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Separation of lactose from human milk oligosaccharides with simulated moving bed chromatography

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Abstract

The successful separation of the disaccharide lactose from a complex mixture of human milk oligosaccharides (HMOS) with the continuous chromatography of simulated moving bed (SMB) technique is described. Since lactose is the main carbohydrate in human milk with well-known functions for the infant, it is necessary to separate it from the rest of the oligosaccharides to divide them into less complex fractions and analyse their partial unknown functions. For separation of lactose from HMOS two different stationary phases (size-exclusion gel as well as ion-exchange gel) were used. As the main result, it is shown that a size-exclusion gel with the particle size of 50–100 μm and porosity of 50 Å was the preferred stationary phase for our separation process with almost complete lactose separation and stable conditions.

Keywords: Simulated moving bed chromatography; Human milk oligosaccharides; Lactose separation; Size-exclusion gel; Ion-exchange gel

1. Introduction

Human milk is the preferred nutrition during the first few months of life [1]. It contains considerable amounts of oligosaccharides [2,3], indicating that they play an important physiological role. It is widely accepted that human milk oligosaccharides (HMOS) contribute to the establishment of a particular intestinal flora (dominated by bifidobacteria), the postnatal development of the immune system, the protective effect of human milk against viral and bacterial infections, and the enhancement of the bioavailability of minerals. The anti-infective effect of human milk has been partly attributed to the high amount of free oligosaccharides as well as glycoconjugates, because these structures might prevent intestinal attachment of infectious agents by acting as receptor analogues [4–7]. The composition of human milk oligosaccharides is very complex. As HMOS are resistant against digestion, they can be detected in the faeces as well as in the urine of breast fed infants [8,9]. There might be even longer lasting positive effects with regard to later life (e.g. less infections) via programming of specific developments. The concentration of free human milk oligosaccharides is in the range of 10–12 g/l [10]. However, the main carbohydrate is lactose (approximately 70 g/l), which is digested and absorbed by the infant and thus contributing to the energy supply.

Oligosaccharides with a potential physiological benefit could be found in animal milks. Some of the oligosaccharides detected in domestic animal milks have structural features in common with human milk oligosaccharides. However, total amounts and individual structures are still different to HMOS. The concentration of oligosaccharides in the milk of the most relevant domestic mammalians is 10–100-fold lower, compared to human milk [11]. Nevertheless, it may be possible to accumulate these oligosaccharides of animal milks in a production scale with specific chromatographic techniques, in order to obtain considerable amounts applicable for dietetic nutrition.

New preparation methods have been developed, which allow purification of oligosaccharides structures as a prerequisite for identifying their biological effects [12]. Additionally, new analytical techniques have significantly improved our knowledge about the structures of milk oligosaccharides [2,13,14]. In the last years, chromatographic separation processes are increasingly used for the separation and cleaning of pharmaceuticals and fine chemicals [15]. However, several

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chromatographic techniques have also been used to separate oligosaccharides into less complex fractions or even single structures with the objective to characterize their biological functions [16]. These techniques include, for example, thin-layer chromatography (TLC) [5], size-exclusion chromatography (SEC) [17], reversed-phase high-performance liquid chromatography (RP-HPLC) [13,18] and ion-exchange chromatography (IEC) [16]. However, simulated moving bed (SMB) chromatography has the potential of increased productivity and reduced mobile phase consumption in comparison to batch elution chromatography. It allows the continuous counter-current separation of a feed into two streams of products (raffinate and extract). The SMB technology is applicable for all kinds of chromatography. It has been used for many years in the sugar industry for large scale separations (e.g. palatinose-trehalulose fractionation) [19–21].

The predominance of lactose in the carbohydrate fraction of milk makes it difficult to separate and analyze the complex oligosaccharides with the intention to identify their biological functions. The purpose of the work presented is the separation of lactose from a *complex* mixture of human milk oligosaccharides. This is in contrast to classical applications of SMB chromatography in the separation of carbohydrates, which were usually less complex and often binary mixtures [22]. Different stationary phases like size-exclusion gel as well as ion-exchange (ligand-exchange) gel were applied. Both standard elution chromatography techniques are established for fractionation and analyses of HMOS [16,17,23].

2. Conditions

2.1. Materials

The carbohydrate-mineral fraction of pooled human milk was used as substrate. Proteins and lipids were removed according to Kobata by centrifugation and precipitation. As stationary phase Toyopearl HW40C (Tosoh Bioscience, Stuttgart, Germany) with the particle size of 50–100 μm and Daisin MCI GEL CK08P (Ca^{2+} form, Mitsubishi, Tokyo, Japan) with the particle size of 75–150 μm were used. The IEC (MCI) gel was used for ligand-exchange chromatography separating the molecules according to their size [24]. The regeneration of the MCI gel was realized with 1 M $CaCl_2 \times H_20$ (Merck, Darmstadt, Germany), the regeneration of HW40C with 0.5 M NaOH (Merck), followed by equilibration with deionized water. The mobile phase was deionized water for the MCI gel and 2% 2-propanol (Baker, Deventer, The Netherlands) for HW40C gel.

2.2. Sample preparation

The SMB-feed-samples (a pooled carbohydrate-mineral fraction) were dissolved in deionized water (as a 2% or 10% solution) and sterilized through a 0.2 μm filter (Nalgene Nunc, Rochester, NY, USA) in a sterile 2 l bottle. This feed solution was kept at 50 °C in a water bath and was degassed online by a two-channel degasser (Knauer, K-5020, Berlin, Germany) prior to injection in the SMB.

2.3. SMB chromatography

The principle of SMB chromatography is illustrated in Fig. 1. The columns packed with the respective adsorbent are connected in series. The system is divided into four zones (zones 1–4), which consist of at least one column. The different zones are characterized by specific inlet and outlet streams. Two inlet lines supply continuously the eluent as well as the feed solution to be separated. Two outlet lines are withdrawing the extract with the more retained component and the raffinate with the less retained component as pure products. SMB chromatography is a separation process with a liquid flow of the mobile phase and a simulated counter-current circulation of the solid adsorbent. The flow of the adsorbent is simulated (in contrast to the principle of a true moving bed) by an appropriate shifting of the injection and collection points one column forward in direction of the liquid flow at constant time intervals. The mobile phase that flows out of zone 4 is recycled to zone 1.

For lactose separation a SMB system Licosep–LAB–SMB 12 × 26 1040 (Novasep, Vandoeuvre-les-Nancy, France) was used. Fig. 2 demonstrates the connections of the inlet and outlet lines. Twelve potential columns are connected in series. The recycling pump provides a recirculation flow rate in the system. Each of the columns is connected to the inlet (feed and eluent) and outlet (raffinate and extract) lines, but only 4 of maximum 48 lines (one of each type) are opened at a given time. These opened lines are changing simultaneously at a constant time interval one position forward in the direction of the liquid flow.

In the work presented here eight columns (500 mm × 25 mm i.d.) were used (Kronlab, Sinsheim, Germany): one

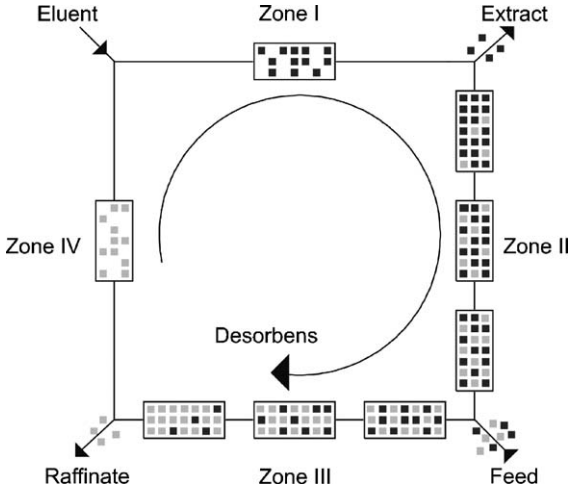

Fig. 1. Principle of the SMB chromatography used for lactose separation: zone 1 and zone 4 includes one column each, zone 2 and zone 3 three columns each (adapted from [19]).

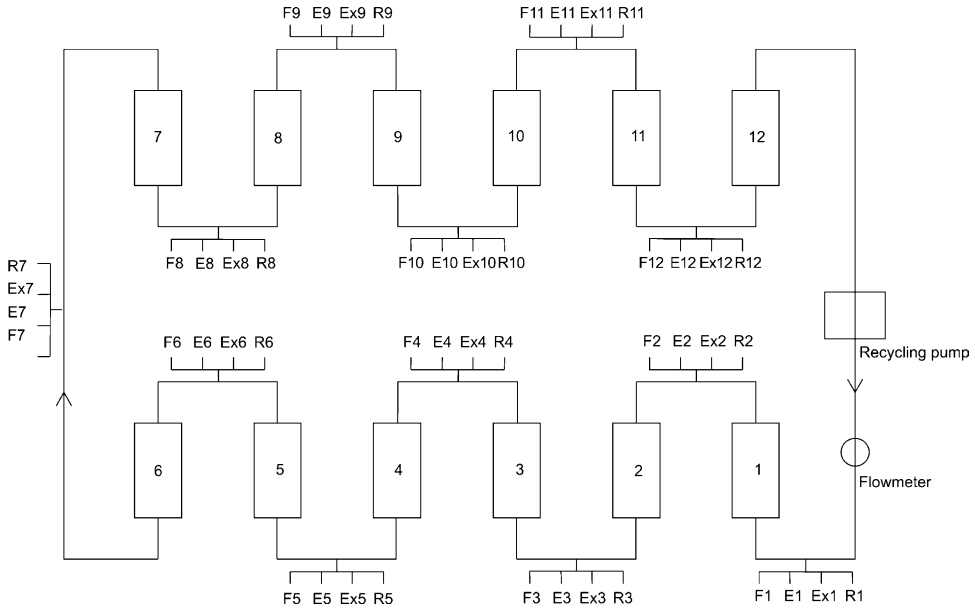

Fig. 2. Diagram of the concept and the internal flows of a Licosep–Lab–SMB-system. The columns are numbered (1–12). All of them are connected to four two-way high-pressure pneumatic valves, one of them coupled to the feed (F), one to the eluent (E), one to the raffinate (R) and one to the extract (Ex). The simultaneous change of the inlet- and outlet-lines leads to the simulated flow of the solid phase. The arrows show the direction of the mobile phase (adapted from Novasep, Vandoeuvre-les-Nancy, France).

in zone 1, three in zone 2 and zone 3, respectively, and one in zone 4. Main components are five dual-head dual-engine pumps (model SD-1, Rainin, Varian, USA). They all have 200 ml/min head sizes except for the feed pump with a 50 ml/min head size. The columns are kept at 50 °C by applying a water bath (Lauda, Lauda-Königshofen, Germany). All of them showed a good reproducibility in their retention volumes (±2% range according to their average values).

2.4. Calculation of the SMB-operating parameters

In order to design a SMB separation process, the internal flow rates of the liquid phase within the four zones and the shifting time period have to be specified to find the less retained component exclusively in the raffinate and the more retained component exclusively in the extract.

Our goal was to separate the disaccharide lactose into the extract and the complex oligosaccharides into the raffinate fraction.

To calculate the SMB-operating parameters, six columns were used in the SMB system on position 1 to position 6 in an open configuration. The eluent was pumped (flow rate 2 ml/min) through the specific valve to the column in position 1, whereas all the other valves and pumps in the system were inactivated, and then the eluate was passed through a refractometer (Knauer, Berlin, Germany). Five-hundred microliter of the sample (see Section 2.2) were applied via injection valve (Rheodyne, Cotati, CA, USA), separated by the stationary phases and detected by RI-detection (integrator (C-R5A, Shimadzu, Tokyo, Japan). The dead time (t_0 = 243 min) and the retention time of the first peak (254 min), of fucosyllactose (406.57 min), of lactose (427.5 min) and of the last detected substance (monosaccharides, 578.73 min) are important for the calculation of the SMB-operating parameters. Lactose is the component with the highest concentration and the one with the highest retardation compared to the rest of the oligosaccharides.

First the porosity ε of the gel was determined:

$$\varepsilon = \frac{t_0 \times M}{V_s} = 0.34 \quad (1)$$

where t_0 = dead time, M = flow rate of the eluent, and V_s = volume of the columns.

After the porosity the retention factors K_i were calculated. Since we worked in the linear range of the adsorption isotherm, the following equation could be used:

$$K_i = \left(\frac{t_R}{t_0} - 1\right) \times \frac{\varepsilon}{1-\varepsilon} \quad (2)$$

where t_R = retention time of the components.; K_1 = 0.0231 (for the first peak); K_2 = 0.3440 (for fucosyllactose); K_3 = 0.3880 (for lactose); K_4 = 0.7060 (for the last peak).

For determining the selectivity α, K_2 and K_3 were needed:

$$\alpha = \frac{K_3}{K_2} = 1.13 \quad (3)$$

Calculation of the flow rate Q of the mobile phase within the zones:

To satisfy the following inequalities and generate equalities, the security factor γ was set up:

$$1 < \gamma < \sqrt{\alpha}, \qquad 1 < \gamma < 1.062$$
$$\rightarrow \gamma = 1,03 \text{ (assumed)} \tag{4}$$

$$\begin{aligned}
&\text{zone I:} && Q\text{I} > s \times K_4 && \rightarrow && Q\text{I} = \gamma \times s \times K_4 && \rightarrow && \frac{Q\text{I}}{s} = 0.73\\
&\text{zone II:} && Q\text{II} > s \times K_2 && \rightarrow && Q\text{II} = \gamma \times s \times K_2 && \rightarrow && \frac{Q\text{II}}{s} = 0.35\\
&\text{zone III:} && Q\text{III} < s \times K_3 && \rightarrow && Q\text{III} = \frac{1}{\gamma} \times s \times K_3 && \rightarrow && \frac{Q\text{III}}{s} = 0.38\\
&\text{zone IV:} && Q\text{IV} < s \times K_1 && \rightarrow && Q\text{IV} = \frac{1}{\gamma} \times s \times K_1 && \rightarrow && \frac{Q\text{IV}}{s} = 0.02
\end{aligned} \tag{5}$$

where s = theoretical flow rate of the stationary phase, and K_i = retention factor.

To determine the theoretical flow rate of the stationary phase s, the following equation was considered (a flow rate of $Q_{\text{exp}} = 2$ ml/min according to the test for calculation the SMB-operating parameters described at the beginning of this paragraph and an average value of $(\bar{Q}/s) = 0.37$ is estimated in consideration of the 1:3:3:1 distribution of columns in zone 1 to zone 4):

$$Q_{\text{exp}} = \left(\frac{\bar{Q}}{s} + \frac{\varepsilon}{1+\varepsilon}\right) \times s, \qquad s = 0.38\,\text{ml/min} \tag{6}$$

Calculation of the switching time Δt:

$$s = \frac{V_{\text{S}} \times (1-\varepsilon)}{\Delta t} \qquad \Delta t = 418 \text{ min} \tag{7}$$

Determination of the flow rate Q with the help of Eq. (4):

$$Q\text{I} = 0.28\,\text{ml/min}, \quad Q\text{II} = 0.13\,\text{ml/min},$$
$$Q\text{III} = 0.14\,\text{ml/min} \quad Q\text{IV} = 0.01\,\text{ml/min}$$

Determination of the different pump flow rates:

$$\begin{aligned}
&Q_{\text{Extract}} = Q\text{I} - Q\text{II} = 5.02\,\text{ml/min},\\
&Q_{\text{Feed}} = Q\text{III} - Q\text{II} = 1.21\,\text{ml/min},\\
&Q_{\text{Raffinate}} = Q\text{III} - Q\text{IV} = 3.84\,\text{ml/min}\\
&Q_{\text{Eluent}} = Q_{\text{Extract}} + Q_{\text{Raffinate}} - Q_{\text{Feed}} = 7.65\,\text{ml/min},\\
&Q_{\text{Recycling}} = 13.42\,\text{ml/min (Eq. (6)!)}, \quad \Delta t = 14.62\,\text{min}
\end{aligned} \tag{8}$$

Originally the flow rates were small (range 0.01–0.28 ml/min) and in contrast, the switching time was long (418 min), therefore, the flow rates were multiplied commonly with the factor 29 and the switching time was divided accordingly. This was the maximum factor applicable due to the pressure limit of the stationary phase (SEC). The resulting flow rate of the feed pump was still too small because of the pump capacity, so it was increased five times whereas the feed concentration was decreased accordingly from 100 to 20 g/l.

Since we have realized the lactose separation in a linear range of adsorption isotherms, it was not necessary to include a simulation-software for calculating the SMB-operating parameters. First of all, our goal was to establish the described separation with the help of SMB chromatography. However, in order to increase the productivity of lactose-reduced HMOS, it would be advisable to use a simulation-software to realize the lactose separation in a non-linear range of adsorption isotherms. The higher productivity could be reached, for example, by increasing the feed concentration. This work is currently performed and will be part of research in the near future.

2.5. Analyses of the products

The products of the process raffinate (all the oligosaccharides except for lactose) and extract (mainly the separated lactose) were analysed by a method of high performance-SEC, respectively. As columns 2 TSK PWXL (300 mm × 7.6 mm i.d.) with one precolumn (Tosoh Bioscience, Stuttgart, Germany) are used, deionized water served as eluent (pump P 580, Gynkotek, Germering, Germany) at a flow rate of 0.4 ml/min and a temperature of 60 °C. The detection was performed by measuring the refractive index (RI; differential refractometer RI-71, Shodex, Germering, Germany).

3. Results and discussion

The analysis of the raffinate and extract with TSK columns shows the successful separation of lactose from human milk oligosaccharides with the help of SMB chromatography. The lactose separation works with both, SEC (HW40C) and IEC (MCI) gel, with similar results of the relative lactose content in the extract and raffinate. With HW40C, the raffinate contents <4% of lactose in comparison to the complex oligosaccharides (neutral and acidic oligosaccharides) (Fig. 3B). By contrast, the extract shows > 96% of the separated lactose (Fig. 3A).

Using MCI, the content of lactose varies in the raffinate from 0 to 20% compared to HMOS (Fig. 4B), while in the extract it has a value of >96% (Fig. 4A).

With regard to the requirements of steady conditions during a longer period of SMB chromatography (for example several weeks), the IEC gel seems to be unsuitable for an efficient lactose separation, due to permanently occurring ion exchanges at the stationary phases (Table 1). During the regeneration of the IEC gel, the stationary phases teared and shrinked, and this was incompatible with the need of constant conditions. As a result, the content of lactose in extract and raffinate shows a larger variation compared to the SEC gel. The yield of raffinate with separated lactose is on average five times smaller with SEC gel, because of the decreased pump flow rates, especially the feed-flow rate, compared to IEC

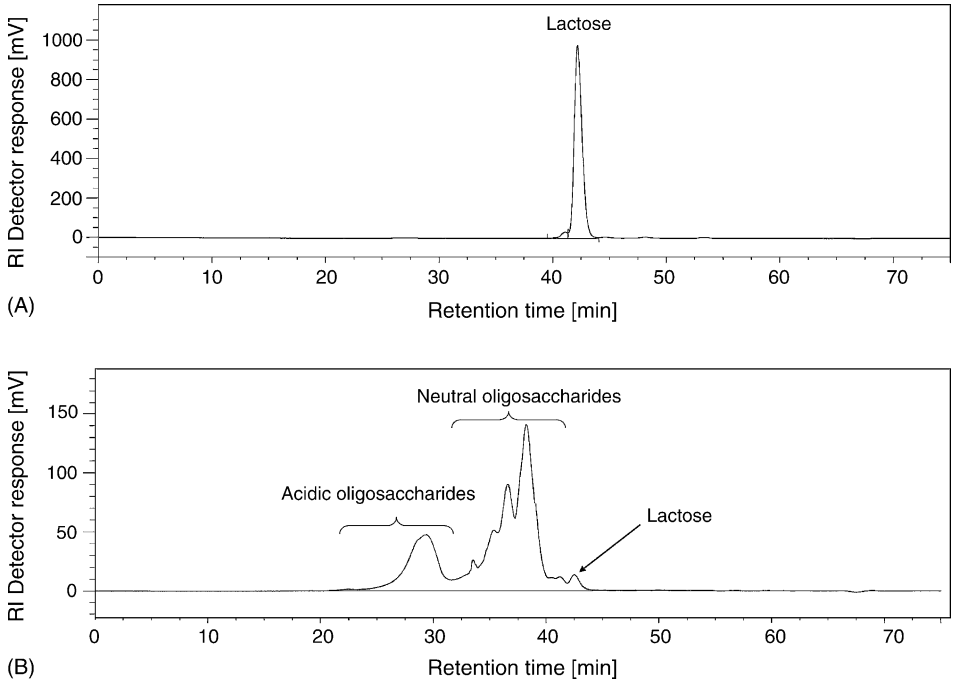

Fig. 3. The extract (A) and raffinate (B) of HMOS separated by SMB chromatography with SEC gel (Toyopearl HW40C) as stationary phase.

gel. These decreased pump-flow rates are the reason for the smaller amount of raffinate (naturally also of extract) within the same time period. It was not possible to increase the flow rate of all pumps simultaneously (especially the recycling pump flow rate), because of the maximum pressure limit of the SEC gel. By contrast, the flow rate with the IEC gel was higher (approximately two times higher, see the recycling flow rate in Table 1) due to the higher particle size. A size-exclusion gel with a particle size of e.g. 150 μm would possibly increase the flow rate of all the pumps in the system, but probably the selectivity of lactose separation would be decreased. This is due to the effect that the greater the parti-

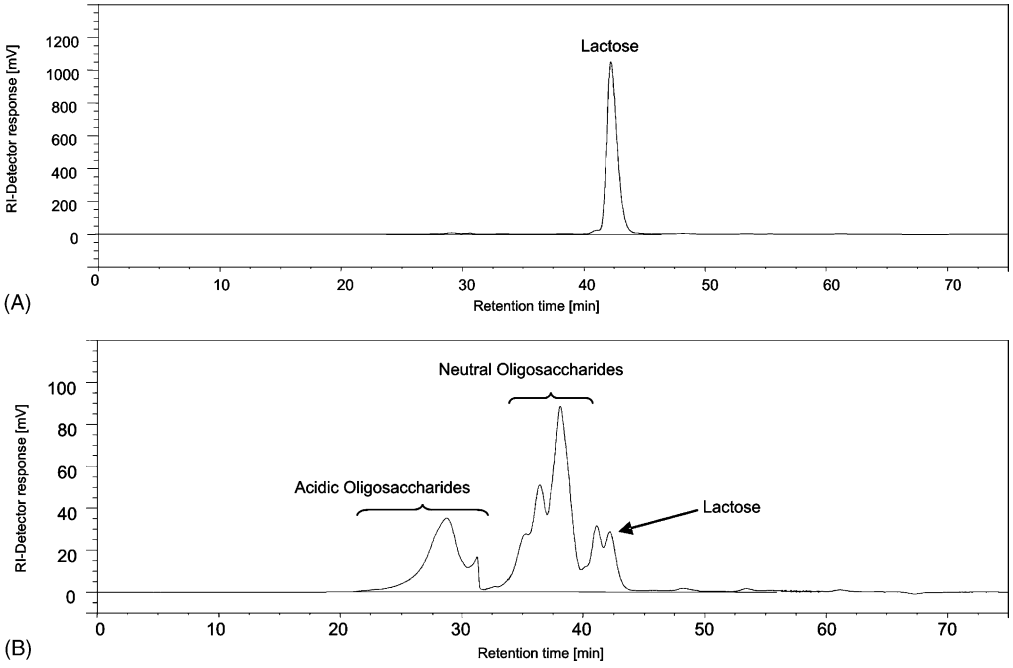

Fig. 4. The extract (A) and raffinate (B) of HMOS separated by SMB chromatography with IEC gel (Daisin MCI GEL CK08P) as stationary phase.

Table 1
Comparison of two different stationary phases for the separation of lactose from HMOS

Characteristic criteria	MCI gel CK08P	HW40C
Method of chromatography	Ligand-exchange chromatography [24]	Size-exclusion chromatography
Sample concentration (g/l)	100–150	20[a]
Mobile phase	Deionized water	2% (v/v) 2-propanol in deionized water
Maximum flow rate per cycle (ml/min)	29.9	14.3
Range of lactose concentration in raffinate (%)	0–20	0–4
Yield of raffinate (g/day)	30–45	4–6
Lactose concentration in extract (%)	>96	>96
Yield of extract (g/day)	90–110	24–28
Changes during regeneration	Rupture and shrinking	No changes
Conclusion	Not suitable for lactose/HMOS separation	Suitable for lactose/HMOS separation

[a] Originally also 100 g/l. Resulting from the new calculation of the SMB parameters the feed flow rate is increased by five times, thus, the feed concentration had to be decreased accordingly to allow for a similar absolute supply of feed between the two experiments.

cle size of the stationary phase, the smaller is the surface of the particles. Therefore, the amount of pores on the surface, which has a great influence on the selectivity, is proportionately decreased.

The lower yield with SEC gel was more than compensated with much more stable conditions for the continuous SMB process. The system is in use with this configuration since nearly 1 year with high purity and reproducibility of the products (raffinate and extract).

4. Conclusions

The separation of lactose from a complex mixture of human milk oligosaccharides was successfully performed by SMB chromatography with two different methods of chromatography: ligand-exchange chromatography (MCI gel), as well as size-exclusion chromatography (HW40C gel).

The flow rates in the system and the yield of lactose were lower when using the SEC gel. However, this gel provided much more stable conditions for the continuous SMB process. Thus, the use of SEC gel for the separation of lactose from HMOS was much more suitable and comfortable compared to the IEC gel.

This applied method for lactose separation with SMB chromatography was successfully performed earlier with low pressure SEC, which also has the potential to fractionate other human milk oligosaccharides [13,16,17]. This means that the probability would be very high to copy these successful separation methods to our SMB system.

The method presented here encourages future attempts to separate lactose from human milk as well as from domestic animal milk oligosaccharides or other lactose containing oligosaccharide mixtures. A transfer of the data to fractionate disaccharides or even larger oligosaccharides from complex oligosaccharide mixtures seems to be possible.

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