; Pedruzzi *et al.*, 2008; Luz *et al.*, 2008; Nobre *et al.*, 2009; Nowak *et al.*, 2009; Vankova *et al.*, 2010). Although many of the studies reported linear adsorption, depending on the composition and concentration of the mixture, non-linear models may be more adequate to describe the equilibrium. Ching *et al.* (1987) found that the equilibrium isotherms determined for fructose and glucose onto a Duolite-Ca^{2+} resin showed an upward curvature when

working at sugar concentration up to 260 $g.L^{-1}$. The same behavior was also found by many other authors working under overloaded conditions (Saska *et al.*, 1992; Lee, 2003; Vente *et al.*, 2005; Nowak *et al.*, 2009).

Equilibrium experimental data obtained in the present work and the respective fittings using linear and anti-Langmuir isotherm models are represented in Figs. 4.1 to 4.3. These figures illustrate the adsorption isotherms of GF_2, GF_3 and GF_4, contained in FOS-pure mixtures and fermentative broth, onto Amberlite, Diaion and Lewatit resins.

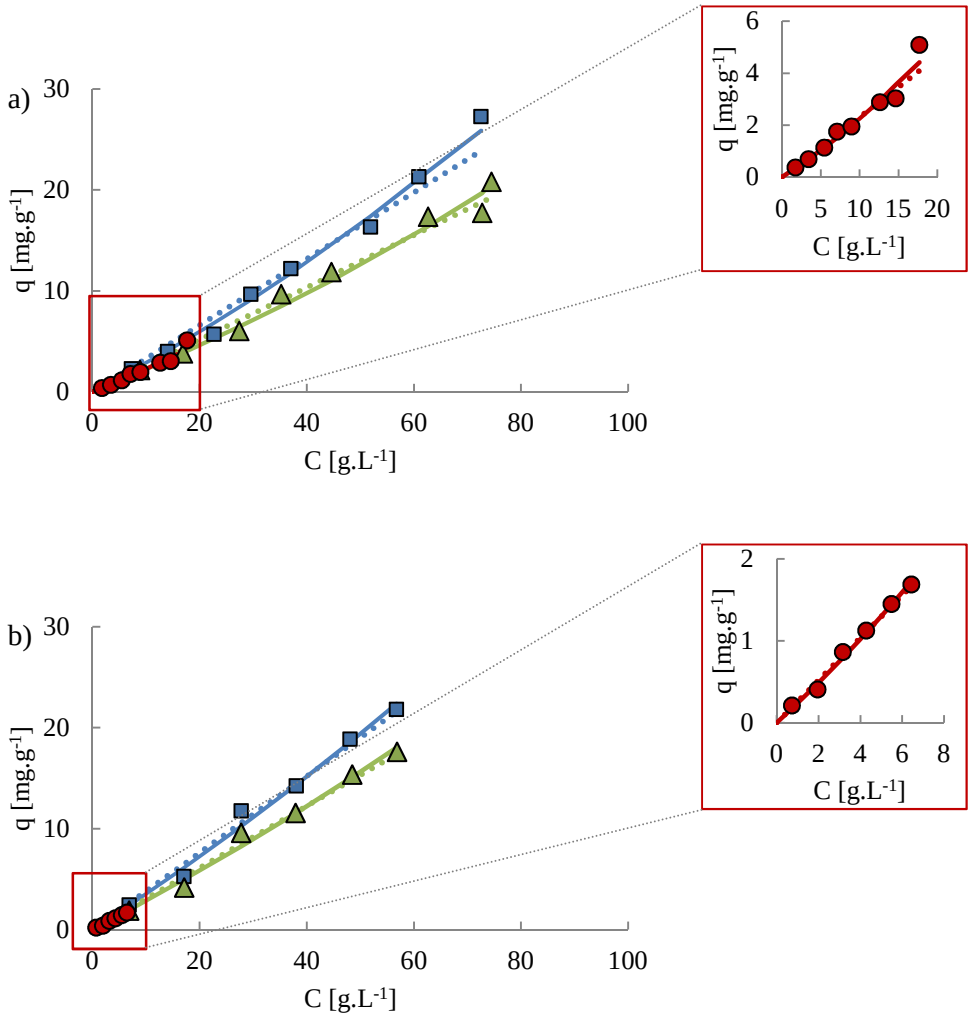

Fig. 4.1. Adsorption isotherms of: a) FOS-pure b) FOS-Ca^{2+} onto Amberlite resin. Symbols represent the experimental data: ■ GF_2, ▲ GF_3, ● GF_4. Lines represent the respective fitting with the isotherm models: (–) Linear and (··) multi-component anti-Langmuir.

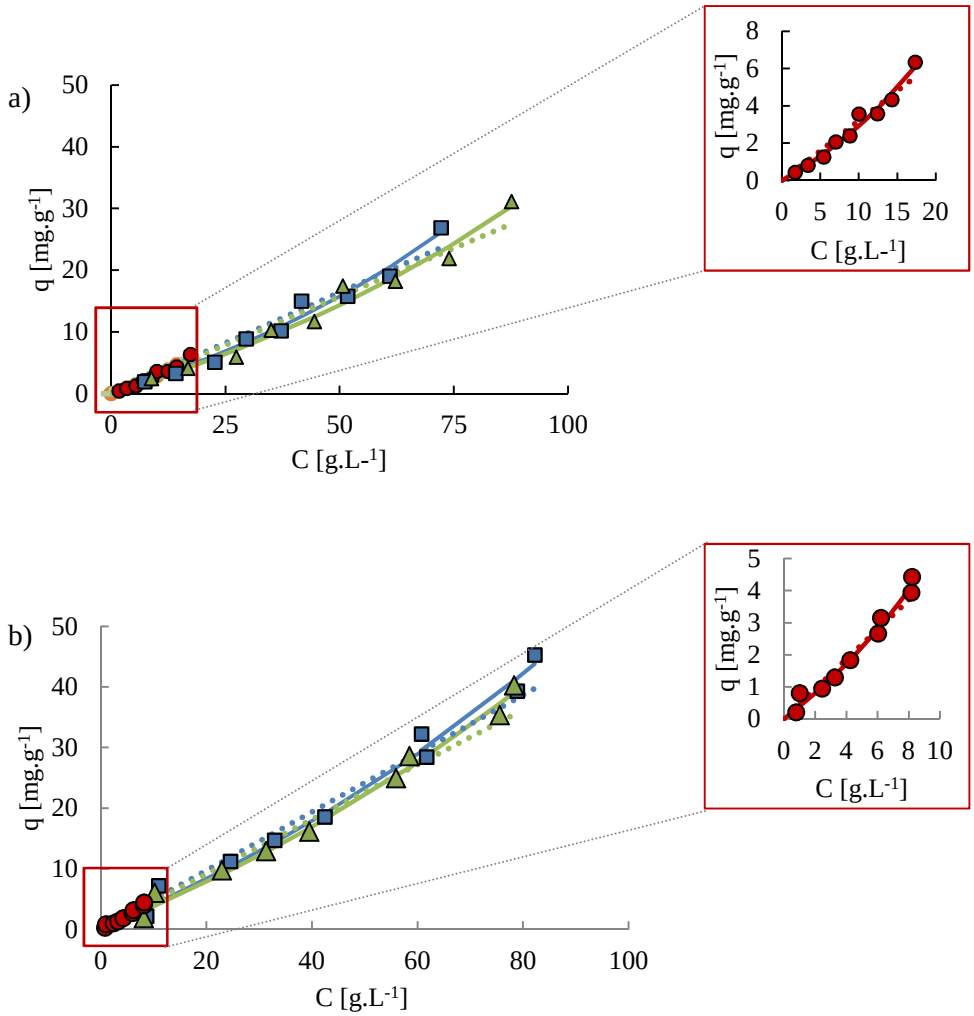

Fig. 4.2 Adsorption isotherms of: a) FOS-pure b) FOS-Na^+ onto Diaion resin. Symbols represent the experimental data: ■ GF_2, ▲ GF_3, ● GF_4. Lines represent the respective fitting with the isotherm models: (–) Linear and (· ·) multi-component anti-Langmuir.

Analyzing the figures, it is possible to observe that the shape of the isotherms determined is linear or with a slight curvature. In the case of non-linear isotherms the curvature exhibits an upward shape. At high solute concentrations, the degree of hydration of the sugar clusters is low. Also, the displacement of the hydration layers of the counter-ions is favoured. Moreover, the swelling pressure decreases leading to the shrinkage of the resin. All these factors, may contribute to the increase of the distribution coefficient and consequently to the upward curvature of the isotherm (Saska *et al.*, 1992; Adachi *et al.*, 1995). In this work, the Langmuir model was used to describe the non-linear isotherms

since it can be applied even when the adsorption parameters of the pure components present in the mixture are unknown (Charton and Nicoud, 1995). As the isotherm curvature is upward, the parameter *a* from the Langmuir isotherm (Eq. 4.2) was set equal to *-1*.

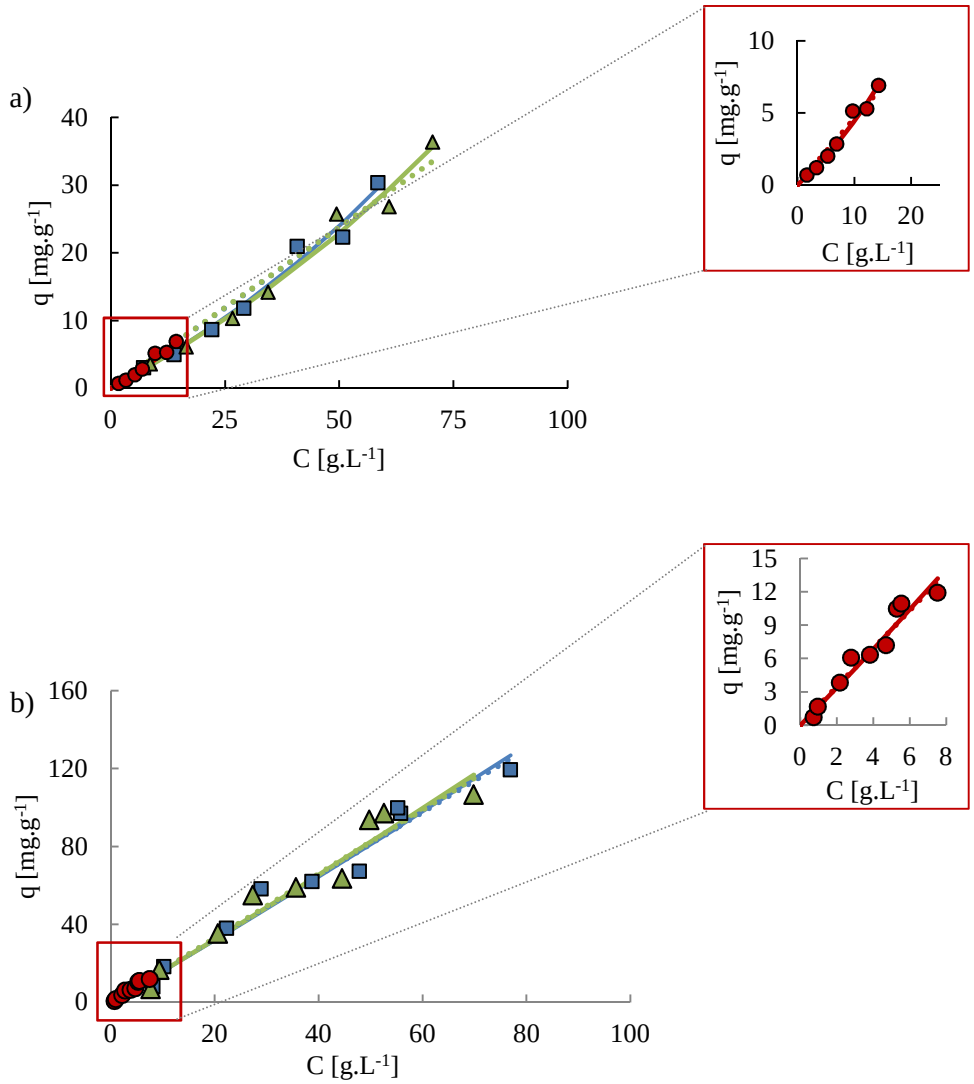

Fig. 4.3 Adsorption isotherms of: a) FOS-pure b) FOS-Na^+ onto Lewatit resin. Symbols represent the experimental data: ■ GF_2, ▲ GF_3, ● GF_4. Lines represent the respective fitting with the isotherm models: (–) Linear and (· ·) anti-Langmuir.

Afterwards, to evaluate if sugar adsorption isotherms were linear or non-linear under the current experimental conditions, data were fitted with both models (Tables 4.2 and 4.3). The performance of each model was compared based on the magnitude of the calculated F-value, which values were always statistically significant ($P < 0.001$). The isotherm parameters were determined by minimizing the HYBRID error function, being the global minimum obtained for each adsorption isotherm also presented in Tables 4.2 and 4.3.

Table 4.2. Adsorption isotherm parameters of FOS-pure mixtures onto ion-exchange resins with the error analysis and Fisher parameter.

Isotherm model	Parameter	Sugar	Resin		
			Lewatit	Diaion	Amberlite
Linear	γ_i (L.kg^{-1})	GF_2	0.45	0.31	0.33
		GF_3	0.45	0.30	0.26
		GF_4	0.44	0.30	0.23
	HYBRID		34.17	35.15	15.99
	F-value		19.40	14.75	30.99
Anti-Langmuir	N (mg.g^{-1})		180.61	121.32	183.36
	$N.K_i$	GF_2	0.36	0.24	0.28
		GF_3	0.36	0.23	0.22
		GF_4	0.35	0.23	0.20
	HYBRID		15.76	15.73	9.66
	F-value		37.92	36.69	55.97

The F-values obtained for the multi-component anti-Langmuir fitting of the FOS adsorption, when using FOS-pure mixtures, were greater than the ones obtained for the linear model for all the resins studied. Therefore, the FOS-pure mixture equilibrium was better described by the multi-component anti-Langmuir model. The adsorption of FOS-Ca^{2+} mixture onto the Diaion resin was also found to be non-linear, therefore it was better represented by the multi-component anti-Langmuir model.

Table 4.3. Adsorption isotherm parameters of FOS-Ca^{2+} and FOS-Na^{+} mixtures onto the respective ion-exchange resins with error analysis and Fisher parameter.

Isotherm model	Parameter	Sugar	Resin		
			Lewatit	Diaion	Amberlite
Linear	γ_i (L.kg^{-1})	GF_2	1.63	0.48	0.38
		GF_3	1.65	0.45	0.31
		GF_4	1.72	0.46	0.26
	HYBRID		146.52	31.91	10.16
	F-value		18.95	31.19	63.61
Anti-Langmuir	N (mg.g^{-1})		5158.59	232.19	307.00
	$N.K_i$	GF_2	1.57	0.39	0.35
		GF_3	1.59	0.36	0.28
		GF_4	1.67	0.37	0.24
	HYBRID		157.53	20.05	9.31
	F-value		16.53	67.73	55.44

On the other hand, according to the F-values, linear isotherms were found to characterize the adsorption of FOS-Ca^{2+} and FOS-Na^{+} mixtures from the fermentative broth onto Amberlite and Lewatit resins. This observation was unexpected since for FOS mixtures from the fermentative broth, higher sugar concentrations have been used and small saccharides and ions were present in the liquid phase, thus a non linear adsorption behavior would be more reasonable rather than a linear adsorption.

4.4.3 EFFECT OF THE FOS MIXTURE

FOS contained in the FOS-Ca^{2+} and FOS-Na^{+} mixtures from the fermentative broth were more adsorbed onto the studied resins than FOS contained in purified mixtures. For sodium resins (Lewatit and Diaion), significant differences were found between the Henry constants determined for GF_2, GF_3 and GF_4, in the FOS-pure (Table 4.2) and the FOS-Na^{+} mixtures (Table 4.3).

The different adsorption degree may be related with the composition of the liquid phase, since FOS-Na^{+} and FOS-Ca^{2+} mixtures contain around 45% (w/w) of FOS but also high

amounts of salts and other sugars, while FOS-pure mixtures are salt free and contain 90% (w/w) of FOS in the total sugar mixture.

Increased sugar concentrations in the liquid phase may accelerate the diffusion of sugars into the solid phase. Moreover, the high ionic strength resulting from the high content in salts from the fermentative broth contributes also to the resin shrinkage phenomena (Adachi *et al.*, 1989). Therefore, both salts presence and high sugar concentrations of the fermentative broth contribute for the displacement of the hygroscopic water within the porous with consequent increase of FOS adsorption.

Furthermore, the presence of additional sugars in the mixture may also contribute for a synergetic effect on the sugar adsorption leading to increased adsorptions as reported by Nobre *et al.* (2009) and Nowak *et al.* (2007).

4.4.4 EFFECT OF THE RESIN STRUCTURE

In this study, two gel-type (Amberlite and Diaion) and one macroporous resins (Lewatit) were used. The adsorption of FOS-pure mixture onto the two gel-type resins was very similar (Table 4.2). For the fermentative broth containing FOS, a different adsorption behavior was observed, however this maybe due to the counter-ion and the liquid phase composition rather than due to the resin structure (Table 4.3). Comparing the adsorption onto the resins with different structures, the macroporous resin showed higher adsorption of FOS than the gel-type ones, especially when using fermentative broth FOS (Tables 4.2 and 4.3).

The water content determined for Lewatit was around 60% while for the other resins was 40%. Therefore, this resin possessed more free water available for partition. Consequently, higher adsorption capacities were expected. Moreover, since the macroporous resin has permanent well-developed pores and channels, those resins are easily penetrated by the sugars, and less affected by the steric effects commonly observed in gel-type resins. This was clearly observed for the FOS fermentative broth mixture. Since the resin does not shrink at high sugar concentrations and at high liquid

phase ionic strengths, the pore blocking and restricted access of sugars to the active sites within the porous plays here a minor role.

4.4.5 EFFECT OF THE COUNTER-ION

Two counter-ions were used in this study, Na^+ and Ca^{2+}. The strength of the complex formed between the cation and the sugar depends on the hydration of the counter-ion, and on the sugar being adsorbed (Angyal, 1989). Calcium resins are known to form strong complexes with fructose, however with FOS the complexes formed are very weak (Howard *et al.*, 1988). Therefore, the interaction of FOS with Na^+ and Ca^{2+} is mainly dependent on the hydration shell of the ion rather than the sugar being adsorbed.

Regarding the two gel-type resins studied, Diaion in Na^+ form and Amberlite in Ca^{2+} form, FOS from the fermentative broth mixture were more adsorbed onto the Na^+ resin than onto the Ca^{2+} one (Table 4.3). Accordingly, Gramblicka *et al.* (2007) showed recently the same trend for a commercial mixture of FOS. Moreover, Vente *et al.* (2005) reported higher capacities for the adsorption of mono- and disaccharides, except fructose, onto a gel-type resin of Na^+ compared to a Ca^{2+} one.

The water content of the resin is mainly present in two different physical stages, as hygroscopic water (free water for partitioning) and hydration water (water around the ion). The hydration water depends on the resin cross-linking and the counter-ion. In aqueous solutions, Na^+ and Ca^{2+} have hydration numbers of 2.0 and 4.3, respectively (Tiihonen *et al.*, 1999). Accordingly, the cation hydration shell increases with the increasing valence of the ion. Therefore, if Ca^{2+} resins have more water around the ion, the free water available for hydrogen bound formation with sugar is smaller. Hence, Na^+ resins show usually greater sugar adsorption compared with the Ca^{2+} ones.

Regarding the adsorption isotherms determined for FOS contained in the purified mixture, almost the same sugar capacity was found for both gel-type resins. Therefore, the results suggest that the presence of salts and other sugars in the mixture have a greater influence on the adsorption than the counter-ion of the resin.

4.4.6 SELECTIVITY

The selectivity is a very important parameter whenever choosing a resin for a given application. The sugar selectivity determined for Lewatit, Amberlite and Diaion resins is given in Table 4.4. These values may be indicative for the separation of FOS from mono- and disaccharides.

Table 4.4. Selectivity values for GF_2, GF_3, and GF_4, contained in purified mixtures or mixtures from the fermentative broth, using Amberlite, Diaion and Lewatit resins.

	Counter-ion		GF_2/GF_3	GF_3/GF_4	GF_2/GF_4
Amberlite	Ca^{2+}	Fermentative broth	1.24	1.18	1.47
		FOS-pure	1.28	1.12	1.43
Diaion	Na^{+}	Fermentative broth	1.07	1.01	1.05
		FOS -pure	1.04	1.02	1.03
Lewatit	Na^{+}	Fermentative broth	1.01	1.05	1.06
		FOS-pure	1.00	1.04	1.04

As expected, the selectivity values determined with the Henry's constants obtained by both linear and multi-component anti-Langmuir models were found to be in agreement and equivalent values were obtained using the isotherm parameters calculated by each model.

Selectivity values determined for FOS from purified and fermentative broth mixtures were similar, hence suggesting that the resins selectivity was not influenced by the liquid phase containing the sugars.

Comparing the selectivity of the Na^{+} resins, macroporous Lewatit and gel-type Diaion, similar values were obtained, thus selectivity was found to be independent of the resin structure.

Sodium resins showed very low selectivity ($\alpha < 1.07$). On the other hand, for the Amberlite calcium resin values about 1.2 for GF_2/GF_3 and 1.5 for GF_2/GF_4 were obtained. Based on the results, although Na^{+} resins showed greater capacities, the Ca^{2+}

resin was the only one demonstrating some potential to selectively adsorb FOS and smaller saccharides. Therefore, the gel-type Ca^{2+} resin Amberlite was shown to be the most suitable resin to be used in the recovery/purification of FOS from fermentative broths.

These results are in accordance with the work from Gramblicka *et al*. (2007) that reported the separation of a commercial FOS mixture using the same resins studied in the current work, and concluded that Amberlite was the most appropriated due to its greater selectivity.

4.5 CONCLUSIONS

The multi-component anti-Langmuir model provided a good correlation for the adsorption of FOS contained in purified mixtures for all the resins studied. The adsorption of fermentative broth FOS (FOS-Na^+) onto Diaion resin was also well fitted by this model. On the other hand, fermentative broth FOS adsorption onto Amberlite and Lewatit resins were better described by the linear model.

Although a higher adsorption capacity was found for FOS from the fermentative broth than for FOS in purified mixtures, the selectivity values were similar.

The adsorption of FOS from the fermentative broth was influenced by the counter-ion, structure and water content of the resin; while the adsorption of FOS from purified mixtures was only influenced by the two last factors.

Studies conducted with FOS purified and from the fermentative broth led to very distinct adsorption behaviours within the same resin. These differences were found to be mainly related to the composition of the liquid phase containing sugars. So, it is possible to conclude that the nature of the fermentative broth obtained at a given fermentation time plays a major role on the sugar adsorption. In conclusion, the FOS adsorption behaviour cannot be predicted by adsorption studies conducted with standard FOS in water matrices. Therefore, to characterize the adsorption of FOS from other types of fermentative broths, for example fermentations conducted with other microorganisms, new adsorption studies should be performed.

Lewatit was the resin that showed the greater capacity. However, due to its low selectivity, the Amberlite resin was elected as the more adequate for the recovery/purification of FOS from a fermentative broth.

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CHAPTER 5

COMPARISON OF ADSORPTION EQUILIBRIUM OF FRUCTOSE, GLUCOSE AND SUCROSE ON POTASSIUM GEL-TYPE AND MACROPOROUS SODIUM ION-EXCHANGE RESINS

ABSTRACT

Adsorption equilibrium of fructose, glucose and sucrose was evaluated on sulfonated poly(styrene-co-divinylbenzene) cation-exchange resins. Two types of resins were used: potassium (K^+) gel-type and sodium (Na^+) macroporous resins. Influence of the cation and effect of the resin structure on adsorption were studied. The adsorption isotherms were determined by the static method in batch mode for mono-component and multi-component sugar mixtures, at 25 and 40ºC, in a range of concentrations between 5 and 250 $g.L^{-1}$. All adsorption isotherms were fitted by a linear model in this range of concentrations. Sugars were adsorbed in both resins by the following order: fructose>glucose>sucrose. Sucrose was more adsorbed in the Na^+ macroporous resin, glucose was identically adsorbed, and fructose was more adsorbed in the K^+ gel-type resin. Data obtained from the adsorption of multi-component mixtures as compared to the mono-component ones showed a competitive effect on the adsorption at 25ºC, and a synergetic effect at 40ºC. The temperature increase conducted to a decrease on the adsorption capacity for mono-component sugar mixtures, and to an increase for the multi-component mixtures. Based on the selectivity results, K^+ gel-type resin seems to be the best choice for the separation of fructose, glucose and sucrose, at 25ºC.

5.1 INTRODUCTION

Complex mixtures are frequently obtained when processes such as fermentative or enzymatic synthesis are used for sugar production. The application of these mixtures in the food industry requires their fractionation in order to meet final product specifications. Separation of glucose (G) from fructose (F) (Azevedo and Rodrigues, 2001), sucrose (S) in molasses (Lefevre, 1962; Neuzil, 1982) and fructose high-fructose corn syrups (Toumi and Engell, 2004), represent major challenges in industrial sugar chromatographic separations. As adsorbents, ion exchange resins of sulfonated poly(styrene-co-divinylbenzene) (PS-DVB) have been largely used in the sugar industry due to their chemical inertness, higher capacity and selectivity (Okada, 1995; Stefansson and Westerlund, 1996; Tiihonen *et al.*, 2002; Luz *et al.*, 2008). According to their structure, the resins are classified in two major groups: gel-type and macroporous (Abrams and Millar, 1997; Sherrington, 1998).

Gel-type resins are translucent and have a smooth and uniform surface. These resins are highly swollen and have a low nominal degree of cross-linking (the DVB content is lower than 12%) resulting in high water retention capacity and fast diffusion kinetics (Abrams and Millar, 1997; Sherrington, 1998). Therefore, they are soft and compressible, restricting their use in packed columns, particularly for large scale applications as back pressure increases with the compression of the particles (Sherrington, 1998).

Macroporous resins are characterized by a permanent well-developed porous structure. They are opaque and their DVB content is greater than 20%. Due to their high degree of cross-linking, they do not show shrinking problems and are resistant to degradation caused by osmotic shock and oxidation (Abrams and Millar, 1997; Sherrington, 1998).

These non-ionic resins can be functionalized with cations, usually calcium, potassium or sodium. These cations form complexes with the hydroxyl group of the adsorbed sugar, leading to a selective adsorption according to the orientation of the hydroxyl group. Thus, the conformation of the sugar and the cation determines the distribution coefficient and the cation-sugar affinity. The carbohydrates separation is mainly based on size

exclusion, hydrophilic or hydrophobic interactions, ligand exchange and ion exchange (Stefansson and Westerlund, 1996; Churms, 1996a; Churms, 1996b).

Gel-type resins in calcium form are the most used in industrial separation of fructose from glucose due to the strong complexes that these resins form with fructose (Beste *et al.*, 2000; Azevedo and Rodrigues, 2001; Luz *et al.*, 2008). Several authors used calcium resins to fractionate mixtures of fructose, glucose and sucrose (Barker *et al.*, 1992; D.Paillat, 1999; Vente *et al.*, 2005a). However, potassium (K^+) and sodium (Na^+) cations are the most recommended ions for the separation of these sugar mixtures due to their advantage in providing higher kinetic rates of adsorption (D.Paillat, 1999; Pedruzzi *et al.*, 2008).

The efficiency of a chromatographic process is largely dependent on the adsorbent used. Adsorption isotherms of the sugars present in a mixture are used for selecting the most adequate adsorbent to be used in the separation, since they describe the equilibrium distribution of a solute between the adsorbent and the liquid phase (Schulte and Epping, 2005). Thus, the evaluation of adsorption isotherms is extremely important as it represents the first step towards scale-up to industrial applications. Dynamic (frontal analysis (Nowak *et al.*, 2007)) and static (adsorption-desorption in column (Azevedo and Rodrigues, 2000; Nowak *et al.*, 2007) and in batch mode (Vente *et al.*, 2005a; Vente *et al.*, 2005b; Gramblicka and Polakovic, 2007) methods have been used to determine the adsorption isotherms.

Parameters such as the structure of the resin, the ionic form (Na^+ or K^+), the components of the mixture (mono and multi-component) and the temperature have been reported to largely influence the adsorption and separation of sugars. There are several studies concerning adsorption of fructose, glucose and sucrose in PS-DVB resins (Vente *et al.*, 2005b; Gramblicka and Polakovic, 2007; Nowak *et al.*, 2007), however, none of these authors addressed all the referred parameters simultaneously. Thus, the aim of this work was to determine the adsorption isotherms of glucose, fructose and sucrose using two PS-DVB ion-exchange resins, namely a macroporous resin in Na^+ form, and a gel-type resin in K^+ form. A static method in batch mode was used to determine the adsorption parameters. Experiments were conducted with mono- and multi-component mixtures of

sugars to evaluate competitiveness between the sugars present in the mixture. Temperature effect (25 and 40ºC) on adsorption and selectivity were also studied for both resins.

5.2 EXPERIMENTAL

5.2.1 MATERIALS

A macroporous resin (Dowex Monosphere 88) in Na^+ form, and a gel-type resin in K^+ form (Dowex Monosphere 99K/320) were purchased from Supelco (Table 5.1).

Table 5.1 Physical and chemical properties of the resins.

Physical and chemical Properties[a]	Dowex Monosphere 88	Dowex Monosphere 99K/320
Ionic form	Na^+	K^+
Structure	Macroporous	Gel-type
Matrix	Styrene-DVB	Styrene-DVB
Functional group	Sulfonate	Sulfonate
Total capacity ($eq.L^{-1}$)	>1.8	>1.5 (H^+ form)
Water content	42-50	57-61 (H^+ form)
Volume median diameter (µm)	500-600	317±15

[a] Data obtained from supplier

Analytical grade fructose, glucose and sucrose were purchased from Panreac, Riedel-de Haën and Merck, respectively, and diluted in Milli-Q pure water.

5.2.2 DETERMINATION OF ADSORPTION ISOTHERMS

Adsorption equilibrium isotherms were determined using a static method, namely the adsorption-desorption in batch mode. Resins were washed several times with Milli-Q pure water until the liquid phase pH achieved a value of 6.0, therefore avoiding further

hydrolysis of sucrose. After equilibrium was reached, the resins were filtered to remove the water excess content. A volume of 3.5 mL of adsorbent, previously weighed, was placed into 15 mL Falcon tubes. Five Falcon tubes were dried at 105ºC in order to determine the resin water content. Next, 3.5 mL of sugar solution was added to the resin and held for 8 h under agitation at 25 or 40ºC. Previous experiments demonstrated that 8 h were enough to reach the equilibrium without occurring sucrose hydrolysis (*data not shown*). These experiments were carried out in triplicate.

For determining the adsorption isotherms, mono- and multi-component sugar solutions were prepared in a range of concentrations between 5 and 250 $g.L^{-1}$. In all the multi-component mixtures, individual sugars mass concentration was the same.

To ensure that the resin is ion saturated after each assay, a regeneration step was conducted with Na_2SO_4 and K_2SO_4, for the Na^+ and K^+ resins, respectively. The amount of ions in solution was controlled by measuring the conductivity after each regeneration step.

5.2.3 SUGARS ANALYSIS

Initial sugar mixture solutions and non-adsorbed sugar concentrations were determined by High-Performance Liquid Chromatography (HPLC) using a Jasco device equipped with a refractive index detector. A Prevail Carbohydrate ES column (5 μm, 250 × 4.6 mm) from Alltech was used. The mobile phase consisted of acetonitrile (HPLC Grade, from Carlo Erba) with 0.04% of ammonium hydroxide (HPLC Grade, from Sigma) in Milli-Q pure water (70:30 v/v). Elution was conducted at a 1 $mL.min^{-1}$ flow rate and room temperature (Dias *et al.*, 2009). A Star Chromatography Workstation software (Varian) was used to record and integrate the refractive index responses.

5.2.4 ADSORPTION PARAMETERS CALCULATION

Calculation of the equilibrium loading, *q*, is the first step to obtain the adsorption isotherms. Equilibrium loading was calculated for each tested concentration on both

resins. The assays were performed for mono- and multi-component sugar mixtures at two different temperatures, namely 25 and 40ºC.

The equilibrium loading representing the mass of sugar adsorbed per mass of resin, is calculated from the following mass balance:

$$q = \frac{(C_i - C)V)}{m} \quad (5.1)$$

Where C_i is the initial concentration of the sugar solution, with a volume V, added to a known amount of adsorbent m, and C is the final concentration of sugars in equilibrium with the adsorbent.

The measured adsorption isotherm data were fitted using a linear regression equation:

$$q = K.C \quad (5.2)$$

allowing the determination of the distribution coefficient, K.

5.2.5 SELECTIVITY OF THE RESIN

To evaluate the efficiency of sugars separation for each resin, at different conditions, the selectivity parameter (α) was calculated. For linear chromatography the selectivity can be expressed as the ratio between distribution coefficients of the sugars j and l (Schulte and Epping, 2005).

$$\alpha_{j/l} = K_j/K_l \quad (5.3)$$

5.2.6 DATA ANALYSIS

Linear regression models were used to fit the experimental data (Eq. 5.2) and their significance was assessed using an ANOVA test. The quality of the fitted models was assessed by their R-square values. Significance of the slope was also determined by a *t*-test. The presence of outliers was investigated. Experimental data showing standardized

residual errors greater than 2.5 times the standard deviation were considered outliers. The normal distribution of the standardized residual errors, a pre-requisite of the linear regression model, was investigated using the Shapiro-Wilk test. Regression data were subjected to a likelihood ratio test of equality (covariance analysis) to determine if the slopes of each linear equation were the same. Statistic analyses were performed using the SPSS 17 Standard Version software (SPSS INC.). All statistical tests were conducted at a 5% significance level.

5.3 RESULTS AND DISCUSSION

5.3.1 ADSORPTION ISOTHERMS

As shown in Figures 5.1 and 5.2, the adsorption isotherms, for fructose, glucose and sucrose for both mono- and multi-component mixtures, were appropriately fitted by linear regression models according to Eq. (5.2). These models were obtained after removing outliers and were found to be highly significant (P<0.001). For all the adsorption isotherms C was found to be a very significant predictor (P<0.001) being the interception values generally not statistically different from zero (P>0.060), as theoretically expected. The linear models obtained explained the variability observed in the experimental q values ($0.977 \leq R^2 \leq 0.999$). Moreover, in general, the residual errors associated to each linear model followed a normal distribution (P>0.075), which is a pre-requirement of the linear regression models. Results obtained are in accordance with the general notions reported in the literature that low initial sugar concentrations conduct to linear adsorption isotherms (Dendene *et al.*, 1995; Luz *et al.*, 2008).

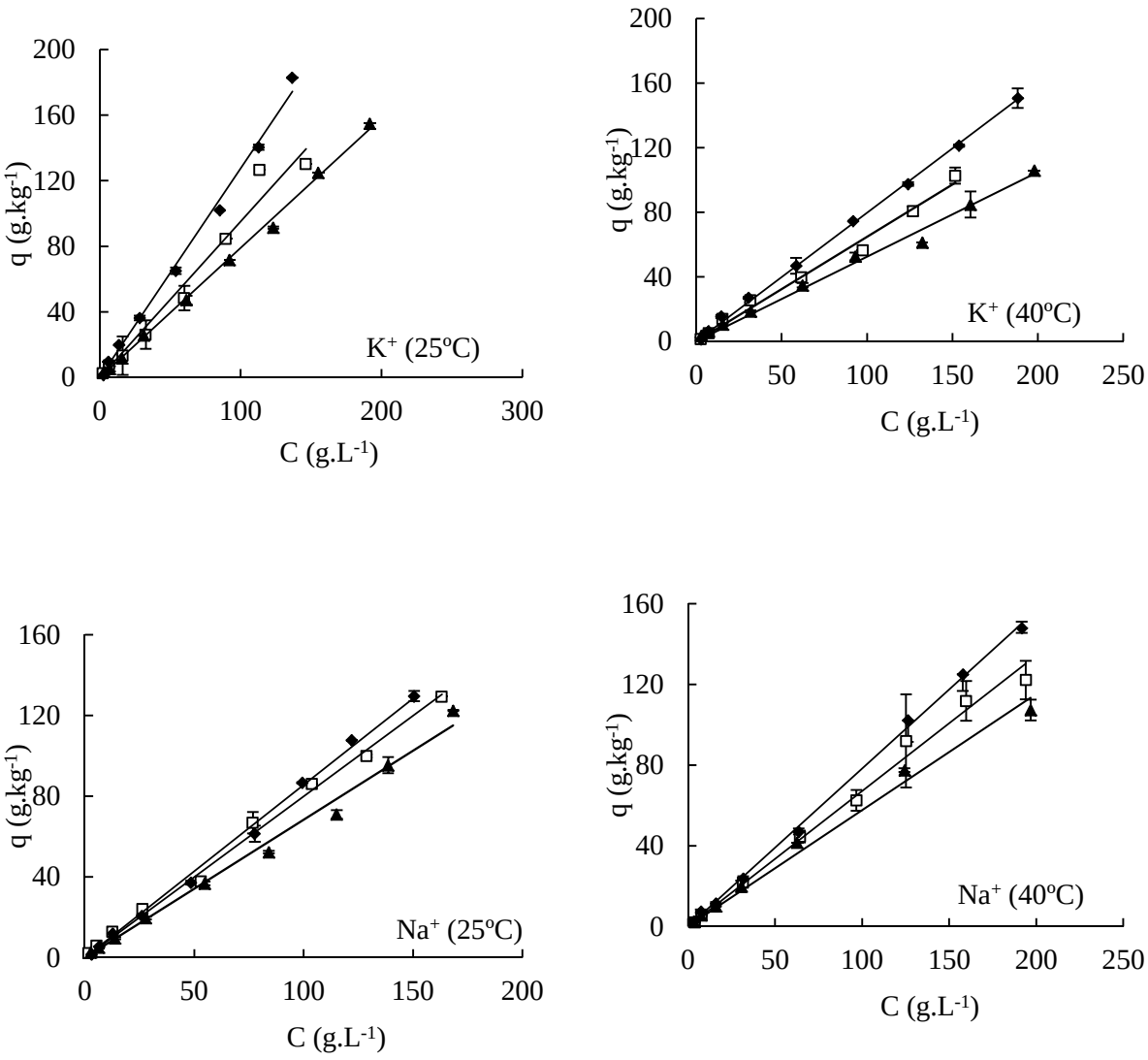

Fig. 5.1 Adsorption isotherms of (◆) fructose, (□) glucose and, (▲) sucrose on Na^+ macroporous and K^+ gel-type resins, at 25 and 40ºC, with mono-component mixtures.

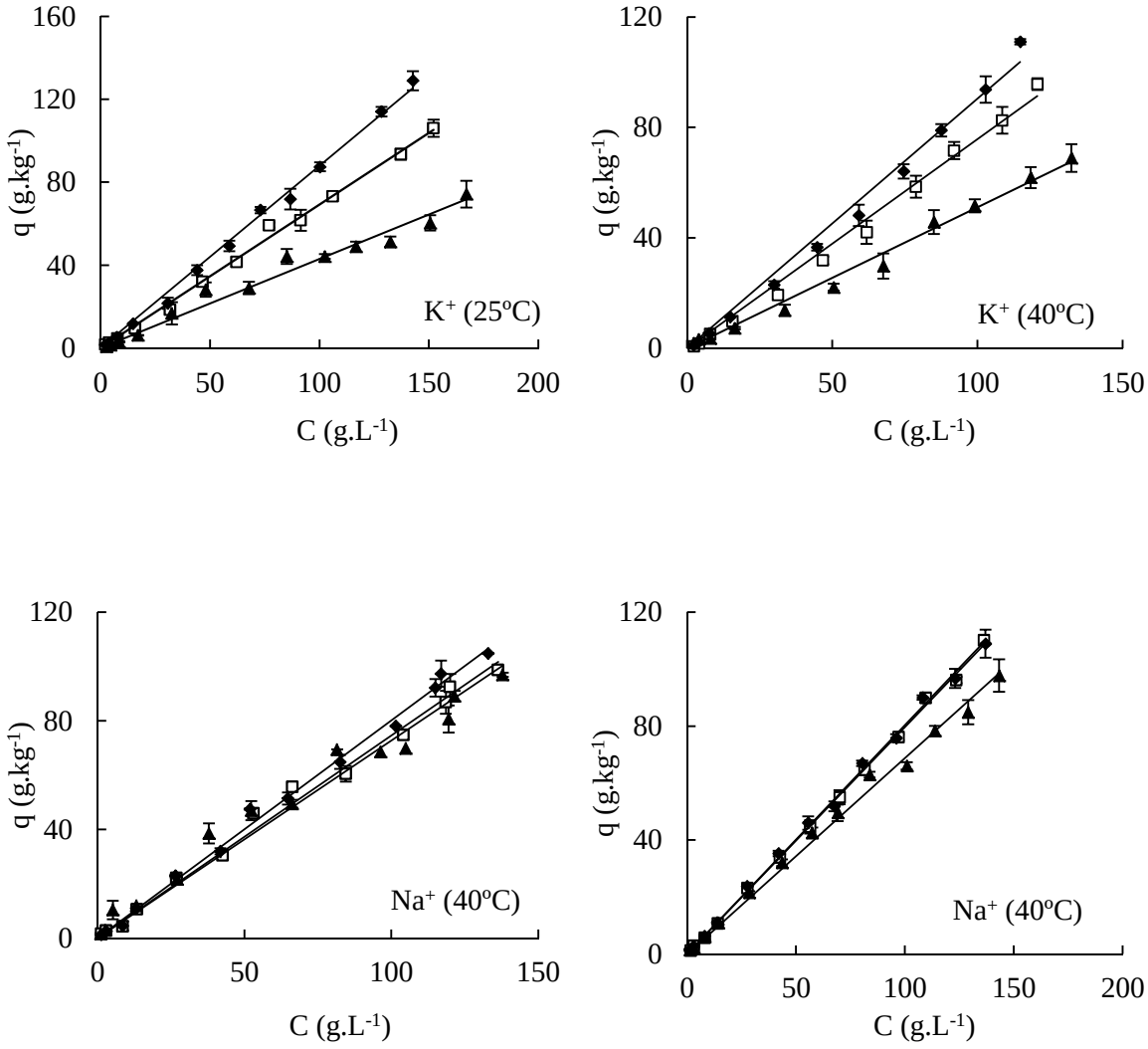

Fig. 5.2 Adsorption isotherms of (◆) fructose, (□) glucose and, (▲) sucrose on Na^+ macroporous and K^+ gel-type resins, at 25 and 40ºC, with multi-component mixtures.

5.3.2 MACROPOROUS RESIN IN SODIUM FORM VERSUS GEL-TYPE RESIN IN POTASSIUM FORM

Potassium and sodium resins are considered non-complex ions, as it has been reported that sugars and univalent cations form weak complexes (Tiihonen *et al.*, 2002). Therefore, for these resins the separation is performed due to the combination of size exclusion and restricted diffusion effects. This separation mechanism is advantageous since it provides higher adsorption kinetic rates as compared with the ones obtained with resins that form strong complexes with sugars, such as calcium resins (Pedruzzi *et al.*, 2008).

In the K^+ and Na^+ resins, the water molecules held in the hydration sphere of the ions are exchanged with some hydroxyl groups of the sugars. Therefore, the number and orientation of the hydroxyl groups determine the relative adsorption of each sugar. The stability of the complex formed depends on the axial-equatorial (eq-ax) sequence of the hydroxyl groups. Glucose and fructose exist as pyranose and furanose ring structures with different mol percentages of α and β forms. The β-D-glucose form does not have any eq-ax and α-D-glucose has one. The α-D-fructose has one eq-ax and β-D-fructose has two (Goulding, 1975). At temperatures between 25 and 40ºC, equilibrium composition for fructose is 10% of α form and 90% of β (Lichtenthaler and Ronninger, 1990); and for glucose is 40% of α and 60% of β (Angyal, 1991). Therefore, for these resins, fructose is expected to be much more adsorbed than glucose. Sucrose does not have any eq-ax oriented group and is excluded from the resin due to its higher molecular size.

The results of the covariance analysis (*data not shown*) showed that, for the multi-component sugar mixtures, the three sugars were adsorbed with different affinities ($P \leq 0.010$) by both the K^+ and Na^+ resins at 25 or 40ºC. Furthermore, as can be observed from Table 5.2, fructose was found to be the most adsorbed sugar, followed by glucose, and finally sucrose. An exception was found for the Na^+ resin at 40ºC, for which fructose and glucose showed similar adsorption rates (K equal to 0.802 and 0.796, respectively, P=0.848) although higher than the ones found for sucrose (K equal to 0.691, P <0.001).

Table 5.2 Distribution coefficients, K ($L.kg^{-1}$), for Na^+ macroporous and K^+ gel-type resins, obtained for multi-component mixtures at 25 and 40ºC.

	K^+				Na^+			
	25 ºC		40 ºC		25 ºC		40 ºC	
F	0.867±0.009	R^2 = 0.996	0.903±0.012	R^2 =0.995	0.799±0.007	R^2 =0.997	0.802±0.006	R^2 =0.998
G	0.692±0.007	R^2 = 0.997	0.757±0.010	R^2 =0.994	0.745±0.008	R^2 =0.996	0.796±0.004	R^2 =0.999
S	0.429±0.009	R^2 = 0.985	0.511±0.008	R^2 =0.991	0.726±0.013	R^2 = 0.989	0.691±0.007	R^2 =0.996

For multi-component mixtures at 25ºC (Table 5.2), glucose and mostly sucrose showed higher distribution coefficients for the Na^+ than for the K^+ resin ($P \leq 0.037$). At 40ºC, glucose showed the same adsorption affinity for both resins ($P=0.670$) (Table 5.2), and sucrose showed a higher adsorption affinity for the Na^+ resin ($P<0.001$). On the other hand, fructose showed a lower adsorption affinity for the Na^+ resin for both temperatures studied ($P<0.001$).

Gramblicka and co-workers (2007) studied recently the adsorption of sugar mixtures at 60ºC using two Na^+ resins with different structures. These authors showed that sucrose adsorption in Na^+ macroporous resin was higher than in Na^+ gel-type. Moreover, they found that glucose and fructose had the same adsorption affinities. Additionally, Vente and collaborators (2005a) studied sugars adsorption using two gel-type resins in Na^+ and K^+ forms at 60ºC. They reported a lower fructose and glucose adsorption in the Na^+ resin in comparison with the K^+ one. Therefore, the results obtained in this work at 40ºC are in general in accordance with those reported in the studies described above.

In summary, sucrose adsorbed better in macroporous and Na^+ resins, glucose showed the same adsorption in macroporous and gel-type resins, and fructose was found to have a higher adsorption in the K^+ gel-type resin.

5.3.3 MONO- VERSUS MULTI-COMPONENT MIXTURES

Assays using multi-component sugar mixtures were performed to evaluate sugars adsorption competitiveness. In general, a decrease in the adsorption loading was found

for the sugars in both resins ($P \leq 0.034$), when comparing results gathered in Table 5.2 with the results from experiments conducted with mono-component mixtures at 25ºC (Table 5.3). An exception was found for sucrose in Na^+ resin, where similar results were obtained for mono- and multi-component mixtures ($P=0.532$). At 40ºC the adsorption of each sugar present in the multi-component mixture was higher than the observed in the mono-component ones ($P \leq 0.019$), except for sucrose in K^+ resin where a similar behavior was found ($P=0.463$).

Table 5.3 Distribution coefficients, K ($L.kg^{-1}$), for Na^+ macroporous and K^+ gel-type resins, obtained formono-component mixtures at 25 and 40 ºC.

	K^+				Na^+			
	25 ºC		40 ºC		25 ºC		40 ºC	
F	1.266±0.016	R^2=0.998	0.794±0.006	R^2=0.999	0.852±0.010	R^2=0.998	0.763±0.005	R^2=0.999
G	0.923±0.031	R^2=0.986	0.645±0.027	R^2=0.977	0.793±0.011	R^2=0.997	0.671±0.017	R^2=0.991
S	0.783±0.008	R^2=0.998	0.529±0.010	R^2=0.995	0.683±0.011	R^2=0.995	0.576±0.013	R^2=0.993

Nowak and co-workers (2007) recently studied the competitiveness between fructose, glucose and sucrose by adding individual sugars to a mixture. These authors reported that the presence of additional sugars in solution increases the specific sugar loading. Their experiments were performed at higher temperatures, namely at 60 and 80ºC, and are in agreement with the results obtained at 40ºC in the present work.

In summary, the results reported in the present study showed that at low temperatures, mixing sugars in the same proportion causes a decrease in the adsorption capacity of each sugar. Furthermore, a competitive effect in the sugars adsorption was found. On the other hand, high temperatures provided a reverse effect, and the presence of other sugars in the mixture conducted to an increase in the adsorption loading. Furthermore, a synergetic effect was found.

5.3.4 TEMPERATURE EFFECT

Several authors reported that the use of temperatures above 70ºC promotes a decrease of the liquid phase viscosity, an increase of the components solubility in the solvent, and

microbial growth inhibition (Beste *et al.*, 2000; Azevedo and Rodrigues, 2001; Luz *et al.*, 2008). Despite these advantages, in the present study the temperatures used were 25 and 40ºC because the maximum operating temperature for the Na^+ resin used was 40ºC, and the range of sugar concentrations studied was low.

Among the several phenomena that contribute to the adsorption mechanism of sugars in PS-DVB ion-exchange resins, the temperature dependence of the distribution coefficient is essentially attributable to complexation and adsorption. The contribution of temperature to the adsorption is correlated with the enthalpy term of the Van't Hoff equation. As a function of the variation of temperature, changes in retention can be caused either by variations in enthalpy or in entropy.

The adsorption capacity found for both resins, at 25 and 40ºC, showed that increasing the temperature promoted a decrease of the distribution coefficients for all the sugars studied in mono-component mixtures (Table 5.3) (P<0.001). These results are in agreement with the results obtained by Dendene and collaborators (1995). These authors compared the adsorption of sugars using Na^+ and K^+ gel-type resins at different temperatures.

As verified by Pedruzzi and co-workers (2008), the adsorption of sugars in PS-DVB resins leads to low values of enthalpy indicating that the adsorption is mainly physique, involving essential size-exclusion and ion-exclusion modes. In these cases, adsorption of sugars decreases at higher temperatures. The decrease of adsorption of chemically bonded sugars is most probably caused by weakening binding forces between the sugar and the resin (Nowak *et al.*, 2007).

Nevertheless, for the Na^+ resin the adsorptions of fructose and sucrose in multi-component mixtures were not significantly affected by the temperature (P≥0.866), and for glucose an increase on the adsorption was observed (P<0.001). Regarding the K^+ resin, a temperature increase resulted in an increase of the adsorption for all the sugars (P≤0.012). These results showed that, for K^+ gel-type and Na^+ macroporous resins, increasing the temperature conducted to a higher sugar adsorption for multi-component mixtures.

Studies using multi-component adsorption of sugars are scarce. The work done by Nowak and co-workers (2007) showed no temperature dependency of the distribution coefficient for glucose and sucrose, and a decrease of the adsorption of fructose was found when increasing the temperature. However, Best and collaborators (2000) showed an increase of glucose adsorption as a result of the temperature increase.

The ion form of the resin, the DVB content and the sugars present in the mixtures seem to have a great influence on the adsorption behavior. In the present study, for Na^+ resin, the adsorption was found to be independent of the temperature, suggesting that this is an entropically determined process rather than enthalpic. However, for K^+ resin, the results showed higher adsorption for higher temperatures probably due to complexation with the cation.

5.3.5 SELECTIVITY

Resin selectivity is a relevant parameter that should be considered whenever selecting the best resin for a certain separation. In the present study, the selectivity values were calculated using the distribution coefficients obtained previously for multi-component mixtures at 25 and 40ºC (Table 5.2) and are presented in Table 5.4. The lowest selectivity was found for fructose/glucose since these sugars have the same molecular weight. Furthermore, the higher selectivity was found for fructose/sucrose due to the strong fructose adsorption onto the resins. These behaviors were found for both resins studied regardless of the temperature used.

Table 5.4 Selectivity values (α) for glucose, fructose, and sucrose adsorbed to Na^+ macroporous and K^+ gel-type resins.

	K^+		Na^+	
	25 ºC	40 ºC	25 ºC	40 ºC
F/G	1.25	1.18	1.07	1.01
G/S	1.61	1.49	1.02	1.15
F/S	2.02	1.76	1.09	1.16

Higher temperatures favored a decrease of the selectivity for the K^+ resin. However, for the Na^+ resin, the selectivity seemed to have not been affected by temperature.

In this work, the selectivity values found for K^+ gel-type resin were higher than for the Na^+ macroporous resin. These results are in accordance with the work of Gramblicka and co-workers (2007) that reported higher selectivity values when working with gel-type resins. Also, Vente and collaborators (2005a) found higher selectivity values for K^+ gel-type resins as compared to Na^+ ones.

5.4 CONCLUSIONS

Fructose, glucose and sucrose adsorption isotherms, determined by the static method in batch mode, for Na^+ macroporous and K^+ gel-type resins, showed linearity for the range of sugar concentrations used. Fructose was found to be the most adsorbed sugar in both resins, followed by glucose and finally sucrose.

Comparing the adsorption of each sugar present in a multi-component mixture, it was found that sucrose was more adsorbed in the Na^+ macroporous resin; glucose showed almost the same adsorption in both resins; and fructose was more adsorbed in K^+ gel-type resin.

A competitive effect on the adsorption was found at 25ºC for the multi-component mixtures as compared to the mono-component ones. However, a reverse effect was found at 40ºC, therefore a synergetic adsorption of sugars in mixtures of multi-components was observed.

The temperature increase conducted to a decrease in the adsorption for mono-component sugar mixtures. However, for multi-component sugar mixtures the temperature increase conducted to an increase of the adsorptions.

Finally, the values of selectivity obtained for K^+ gel-type showed that this resin is probably the best choice to separate mixtures of fructose, glucose and sucrose, operating at 25ºC.

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CHAPTER 6

FRUCTO-OLIGOSACCHARIDES SEPARATION BY SIMULATED MOVING BED

ABSTRACT

On a large scale, fructo-oligosaccharides (FOS) are produced by transfructosylation of sucrose through microbial enzymes. The fermentative broth obtained contains, apart from FOS, small chain saccharides that do not contribute to the beneficial prebiotic activity and therefore must be removed. In this work, the separation of FOS from mono- and disaccharides by Simulated Moving Bed (SMB) chromatography was studied. In order to choose the stationary phase, batch experiments were conducted with two ion-exchange resins: Dowex Monosphere 99K/320 and Dowex 50WX2, both in the potassium form. Both adsorbents presented linear isotherms for all sugars. The Dowex Monosphere 99K/320 resin was chosen for the process modelling and SMB operation. A mathematical model was used to estimate kinetic parameters and simulate the chromatographic elution profile of the fermentative broth loaded onto the Dowex Monosphere 99K/320 resin. Finally, FOS were successfully purified from 37.1 to 62.9% (w/w) using the SMB pilot plant. FOS yield and productivity obtained in the raffinate was 69.4% (w/w) and 82.1 $g.L^{-1}.h^{-1}$, respectively.

6.1 INTRODUCTION

Fructo-oligosaccharides (FOS) are non-digestible sugars known to be selectively fermented by the beneficial bacteria existent in the colon. Consequently increases their growth and/or activity and subsequently improves the health of the host (Gibson, 1999). FOS are considered prebiotics (Roberfroid, 2007) and many health benefits have been associated with their daily intake (Kelly, 2009). Based on that, FOS, namely kestose (GF_2), nystose (GF_3) and fructo-furanosilnystose (GF_4), have been extensively used as food supplements. Therefore, an increased commercial interest on these oligosaccharides has driven the research efforts during the last decade, particularly in the development of efficient recovery processes.

On a large scale, FOS can be produced by fermentation of sucrose through fructosyltransferases enzymes that are present in many microorganisms (e.g. *Aureobasidium pullulans* and *Aspergillus niger)* (Maiorano *et al.*, 2008). The maximum yield obtained by the enzymatic synthesis is around 55-60% of FOS because glucose, a by-product, inhibits the enzymatic reaction (Yun and Song, 1993; Sangeetha *et al.*, 2005). Consequently, the final composition of the broth includes not only FOS, but also di- and monosaccharides, namely sucrose (S), fructose (F) and glucose (G) that do not contribute to the beneficial prebiotic properties and must be removed from the mixture. For this purpose, several recovery and fractionation techniques have been explored (Sanz and Martinez-Castro, 2007; Pinelo *et al.*, 2009).

Simulated Moving Bed (SMB) chromatography is a separation technique developed for the petrochemical industry in the 60s, though it has also been largely used for the fractionation of sugars for more than 30 years (Broughton and Gerhold, 1961; Charton and Nicoud, 1995; Minceva *et al.*, 2003). Lately, the use of SMB was extended to the fine chemistry (Pavone and Hotier, 2000) and pharma industries (Juza *et al.*, 2000), enantiomer separation (Pais *et al.*, 1998) and biotechnology fields (Zhang *et al.*, 2001).

The principle underlying the SMB technology consists in the continuous counter-current motion of the solid phase relatively to a liquid phase, without the real motion of the adsorbent, thus avoiding the technical problems of a true moving bed. The movement of

the adsorbent is actually simulated by the use of a valve system that moves the position of two inlet (feed and eluent) and two outlet streams (raffinate and extract) by switching one column in the direction of the liquid phase flow, at a fixed interval of time (Charton and Nicoud, 1995) (Fig. 6.1).

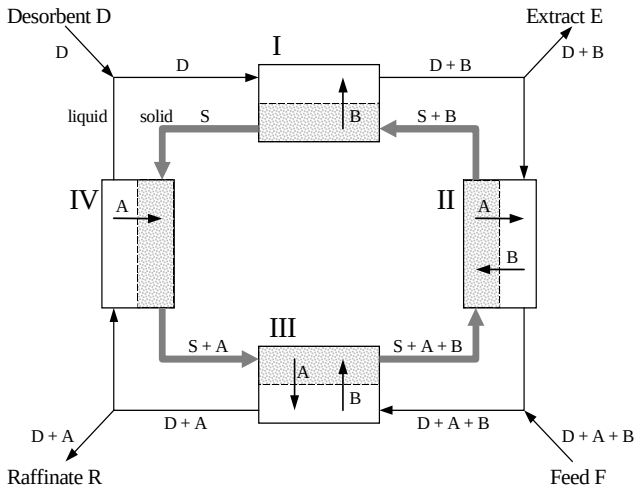

Fig. 6.1 Schematic diagram of a SMB unit with 4-zones.

The main advantage of this technology is that it can be used for difficult separations, using adsorbents with low selectivities and with a small number of theoretical plates (Charton and Nicoud, 1995). Therefore, the peaks obtained in the chromatographic profile do not have to be completely separated, just at specific locations of the unit where the outlet streams, raffinate and extract, are collected. Furthermore, as the system is continuous, high productivities per unit time and unit mass of adsorbent can be obtained with low eluent consumption (Mazzotti *et al.*, 1997; Gomes *et al.*, 2006).

The number of reports on the adsorption of oligosaccharides onto ion-exchange resins for SMB applications is limited (Kawase *et al.*, 2001; Gramblicka and Polakovic, 2007; Vankova and Polakovic, 2010; Vankova *et al.*, 2010a; Vankova *et al.*, 2010b). Geisser and co-workers (2005) were the only ones reporting the separation of a complex mixture of oligosaccharides using a SMB plant. These authors studied the separation of lactose from the oligosaccharides mixture in human milk. Regarding the applications of SMB

for separating sugars, most studies were conducted with binary mixtures that are less complex than a fermentative broth mixture composed of several sugars and salts (Beste *et al.*, 2000; Azevedo and Rodrigues, 2001; Coelho *et al.*, 2002; Lee, 2003). Therefore, until now, no work has reported the separation of FOS from the smaller saccharides in a fermentative mixture using a SMB plant (Vankova and Polakovic, 2010).

The first step to study the separation of any compound by SMB is to choose an efficient adsorbent. Ion exchange resins of sulfonated poly(styrene-*co*-divinylbenzene) (PS-DVB) have been largely used as adsorbents in the sugar industry due to their chemical inertness, higher capacity and selectivity (Tiihonen *et al.*, 2002; Luz *et al.*, 2008). These non-ionic resins can be functionalized with cations, such as potassium. Potassium cations form weak complexes with sugars providing high kinetic adsorption rates (Pedruzzi *et al.*, 2008). Hence, potassium resins have proven to be suitable for the separation of sugar (Vente *et al.*, 2005a; Pedruzzi *et al.*, 2008; Nobre *et al.*, 2009).

The main goal in this work is to evaluate the separation of FOS from the mono- and disaccharides contained in a fermentative broth mixture by SMB. The work has been planned in three distinct stages. Initially, experiments were conducted in a single column in order to select the best stationary phase between a Dowex Monosphere 99K/320 and a Dowex 50WX2 resins in potassium form. Using the selected resin, the equilibrium adsorption isotherms were determined for each single sugar. Afterwards, a model of the chromatographic elution on a single column was developed. Kinetic and adsorption parameters were identified. Finally, the separation of FOS from sucrose, glucose and fructose (SGF) was evaluated in a SMB pilot plant.

6.2 MATERIAL & METHODS

6.2.1 ADSORBENT

The gel-type PS-DVB resins Dowex Monosphere 99K/320 (in potassium form) and Dowex 50WX2 (with 200-400 mesh, in hydrogen form) were purchased from Supelco. Physicochemical properties of the resins are summarized in Table 6.1

Table 6.1 Physicochemical properties of resins.

PHYSICOCHEMICAL PROPERTIESA	DOWEX 50WX2 200-400	DOWEX MONOSPHERE 99K/320
Ionic form	H^+	K^+
Structure	Gel-type	Gel-type
Matrix	Styrene-DVB	Styrene-DVB
Functional group	Sulfonate	Sulfonate
Cross-linkage	2%	6%
Total capacity (eq/L)	0.6 (H^+ form)	>1.5 (H^+ form)
Water content	74-82% (H^+form)	57-61% (H^+ form)
Particle size	37-74 µm (200-400 mesh)	317+15 µm

[a] Data obtained from supplier

6.2.2 COLUMN PACKING

Columns were packed by the slurry method on Superformance® glass columns SP 300x16 from Götec Labortechnik. The columns have 1.6 cm of internal diameter and are equipped with two pistons that allow the length adjustment between 20-30 cm, and a thermostatic jacket for temperature control.

Dowex 50WX2 resin, purchased in H^+ form, was converted to the K^+ form by eluting a 0.5 M K_2SO_4 solution at 3 $mL.min^{-1}$ flow-rate. The procedure was monitored by measuring the pH on the column outlet. When the pH reached the pH of the feed

solution, the cation conversion was assured. Afterwards, the resin was re-equilibrated with MilliQ pure-water.

The analytical system used was a Waters® High-Performance Liquid Chromatography (HPLC) system equipped with refractive index (RI) and ultraviolet (UV) detectors.

6.2.3 SUGAR MIXTURE

The fermentative broth, containing FOS and small saccharides, was produced in a Lab Pilot Fermenter of 75 L (Type LP351, Bioengineering). Fermentation was conducted with *Aureobasidium* sp. fungi according to previously established conditions (28 ºC, 300 rpm and pH 5.0) (Rocha *et al.*, 2009).

At 50 h of fermentation, the biomass was removed by centrifugation at 35.000 rpm in a continuous flow centrifuge (CEPA, Carl Padberg, Zentrifugenbau GmbH) fed at 100 $mL.min^{-1}$, followed by ultra-filtration of the fermentative broth through a Pellicon ultrafiltration cassette with 10 kDa cut-off (Millipore).

Based on the initial medium composition, the cations expected to be present in the fermentative broth mixture are: sodium, potassium and some traces of magnesium. In order to avoid any cation exchange between the resin and the fermentative broth, the mixture was pre-treated to guarantee that only the present cation (at least 90%) in the final broth was potassium. A 200 mL volume of fermentative broth was passed in consecutive batches steps through a column filled with 100 mL of Dowex Monosphere 99/K 320 at $5mL.min^{-1}$. Between each batch, the resin was washed with Milli-Q pure water and regenerated to K^+ form with 150 mL of a 0.5 M K_2SO_4 solution. The ion exchange procedure was monitored by measuring the conductivity at the column outlet.

Samples were collected for the determination of sodium, potassium and magnesium by flame atomic absorption spectrophotometry.

6.2.4 SUGARS ANALYSIS

Sugars were analysed by HPLC with a Prevail Carbohydrate ES column (250 × 4.6 mm) from Alltech, using a mobile phase consisting of acetonitrile (HPLC grade; Carlo Erba) and water (70:30 v/v), with 0.04% of ammonium hydroxide in water (HPLC grade; Sigma). Elution was conducted at 1 $mL.min^{-1}$ flow-rate and room temperature, and sugars were detected by RI (Dias *et al.*, 2009; Nobre *et al.*, 2009).

FOS, namely GF_2, GF_3 and GF_4, were purchased from Wako (Wako, Chemicals GmbH). Sucrose, fructose and glucose (analytical grade) were obtained from Merck.

6.2.5 RESIN SELECTION

In order to analyze the suitability of resins for the separation of FOS from the mono- and disaccharides present on the fermentative broth, 10 μL volume of the broth were loaded in the packed columns. The experiments were performed at a 2 $mL.min^{-1}$ flow-rate and 21ºC, and the elution profiles were measured and recorded with the RI detector.

Tracer pulse experiments were conducted with blue-dextran in order to determine the void fraction (ε) of resins. A 10 μL volume of a 5 $g.L^{-1}$ blue-dextran solution was loaded in each column at a 2 $mL.min^{-1}$ flow-rate (Q). The retention time (t_0) and concentration at the exit of the column were recorded by the UV detector set at 211.4 nm. The void volume was determined by the following equation:

$$\varepsilon = \frac{(t_0 . Q - V_d)}{V_c} \tag{6.1}$$

where V_c and V_d are the column and the void volumes, respectively.

The total bed porosity (ε_T) was determined by injecting a K_2SO_4 pulse, a non-retained pore-penetrating tracer substance. Using the retention time recorded by the RI detector, ε_T was further calculated by Eq. (6.1). ε_T represents the sum of the void within the particles as well as between particles. The particle porosity was afterwards calculated by the following equation:

$$\varepsilon_p = \frac{(\varepsilon_T - \varepsilon)}{(1 - \varepsilon)} \tag{6.2}$$

The separation efficiency was evaluated by the retention factor ($k_{s,i}$) and the separation factor (α) obtained for each resin according to the following equations:

$$k_{s,i} = \frac{(t_i - t_0)}{t_0} \tag{6.3}$$

$$\alpha = \frac{k_{s,i}}{k_{s,j}} \qquad \text{where } i \neq j \text{ and } k_{s,j} > k_{s,i} \tag{6.4}$$

where t_i is the retention time obtained for the sugar i, using the RI detector.

For the quantitative determination of the column efficiency or the number of theoretical plates (N), an amount of 10 μL of fermentative broth was injected into the packed column at five different flow-rates: 0.5, 1, 1.5, 2, 2.5 mL.min^{-1}. From the resulting chromatogram the column efficiency was calculated for FOS and SGF, respectively. Considering that peaks are symmetrical and perfectly Gaussian, the column efficiency was calculated using the retention time and width at half height ($w_{1/2,i}$) of each peak (Schulte and Epping, 2005):

$$N_i = 5.54 \left(\frac{t_i}{w_{1/2,\ i}}\right)^2 \tag{6.5}$$

To compare the efficiency of columns with different lengths (L), the efficiency per meter ($N_{L,i}$) was calculated by the following equation:

$$N_{L,i} = \frac{N_i}{L} = \frac{1}{HETP} \tag{6.6}$$

The height of an equivalent theoretical plate (*HETP*) was calculated using the determined $N_{L,i}$ value (Eq. (6.6)).

The resolution (R_s) is defined by the ratio of the distance between the retention times of the components, FOS (t_A) and SGF (t_B), and the arithmetic mean of the two peak widths ($w_{1/2,i}$):

$$R_S = 1.18\frac{(t_B - t_A)}{(w_{1/2,A} + w_{1/2,B})} \quad (6.7)$$

6.2.6 ADSORPTION ISOTHERMS

The adsorption equilibrium isotherms for FOS and SGF present in the fermentative broth were determined by the Retention Time Method (RTM) for both resins, and for the selected resin by the column adsorption-desorption method (Vente *et al.*, 2005b).

6.2.6.1 RTM method

A 10 µL volume of fermentative broth was loaded onto the column at 2 mL.min^{-1} flow-rate and 21ºC. Using the RI detector, the retention time of FOS and SGF was determined. Since this method is only valid under linear conditions, several concentrations were studied to assure that the retention time was not affected by the concentration.

The adsorption constants (H_i) were estimated from the retention times (t_i) according to the following equation:

$$H_i = \left(\frac{\varepsilon}{1-\varepsilon}\right)\left(\frac{t_i}{t_0} - 1\right) \quad (6.8)$$

6.2.6.2 Adsorption-desorption method

The packed column was connected to an HPLC system and the breakthrough curves were monitored by the UV detection at 211.4 nm, since the RI signal is saturated for concentrations higher than 6 g.L^{-1}. Samples were collected during the process and the total sugar concentration was determined using an Abbe refractometer. Equilibrium experiments were conducted at 30ºC and flow-rate of 5mL.min^{-1}.

After equilibration with Milli-Q pure water, the column was fed with diluted pre-treated fermentative broth containing G, F, S, GF_2, GF_3 and GF_4. The fermentative broth was loaded until the UV detector signal and the global concentration measured by

refractometry was constant, thus ensuring the equilibrium between the liquid and solid phases.

Afterwards, the column was disconnected from the HPLC and closed, and the system was washed with Milli-Q pure water to remove all the remaining sugars. Next, the column was reconnected and the sugars were desorbed from the column with Milli-Q pure water. This procedure was stopped when the RI signal reached zero. The collected effluent was then analyzed by HPLC to determine the total concentration of each sugar in equilibrium inside the column.

Different dilutions of the fermentative broth, consequently different initial sugars concentration, were studied.

The mass of sugar adsorbed per mass of resin (q_i) was calculated from the mass balance presented below:

$$m_i = \varepsilon.V_c.C_i + (1-\varepsilon).V_c.q_i \quad (6.9)$$

where m_i is the mass of total sugars inside the column after the equilibrium and C_i the initial load concentration of the component *i*.

Adsorption equilibrium isotherms were fitted by the linear model that follow Henry's law and correlate the concentration of adsorbate into the stationary phase, q_i, with the concentration in liquid phase, C_i. The isotherm is represented by the following equation:

$$q = \gamma_i.C, \text{ i} = 1, \ldots, \text{n} \quad (6.10)$$

where γ_i, the slope of the isotherm, represents the distribution coefficient (Henry's constant).

6.2.7 SINGLE CHROMATOGRAPHY COLUMN MODELLING

Regarding the adsorption kinetics, the chromatographic elution profile of the fermentative broth loaded onto the Dowex Monosphere 99K/320 resin was simulated by the kinetic and Linear Driving Force (LDF) models and compared with the experimental

data. In this study, a binary separation was envisaged, namely the separation of FOS from SGF. Therefore, the model parameters were identified and estimated for these two peaks. The isotherm parameters, porosity, fluid velocity, diffusion and mass transference coefficients were experimentally determined from batch experiments. However, the methods used to determine these parameters are normally based on ideal assumptions. In this work, a model identification procedure was developed in order to determine a set of estimated parameters that could lead to a minimum distance between the simulated and the experimental data using the least-squares approach.

6.2.7.1 LDF Model

The LDF model, first introduced by Glueckauf and Coates (1947) describes well the intra-particle mass transfer in linear chromatographic systems (Gomes *et al.*, 2006). The LDF model assumes a parabolic concentration profile in the pore of the adsorbent. The mass balance for a component *i* in the liquid phase is described by the model as follows:

$$\frac{\delta c_i}{\delta t} + \frac{1-\varepsilon}{\varepsilon}\frac{\delta q_i}{\delta t} = D_{L,i}\frac{\delta^2 c_i}{\delta z^2} - u\frac{\delta c_i}{\delta z} \tag{6.11}$$

where $D_{L,i}$ is the diffusion coefficient, u the fluid velocity and z the axial coordinate. The index i represents the components SGF and FOS.

The diffusion rate into the particle is proportional to the amount required to produce the equilibrium, therefore the solid phase mass balance equation is described as follows:

$$\frac{\delta q_i}{\delta t} = k_{F,i}(q_i^{eq} - q_i) \tag{6.12}$$

where $k_{F,i}$ represents the mass transfer coefficient, and q_i^{eq} the adsorbed equilibrium concentration.

6.2.7.2 Kinetic Model

In the kinetic model all the dispersion effects are lumped in a LDF mass transfer coefficient. Therefore, the mass balance in the liquid phase is described by the following equation:

$$\frac{\delta c_i}{\delta t} + \frac{1-\varepsilon}{\varepsilon}\frac{\delta q_i}{\delta t} = -u\frac{\delta c_i}{\delta z} \tag{6.13}$$

and in the solid phase is given by:

$$\frac{\delta q_i}{\delta t} = {k_i}^{rel} u(q_i^{eq} - q_i) \tag{6.14}$$

where k_i^{rel} is the relative mass transfer coefficient.

6.2.7.3 Equilibrium Dispersive Model

The LDF and kinetic parameters were determined by a relation obtained in linear conditions between the parameters of an Equilibrium Dispersive (ED) and a LDF model Eq. (6.23). In the Equilibrium Dispersive (ED) model the effects of mass transfer resistance and the axial dispersion are lumped into an apparent axial dispersion term ($D_{app,i}$). The mass balances for the liquid and solid phases are described as following:

$$\frac{\delta c_i}{\delta t} + \frac{1-\varepsilon}{\varepsilon}\frac{\delta q_i}{\delta t} = D_{app,i}\frac{\delta^2 c_i}{\delta z^2} - u\frac{\delta c_i}{\delta z} \tag{6.15}$$

$$q_i^{eq} = q_i \tag{6.16}$$

where $D_{app,i} = \frac{L.u}{2N_i}$ (6.17)

6.2.7.4 Initial and boundary conditions

The initial condition describes the state of the column at the beginning of the experiment. In the initial state, inside the column there is only the stationary phase and pure solvent, therefore initial conditions are given by:

$$C_i\,(z,\, t = 0) = 0 \quad \text{for } 0 \leq z \leq L \tag{6.18}$$

When numerical solutions are used to solve systems of Partial Differential Equations (PDEs), it is critical to use a well defined boundary condition. The rectangular boundary condition is often used because it simulates the opening and closing of an ideal mechanical valve. However, in reality the boundary condition is affected by the physical performance of the valve and by the dispersion phenomena. Boundary conditions at the column inlet are therefore described as follows:

$$C_i\,(0,t) = C_i^o \Phi(t) \tag{6.19}$$

$$\phi(t) = \begin{cases} (t - t_d) < t_p, 1\text{-}e^{\left(-\frac{t-t_d}{\tau}\right)} \\ (t - t_d) \geq t_p, 1\text{-}e^{\left(-\frac{t-t_d}{\tau}\right)} - \left(1\text{-}e^{\left(-\frac{t-t_d-t_p}{\tau}\right)}\right) \end{cases} \tag{6.20}$$

where C_i^0 represents the concentration of the component *i* in the feed, $\Phi(t)$ is the injection profile , t_d the time delay, t_p the injection duration and τ the time constant of the valve which controls the rise time of the pulse.

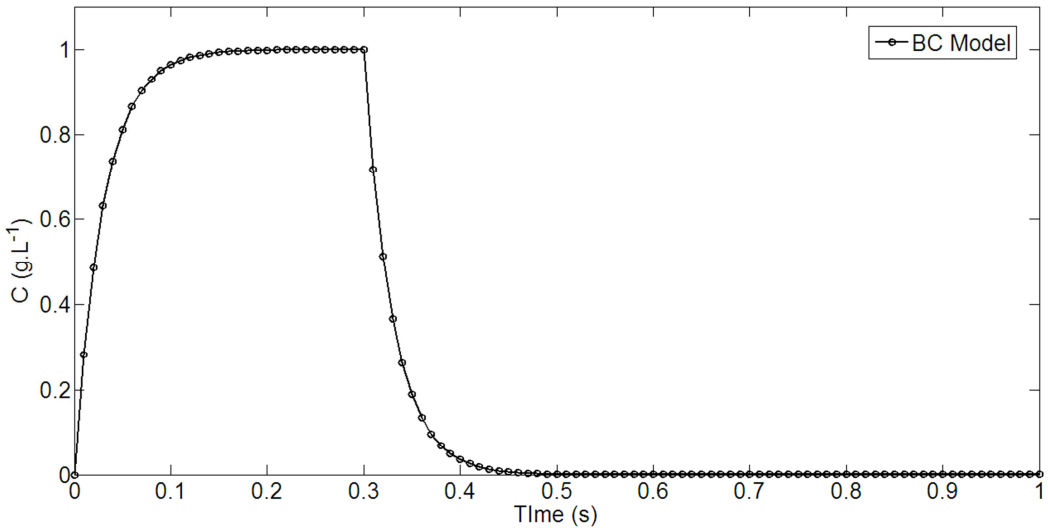

Fig. 6.2 Boundary condition model.

In a HPLC set-up, the sample mixture is first injected into a closed loop with a known volume (V_{Loop}). Afterwards the loop is filled, a valve opens the loop, and the sample is loaded into the column. In this case, the injection time is determined using the following equation:

$$t_p = \frac{V_{Loop}}{Q} \tag{6.21}$$

By adjusting the τ parameter, the shape of the boundary condition is changed, while the amount of mass injected into the column remains constant. The t_d parameter accounts for the delay introduced by the dead-volume between the loop and the column, and is obtained directly from the chromatograms.

6.2.7.5 Initial estimates

The parameters needed for the LDF and kinetic models are the void volume, diffusion coefficients, internal fluid velocity, mass transfer coefficients and the isotherm parameters. The void volume and the isotherm parameters were determined from

experiments performed in the column. The fluid velocity was calculated by the following equation:

$$u = \frac{Q}{\varepsilon V_c} \tag{6.22}$$

Finally, the initial estimates for the diffusion ($D_{L,i}$) and mass transference ($k_{F,i}$) coefficients were determined by the following equation:

$$D_{app,i} = D_{L,i} + \frac{k'_{0,i}}{\left(1 + k'_{0,i}\right)^2}\frac{1}{k_{F,i}}u^2 \tag{6.23}$$

where $k'_{0,i} = \frac{(1-\varepsilon)}{\varepsilon}H_i$ (6.24)

Eq. (6.23) results from the relation obtained in linear conditions between the parameters of an ED and a LDF model (Guiochon *et al.*, 1994; Grosfils *et al.*, 2007).

For the LDF model, the diffusion ($D_{L,i}$) and mass transference coefficients ($k_{F,i}$) were experimentally determined by the injection of sugars at different fluid velocities. $D_{app,i}$ was plotted against u^2 and the $D_{L,i}$ and $k_{F,i}$ values were afterwards determined by the *y*-intercepts and slope, respectively.

For the kinetic model, no diffusion is considered and, $D_{L,i}$ is set to zero. Therefore, the $k_{F,i}$ parameter is easily determined from Eq. (6.23).

6.2.7.6 Calibration curves

Eluted sugars are detected by RI at the outlet of the column. Hence, the data collected from the sugars profile concentration is given as a RI signal along the time. In order to convert the RI signal into concentrations, calibration curves for fructose, glucose, sucrose and the SGF mixture were performed. The SGF mixture was prepared in such a way that the concentration of each sugar in the mixture had the same proportion as in the fermentative broth.

6.2.7.7 Numerical solution

Except for a few simple cases, no analytical methods enable a solution for a system modelled by PDEs; therefore numerical techniques must be used. These numerical solutions can be derived using different methods. The simulator used in the current work is based on the "method of lines". The principle behind this technique consists in the replacement of a spatial variable by a grid of small equal segments, and the replacement of the spatial differential terms by a finite difference approximation in the LDF and kinetic models (Eqs. (6.11) and (6.13)). In this way, the PDEs are transformed in Ordinary Differential Equations (ODEs) which are easily solved by readily available solvers.

The spatial derivatives are approximated using differentiation matrices according to:

$x_z = D_1.x$ (for the first order derivative terms) (6.25)

$x_{zz} = D_2.x$ (for the second order derivative terms) (6.26)

For this simulation, two finite difference schemes from Matlab MatMol toolbox (www.matmol.org; (Wouwer *et al.*, 2004)) were selected, namely a *four point biased upwind* scheme on a uniform grid for the first derivative and a *five point centered* scheme on a uniform grid for the second derivative. For a small number of discretization points, the numerical solution developed spurious oscillations in the concentration profiles, to avoid this situation, a slope limiter scheme (*using a Koren limiter)* from MatMol, was used to approximate the first order derivative.

By increasing the number of discretization points, the error introduced by the finite difference scheme can be reduced. Once the number of discretization points have been chosen in such a way that the accuracy and the simulation time are compromised, the parameter identification algorithm can be used. The parameter identification problem is known as an inverse problem because it uses the measurements to adjust the parameters of the model, reshaping the numerical solution to obtain a closer fit. The parameter identification algorithm is based on the minimization of a cost function (J) measuring the deviation between the model prediction C_{sim} and the measured C_{Exp} outputs, in the least square sense:

$$J = \sum_{i=1}^{n} \left(C_{Sim} - C_{Exp}\right)^2 \quad (6.27)$$

Minimization is achieved using the Matlab optimization toolbox. The identification algorithm is initiated using the experimental estimated parameters.

6.2.8 SMB SEPARATION

6.2.8.1 Principle

The basic principle of a SMB is to promote a counter-current flow between the solid and liquid phases without having a true moving bed (TMB) process. Instead of a moving bed, the SMB consists in a series of fixed columns with an appropriated shift of injection and collection points (Ruthven and Ching, 1989). The SMB and TMB principles are very similar. In fact, an SMB with a high number of columns per zone has a performance very close to a TMB one (Charton and Nicoud, 1995). Therefore, the steady state separation performance of an SMB unit can be predicted by using the simpler model of the equivalent TMB (Mazzotti *et al.*, 1997). In order to convert the operating conditions of a TMB unit into an equivalent SMB, the following conversion rules are used (Ruthven and Ching, 1989):

$$V_j^{TMB} = S_j^{TMB} . V_{SMB} \quad (6.28)$$

$$Q_S = \left[\frac{(1-\varepsilon).V_c}{t^*}\right] \quad (6.29)$$

$$Q_j^{SMB} = \left[Q_j^{TMB} + \frac{Q_s.\varepsilon}{1-\varepsilon}\right] \quad (6.30)$$

where V_j^{TMB} and V_{SMB} are the volumes of the *j*th section of the TMB unit and the volume of the fixed bed of the SMB unit, respectively; S_j is the number of subsections in the *j*th section of the SMB unit; t^* represents the switching time of the SMB unit and Q_S, Q_j^{SMB} and Q_j^{TMB} are the flow-rates of the solid phase, SMB unit and the equivalent TMB unit, respectively.

The TMB used was the classical 4-zone process as it is schematically represented in Fig. 6.3. Each zone has a specific function in order to split the feed stream into two components: FOS (component A) and SGF (component B). The 4-zones are separated by the position of the inlet and outlet streams. The eluent flow circles from the zone I to IV and is afterwards recycled to zone I; the adsorbent circulates in counter-current from IV to I where is recycled to zone IV. The inlet streams consist in the feed (F) and eluent/desorbent (D). The outlet streams consist in the raffinate (R) and extract (E). In the process used in the current work, the feed solution corresponds to the treated fermentative broth, and the eluent, to ultra-pure water. The feed stream is injected between the zones II and III, and the eluent between the zones IV and I. The less retained component is FOS. Therefore, this component is eluted with the eluent and collected between zones III and IV. SGF is more retained, therefore it is collected between zones II and I. Zones II and III are the separation zones, while I and IV are the regeneration zones where the adsorbent and the eluent are cleaned of solute, avoiding the cross contamination.

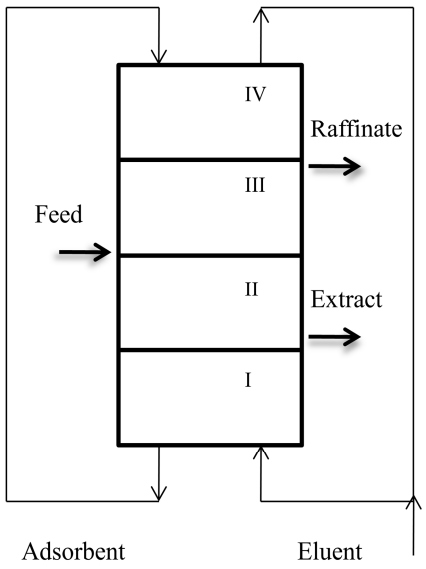

Fig. 6.3 Schematic representation of the 4-zone True Moving Bed principle.

In order to obtain an appropriated separation, each zone must have a specific flow-rate. All the 4-zone flow-rates are related to the inlet/outlet ports by the following mass balances:

$$Q_{II} = Q_I - Q_E \quad (6.31)$$

$$Q_{III} = Q_{II} + Q_F \quad (6.32)$$

$$Q_{IV} = Q_{III} - Q_R \quad (6.33)$$

$$Q_I = Q_{IV} + Q_D \quad (6.34)$$

The inlet and outlet flow-rates are related by:

$$Q_E + Q_R = Q_F + Q_D \quad (6.35)$$

6.2.8.2 Flow-rates determination by the Equilibrium theory

For a linear adsorption equilibrium system, the region where the operating conditions correspond to the complete separation between FOS and SGF can be determined by the Henry constants. These conditions do not depend on the feed concentration. Based on the equilibrium theory, and assuming that the dispersion and the mass transfer effects are negligible, the following inequalities must be fulfilled to determine the complete separation region (Mazzotti *et al.*, 1997):

$$H_B < m_1 \quad (6.36)$$

$$H_A < m_2 < m_3 < H_B \quad (6.37)$$

$$m_4 < H_A \quad (6.38)$$

where m_i are the following dimensionless flow-rate ratios:

$$m_i = \frac{Q_i}{Q_s}, \quad \text{for } i = 1, 2, 3, 4 \quad (6.39)$$

The operating points from the triangle-shaped region of the (m_2, m_3) plane in the Fig. 6.4 correspond to the complete separation region. As shown in Fig. 6.4, the (m_2, m_3) plane is

divided into more three different regions where the complete separation is not achieved (Ching *et al.*, 1985; Mazzotti *et al.*, 1997). In the region where $m_3 > H_B$ and $H_A < m_2 < H_B$, the purity of the extract stream is 100%. However, the raffinate is contaminated with the most retained component (SGF). On the other hand, on the region $m_2 < H_A$ and $H_A < m_3 < H_B$ the purity of the raffinate stream is 100% and the extract is contaminated by FOS. On the region corresponding to $m_3 > H_B$ and $m_2 < H_A$ none of the streams is pure. Hence, SGF and FOS are present in both streams. The optimum operating point from the equilibrium theory lies on the vertex of the triangle.

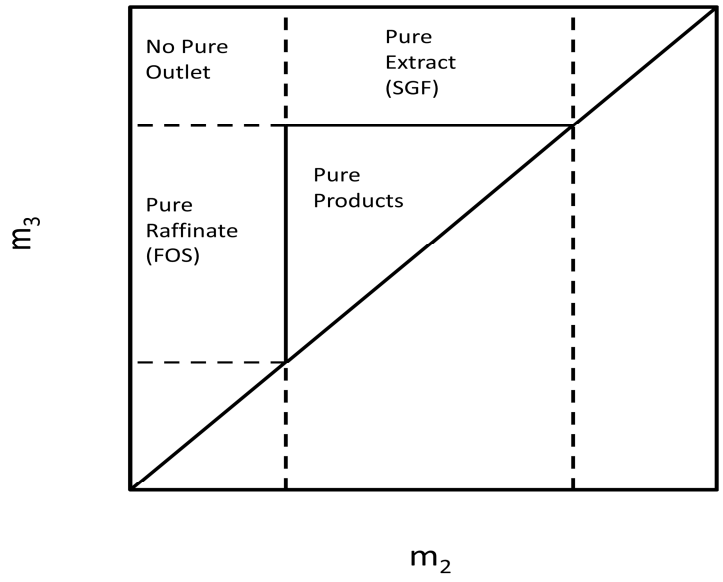

Fig. 6.4 Triangular diagram obtained from the equilibrium theory representing the separation regions of the (m_2, m_3) plane for a system described by the linear adsorption isotherm.

In order to determine the values of the flow-rates m_i, a safety factor (β) should be introduced:

$$m_1 = \beta H_B \tag{6.40}$$

$$m_2 = \beta H_A \tag{6.41}$$

$$m_3 = \frac{H_B}{\beta} \tag{6.42}$$

$$m_4 = \frac{H_A}{\beta} \tag{6.43}$$

If β is close to 1.00, the TMB or equivalent SMB is working under its maximum possibilities and the system becomes very sensitive to the flow-rates and number of plates. Therefore, to operate with a more robust system, even with less productivity, β must be higher than 1.00 and smaller than $\sqrt{\alpha}$. The flow-rate ratios are independent on the size and productivity of the SMB unit.

Finally, the flow-rates on each zone of the TMB can be determined by the following relationship:

$$Q_{jTMB} = m_j Q_s \tag{6.44}$$

The inlet and outlet flow-rates can be determined by the following equations:

$$Q_F = \left[\frac{H_B}{\beta} - H_A.\beta\right] Q_S \tag{6.45}$$

$$Q_D = Q_S \left[\beta.H_B - \frac{H_A}{\beta}\right] \tag{6.46}$$

$$Q_E = Q_S[(H_B - H_A)\beta] \tag{6.47}$$

$$Q_R = Q_S \left[\frac{(H_B - H_A)}{\beta}\right] \tag{6.48}$$

In the current work, the flow-rate conditions were chosen to select operating points from the complete separation region of the (m_2, m_3) plane. From the Henry constants, and using a determined value for the security factor (β), it was possible to predict several sets of values which guarantee the complete regeneration of the solid and liquid phases. Based on these values, a set of four flow-rate ratios m_j were determined and an operating point was selected. By choosing a value for the switching time period (t^*), the solid flow-rate (Q_s), and finally the respective inlet/outlet and internal operating flow-rates of the SMB unit, were calculated.

It is worth pointing out that the choice of the operating conditions may guarantee not only the desired separation, but also the proper operation of the SMB unit. The system works with preparative pumps, therefore the flow-rate must be high enough to assure that the pump delivers with accuracy. On the other hand, the pressure drop across the packed bed should not be higher than the 60 bar, which is the maximum back pressure allowed in this system.

6.2.8.3 Performance parameters

In order to compare the quality of the separation obtained by several SMBs a set of parameters should be determined. Since the eluent is water, the main economic criteria to characterize the SMB separation is the amount of product produced per unit of time and volume of stationary phase, defined by the productivity (($Pr_{i,j}$) $\left[\frac{kg}{s.m^3}\right]$). However, other parameters should be considered when characterizing the SMB performance, such as purity ($Pu_{i,j}$) [%], recovery/yield ($Y_{i,j}$) [%],and dilution ($D_{i,j}$) [%]. These set of parameters are defined by the following equations:

$$Pu_{i,j} = 100\frac{c_{i,j}}{c_{A,j} + c_{B,j}} \tag{6.49}$$

$$Y_{i,j} = 100\frac{c_{i,j}Q_j}{c_{i,F}Q_F} \tag{6.50}$$

$$Pr_{i,j} = \frac{c_{i,j}Q_j}{N_c(1-\varepsilon)V_c} \tag{6.51}$$

$$Di_{i,j} = 100\left(1 - \frac{c_{i,j}}{c_{i,F}}\right) \tag{6.52}$$

where *i* represents the component from the mixture FOS/SGF and *j* the extract/raffinate.

6.2.8.4 SMB plant

The SMB pilot plant used in the current work was a Varicol LAB by Novasep equipped with five Armen pumps. The pumps used to generate feed (head size 50 $mL.min^{-1}$), eluent, extract and raffinate (head size 250 $ml.min^{-1}$, each), were equipped with two pistons. A fifth pump was used to recycle the liquid stream inside the SMB (head size of 500 $ml.min^{-1}$). The maximum working pressure allowed by the system is a 60 bar.

Eight jacked Superformance® glass columns SP 300x16, from Götec Labortechnik, were linked in series and connected to the SMB plant. The column jackets were connected to each other by silicone tubes and to a thermostat bath, keeping the system at 30ºC. Columns were packed with Dowex Monosphere 99K/320 resin. The columns configuration per zone used in the separation was 2-2-2-2, for all the experiments.

The columns were connected to electronically controlled valves that switch on/off the feed and the liquid phase, to the withdrawn ports or to the next column. The process is automatically controlled. The recycle pump is located between zone I and IV and has the same flow-rate as the stream in zone I of the SMB.

6.3 RESULTS & DISCUSSION

6.3.1 SELECTION OF THE STATIONARY-PHASE

Since the main purpose of this study was the purification of FOS from a mixture in a fermentative broth by SMB, the first step was to choose an adequate resin and eluent. Two cationic resins in potassium form were studied, Dowex Monosphere 99K/320 and a Dowex 50WX2. Bearing in mind that the sugars are soluble in water and the purified mixture of FOS should be food grade, ultra-pure Milli-Q water was chosen as an adequate eluent. Furthermore, using water as eluent is an advantage for the global economy of the process (Pedruzzi *et al.*, 2008).

6.3.1.1 Fermentative broth characterization

The fermentative broth used in the separation experiments was found to contain 3.30 $g.L^{-1}$ (Na^{+}), 1.69 $g.L^{-1}$ (K^{+}) and 0.02 $g.L^{-1}$ (Mg^{2+}). As sodium represents 66% of the total ion content of the fermentative broth, and cationic-exchange resins in the potassium form were used, the removal or decrease of the sodium content in the mixture was required. Therefore, a pre-treatment was conducted to exchange sodium by potassium.

Potassium, sodium and magnesium contents determined in the column outlet effluent during the ion exchange procedure are represented in Fig. 6.5. This procedure enabled a 9.6 $g.L^{-1}$ concentration of potassium, representing 98% of the total ions content, in the global volume of treated fermentative broth mixture.

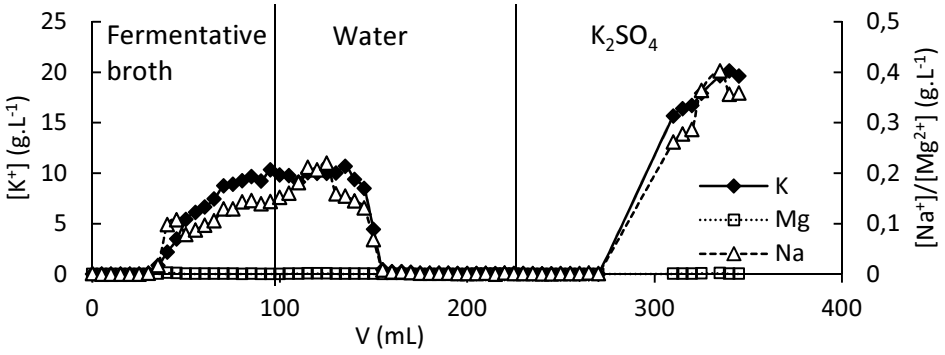

Fig. 6.5 Potassium, sodium and magnesium concentration profiles obtained on the eluent collected during the cation exchange procedure conducted to pre-treat the fermentative broth prior to the separation experiments.

The concentration of sugars determined in the treated fermentative broth, used in the batch experiments and separation assays, was: 15 $g.L^{-1}$ of F, 60 $g.L^{-1}$ of G, 10 $g.L^{-1}$ of S, 18 $g.L^{-1}$ of GF_2, 38 $g.L^{-1}$ of GF_3 and 8 $g.L^{-1}$ of GF_4. Mono- and disaccharides (SGF) were found to represent 57% of the fermentative broth mixture and 70% of these sugars were glucose. FOS represents 43% of the total sugars, and GF_3 is the sugar with higher concentration in the mixture, representing 60% of the total FOS content.

6.3.1.2 Resins characterization

Pulse tests with blue dextran, single sugars and the fermentative broth mixture were performed for the two resins studied.

Fig. 6.6 and Fig. 6.7 show the elution profiles obtained for the injection pulses of 10 µL of blue dextran and single sugars (F (16 $g.L^{-1}$), G (16 $g.L^{-1}$), S (16 $g.L^{-1}$), GF_2 (30 $g.L^{-1}$), GF_3 (50 $g.L^{-1}$) and GF_4 (15 $g.L^{-1}$)) on a Dowex 50WX2 and Dowex Monosphere 99K/320 resin, respectively, both in K^+ form. Fig. 6.8 shows the elution profiles obtained for an injection pulse of 10 µL of fermentative broth onto each resin studied.

For both resins, sugars were found to be eluted in order of decreasing molecular size (Fig. 6.6 and Fig. 6.7). Therefore, the retention time of each single sugar increased in the following order: $GF_4 < GF_3 < GF_2 < S < G < F$. These resins work as a molecular sieve and sugars were separated by size exclusion. Although G and F have the same molecular weight, the different orientation of the OH groups determines the relative adsorption of each sugar (Goulding, 1975). Fructose has more eq-ax sequences in its configuration, therefore, it is expected to adsorb more than glucose (Nobre *et al.*, 2009).

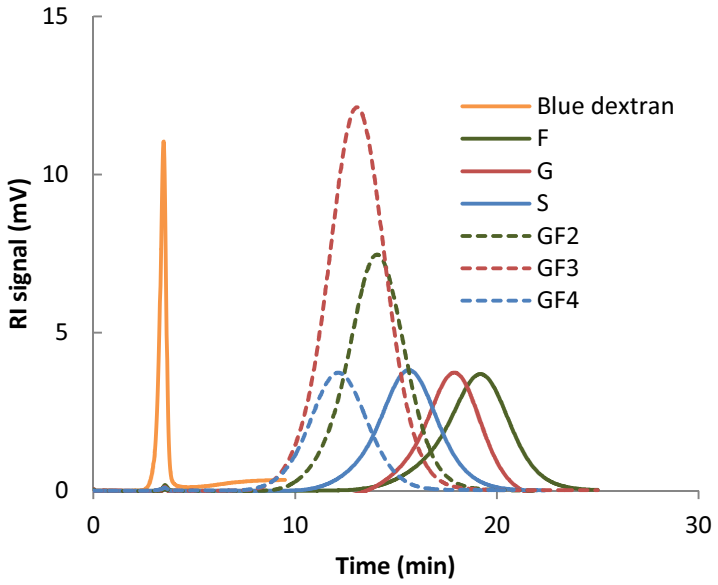

Fig. 6.6 Elution profiles of single component pulses of blue dextran, F, G, S, GF_2, GF_3 and GF_4 on a Dowex 50WX2 resin in K^+ form (21.4 x 16 mm) at a flow-rate of 2 $mL.min^{-1}$ and 21 ºC.

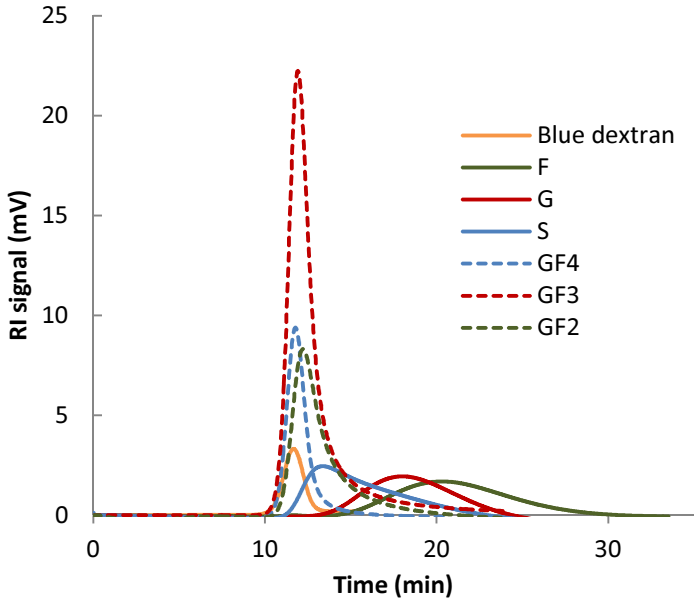

Fig. 6.7 Elution profiles of single component pulses of blue dextran, F, G, GF, GF_2, GF_3 and GF_4 on a Dowex Monosphere 99K/320 resin (29.5 x 16 mm) at a flow-rate of 2 mL.min^{-1} and 21 ºC.

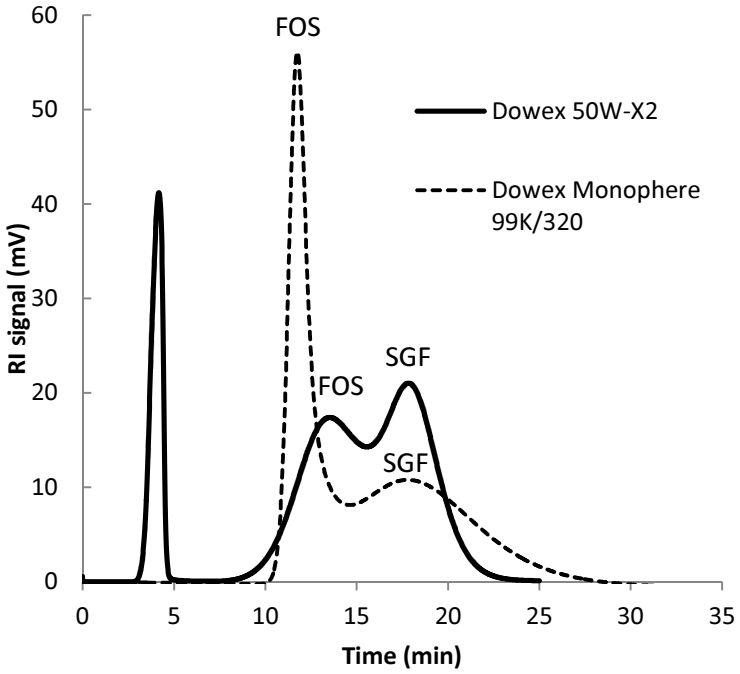

Fig. 6.8 Elution profile of a pulse of the fermentative broth on a Dowex Monosphere 99K/320 or a Dowex 50WX2 resin, both in K^+ form, at a flow-rate of 2 mL.min^{-1} and 21 ºC.

Properties of the stationary phase as the chemical structure, particle size, counter-ion and the degree of cross-linking, contribute to the different adsorption behaviours of the sugars. The resins studied in the current work have the same chemical structure (gel-type) and the same counter-ion (K^+). The differences between them are in the particle size and the degree of cross-linking. The Dowex Monosphere 99K/320 has a larger particle size and a degree of cross-linking of 6%, while Dowex 50WX2 has a 2% DVB content.

Resins with small degree of cross-linking have more elasticity and are more prone to swelling, therefore the water content available for partitioning will also be higher. These resins are more accessible for the saccharides resulting in an increase in sorption and faster kinetic diffusions (Adachi *et al.*, 1989a; Adachi *et al.*, 1989b). On the other hand, these resins are softener and more compressible than the ones with high cross-linking degrees, which results in less resistance to osmotic shocks and mechanical attrition (Sherrington, 1998). The particle size is also an important parameter for choosing an adequate resin. Larger particle sizes cause slower kinetics and broader peaks; however the resistance to the high back pressure caused by the high flow-rates used in the SMB system is also improved. Therefore, these two factors have to be balanced in order to make a rational choice of the resin to be used.

The adsorption constants of the single sugars, as well as the FOS and SGF were determined by the RTM method from the retention times previously obtained (Figs. 6.6 to 6.8). For each resin the values of the adsorption constants (H_i), retention factors (k_i) and separation factor (α) are summarized in Table 6.2.

Table 6.2 Retention factors ($k_{s,i}$), separation factors (α) and adsorption constants (H_i) for F, G, GF, GF_2, GF_3, GF_4, SGF and FOS using a Dowex Monosphere 99K/320 or a Dowex 50WX2 resin.

										α
Resin		F	G	GF	GF_2	GF_3	GF_4	SGF	FOS	FOS/SGF
Dowex Monosphere 99K/320	$k_{s,i}$	0.74	0.54	0.14	0.05	0.02	0.01	------	------	------
	H_i	0.468	0.342	0.091	0.029	0.014	0.006	0.331	0.005	73.6
Dowex 50WX2	$k_{s,i}$	4.45	4.08	3.44	2.99	2.71	2.44	------	------	------
	H_i	0.812	0.745	0.628	0.547	0.495	0.446	0.742	0.519	1.4

For the Dowex Monosphere 99K/320, the retention factors found for FOS were very small (< 0.1). The retention time obtained for FOS (t_{FOS}: 11.75 min at a flow-rate of 2 $mL.min^{-1}$) was similar to the dead time of this resin (t_0: 11.67 min). Hence, FOS are almost completely excluded from the particle pores due to their high molecular weight. The increased percentage of DVB of this resin implies a more closed pore structure and therefore, the entrance of the high sugars inside the pores is blocked.

Comparing the results obtained for the two resins, it was found that the Dowex 50WX2 resin retention factors are much higher than the Dowex Monosphere 99K/320 ones. The smaller content in DVB of the Dowex 50WX2 resin provide its higher water content, thus resulting in an increased sorption by partition.

On the other hand, the low adsorption of FOS onto the Dowex Monosphere 99K/320 resin results in a very high separation factor ($\alpha \approx 73.6$).

Column efficiency is a measure of the dispersion of a peak and is expressed as the number of theoretical plates (N). The efficiency of a column can be also measured by stating the plate height. The higher the N value (the more plates the better), or the lower the height of an equivalent theoretical plate (HETP), the more efficient is the column to separate any particular sugar. Column efficiency is dependent on many factors such as column length, particle size, packing quality, flow-rate, dead volume, temperature and the retention factor.

Results presented in Table 6.3 show that the number of theoretical plates for FOS and SGF decreases with increasing flow-rates, for both resins. Lower flow-rates enable more time for the solute to reach the equilibrium between liquid and solid phases, resulting in a smallest peak broadening (Fritz and Gjerde, 2009).

The higher the efficiency of a column, the closer the peak shape to the ideal rectangular elution profile of an ideal chromatogram leading to a narrower peak width (Schulte and Epping, 2005). The FOS peak obtained with Dowex Monosphere 99K/320 resin is very sharp (Fig. 6.8), leading to a small peak width ($w_{1/2,\ FOS}$: 1.1 min at 2 $mL.min^{-1}$), which results in a high efficiency of this resin to separate FOS. On the other hand, the SGF

peak is relatively broad, resulting in a high width of the respective peak ($w_{1/2,\ SGF}$: 8.3 min at 2 mL.min^{-1}), and therefore, in a smaller N (Table 6.3).

For the Dowex 50WX2 resin, the differences observed between the elution peaks of the two sugar groups are more related with the differences between their retention times since the peak widths were found to be similar ($w_{1/2,\ FOS}$: 4.1 min; $w_{1/2,\ SGF}$: 3.6 min at 2 mL.min^{-1}). Therefore, since SGF is the most retained component a higher N was obtained for this particular group of sugars.

Table 6.3 Column efficiency (N_i; N_L) and height of a theoretical plate (HETP) determined for SGF and FOS from an injection of fermentative broth onto a Dowex Monosphere 99K/320 or a Dowex 50WX2 resin.

Resin	Sugar	Q (mL.min^{-1})	0.5	1	1.5	2	2.5
Dowex Monosphere 99K/320	FOS	N	657.6	612.5	583.9	652.9	525.7
		HETP (cm)	0.045	0.048	0.051	0.045	0.056
	SGF	N	82.6	48.4	32.3	25.6	17.8
		HETP (cm)	0.36	0.61	0.91	1.2	1.6
Dowex 50WX2	FOS	N	117.5	91.4	73.0	60.1	51.8
		HETP (cm)	0.18	0.23	0.29	0.36	0.41
	SGF	N	266.8	208.6	163.3	136.8	118.2
		HETP (cm)	0.08	0.10	0.13	0.15	0.18

The chromatographic resolution is typically used to describe the degree of separation of two adjacent peak profiles. Using the elution profiles given in Figs. 6.9 and 6.10, the resolutions for the two resins could be determined. In these experiments, the fermentative broth was eluted at five different flow-rates. For the elution conditions used, the peak shape of the eluted sugars (SGF and FOS) was considered symmetrical, and therefore Eq. (6.7) was employed.

For both resins, comparing the resolution with the different flows studied, a decrease in resolution with increasing flow-rate is evident (Fig. 6.11). As previously shown, column efficiency tends to decrease by increasing flow-rate, therefore this was expected.

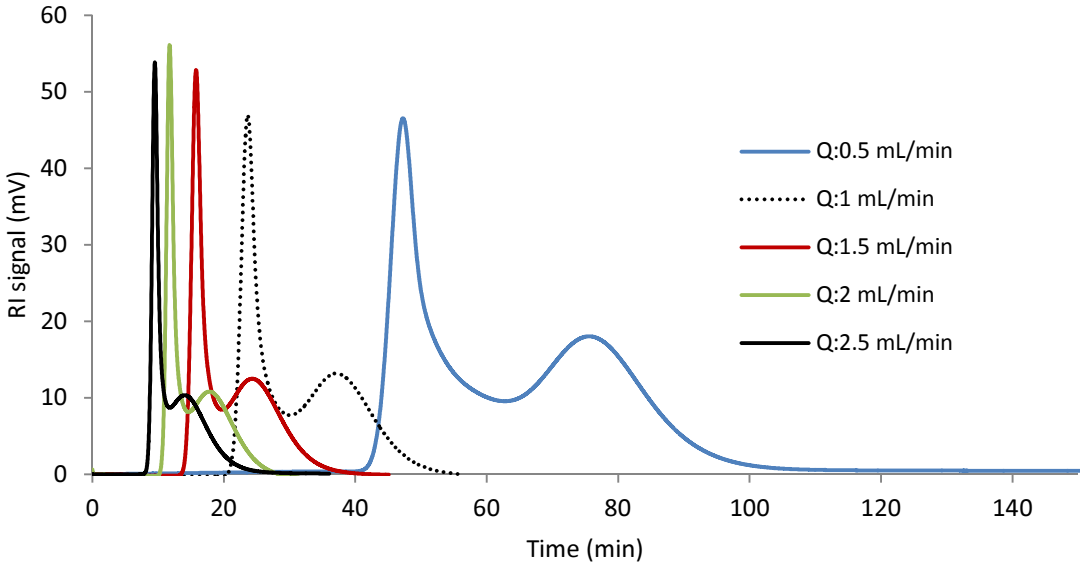

Fig. 6.9 Elution profile of a pulse of the fermentative broth on a Dowex Monosphere 99K/320 resin, at 0.5, 1, 1.5, 2 and 2.5 mL.min^{-1}.

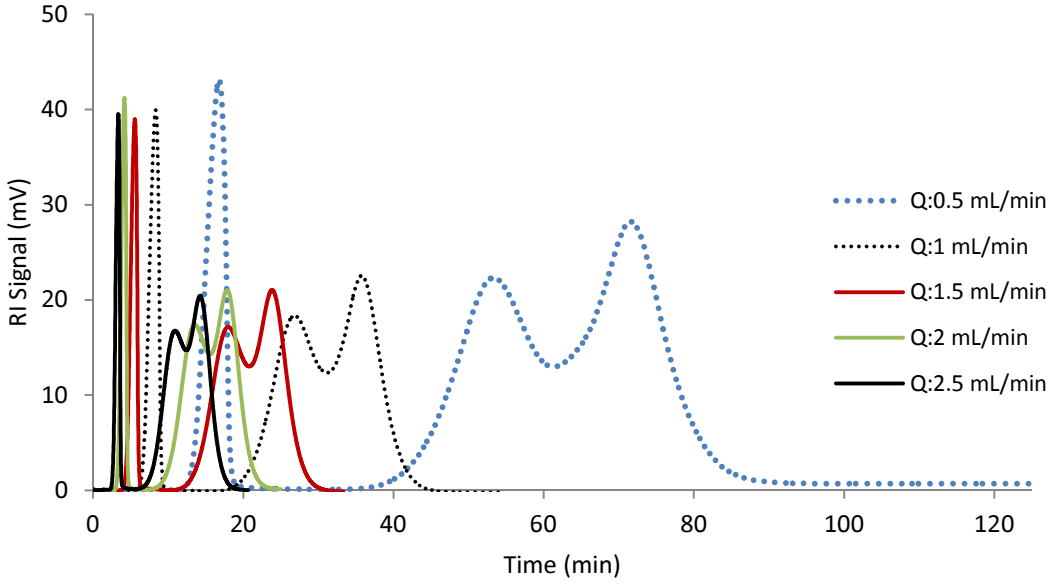

Fig. 6.10 Elution profile of a pulse of the fermentative broth on a Dowex 50WX2 resin, at 0.5, 1, 1.5, 2 and 2.5 mL.min^{-1}.

The resolution depends on three parameters: selectivity, retention factor and column efficiency. To obtain high resolution, the three terms must be maximised. Dowex

Monosphere 99K/320 resin presented higher values of resolution at the same flow-rate conditions compared to Dowex 50WX2. The selectivity and column efficiency of this resin to separate FOS is much higher than Dowex 50WX2, hence, these parameters contributed for the increase in resolution at small flows. On the other hand, for the highest flow-rates studied, similar resolution values were obtained for both resins, a consequence of the small retention factor and smallest column efficiency for Dowex Monosphere 99K/320 resin to separate SGF.

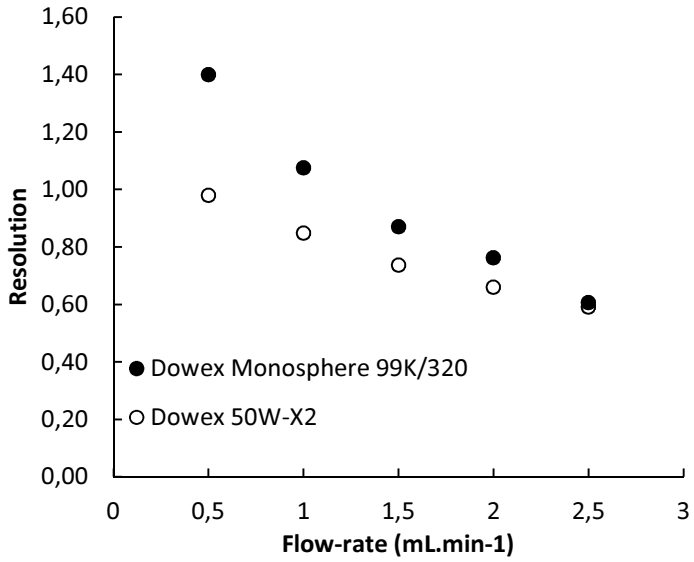

Fig. 6.11 Chromatographic resolution of the Dowex Monosphere 99K/320 and Dowex 50WX2 resins at different flow-rates.

In order to check the mechanical resistance of the resins at high flow-rate working conditions, each column filled with the two different resins was connected to the SMB system and the pressure drop and resin shrinking was monitored. Since the Dowex 50WX2 resin has a much smaller content in DVB, it was observed a very high shrinkage effect at a flow-rate of 18 $mL.min^{-1}$. Consequently, this phenomenon caused a high pressure drop in the system. Because the maximum pressure supported by the SMB is 60 bar, the experiments conducted with Dowex 50WX2 resin were not possible to work at flow-rates greater than 20 $mL.min^{-1}$.

Although several authors studied sugars separation using Dowex 50W resins with different contents of DVB (2, 4, 6, 8 and 12%), none of them used flow-rates above 10 $mL.min^{-1}$ (Dendene *et al.*, 1995; Adachi and Matsuno, 2000; Lee, 2003; Vente *et al.*, 2005a; Pedruzzi *et al.*, 2008). Lee (2003) used a Dowex 50WX12 resin in the calcium form in a SMB system. However, the eluent flow-rate used in the separation was 3.78 $mL.min^{-1}$. Therefore, no issues related with the pressure increase in the system have been reported. All the other mentioned studies were carried out in batch columns connected to HPLC systems, so in operational conditions that differ greatly from the real SMB.

On the other hand, the pressure drop tests performed with the Dowex Monosphere 99K/320 resin showed a stable resin bed length in the column for flow-rates up to 35 $mL.min^{-1}$. The high amount of DVB content and the larger particle size of the resin contribute to its higher mechanical resistance, thus enabling the use of reasonable flow-rates for SMB operations. Tests conducted with a mobile phase flow-rate of 35 $mL.min^{-1}$ caused a system pressure drop of 40 bar.

In summary, comparing with Dowex Monosphere 99K/320, the Dowex 50WX2 resin provided higher retention factors and similar resolution at highest flow-rates concerning the separation of FOS from SGF sugars contained in a fermentative broth. However, this resin does not have enough mechanical resistance to be used in the high flow-rates required for an SMB operation. Therefore, the Dowex Monosphere 99K/320 resin was selected for the following separation tests performed in the SMB plant.

6.3.1.2.1 Adsorption equilibrium isotherms

Since the Dowex Monosphere 99K/320 resin was shown to be suitable for the FOS purification in a SMB system, the resin adsorption equilibrium and kinetic parameters were determined. The intra-particle porosity for this resin at 30 ºC was found to be 0.007.

Adsorption equilibrium isotherms were determined by the adsorption-desorption method using the fermentative broth mixture. As shown in Fig. 6.12, the sugars were linearly adsorbed onto the resin. The distribution coefficients determined for each single sugar (γ) are presented in Table 6.4.

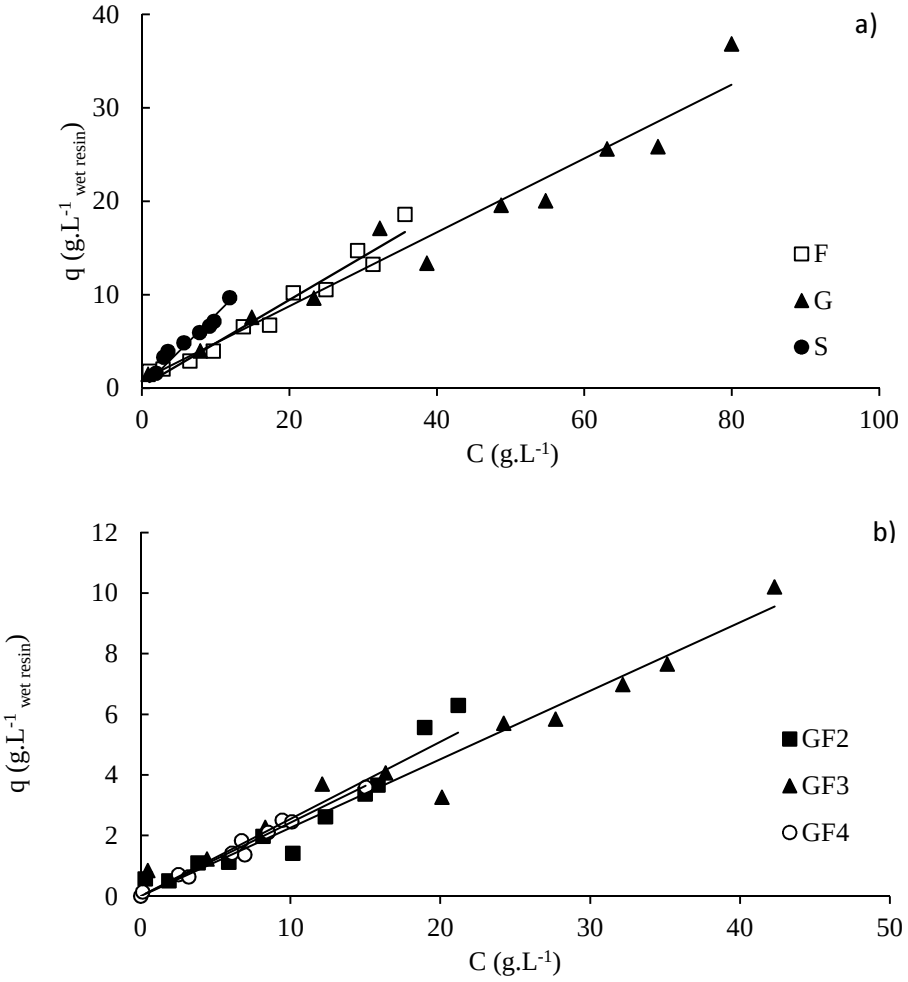

Fig. 6.12 Adsorption equilibrium isotherms of (a) fructose, glucose and sucrose, and (b) GF_2, GF_3 and GF_4, onto a Dowex-Monosphere 99K/320 resin at 30 ºC.

Table 6.4 Distribution coefficients for sugars contained in the fermentative broth mixture (γ_i) and the respective coefficient of determination (R^2) found for the linear approach.

Sugar	γ_i	R^2
F	0.47	0.96
G	0.41	0.95
GF	0.79	0.93
GF_2	0.25	0.90
GF_3	0.23	0.95
GF_4	0.24	0.98

Mono- and disaccharides were found to be more adsorbed onto the resin than FOS, thus being in accordance with results obtained by the RTM method. Nevertheless, the distribution coefficient found for sucrose was much higher than the one obtained for the monosaccharides, hence possibly this value has been over quantified. The distribution coefficients obtained for the sugar mixtures were approximately $\gamma_{FOS}\approx 0.2$ and $\gamma_{SGF}\approx 0.4$.

6.3.2 MODELLING

6.3.2.1 Calibration coefficients

Eluted sugars were detected by RI at the column outlet. Therefore, the data collected from the sugars profile concentration is given as a RI signal along the time. In order to convert the RI signal to concentrations, calibration curves for the different sugars were performed.

Fig. 6.13 shows the calibration curves obtained for F, G, S and SGF mixture, respectively. The following equations and respective regression coefficients were obtained:

$$RI_{signal,F} = 114.04 C_F \quad (R^2 = 1.00) \quad (6.53)$$

$$RI_{signal,G} = 114.80 C_G \quad (R^2 = 1.00) \quad (6.54)$$

$$RI_{signal,S} = 111.15 C_S \quad (R^2 = 1.00) \quad (6.55)$$

$$RI_{signal,SGF} = 114.48 C_{SGF} \quad (R^2 = 1.00) \quad (6.56)$$

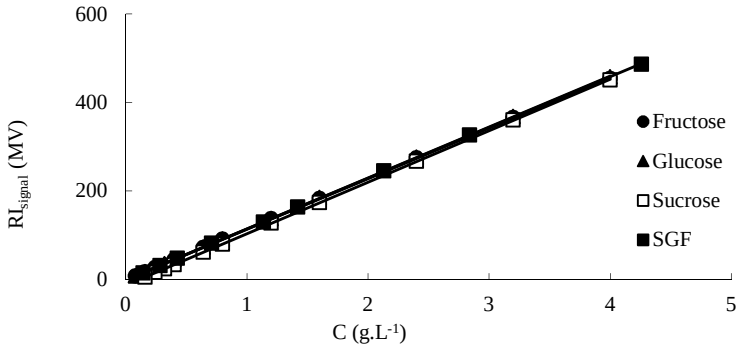

Fig. 6.13 Calibration curves obtained for fructose, glucose, sucrose and the mixture SGF.

As commercial standards of FOS are only available in analytical amounts, it was not possible to determine the calibration curve for this mixture. However, since the calibration curves of the pure mono- and disaccharides (F, G and S) were very similar, it is possible that the FOS calibration curve will not deviate much from the one obtained for the equivalent SGF mixture. Therefore, the calibration curve obtained for the SGF mixture was used for the FOS mixture as well.

6.3.2.2 Initial parameter estimates

In order to determine the kinetic parameters of the LDF model, the $D_{app,i}$ values, experimentally determined, were plotted against u^2 (Fig. 6.14), and the following relationships were obtained:

$$D_{app,B} = 13.26u^2 + 5E - 8 \qquad (R^2 = 0.999) \qquad (6.57)$$

$$D_{app,A} = 0.42u^2 + 3E - 8 \qquad (R^2 = 0.971) \qquad (6.58)$$

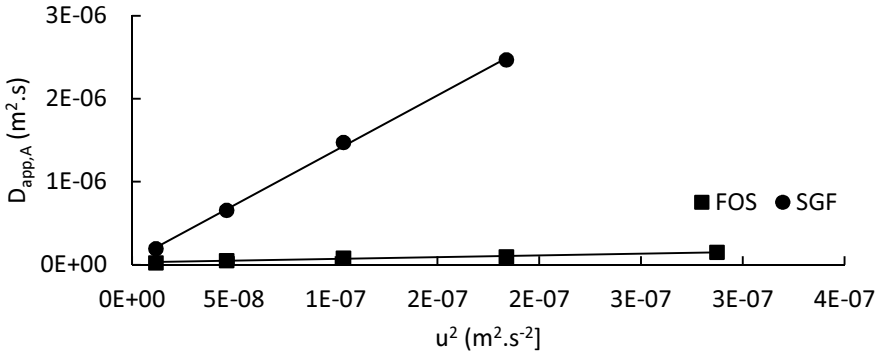

Fig. 6.14 Determination of the kinetic parameters for the FOS and SGF mixtures.

The kinetic parameters determined experimentally and later used as initial parameter estimates in the kinetic and LDF models are presented in Table 6.5.

Table 6.5 Kinetic parameters for the LDF and kinetic models. (A: FOS; B: SGF)

Kinetic model		LDF model	
$K_{F,A}$ (s^{-1})	0.013	$K_{F,A}$ (s^{-1})	0.003
$K_{F,B}$ (s^{-1})	0.016	$K_{F,B}$ (s^{-1})	2.994
		$D_{L,A}$ ($m^2.s^{-1}$)	2.7 x10^{-08}
		$D_{L,B}$ ($m^2.s^{-1}$)	5.1 x10^{-08}

6.3.2.3 Numerical solution

6.3.2.3.1 Boundary condition

The numerical solution is very sensitive to the boundary condition. The most critical parameters of the model are the injection time (t_p), which is directly dependent on the loop volume and on the flow-rate; and τ, which sets the rise and fall times of the boundary condition (Eq. (6.20)). In order to evaluate its influence on the simulation results, several values of t_p ($0.30 > t_p > 0.48$ s, corresponding to $10 > V_{Loop} > 16$ µL) and τ ($0.01 > \tau > 1.5$) were computed. Simulations were performed at a 2 mL.min^{-1} flow-rate.

Fig. 6.15 shows the influence of varying t_p between 0.30 and 0.48 s on the simulated chromatographic profile. The increase of t_p, in the boundary condition model leads to an increase of the amount of mass injected in the column, which is directly related to an increase of the injected volume, therefore the RI signal, reflecting the concentration of injected sugars, also increases. Results show that a small increase of the t_p parameter had a high influence on the final chromatographic profile.

The volume of mass injected in the column remains constant, and the width of the injection pulse is very small compared to the width of the output peaks, therefore the variation of τ will not influence significantly the chromatographic profile obtained. In the identification algorithm a value of τ equal to 0.01 was used.

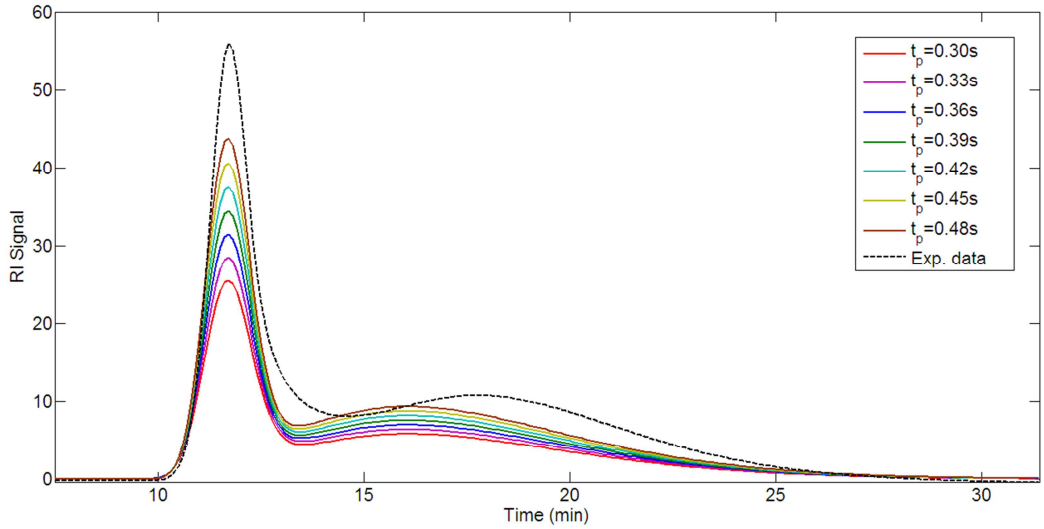

Fig. 6.15 Influence of the injection time on the numerical solution.

Fig. 6.16 shows that the variation of τ has a very low influence on the numerical solution.

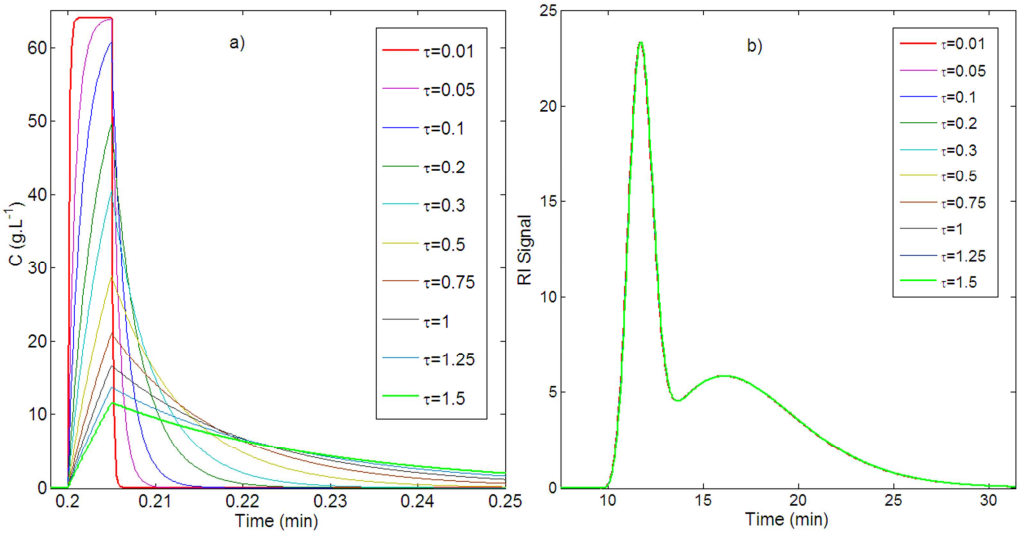

Fig. 6.16 Shape of the boundary condition for FOS a) and numerical solution obtained for the fermentative broth b).

6.3.2.3.2 Convergence tests

Simulations using an increasing number of discretization points were performed to verify the convergence of the numerical solution with finite differences (FD) and slope limiter (SL) in the kinetic and LDF models (Fig. 6.17 to Fig. 6.20).

The peak shape of FOS was found to be very sharp, consequently, for the kinetic model, a small value for the axial dispersion term was included in the model (9 x10^{-08} $m^2.s^{-1}$) to alleviate oscillations.

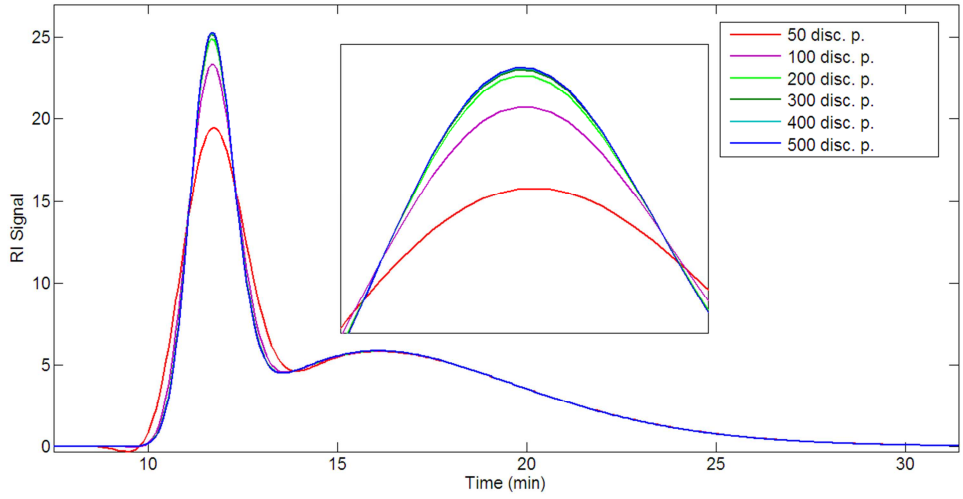

Fig. 6.17 Convergence test for the kinetic model with finite differences.

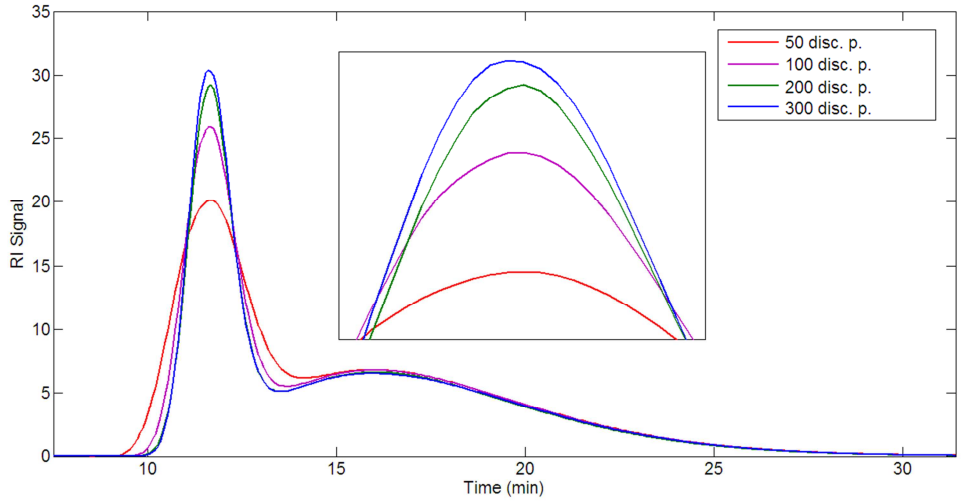

Fig. 6.18 Convergence test for the kinetic model with slope limiter.

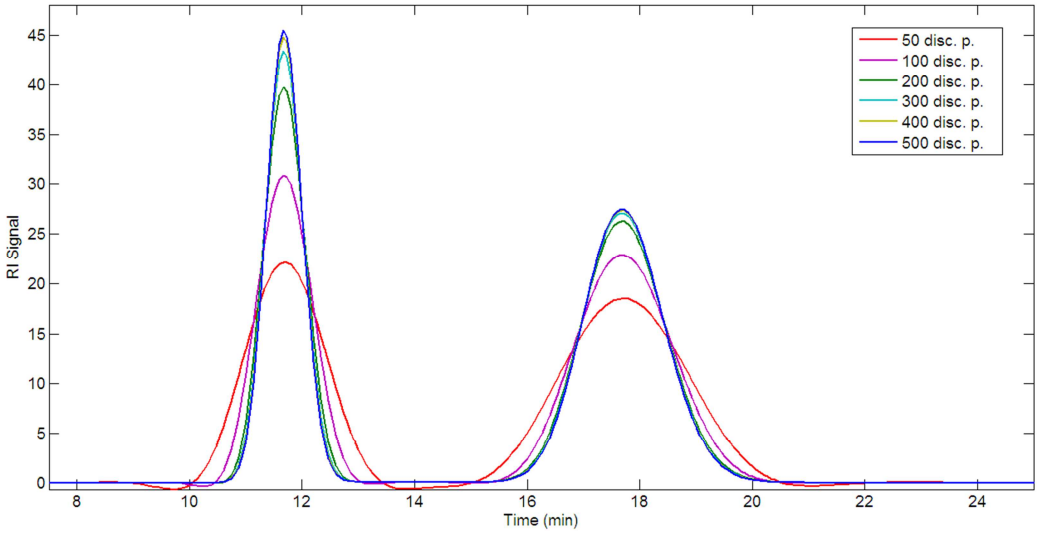

Fig. 6.19 Convergence test for the LDF model with finite differences.

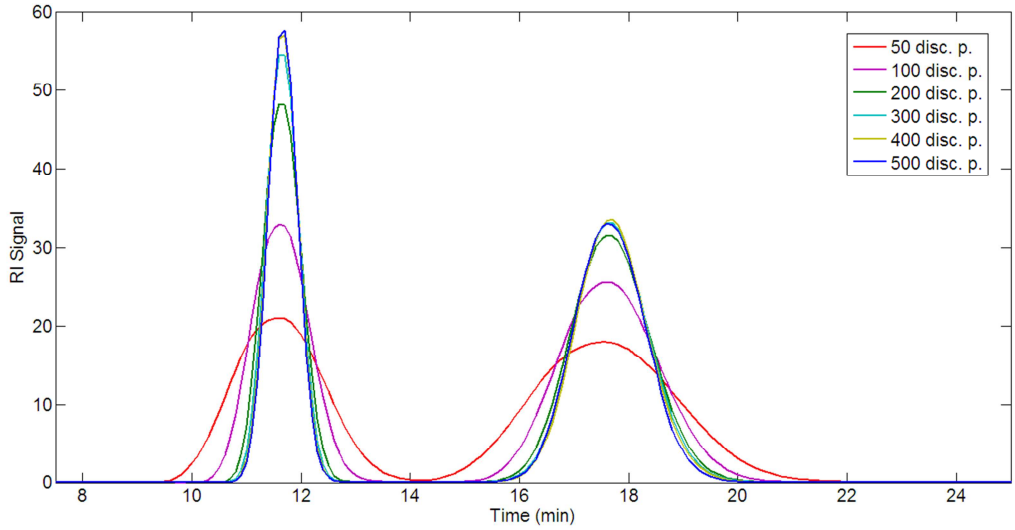

Fig. 6.20 Convergence test for the linear driving force model with slope limiter.

The amount of mass entering ($m_{i,\ IN}$) and exiting ($m_{i,\ OUT}$) the column was computed using the following equations, respectively:

$$m_{i,IN} = \int_0^{t_{SIM}} C_i(0,t)Qdt \tag{6.59}$$

$$m_{i,OUT} = \int_0^{t_{SIM}} C_i(L,t)Qdt \tag{6.60}$$

where $i = A, B$ and t_{SIM} is the total simulation time. All the simulations were performed on a personal computer equipped with an Intel Core2 Quad core processor running at 3GHz. Matlab was configured to utilize all the four cores.

The input mass of the components A and B (FOS and SGF) was 0.64 and 0.85 mg, respectively. The computed output masses obtained for the different schemes and models is presented in Tables 6.6 and 6.7.

Table 6.6 Performance parameters for the kinetic model.

		Discretization points						
		50	**100**	**150**	**200**	**300**	**400**	**500**
FD	Error	451	43	8	2	0.1	0.01	0.00
	$m_{A,OUT}$ (mg)	0.6397	0.6431	0.6436	0.6436	0.6438	0.6439	0.6439
	$m_{B,OUT}$ (mg)	0.8478	0.8480	0.8480	0.8480	0.8482	0.8483	0.8483
	Simulation Time (s)	12.79	40.17	84.98	147.12	356.04	683.01	1146.77
SL	Error	1615	272	62	29	0.00	-	-
	$m_{A,OUT}$ (mg)	0.7610	0.7512	0.7387	0.7283	0.7503	-	-
	$m_{B,OUT}$ (mg)	1.0092	0.9896	0.9727	0.9507	0.9566	-	-
	Simulation Time (s)	44.00	161.03	355.34	629.46	1481.47	-	-

Using the FD method, the differences observed between the mass at the output and input were much smaller than the ones obtained with the SL method (Fig. 6.17 to 6.20 and Tables 6.6 and 6.7). Therefore, FD gave results that are more realistic than the SL since in the mass balance the input should be equal to the output. For the identification algorithm the FD scheme was used.

The error values presented in Tables 6.6 and 6.7 were computed using the sum of squared errors, where the residual correspond to the difference between the studied chromatogram and the chromatogram obtained for the largest amount of discretization points. Results show that for more than 200 discretization points the error decreased considerably for both models (Tables 6.6 and 6.7) and the results converged to almost the same chromatographic profile (Figs. 6.17 to 6.20).

Table 6.7 Performance parameters for the linear driving force model

		Discretization points						
		50	**100**	**150**	**200**	**300**	**400**	**500**
FD	Error	5933	1935	654	237	29	3	0.00
	$m_{A,OUT}$ (mg)	0.6423	0.6411	0.6420	0.6422	0.6421	0.6421	0.6422
	$m_{B,OUT}$ (mg)	0.8502	0.8502	0.8530	0.8537	0.8542	0.8543	0.8543
	Simulation Time (s)	12.91	39.91	94.99	154.74	376.59	727.82	1229.82
SL	Error	10080	4028	1480	574	92	23	0.00
	$m_{A,OUT}$ (mg)	0.7718	0.7624	0.7546	0.7465	0.7297	0.7147	0.7012
	$m_{B,OUT}$ (mg)	1.0249	1.0172	1.0016	0.9919	0.9685	0.9502	0.9410
	Simulation Time (s)	30.39	101.33	179.17	406.98	1202.99	2661.91	4589.94

From the error analysis it was also possible to observe that the chromatographic profile is much less sensitive to a variation of the number of discretization points when using the kinetic model than when using the LDF model.

The simulation time increases significantly with the number of discretization points; therefore, a compromise between the simulation time and the accuracy must be considered, especially when a large number of simulations are needed, as in the parameter identification case. For this reason, 150 discretization points were chosen to perform the following simulations, since it represents a good compromise between the included error and the simulation time.

6.3.2.3.3 Sensitivity analysis of the isotherm and kinetic parameters

A sensitivity analysis was performed in order to quantify the influence of the parameter errors in the numerical solution. A 10% variation of the isotherm parameters (obtained by the RTM method), as well as of the experimental kinetic parameters, was studied.

The deviation was evaluated using the sum of squared errors, where the residual corresponds to the difference between the chromatographic profiles obtained with different parameters and the experimental chromatographic profile.

Comparing the two schemes used (FD and SL), it was found that the SL is more sensitive to small variations than the FD. Therefore, the FD scheme proved again to be a more robust scheme. Analyzing the sensitivity for each studied parameter, it was found that the H_B parameter is the most sensitive to small variations. Finally, the LDF model was the least sensitive to small variations when using the FD approach, except to the H_B parameter. Nevertheless, when using the SL scheme, the kinetic model showed a smaller variation of the parameters values (Table 6.8).

Table 6.8 Sensitivity analysis.

		Kinetic model		**LDF model**	
Parameter	**Variation**	FD	SL	FD	SL
H_A	+10%	0.2	5.1	0.02	8.2
	-10%	0.1	2.8	0.02	7.1
H_B	+10%	29.5	50.6	2465	3251
	-10%	35.2	41.5	2793	3563
K_A / $K_{F,A}$	+10%	0.1	4.6	0.02	8.2
	-10%	0.06	3.1	0.02	7.1
K_B / $K_{F,B}$	+10%	3.8	10.5	0.3	6.6
	-10%	4.9	8.4	0.4	9.5
$D_{L,A}$	+10%	-	-	7.5	18.6
	-10%	-	-	8.9	6.4
$D_{L,B}$	+10%	-	-	4.4	17.2
	-10%	-	-	5.0	7.8

6.3.2.4 Parameters identification

The estimated parameters obtained by the kinetic and LDF models, and the correspondent chromatographic profiles, are presented in Table 6.9, and Figs. 6.21 and 6.22, respectively.

Table 6.9 Identification results for the kinetic and LDF models.

	Kinetic model			**LDF model**		
Parameters	**Initial**	**Final**	**|Variation|**	**Initial**	**Final**	**|Variation|**
H_A	0.0045	0.0036	0.0009	0.0045	0.00067	0.004
H_B	0.33	0.31	0.02	0.33	0.26	0.07
K_A / $K_{F,A}$ (s^{-1})	0.013	0.037	0.02	0.003	0.005	0.002
K_B / $K_{F,B}$ (s^{-1})	0.017	0.013	0.004	3.0	2.5	0.5
$D_{L,A}$ ($m^2.s^{-1}$)	-------	-------	-------	2.7×10^{-08}	5.0×10^{-08}	2×10^{-08}
$D_{L,B}$ ($m^2.s^{-1}$)	-------	-------	-------	5.1×10^{-08}	1.8×10^{-06}	2×10^{-06}

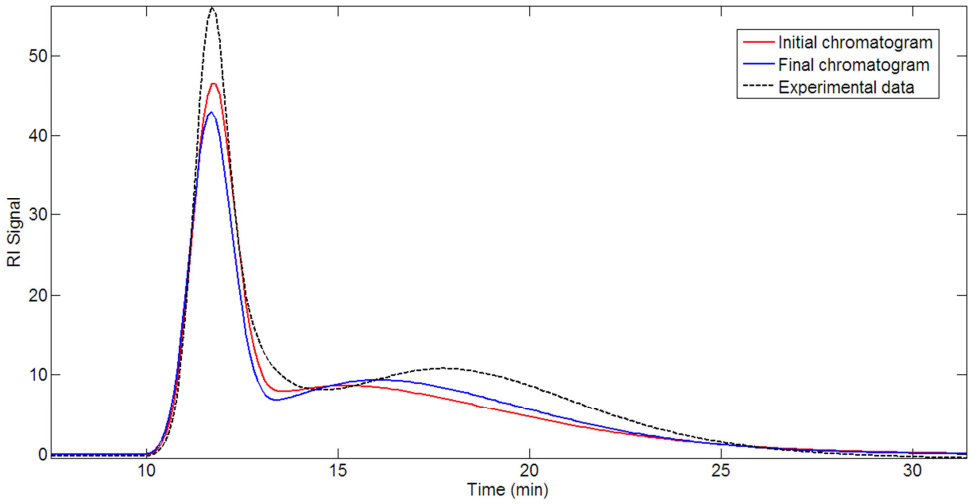

Fig. 6.21 Identification results for the kinetic model with finite difference schemes

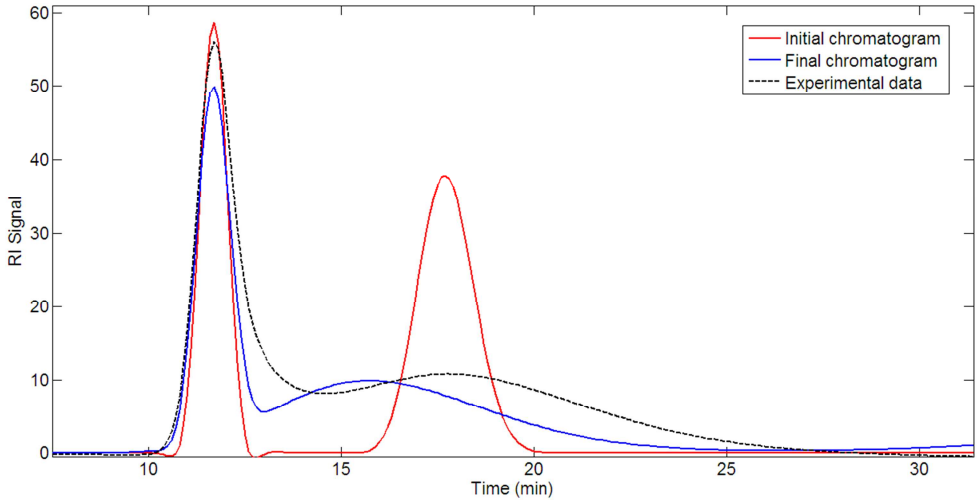

Fig. 6.22 Identification results for the linear driving force model with finite difference schemes

In the kinetic model, the initial estimated parameters were closer to the final ones that were determined by the identification algorithm, when compared to the LDF model (Table 6.9). Therefore, the initial value for the cost function was also smaller (Table 6.10). Additionally, the number of parameters that need to be adjusted with the LDF model (6) is higher than the one with the kinetic model (4). As a result, a high number of iterations were required to complete the identification algorithm with the LDF model, compared to the kinetic model (Table 6.10). Consequently, more time was needed to finish the parameter identification procedure. It is also more difficult to estimate a larger number of parameters when limited experimental information is available (enough amount information may be lacking to enable finding unique estimates for these parameters).

Table 6.10 Cost function values at the start and end of the parameter identification.

Model	J_i	J_f	**Iterations**	**Simulations**	**Time**
Kinetic model	2586.4	1974.3	148	359	8h 29min
LDF model	17143.0	1988.5	232	425	11h 13min

The results of the current work are in accordance with the study reported by Grosfils *et al.* (2007) that also chose the kinetic model to describe a batch chromatographic process for the separation of a binary mixture, instead of the LDF model.

6.3.3 SMB EXPERIMENTS

The void fraction values and the Henry constants were determined for the eight single columns. The average value determined for the void fraction was 0.382 $\pm$ 0.004 (2%). The Henry constant determined by the RTM method for FOS and SGF was 0.005 and 0.331, respectively (Chapter 6.3.1.2.1). Since the adsorption equilibrium values are relatively different from the ones obtained by the adsorption/desorption method ($\gamma_{FOS} \approx 0.2$; $\gamma_{SGF} \approx 0.4$), both coefficients were taken into account for the selection of the ideal separation region and operating points (Fig. 6.23).

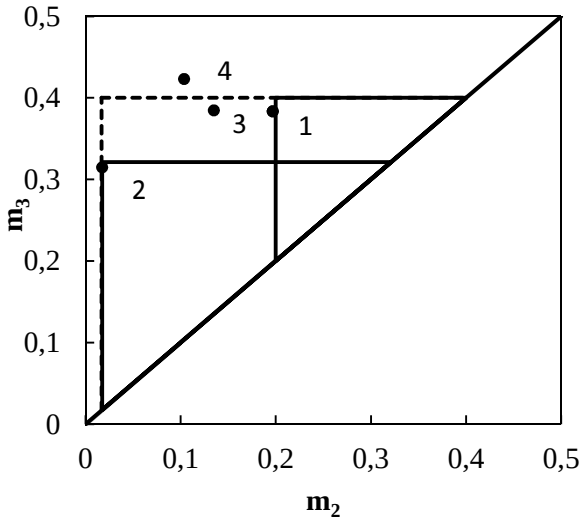

Fig. 6.23 Separation region and operating points selected for the SMB experiments.

One operating point in each triangle was chosen. Operating points 1, 2 and 3 were selected inside the triangles, and number 4 was selected outside the triangle, specifically

in the pure extract region. The respective (m_2, m_3) coordinates are presented in Table 6.11.

Table 6.11 Dimensionless flow-rate ratios selected for the experiences performed in the SMB plant.

	1	2	3	4
m_1	0.413	0.328	0.416	0.458
m_2	0.197	0.018	0.135	0.104
m_3	0.383	0.315	0.385	0.423
m_4	0.021	0.017	0.125	0.096

The operating flow-rates used in each set of experiments are shown in Table 6.12. It is important to note that the flow-rate of zone I is equivalent to the flow delivered by the recycle pump, since this pump is fixed between zones IV and I. The switching time was set to 1.5 min in all the experiments.

All the SMB experiments were performed with treated fermentative broth. The feed concentration of FOS and SGF was 122 ± 3 and 74 ± 2 g.L^{-1}, respectively.

Table 6.12 Operating parameters of the experiences performed in the SMB plant: switching time; internal flow-rates and operating pump flow-rates for feed, raffinate, desorbent, eluent and recycling streams (mL.min^{-1}).

$SMB_{exp.}$	Q_F	Q_R	Q_D	Q_E	$Q_{I=rec.}$	Q_{II}	Q_{III}	Q_{IV}	t* (min)
1	3.8	7.4	8.0	4.4	21.0	16.6	20.4	13.0	1.5
2	6.1	6.1	6.3	6.3	19.3	12.9	19.0	12.9	1.5
3	5.1	5.3	5.9	5.7	21.1	15.3	20.4	15.1	1.5
4	6.5	6.7	7.4	7.2	21.9	14.7	21.2	14.5	1.5

The extract and raffinate streams were collected during a complete cycle. Afterwards, the sugar concentrations collected during each cycle were analyzed by HPLC. From Fig. 6.24 it was possible to conclude that the SMB experiments reached the steady state after approximately 9 cycles.

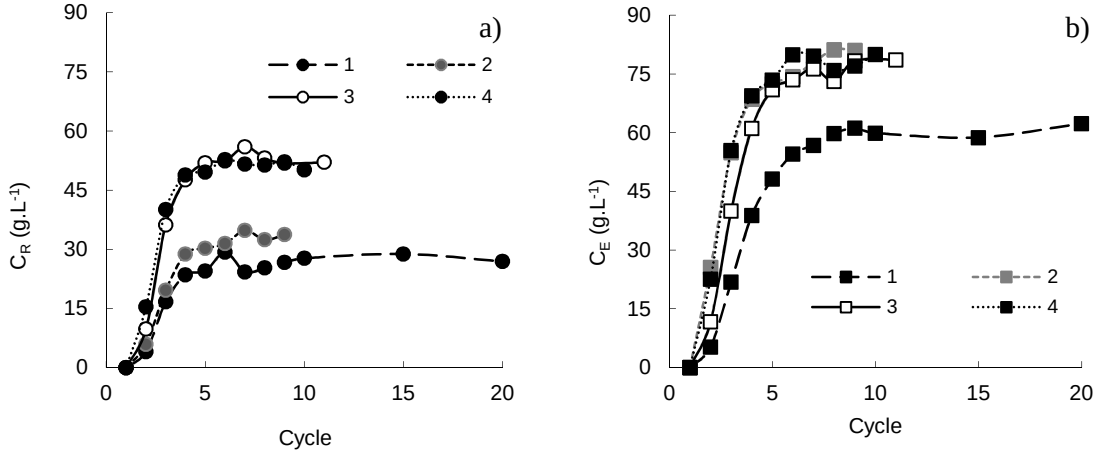

Fig. 6.24 Average concentrations of a) FOS (•) and b) SGF (■) collected per cycle, in the raffinate and extract streams, respectively, for the four (1, 2, 3 and 4) SMB experiments.

The sugar concentration of the raffinate and extract collected at the steady state, with the respective flow-rates used in each set of experiments, allowed the evaluation of the chromatographic separation performance. Table 6.13 summarizes the calculated parameters, namely purity, yield, productivity and dilution, for FOS collected in the raffinate (R_{FOS}) and SGF in the extract (E_{SGF}).

As previously mentioned, the main goal of this SMB separation is to purify FOS from a fermentative broth. From the results, it was possible to observe that in the experiment 1, a higher purity degree for the SGF mixture was obtained. However, the FOS purity and productivities obtained were very low, and the final product is much more diluted when compared with the other experiments. The experiment 3 was the one that showed the highest FOS purities in the raffinate stream (FOS purity 65.0%). Moreover, the yields obtained were also high and, the product dilution, that influences the cost of the further operations as evaporation and concentration, was found to be small. Nevertheless, in the experiment 4, approximately the same purity, yield and dilution values were obtained, but the productivity for both components was slightly higher when compared with the other experiments.

Although the operating point of the experiment 4 is located in the pure extract region, the extract stream only achieved 79.3 % of purity. Therefore, the dispersion and mass transfer effects, assumed as negligible by the triangle theory, may in fact, influence the performance of the chromatographic separation.

Based on the results, the experimental conditions of the experiment 4 were considered to be the best ones for the separation of FOS from the mixture using this SMB plant.

Table 6.13 Performance of the SMB separation.

$SMB_{exp.}$	Pu (%)		Yi (%)		Pr ($g.L^{-1}.h^{-1}$)		Di	
	R_{FOS}	E_{SGF}	R_{FOS}	E_{SGF}	R_{FOS}	E_{SGF}	R_{FOS}	E_{SGF}
1	51.8	82.1	73.5	60.8	48.9	67.3	62.2	47.5
2	55.9	65.9	46.9	72.1	50.2	125.7	53.2	30.9
3	65.0	78.9	69.8	70.5	67.6	110.5	32.9	37.4
4	62.9	79.3	69.4	70.5	82.1	141.5	32.3	36.4

Fig. 6.25 shows the average sugar concentrations per cycle in the raffinate and extract streams for the experiment 4. The profile obtained for the raffinate (Fig. 6.25 a)) shows that GF_3 (nystose) is the sugar present in highest concentrations. However, the content in fructose and glucose is still high on this stream, thus these are the principal contaminants of the raffinate. The amount of fructose and glucose in the feed mixture is very high (Table 6.14), representing 57% of the total sugars. Consequently, it was very difficult to remove these sugars from the FOS mixture.

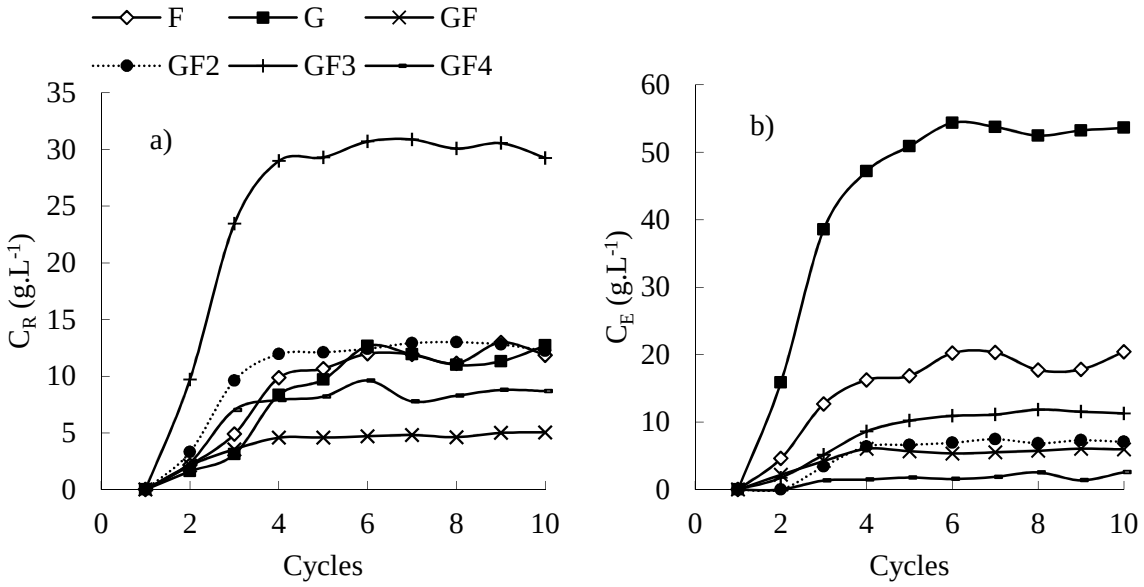

Fig. 6.25 Average sugar concentrations per cycle in the raffinate a) and extract b) streams for the SMB experiment 4.

The SMB chromatographic method was found to be adequate for the purification of FOS from a fermentative broth. Nevertheless, since the purities obtained in the working current are still far from 100%, further experiments should be conducted to optimize the SMB operation.

Table 6.14 Percentage of sugars on the raffinate and extract collected at the steady state on the experiment 4 and the respective percentage of sugar feed.

(%)	F	G	GF	GF_2	GF_3	GF_4	SGF	FOS
C_R	14.9	15.9	6.3	15.3	36.7	10.9	37.1	62.9
C_E	20.2	53.1	5.9	7.0	11.2	2.5	79.3	20.7
C_F	18.7	38.7	5.5	10.2	21.3	5.6	62.9	37.1

In all the experiments performed, the pressure of the system was always very close to the system's limits. Additionally, the choice of the flow-rates in each set of operating conditions was very limited. An alternative to avoid the pressure issues could be increasing the internal diameter of the tubes that connect the columns in series.

With the current SMB set-up it was also not possible to determine the internal sugar concentrations profile. For this reason, it is difficult to evaluate the precise causes behind the small FOS purities obtained in the process. It is possible that the SGF mixture was not completely desorbed in zone IV, and consequently contaminated the zone I thus decreasing the purity of FOS in the raffinate. The knowledge of the internal sugar concentrations in further experiments could help to select better operating points for the envisaged separation.

6.3.4 CONCLUSIONS

The performance of two potassium gel-type resins (Dowex 50WX2/Dowex Monosphere 99K/320) on the separation of FOS from a mixture of glucose, fructose and sucrose was evaluated. Although Dowex 50WX2 resin showed high efficiency on the separation, it was not resistant enough to the back pressure of the SMB system operating at high flow-rates. Therefore, the Dowex Monosphere 99K/320 resin was selected for the modelling and SMB operation.

Linear equilibrium adsorption was obtained for all the sugars of the fermentative broth in the range of concentrations used with the Dowex Monosphere 99K/320 resin.

From the simulation analysis, it was chosen the boundary condition parameters (t_p and τ), the number of discretization points (150) and a finite difference scheme, conducting to good identification of the model parameters that were determined. Finally, the kinetic model was considered an appropriate method for modelling the experimental chromatographic profile of a fermentative broth mixture.

The performance of a SMB chromatographic plant in the separation of FOS from SGF was evaluated. The triangle theory was used to choose several operating points. FOS were successfully purified from 37.1 to 62.9% using the abovementioned SMB unit. Main contaminants of the raffinate stream were the monosaccharides. The FOS yield and productivity in the raffinate was 69.4% and 82.1 $g.L^{-1}.h^{-1}$, respectively.

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CHAPTER 7

CONCLUSIONS AND FUTURE WORK

1.1 CONCLUSIONS

The main purpose of this thesis was the recovery of fructo-oligosaccharides (FOS) from sugar mixtures obtained by fermentation. Liquid chromatography was the separation method chosen among the downstream techniques available. Two different adsorbents were studied, namely the activated charcoal and several cationic exchange resins. The main conclusions drawn from this thesis are summarized below:

- The use of an activated charcoal single column to adsorb FOS with a gradient of water/ethanol as desorbent, showed to be a simple and very efficient method to separate and desalt FOS contained in fermentative broths. With the proposed process, 74.5% (w/w) of FOS were recovered with 92.9% (w/w) of purity. Moreover, fractions with purity up to 97% (w/w) of FOS were obtained.

- In the sugar industry the most used resins are the gel-type in Ca^{2+} form. Therefore, the performance of a Dowex Monosphere Ca resin in the separation of FOS was evaluated. FOS adsorption showed to be influenced by the components of the fermentative broth. FOS isotherms in purified mixtures were fitted with a model derived from the anti-Langmuir isotherm while FOS isotherms contained in a fermentative broth were better fitted with the Toth and Langmuir isotherms. For both studied mixtures, the adsorption capacity of individual sugars was primarily determined by their molecular size, and was significantly dependent on the liquid phase composition, whereas the selectivity was essentially constant.

- Three commercial resins, namely Lewatit (S2568), Amberlite (CR1320Ca) and Diaion (UBK530) were also evaluated concerning FOS separation. Results showed that the adsorption behavior of FOS depends mainly on the composition of the liquid phase containing sugars. Therefore, it was possible to conclude that FOS adsorption behaviour cannot be predicted by adsorption studies conducted with standard FOS in water matrices. Also, to characterize the adsorption of FOS from other types of fermentative broths, for example fermentations conducted with other microorganisms, new adsorption studies should be performed.

The adsorption of purified FOS, in aqueous solutions, was better described by the multi-component anti-Langmuir isotherm. While for FOS contained in the fermentative broth linear isotherms were chosen, except for the Diaion (UBK530) resin.

Although a higher adsorption capacity was found for FOS from the fermentative broth than for purified FOS in aqueous solutions, due to the presence of salts and other sugars, the selectivity values were similar.

The adsorption of FOS from the fermentative broth was influenced by the counter-ion, structure and water content of the resin; while the adsorption of FOS from purified mixtures was only influenced by the two last factors. FOS from fermentative broth mixture were more adsorbed onto the Na^+ resin than onto the Ca^{2+} one. Additionally, the macroporous Lewatit (S2568) resin was the one that showed the greater capacity, due to its high water content.

However, the Amberlite (CR1320Ca) resin was elected as the more adequate for the recovery/purification of FOS from a fermentative broth due to its higher selectivity.

- The performance of a macroporous resin in Na^+ form and a gel-type, in K^+ form, in sugars separation, at different temperatures and with mono and multi-component mixtures, was studied and compared. Based in the selectivity values, it was found that Dowex Monosphere 99K/320 gel-type resin, in K^+ form, was the best choice to separate a mixture of fructose, glucose and sucrose, operating at 25ºC.

  A competitive effect on the adsorption was found at 25ºC for the multi-component mixtures as compared to the mono-component ones. However, a reverse effect was found at 40ºC.

  The temperature increase from 25 to 40ºC led to a decrease in the adsorption for mono-component sugar mixtures. However, for multi-component sugar mixtures a reverse effect was obtained.

- Since a good performance was obtained with a gel-type resin, in K^+ form, on sugars separation, adsorption equilibrium, kinetic and mechanical resistance studies were conducted with a Dowex Monosphere 99K/320 and a Dowex 50WX2 resins, both gel type and in K^+ form. The Dowex 50WX2 resin provided higher retention factors and similar resolution at highest flow-rates concerning the separation of FOS from mono- and disaccharides contained in a fermentative broth. However, this resin did not show sufficient mechanical resistance to be used in a SMB plant. Therefore, the Dowex Monosphere 99K/320 resin was selected for the separation tests in the SMB plant.

  The fermentative broth chromatographic elution on a single column was modelled and kinetic and adsorption parameters were identified.

  FOS were successfully purified from 37.1 to 62.9% (w/w) using the SMB pilot plant. Furthermore, the FOS yield and productivity obtained in the raffinate was 69.4% (w/w) and 82.1 $g.L^{-1}.h^{-1}$, respectively.

1.2 FUTURE WORK

Many questions have been raised during the development of this thesis that suggest some future directions in FOS purification research as listed below:

- Increase the internal diameter of the tubes that connect the SMB columns in series to decrease the global pressure drop of the SMB plant, enabling the operation with a wider range of flow-rates.
- Implement in the SMB plant a collecting port between two columns to allow the determination of the internal sugar concentration profile.
- Study the salts influence in the chromatographic profile of the fermentative broth.
- Study the influence of resin shrinking on the accuracy of determined adsorption isotherms.
- Model the separation of FOS from mono- and disaccharides, by a true moving bed (TMB) approach and a real SMB process, to predict the adequate SMB operating conditions.
- Validate, with experimental data, the above estimated parameters in the pilot SMB plant.
- Since the concentration and proportion of sugars from the fermentative broth may vary between fermentations, it will be interesting to study the influence of these variations in the separation performance.
- Since the Dowex 50WX2 resin showed a great performance in the separation of salts from the fermentative broth mixture, with salts being the less retained compounds and having no commercial value, the use of a three-zone SMB process may be an economical alternative to desalt the fermentative broth.

Results gathered in this thesis are very promising and have led to interesting new questions that warrant further research.