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(54) Title: PROCESS FOR PURIFICATION OF A NEUTRAL HUMAN MILK OLIGOSACCHARIDE USING SIMULATED MOVING BED CHROMATOGRAPHY

(57) Abstract: The present application discloses a process for the purification of a neutral human milk oligosaccharide (neutral HMO). The process uses simulated moving bed (SMB) chromatography which allows the continuous purification of large quantities of HMOs with high purity. Contrary to chemical synthesis routes of neutral HMOs, and their subsequent purification, the presented process allows the provision of HMOs free of noxious chemicals, such as e.g. trace amounts of heavy metals or organic solvents. The individual neutral HMO product may be obtained in solid form by spray drying or as a concentrated syrup. The provided neutral HMO is very well-suited for use in food applications.

Process for purification of a neutral human milk oligosaccharide using simulated moving bed chromatography

The present application discloses a process for the purification of a neutral human milk oligosaccharide (neutral HMO). The process uses simulated moving bed (SMB) chromatography which allows the continuous purification of large quantities of HMOs with high purity. Contrary to chemical synthesis routes of neutral HMOs, and their subsequent purification, the presented process allows the provision of HMOs free of noxious chemicals, such as e.g. trace amounts of heavy metals or organic solvents. The individual neutral HMO product may be obtained in solid form by spray drying or as a concentrated syrup. The provided neutral HMO is very well-suited for use in food applications.

Human milk represents a complex mixture of carbohydrates, fats, proteins, vitamins, minerals and trace elements. By far the most predominant fraction is represented by carbohydrates, which can be further divided into lactose

and more complex oligosaccharides. Whereas lactose is used as an energy source, the complex oligosaccharides are not metabolized by the infant. The fraction of complex oligosaccharides accounts for up to 1/10 of the total carbohydrate fraction and consists of probably more than 150 different oligosaccharides. The occurrence and concentration of these complex oligosaccharides are specific to humans and thus cannot be found in large quantities in the milk of other mammals, such as for example domesticated dairy animals.

The existence of these complex oligosaccharides in human milk has been known already for a long time and the physiological functions of these oligosaccharides have been subject to medical research for many decades. For some of the more abundant human milk oligosaccharides, specific functions have already been identified.

The limited supply and difficulties of obtaining pure fractions of individual HMOs led to the development of chemical routes to some of these complex molecules. However, synthesis of HMOs by chemical synthesis, enzymatic synthesis or fermentation has proved to be challenging. At least large-scale quantities as well as qualities adequate for food applications have not been able to be provided to date. In this regard, particularly chemical synthetic routes of specific HMOs (e.g. the HMO 2´-fucosyllactose; see WO 2010/115935 A1) involve several noxious chemicals, which involve the risk of contamination of the final product.

Due to the challenges involved in the chemical synthesis of human milk oligosaccharides, several enzymatic methods and fermentative approaches have been developed. However, these methods yield complex mixtures of oligosaccharides i.e. the desired product is contaminated with starting material such as lactose, biosynthetic intermediates and substrates such as individual monosaccharides and polypeptides etc.

Processes in the state of the art for purifying individual oligosaccharide products from these complex mixtures are technically complex and also uneconomical for food applications. For the purification of the disaccharides lactose or sucrose from complex mixtures such as whey or molasses, industrial scale processes have been developed which involve multiple crystallizations. The

disadvantage of said methods is that they are elaborate and only lead to low yields.

For the purification of complex oligosaccharides, such as certain HMOs, gel-filtration chromatography has been the method of choice until now. The disadvantage of gel-filtration chromatography is that it cannot be efficiently scaled up and it is unsuitable for continuous operation. Thus, gel-filtration chromatography is not economical and renders it impossible to provide certain HMOs - like the HMO 2′-fucosyllactose - in reasonable amounts and quality to use them in human food.

Simulated moving bed (SMB) chromatography has its roots in the petrochemical and mineral industries. Today, SMB chromatography is used by the pharmaceutical industry for the separation of enantiomers from racemic mixtures. SMB chromatography has already been used for the separation of the monosaccharide fructose from fructose-glucose solutions and for the separation of the disaccharide sucrose from sugar beet or sugar cane syrups on large-scale. However, SMB chromatography has not yet been used for the purification of HMOs, or any other complex oligosaccharide, from fermentations yet.

Simulated moving bed (SMB) chromatography was developed as a continuous separation process analogous to continuous chemical separation processes such as rectification. In rectification, a countercurrent is established between the liquid and the gaseous phase, which allows then the continuous application of feed and withdrawal of product(s). In addition, counter-current chromatographic operations in theory should achieve separations superior to conventional cross–current operations. However, chromatographic counter-current operations would require the mobile and stationary phases to move in opposite directions. Thus, SMB chromatography was developed as a practical solution to the difficulties related to the concept of moving solid chromatography material in a continuous chromatographic separation process.

The standard SMB concept involves four different zones with four externally applied streams: a feed stream comprising the components to be separated, a desorbent or mobile phase stream, an extract and a raffinate stream (with the raffinate stream representing the less retained component(s)). These liquid

streams divide the SMB system into four different zones (each zone or section can comprise one or more columns) with the following tasks: zone I is required for the regeneration of the solid phase, the purpose of zone II is the desorption of the less strongly desorbed material, the task of zone III is the adsorption of the strongly adsorbed material and finally the task of zone IV is the adsorption of the less adsorptive material. Thus, more strongly adsorbing components establish a concentration wave in zone II and are transported to the extract port whereas less strongly adsorbing components migrate towards the raffinate port.

In principle, zone I and IV serve for regeneration of the solid phase (regeneration zones) whereas zones II and III can be regarded as the actual separation zones of the system (separation zones). In addition to the four liquid streams and resulting zones, the system contains (for the closed loop operation) a recycling pump for the mobile phase (desorbent), passing the mobile phase through the fixed zones in one direction. Counter-current flow is then achieved by the periodical shifting and continuous supply or withdrawal of feed, desorbent and products sequentially from one column to the next in the system.

Besides the standard closed loop, 4 zone SMB system, open loop, 3 zone systems can be used as well. The 3 zone open loop systems are economical in the case where fresh solvent is rather inexpensive e.g. where water or water/ethanol is used as mobile phase. By using a 3 zone open loop configuration, the regeneration of the liquid phase is no longer needed, thus making zone IV obsolete.

Besides the standard SMB systems for the separation of a two component mixture also eight-zone closed loop or five zone open loop SMB systems have been developed for the separation of more than 2 components.

Due to the continuous mode of operation and also the possibility of using rather larger column sizes and recycling of the mobile phase, the SMB system can in principle be scaled into production volumes of 100s of tons.

Starting from this prior art, the technical problem is the provision of a novel

process to provide a neutral HMO in large amounts, with high purity and free of noxious chemicals.

The technical problem is solved by the process according to claim 1, the neutral HMO according to claim 14 and the use of a neutral HMO according to claim 18. The dependent claims display advantageous embodiments.

The present invention provides a process for purification of a neutral HMO (e.g. 2′-fucosyllactose) in a continuous chromatography (or in a continuous manner) from a crude solution comprising the neutral HMO (e.g. 2′-fucosyllactose) and contaminants, wherein the crude solution comprising the neutral HMO (e.g. 2′-fucosyllactose) and contaminants comprises or consists of a solution which is selected from the group consisting of microbial fermentation extract, biocatalysis reaction solution, chemical synthesis solution, and combinations thereof and wherein the purity of the neutral HMO (e.g. 2′-fucosyllactose) in the solution is <80 %. The process is characterized in that the crude solution is applied to at least one purification step using simulated moving bed chromatography. In this way, a purified solution comprising the desired neutral HMO (e.g. 2′-fucosyllactose) with a purity of ≥80 % is provided.

The process for purification of a neutral HMO in a continuous chromatography may be a process for purification of a neutral HMO in a continuous manner. In this regard, the neutral HMO may be 2′-fucosyllactose or lacto-*N*-tetraose.

The Applicant has discovered that with the developed process involving a purification step using simulated moving bed chromatography, HMOs may be provided with high purity, without heavy metal contaminants and in a continuous manner. Thus, large amounts of high-quality HMOs may be provided in a very convenient and economical way, e.g. from a crude solution from microbial fermentation. The inventive process also turned out to be highly stable even without a step of regeneration of the column material (e.g. cationic column material) used in the simulated moving bed chromatography step. In fact, the whole process can be operated in a stable and continuous manner for several months.

In a preferred embodiment, the purity of the neutral HMO in the crude solution is $\leq$70 %, $\leq$60 %, $\leq$50 %, $\leq$40 %, $\leq$30 %, $\leq$20 %, $\leq$10 % or $\leq$5 % and/or the purified solution contains the HMO with a purity of $\geq$80 %, preferably of $\geq$90 %. The term "crude solution" refers to a solution containing neutral HMO before the purification step of single moving bed chromatography whereas the purified solution refers to a solution after the step of single moving bed chromatography.

The at least one simulated moving bed chromatography step may have

i)   at least 4 columns, preferably at least 8 columns, more preferably at least 12 columns, wherein at least one column comprises a weak or strong cation exchange resin, preferably a cation exchange resin in the H^+-form or Ca^{2+}-form; and/or

ii)   four zones I, II, III and IV with different flow rates; and/or

iii)   an eluent comprising or consisting of water, preferably ethanol and water, more preferably 5-15 vol.-% ethanol and 85-95 vol.-% water, most preferably 9-11 vol.-% ethanol and 89-91 vol.-% water, wherein the eluent optionally further comprising sulphuric acid, preferably $\leq$10 mM sulphuric acid; more preferably $\leq$2-5 mM sulphuric acid; and/or

iv)   an operating temperature of 15° to 60 °C, preferably 20° to 55 °C, more preferably 25° to 50 °C.

If the HMO to be purified is 2´-fucosyllactose, the at least one simulated moving bed chromatography step may have

i)   four zones I, II, III and IV with different flow rates, wherein the flow rates are preferably: 28-32 ml/min in zone I, 19-23 ml/min in zone II, 21-25 ml/min in zone III and/or 16-20 ml/min in zone IV; and/or

ii)   a feed rate of 2-4 ml/min, preferably 3 ml/min; and/or

iii)   an eluent flow rate of 10-13 ml/min, preferably 11.5 ml/min; and/or

iv)   a switching time of 16-20 min, preferably 17-19 min, more preferably 18 min.

Preferably, at least one of the columns comprises 0.1 to 5000 kg of cation exchange resin, preferably 0.2 to 500 kg of cationic exchange resin, more preferably 0.5 to 50 kg of cation exchange resin, most preferably 1.0 to 20 kg of cation exchange resin.

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Importantly, scaling-up of the amount of cation exchange material, the flow rate in the different zones, the feed rate, the eluent flow rate and/or the switching time is possible. The scaling-up may be by a factor of 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 1000 or all possible scaling factors

10   in between said values.

In the columns, a strong cation exchange resin may be used as stationary phase. Preferably, the cation exchange resin is a sulfonic acid resin, more preferably a Purolite® PCR833H (Purolite, Ratingen, Germany), Lewatit MDS

15   2368 and/or Lewatit MDS 1368 resin. If a cation ion exchange resin is employed in the columns, it may be regenerated with sulphuric acid. Sulphuric acid can be employed in the eluent, preferably at concentration of 10 mM sulphuric acid or less. The (strong) cation exchange resin may be present in H^+-form or in Ca^{2+}-form.

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Operating temperatures above 60°C are not preferred during simulated moving bed chromatography. It was found that especially in the presence of a strong cation ion exchange resin (in H^+-form or Ca^{2+}-form) as stationary phase, the applied neutral oligosaccharides were significantly destabilized i.e. depol-

25   ymerized which was detrimental to the final yield of the neutral HMO.

In an advantageous embodiment of the invention, the invention is characterized in that the purified solution is applied to at least one further purification step using simulating moving bed chromatography, wherein a purified solu-

30   tion comprising the neutral human milk oligosaccharide with a purity of ≥90 %, preferably ≥92%; more preferably ≥ 93% is provided. In particular, the invention yields a HMO product free of recombinant DNA, and free of host strain proteins.

35   The further simulated moving bed chromatography may have

i)   at least 4 columns, preferably at least 8 columns, more preferably at least

12 columns, wherein at least one column comprises a weak or strong cation exchange resin, preferably a cation exchange resin in the H^+-form or Ca^{2+}-form; and/or

ii)   four zones I, II, III and IV with different flow rates, and/or

iii)   an eluent comprising or consisting of water, preferably ethanol and water, more preferably 5-15 vol.-% ethanol and 85-95 vol.-% water, most preferably 9-11 vol.-% ethanol and 89-91 vol.-% water, wherein the eluent optionally further comprises sulphuric acid, preferably ≤10 mM sulphuric acid; more preferably ≤2-5 mM sulphuric acid, and/or

iv)   an operating temperature of 15° to 60 °C, preferably 20° to 55 °C, more preferably 25° to 50 °C.

If the HMO to be purified is 2′-fucosyllactose, the further simulated moving bed chromatography step may have

i)   four zones I, II, III and IV with different flow rates, wherein the flow rates are preferably: 28-32 ml/min in zone I, 19-23 ml/min in zone II, 21-25 ml/min in zone III and/or 16-20 ml/min in zone IV; and/or

ii)   a feed rate of 2-4 ml/min, preferably 3 ml/min; and/or

iii) an eluent flow rate of 10-13 ml/min, preferably 11.5 ml/min; and/or

iv)   a switching time of 16-20 min, preferably 17-19 min, more preferably 18 min.

Particularly, at least one of the columns contains 0.1 to 5000 kg of cation exchange resin, preferably 0.2 to 500 kg of cation exchange resin, more preferably 0.5 to 50 kg of cation exchange resin, most preferably 1.0 to 20 kg of cation exchange resin.

Importantly, scaling-up of the amount of cation exchange material, the flow rate in the different zones, the feed rate, the eluent flow rate and/or the switching time is possible. The scaling-up may be by a factor of 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 1000 or all possible scaling factors in between said values.

After a purification step using simulated moving bed chromatography, the pH of the purified solution may be adjusted to pH 7, preferably by adding a base, more preferably by adding NaOH (e.g. 0.2 M NaOH).

According to the invention, the crude solution containing the neutral HMO and contaminants comprises or consists of a solution which is selected from the group consisting of microbial fermentation, microbial fermentation extract, biocatalysis reaction solution, chemical synthesis solution and combinations thereof. Fermentation as a source of the neutral HMO has the advantage that it is more cost-effective than chemical synthesis or biocatalysis i.e. enzymatic synthesis. Thus, a microbial fermentation extract is preferred.

Preferably, before applying the solution to the at least one simulating moving bed chromatography step, the solution (preferably a microbial fermentation solution) comprising the neutral HMO is

i)   filtered or centrifuged to remove the biomass and/or any insoluble material, preferably filtered with activated carbon, charcoal, celite and/or by cross-flow filtration to remove any insoluble material and organic contaminants, more preferably filtered by cross-flow filtration, most preferably filtered by cross-flow filtration using a microfiltration membrane; and/or

ii)   applied to at least one purification step using cation and/or anion exchange chromatography, preferably first at least one cation exchange chromatography step and then at least one anion exchange chromatography step.

In a further preferred embodiment, before applying the solution to at least one purification step using simulated moving bed chromatography or after a purification step using simulated moving bed chromatography, the solution containing the neutral HMO is electrodialysed and/or diafiltered, preferably diafiltered with a nanofiltration membrane, more preferably diafiltrated with a nanofiltration membrane having a size exclusion limit of ≤ 20 Å. Most preferably, the solution is dialysed to a conductivity of ≤ 15 mS/cm, preferably ≤ 10 mS/cm, more preferably ≤ 5 mS/cm.

If the crude solution is dialysed before applying the solution to at least one purification step using simulated moving bed chromatography, major contaminants depend on the origin of the neutral HMO fractions (i.e. chemical synthesis, biocatalysis or fermentation). Typical contaminants are monosaccharides (e.g. glucose, galactose, fucose, etc.), disaccharides (e.g. lactose) and by-products (e.g. lactulose). In case where fermentation was used as a source of the neutral HMO, the crude solution usually comprises the employed carbon source (e.g. glycerol, sucrose and/or glucose) as well as by-products of the employed microbes (e.g. higher molecular mass oligosaccharides) as contaminants. As further contaminants, also oligosaccharides may be present which are generated due to the promiscuity of the used glycosyltransferases (e.g. glycosyltransferases in the synthesizing cell which convert the desired product, the substrate or an intermediate product into a contaminating oligosaccharide). Said contaminants can efficiently be removed by a purification step using simulated moving bed (SMB) chromatography.

After dialysis, preferably after electrodialysis and/or diafiltration (optionally before applying the solution to the SMB chromatographic process), the solution comprising the HMO may be concentrated, in particular

i)   to a concentration of $\geq$ 50 g/l, $\geq$ 100 g/l, preferably $\geq$ 200 g/l, more preferably $\geq$ 300 g/l; and/or

ii) employing a vacuum concentrator; and/or

iii) by nanofiltration; and/or

iv) at a temperature of 4° to 50 °C, preferably 10° to 45 °C, optionally 20° to 40 °C or 30° to 35 °C.

More preferably, the HMO-comprising fraction is concentrated by employing nanofiltration. The nanofiltration step can be further used to dialyse away contaminating salts. Here, the HMO fraction may firstly be concentrated by nanofiltration and the resulting concentrated HMO fraction is then subsequently diluted with water, preferably double-distilled H_2O (ddH_2O) or deionized water, and then the diluted HMO fraction may again be concentrated using a nanofiltration membrane.

Concentration by nanofiltration is particularly preferred because an exposure of the neutral HMOs to high temperatures may be dispensed with. Thus, said method of concentration is less destructive to the structure of HMOs than a heat treatment i.e. it does not induce thermal damage to the neutral HMOs during concentration. An additional advantage of nanofiltration is that it can be used both for concentrating and for dialysing (diafiltering) the neutral HMOs. In other words, a membrane used in nanofiltration does not have to be exchanged if the concentration step and the dialysis step are implemented in succession in the inventive process. In addition, the salt concentration of the solution containing neutral HMOs can be significantly reduced. This saves material and time and makes the whole process more economical. Preferably, nanofiltration is combined with electrodialysis. This combination turned out to give excellent results in concentrating and desalting.

In a preferred embodiment of the invention, the purified solution is sterile filtered and/or subjected to endotoxin removal, preferably by filtration of the purified solution through a 3 kDa filter.

The purified solution may be spray-dried, particularly spray-dried at a concentration of the neutral HMO of 20-60 % (w/v), preferably 30-50 % (w/v), more preferably 35-45 % (w/v), an inlet temperature of 110-150 °C, preferably 120-140 °C, more preferably 125-135 °C and/or an outlet temperature of 60-80 °C, preferably 65-70 °C.

In a preferred embodiment of the inventive process, the neutral HMO which is to be purified is a neutral HMO having more than 3 monosaccharide units, preferably a neutral human milk trisaccharide, tetrasaccharide, pentasaccharide or hexasaccharide. More preferably, the neutral HMO is selected from the group consisting of 2'-fucosyllactose, 3-fucosyllactose, 2',3-difucosyllactose, lacto-*N*-triose II, lacto-*N*-tetraose, lacto-*N-neo*tetraose, lacto-*N*-fucopentaose I, lacto-*N-neo*fucopentaose I, lacto-*N*-fucopentaose II, lacto-*N*-fucopentaose III, lacto-*N*-fucopentaose V, lacto-*N-neo*fucopentaose V, lacto-*N*-difucohexaose I, lacto-*N*-difucohexaose II, 6'-galactosyllactose, 3'-galactosyllactose, lacto-*N*-hexaose, lacto-*N-neo*hexaose, *para*-lacto-*N*-hexaose, *para*-lacto-*N-neo*hexaose and difucosyl-lacto-*N-neo*hexaose. Most preferably, the neutral HMO is 2'-fucosyllactose or lacto-*N-neo*tetraose.

Optionally, it is also possible that the neutral HMO is not 2´-fucosyllactose.

In an advantageous embodiment of the invention, the inventive process is characterized in that the crude solution comprising the neutral human milk oligosaccharide and contaminants comprises or consists of a microbial fermentation extract, wherein the microbial fermentation extract is obtained in at least one step of microbial fermentation which is preferably followed by at least one step of

a)   filtration of a solution, preferably the crude solution, to separate soluble material from insoluble material after the microbial fermentation; and/or

b)   ion exchange chromatography, preferably cation exchange chromatography, more preferably cation exchange chromatography followed by anion exchange chromatography, of a solution, preferably of a solution obtained in step a); and/or

c)   concentration of a solution, preferably a solution obtained in step b), more preferably by evaporation of water and/or by nanofiltration, optionally by concentrating more than once; and/or

d)   dialysis of a solution, preferably of a solution obtained in step c), more preferably by electrodialysis and/or diafiltration, most preferably diafiltration with a nanofiltration membrane, optionally by dialysing more than once; and/or

e)   chromatography of a solution using simulated moving bed chromatography, preferably a solution obtained in step d); and/or

f)   filtration of a solution, preferably a solution obtained in step e), to separate neutral human milk oligosaccharide from coloured contaminants, more preferably by filtration through activated carbon; and/or

g)   spray-drying a purified solution comprising the neutral human milk oligosaccharide, preferably a purified solution obtained in step f).

Most preferably, all steps a) to g) are implemented in succession. The implementation of all steps a) to g) in succession has been found to be the most advantageous embodiment of the inventive process. Said process is cost and time efficient and enables the provision of large quantities of highly pure,

spray-dried (i.e. amorphous) neutral HMOs from microbial fermentation (extracts). In particular, the concentration and desalting steps of the HMO solution using nanofiltration represent extremely cost efficient and gentle operating steps preventing undesired by-product formation.

The invention thus provides a neutral HMO which is producible with the inventive process. The neutral HMO (e.g. 2′-fucosyllactose or lacto-*N*-tetraose) is preferably spray-dried. The purified neutral HMO has the advantage of being highly pure and being free of heavy metal contaminants, and/or organic solvents.

The neutral HMO according to the invention can have

i)   a solid granule form; and/or

ii)   a glass transition temperature of 60 to 90 °C, preferably 62 to 88 °C, more preferably 64 to 86 °C, determined by differential scanning calorimetry; and/or

iii) a particle size of 5 to 500 μm, preferably 10 to 300 μm, determined by laser diffraction; and/or

iv)   a mean particle size of 10 to 100 μm, preferably 20 to 90 μm, more preferably 30 to 80 μm, most preferably 40 to 70 μm, determined by laser diffraction; and/or

v)   an amorphous state, preferably an amorphous state with no characteristic peaks of crystalline matter in X-ray powder diffraction, and/or

vi)   a moisture content of $\leq 10\%$, preferably $\leq 8\%$, more preferably $< 5\%$.

The neutral HMO may be used in medicine, preferably in prophylaxis or therapy of gastrointestinal disorders. It can also be used in nutrition, preferably medicinal nutrition or dairy nutrition (e.g. cereal products).

In a preferred embodiment of the invention, the neutral human milk oligosaccharide is a neutral HMO having more than 3 monosaccharide units, preferably a neutral human milk trisaccharide, tetrasaccharide, pentasaccharide or hexasaccharide. More preferably, the neutral HMO is selected from the group

consisting of 2'-fucosyllactose, 3-fucosyllactose, 2',3-difucosyllactose, lacto-*N*-triose II, lacto-*N*-tetraose, lacto-*N-neo*tetraose, lacto-*N*-fucopentaose I, lacto-*N-neo*fucopentaose I, lacto-*N*-fucopentaose II, lacto-*N*-fucopentaose III, lacto-*N*-fucopentaose V, lacto-*N-neo*fucopentaose V, lacto-*N*-difucohexaose I, lacto-*N*-difucohexaose II, 6'-galactosyllactose, 3'-galactosyllactose, lacto-*N*-hexaose, lacto-*N-neo*hexaose, *para*-lacto-*N*-hexaose, *para*-lacto-*N-neo*hexaose and difucosyl-lacto-*N-neo*hexaose. Most preferably, the neutral HMO is 2'-fucosyllactose or lacto-*N-neo*tetraose.

Optionally, it is also possible that the neutral HMO is not 2'-fucosyllactose.

Furthermore, it is proposed to use the neutral HMO according to the invention as additive in food, preferably as additive in human food and/or pet food, more preferably as additive in human baby food.

With reference to the following Figures and Examples, the subject according to the invention is intended to be explained in more detail without wishing to restrict said subject to the special embodiments shown here.

Figure 1 schematically illustrates one purification step using simulated moving bed chromatography. The simulated moving bed chromatography may have e.g. 12 columns in a serial arrangement 1, wherein the arrangement is divided into four different zones I, II, III and IV. The crude solution containing the neutral HMO 2'-fucosyllactose and contaminants is applied between zone II and III to the feed entry 2. Extract is removed from exit 4 between zone I and zone II whereas raffinate is removed at exit 3 between zone III and zone IV. Raffinate at exit 3 contains the purified HMO 2'-fucosyllactose whereas extract at exit 4 contains low molecular weight contaminants (e.g. monosaccharides and disaccharides).

Figure 2 schematically illustrates two subsequent purification steps using simulated moving bed chromatography. Each simulated moving bed chromatography may have e.g. 12 columns in a serial arrangement 1, 1', wherein each arrangement is divided into four different zones Ia, IIa, IIIa and IVa or zones Ib, IIb, IIIb and IVb, respectively. The crude solution comprising the neutral HMO 2'-fucosyllactose and contaminants is applied between zone IIa and

IIIa of the first arrangement 1 to the feed entry 2. Extract is removed at exit 4 between zone Ia and zone IIa whereas raffinate leaves the first serial arrangement 1 at exit 3 between zone IIIa and zone IVa and is applied to the second serial arrangement 1´ between zone IIb and IIIb. In the second serial arrangement 1´, extract is removed at exit 6 whereas raffinate is removed at exit 5 between zone IIIb and zone IVb. Raffinate at exit 5 contains highly purified 2´-fucosyllactose whereas extract at exit 6 contains high molecular weight contaminants (e.g. higher oligosaccharides).

Figure 3 schematically illustrates a preferred purification scheme according to the present invention. Firstly, a solution 7 containing the neutral HMO 2´-fucosyllactose and contaminants is applied to an electrodialysis step 8 until a conductivity of $\leq$ 0.5 mS/cm is obtained. Said solution is concentrated until the solution has reached a concentration of 2´-fucosyllactose of approx. 40% (w/v). Subsequently, said solution is applied to at least one purification step using simulated moving bed chromatography 9. After the simulated moving bed chromatography, a purified solution comprising 2´-fucosyllactose with high purity is obtained. Said pure solution is subjected to sterile filtration 11 (preferably also endotoxin removal). Before sterile filtration 11, an additional step of electrodialysis 10 with subsequent concentration may optionally be performed. After sterile filtration 11, the purified solution comprising 2´-fucosyllactose is subjected to spray drying 12 and pure, spray dried 2´-fucosyllactose 13 is obtained in solid granule form.

Figure 4 shows the result of an X-ray powder diffraction analysis of two samples of spray-dried 2´-fucosyllactose according to the present invention (sample #1 and sample #2). The two obtained diffractograms reveal that both sample #1 and sample #2 are in the fully amorphous state (no characteristic peaks of crystalline matter).

Figure 5 shows the particle size distribution of spray-dried 2´-fucosyllactose according to the present invention (sample #1 and sample #2) determined by laser diffraction. A mean particle size of approx. 68 µm was determined for sample #1. Sample #2 had a mean particle size of approx. 44 µm. Both values are considered to be high for a spray-dried product.

Figure 6 shows the biosynthesis of the neutral human milk tetrasaccharide lacto-*N-neo*tetraose 17. The biosynthesis starts with the disaccharide lactose 14 which is converted by β-1,3-*N*-acetyl-glucosamintransferase 15 to the trisaccharide lacto-*N*-triose 16. The trisaccharide lacto-*N*-triose 16 is subse-
5   quently converted into the tetrasaccharide lacto-*N-neo*tetraose 18 by the en- zyme 1,4-galactosyltransferase 17. *In vivo*, a certain percentage of lacto-*N- neo*tetraose 18 is further converted into larger oligosaccharides 19, 20, 21 by the enzymes β-1,3-*N*-acetyl-glucosamintransferase 15 and 1,4- galactosyltransferase 17. If the aim of the inventive process is the purification
10   of lacto-*N-neo*tetraose 17, the larger oligonucleotides as well as the smaller oligonucleotides (educts) lactose 14 and lacto-*N*-triose 16 may be present as contaminants 22 in a crude solution containing lacto-*N-neo*tetraose 17. The inventive process enables efficient removal of said contaminants 22.

15   Figure 7 shows a comparison of two different nanofiltration membranes for the concentration of a 2'-Fucosyllactose containing solution by nanofiltration (VCF: volumetric concentration factor, Flux: expresses the rate at which water permeates the nanofiltration membrane). Alfa Laval nanofiltration membrane type NF99 (NF) and Alfa Laval nanofiltration membrane type NF99HF were
20   used as nanofiltration membrane. It can be seen that at VCF ≤ 6, the NF99HF nanofiltration membrane allows a higher flux, i.e. a faster concentration of the solution.

Figure 8 shows the HPLC analysis of the feed (Figure 8A) and raffinate (Figure
25   8B) of the SMB chromatography of Example 6 (saccharose was used as inter- nal standard; see Fig. 8A). As analytical column, a ReproSil Carbohydrate col- umn (amino functionalized silica column; 5 μm; 250 x 4.6 mm; Dr. Maisch GmbH; Ammerbuch) was employed with a flow rate of 1.4 ml/min. As eluent, a mixture of acetonitrile and water (68 vol.-% : 32 vol.-%) was used. Elution
30   was isocratic. The injection volume was 20 μl. Oven temperature was 35 °C. In the SMB chromatography, a strong cationic exchange resin was used which was present in the H$^+$-form.

Figure 9 shows the HPLC analysis of the extract of the SMB chromatography of
35   Example 6. As analytical column, a ReproSil Carbohydrate column (amino functionalized silica column; 5 μm; 250 x 4.6 mm; Dr. Maisch GmbH;

Ammerbuch) was employed with a flow rate of 1.4 ml/min. As eluent, a mixture of acetonitrile and water (68 vol.-% : 32 vol.-%) was used. Elution was isocratic. The injection volume was 20 µl. Oven temperature was 35 °C. In the SMB chromatography, a strong cationic exchange resin was used which was present in the H^+-form.

Figure 10 shows the HPLC analysis of the extract of the second SMB chromatography (= further SMB chromatography according to the invention) of Example 6. As analytical column, a ReproSil Carbohydrate column (amino functionalized silica column; 5 µm; 250 x 4.6 mm; Dr. Maisch GmbH; Ammerbuch) was employed with a flow rate of 1.4 ml/min. As eluent, a mixture of acetonitrile and water (68 vol.-% : 32 vol.-%) was used. Elution was isocratic. The injection volume was 20 µl. Oven temperature was 35 °C. In the SMB chromatography, a strong cationic exchange resin was used which was present in the H^+-form.

Figure 11 shows the HPLC analysis of the feed (Figure 11A) and raffinate (Figure 11B) of the SMB chromatography of Example 7. As analytical column, a ReproSil Carbohydrate column (amino functionalized silica column; 5 µm; 250 x 4.6 mm; Dr. Maisch GmbH; Ammerbuch) was employed with a flow rate of 1.4 ml/min. As eluent, a mixture of acetonitrile and water (68 vol.-% : 32 vol.-%) was used. Elution was isocratic. The injection volume was 20 µl. Oven temperature was 35 °C. In this SMB chromatography, a strong cationic exchange resin was used which was present in the Ca^{2+}-form.

Figure 12 shows the HPLC analysis of the extract of the SMB chromatography of Example 7. As analytical column, a ReproSil Carbohydrate column (amino functionalized silica column; 5 µm; 250 x 4.6 mm; Dr. Maisch GmbH; Ammerbuch) was employed with a flow rate of 1.4 ml/min. As eluent, a mixture of acetonitrile and water (68 vol.-% : 32 vol.-%) was used. Elution was isocratic. The injection volume was 20 µl. Oven temperature was 35 °C. In this SMB chromatography, a strong cationic exchange resin was used which was present in the Ca^{2+}-form.

Example 1: Purification of 2'-fucosyllactose using simulated moving bed chromatography (SMB chromatography)

A clear, particle-free solution containing 2'-fucosyllactose at a concentration of 250 g/L was electrodialysed to 0.5 mS/cm using a PC-Cell BED 1-3 electrodialysis apparatus (PC-Cell, Heusweiler, Germany) equipped with PC-Cell E200 membrane stack. Said stack comprised the following membranes: cation exchange membrane CEM: PC SK and the anion exchange membrane AEM:PcAcid60 having a size exclusion limit of 60 Da.

For SMB purification, the 2'-fucosyllactose solution was concentrated to 300 g/L employing a vacuum concentrator at 40 °C. For the SMB chromatography, a closed loop SMB system equipped with 12 columns (Prosep® columns with the dimensions: 40 mm x 740 mm (Latek, Eppelheim, Germany)) arranged in 4 zones was employed. Each column comprised 760 g of Purolite® PCR833H+ (Purolite, Ratingen, Germany) strong cation ion exchanger resin.

The system was operated at a temperature of 25°C with the following set flow parameters: flow rate zone I was 30.00 ml/min, flow rate zone II was set to 21.00 ml/min, flow rate zone III was 21.48 ml/min, flow rate of zone IV was set to 18.44 ml/min, feed was set to 3.00 ml/min, eluent flow was set to 11.56 ml/min and switching time was set to 17.92 min. As eluent, water with 10 % (v/v) food grade ethanol was used.

Major contaminants such as lactose, monosaccharides such as fucose, glucose, galactose and glycerol, were fractioned into the extract. 2'-fucosyllactose and larger oligosaccharide contaminants (e.g. difucosyllactose) were fractioned into the raffinate.

The 2'-fucosyllactose was marginally diluted through the SMB purification step – the concentration of 2'-fucosyllactose in the raffinate was determined at 200g/l. The pH of the raffinate was adjusted to pH 7 by using 0.2 N NaOH. At the described settings the SMB systems could be continuously operated for at least 3 months.

Then, the obtained solution was again subjected to electrodialysis until a con-

ductivity of less than 0.5 mS/cm was obtained and concentrated to obtain a
40% (w/v) 2'-fucosyllactose solution.

5   The solution was then subjected to sterile filtration and endotoxin removal by
passing the solution through a 3 kDa filter (Pall Microza ultrafiltration hollow
fiber module SEP-2013, Pall Corporation, Dreieich).

Using this protocol 2'-fucosyllactose with a purity of 90.4 % could be obtained.
Major contaminants were 3'-fucosyllactose (2.6%), difucosyllactose (1.5%)
10   and lactose (1.4%). The yield of the purification was approximately 80%.

**Example 2: Purification of 2'-fucosyllactose using multicomponent SMB
chromatographic separation.**

15   A clear, particle-free solution comprising 2'-fucosyllactose at a concentration
of 250g/L was electrodialysed to 0.5 mS/cm using a PC-Cell BED 1-3
electrodialysis apparatus (PC-Cell, Heusweiler, Germany) equipped with PC-
Cell E200 membrane stack. Said stack comprised the following membranes:
cation exchange membrane CEM:Pc SK and anion exchange membrane
20   AEM:Pc Acid 60 which possess a size exclusion limit of 60 Da.

For SMB purification, the 2'-fucosyllactose solution was concentrated to 300
g/L employing a vacuum concentrator at 40 °C. For the SMB chromatography,
a close loop multicomponent SMB System equipped with 24 columns
25   (Prosep® columns with the dimensions: 40 mm x 740 mm (Latek, Eppelheim,
Germany)) arranged in 2 x 4 zones was employed. Each column contained 760
g of Purolite® PCR833H+ (Purolite, Ratingen, Germany) strong cation ion ex-
changer resin.

30   The system was operated at 25 °C with the following set flow parameters:
flow rate zone Ia was 30.00 ml/min, flow rate zone IIa was set to 21.00
ml/min, flow rate of zone IIIa was set to 21.48 ml/min, flow rate of zone IVa
was set to 18.44 ml/min, feed was set to 3.00 ml/min, eluent flow was set to
11.56 ml/min and switching time was set to 17.92 min.

35

The raffinate of the first separation was passed on at a flow rate of 3.04

ml/min to a second separation step. The flow rate of zone Ib was kept at 30 ml/min, flow rate of zone IIb was set to 19.61 ml/min, flow rate of zone IIIb was set to 21.63 ml/min, flow rate of zone IVb was set to 18.46 ml/min, eluent flow was set similarly to 11.56 ml/min and switching time of zones Ib to IVb was 10.46 min.

As eluent water with 10 % (v/v) food grade ethanol was used.

In the multicomponent separation, contaminants such as lactose, monosaccharides such as fucose, glucose, galactose and glycerol were found in the extract of the first separation step and larger oligosaccharide contaminants (e.g. difucosyllactose) were fractioned into the raffinate of the second separation step.

2'-fucosyllactose was fractioned into the raffinate of the first separation step and the extract of the second separation step and was thus free of low and high molecular weight contaminants. 2'-fucosyllactose was only marginally diluted through the SMB purification step – the concentration of 2'-fucosyllactose in the extract of the second purification step was determined at 200g/l.

The pH of the raffinate after the first separation step was adjusted to pH 7 by using 0.2 N NaOH.

Using this protocol, 2'-fucosyllactose with a purity of 93.0% could be obtained. Major contaminants were 3'-fucosyllactose (1.1%), difucosyllactose (0.9%) and lactose (1.0%).

Example 3: Obtaining 2'-fucosyllactose in solid form by spray drying

The 2'-fucosyllactose fractions obtained by SMB chromatography were again subjected to electrodialysis treatment until a conductivity of less than 0.5 mS/cm was obtained. The fractions were then concentrated under vacuum to obtain 2'-fucosyllactose fractions containing 40% (w/v) 2'-fucosyllactose. The solutions were subsequently subjected to sterile filtration and endotoxin removal by passing the solution through a 3 kDa filter (Pall Microza ultrafiltra-

tion hollow fiber module SEP-2013, Pall Corporation, Dreieich, Germany).

The thus-obtained sterile 2'-fucosyllactose solutions were then spray dried using a NUBILOSA LTC-GMP spray dryer (NUBILOSA, Konstanz, Germany). For spray-drying of the 2'-fucosyllactose, the 40% (w/v) solution was passed under pressure through the spray dryer nozzles with an inlet temperature set to 130°C. The flow was adjusted to maintaining an outlet temperature between 66°C to 67°C.

Using these settings a spray dried powder with less than 5% moisture could be obtained. The moisture contents were determined by Karl-Fischer titration.

Example 4: Characterisation of the spray-dried 2´-fucosyllactose

1. Differential scanning calorimetry (DSC)

A Mettler Toledo 821e (Mettler Toledo, Giessen, Germany) was used to determine thermal events of two samples (sample #1 and sample #2) of spray-dried 2´-fucosyllactose.

Approximately 25 mg of a spray-dried sample was analyzed in crimped Al-crucibles (Mettler Toledo, Giessen, Germany). The samples were cooled to 0 °C at 10 K/min and reheated to 100 °C at a scanning rate of 10 K/min. After cooling down the samples to 0 °C in a second heating cycle the samples were heated to 150 °C The midpoint of the endothermic shift of the baseline during the heating scan was taken as Tg. Exothermic and endothermic peaks are reported by means of the peak temperature and the normalized energy of the event. The results of the DSC analysis of the samples are summarized in Table 1.

sample	1st heating scan					2nd heating scan			
	Tg	Endotherm		exotherm		Tg1	Tg2	exotherm	
	°C	°C	J/g	°C	J/g	°C	°C	°C	J/g
#1	63.9	87.9	-0.5	82.8	0.7	67.4	122.6	n.d.	n.d.
#2	87.1	n.d.	n.d.	n.d.	n.d.	84.6	n.d.	125.9	1.1

n.d.: not detected
Table 1

DSC analysis of the sample #1 revealed a main glass transition (Tg) at 67.4 °C in the 2nd heating scan. A small second Tg was also detected at 122.6 °C in the 2nd heating scan. The main glass transition was detected in the first 1st heating scan followed by an exo- and endothermic event at temperatures above the Tg. These events are attributed to relaxation effects in the sample.

DSC analysis of the sample #2 showed a substantial higher glass transition (Tg) at 84.6 °C in the 2nd heating scan which was also detected in the 1st heating scan. This may point to a lower residual water content of sample #2 compared to sample #1. Since the glass transition was detected close to the final temperature of the 1st heating scan, potential relaxation phenomena could not be detected. In this sample, a second glass transition could not be detected although a small exothermic peak at 125.9 °C was visible in the 2nd heating scan.

2. X-ray powder diffraction

Wide angle X-ray powder diffraction (XRD) was used to study the morphology of the samples #1 and #2. The X-ray diffractometer Empyrean (Panalytical, Almelo, The Netherlands) equipped with a copper anode (45 kV, 40 mA, $K_{\alpha 1}$ emission at a wavelength of 0.154 nm) and a PIXcel3D detector was used. Approximately 100 mg of the spray-dried samples was analyzed in reflection mode in the angular range from 5-45° 2θ, with a step size of 0.04° 2θ and a counting time of 100 seconds per step.

XRD analysis of samples #1 and #2 revealed the fully amorphous state of both samples and showed no characteristic peaks of crystalline matter (see Figure 4 for an overlay of both diffractograms).

3. Laser diffraction

Laser-diffraction measurements were performed using a Partica LA-950 Laser Diffraction Particle Size Distribution Analyzer (Horiba, Kyoto, Japan) equipped with a 605 nm laser diode for detecting particles >500 nm and 405 nm blue light emitting diode (LED) for detecting particles < 500 nm. Isooctane was used as dispersion medium (refractive index of 1.391). Since the refractive

index of the samples was unknown, the refractive index of sugar (disaccharide) particles was used (1.530). The samples were dispersed in isooctane by ultrasonication for up to 5 minutes. Prior to measurement, the system was blanked with isooctane. The dispersion of each sample was measured 3 times and the mean values and the standard deviation are reported.

The mean particle size (weighted average of particle sizes by volume) and the mode particle size (peak of the distribution) were recorded. In addition to the particle distribution by volume (q%), the result were recorded as:

d(v, 10):   particle diameter corresponding to 10% of the cumulative un dersize distribution by volume

d(v, 50):   particle diameter corresponding to 50% of the cumulative un dersize distribution by volume

d(v, 90):   particle diameter corresponding to 90% of the cumulative un dersize distribution by volume

The particle size distribution for sample #1 and #2 is shown in Figure 5. The mode size, which represents the particle size of the highest intensity, is comparable for both samples. Overall, the mean particle size of 67.85 μm (sample #1) and 43.65 μm (sample #2), respectively, is regarded as unusually high for spray-dried particles. The fraction detected at higher particle diameters is probably caused by agglomerated powder particles.

Table 2 summarizes the particles size characteristics of sample #1 and #2.

Size	sample #1	sample #2
Mean [μm]	67.84 ± 38.12	43.65 ± 0.57
Mode [μm]	12.60 ± 0.07	13.92 ± 0.01
D10 [μm]	10.39 ± 0.17	10.65 ± 0.01
D50 [μm]	25.67 ± 4.41	19.68 ± 0.03
D90 [μm]	68.13 ± 26.30	52.37 ± 0.76

Table 2

Example 5: Volume reduction and desalting of 2'-fucosyllactose comprising supernatant using nanofiltration

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For the concentration and desalting of 2'-fucosyllactose containing culture supernatant, an Alfa Laval M-20 membrane filtration module equipped with either a NF99 (Alfa Laval NF99) or NF99HF (Alfa Laval NF99HF) nanofiltration membrane was employed. The used 2'-fucosyllactose containing culture su-
10   pernatant (containing 25 g/l 2'-fucosyllactose) was separated from the fermentation microbial biomass by using cross-flow filtration. The molecular cut-off of the cross-flow filtration membrane was 150 kDa (StrassBurger Filter Micro Cross Module® FS10LFC-FUS1582).

15   The cell free filtrate was then treated with cation ion exchanger (Lewatit S2568 in proton form (Lanxess)) and anion ion exchanger (Lewatit S6368 in carbonate form (Lanxess)) before being subject to nanofiltration. The solution was neutralized after each ion exchanger step using sodium hydroxide solution or hydrochloric acid, respectively. Inlet pressure of the membrane mod-
20   ule was 42 bar and outlet pressure was 40 bar, flow rate of the feed within the membrane module was 8 liter/min. The permeate was removed from the process, whereas the retendate was fed back into reservoir and membrane module. The volume of the reservoir connected to the membrane stack was 6 liters. During the concentration of the 2'-fucosyllatose-comprising solution the
25   reservoir was continuously filled with 2'-fucosyllactose culture supernatant solution until an 8-fold concentrated solution was obtained.

Figure 7 shows the obtained flux (L/m^2/h) plotted against the volume concentration factor (VCF) of the two employed membranes. Having concentrated
30   the 2'-fucosyllactose concentration 8-fold to approximately 200 g/l 2'-fucosyllactose, the concentrated solution was diafiltered for desalting by adding deionized water at the same rate as permeate was obtained from the membrane module. Using the diafiltration step, the conductivity of the 2'-fucosyllactose concentrate could decrease from 25 mS to less than 7 mS using
35   the HF99HF membrane.

By using this nanofiltration approach, the 2'-fucosyllactose solution could be concentrated 8-fold to a 2'-fucosyllactose concentration of $\geq$200 g/l 2'-fucosyllactose under mild conditions (avoiding thermal exposure), with the diafiltration step a large fraction of the salt content could be removed. The 2'-fucosyllactose concentrate was subjected to electrodialysis for further reduction of the salt content.

Example 6: Purification of lacto-*N*-tetraose using two simulated moving bed chromatography steps and an ion exchanger resin in H$^+$ form.

A clear particle free lacto-*N*-tetraose solution (30 g/l) obtained from bacterial fermentation was electrodialysed to a conductivity of 0.5 mS/cm using a PC-Cell electrodialysis apparatus (see above). For SMB chromatography, the lacto-*N*-tetraose solution was concentrated to 50 g/l under vacuum at 40°C. Alternatively, the lacto-*N*-tetraose containing solution can be desalted and concentrated using a nanofiltration membrane (e.g. nanofiltration membrane HF99HF from Alfa Laval).

For SMB chromatography, a closed loop SMB system equipped with 12 columns (Prosep® glas columns with the dimensions: 40 mm x 740 mm (Lartek, Eppelheim, Germany) arranged in 4 zones was employed. Each glass column contained 760 g of Purolite® PCR833H+ strong cation ion exchanger resin. The strong cationic exchanger resin was present in the H$^+$-form.

The system was operated at 25°C with the following parameter settings: flow rate of zone I was set to 30.00 ml/min, flow rate of zone II was set to 19.07 ml/min, the flow rate of zone IV was set to 18.44 ml/min. Feed was kept at 1 ml/min and eluent flow was set to 11.56 ml/min with a switching time of 17.92 min. As eluent water with 10 % (v/v) food grade ethanol was used.

Under these parameters, the lacto-*N*-tetraose and larger neutral oligosaccharides were fractioned into the raffinate. The purity of lacto-N-tetraose was 86.3% instead of 33.4% at the SMB feed (see Fig. 7A for HPLC analysis of the SMB feed and Fig. 7B for HPLC analysis of the SMB raffinate). Contaminants such as lactose, monosaccharides and glycerol were found in the SMB extract fraction (see Fig. 8 for HPLC analysis of the SMB extract fraction). The raffinate

containing the lacto-*N*-tetraose was adjusted to neutral pH using a 0.2 N
NaOH solution.

The pH of the obtained extract containing the lacto-*N*-tetraose was adjusted
to pH 7 by using 0.2 N NaOH. Then, the obtained solution was subjected to
electrodialysis until a conductivity of less than 0.5 mS was again obtained. The
solution was then concentrated under vacuum to approx. 50 g/l lacto-*N*-
tetraose and then sterile-filtered by passing through a 3 kDa cross-flow filter
(Pall Microza ultrafiltration hollow fiber module SEP-2013, Pall Corporation,
Dreieich).

In order to separate away larger oligosaccharide contaminants from the lacto-
N-tetraose, a second SMB chromatography separation was performed. Using
the same SMB system the parameters were changed as follows: flow rate
zone I 30 ml/min, flow rate zone II 19.27 ml/min, flow rate zone IV 17.30
ml/min. Feed was set to 2.08 ml/min and eluent flow 12.70 ml/min. Raffinate
flow was 4.04 ml/min and extract flow was 10.73 ml/min. Switching time of
the SMB separation was set to 10.46 min. As eluent again a water/ethanol
mixture 9:1 (v/v) was employed. The HPLC analysis of the extract of the se-
cond lacto-*N*-tetraose separation by SMB chromatography is shown in Fig. 10.

Under these conditions, the lacto-*N*-tetraose was fractioned into the extract
and 5 to 10% of the total amount of larger neutral oligosaccharides was found
in the raffinate. Using this protocol (amino functionalized silica column after
second SMB step), lacto-*N*-tetraose with a purity of 93.1 % could be obtained.

**Example 7: Purification of lacto-*N*-tetraose using simulated moving bed
chromatography with an ion exchanger resin in Ca^{2+} form**

A clear particle free lacto-*N*-tetraose solution (30 g/l) obtained from bacterial
fermentation was electrodialysed to a conductivity of 0.5 mS/cm using a PC-
Cell electrodialysis apparatus (see above).

For SMB chromatography with calcium as counter-ion, the lacto-*N*-tetraose
solution was concentrated to 50 g/l under vacuum at 40°C. For SMB chroma-
tography a close loop SMB system equipped with 12 columns (Prosep® glas

columns with the dimensions: 40 mm x 740 mm (Lartek, Eppelheim, Germany)) arranged in 4 zones was employed.

Each column contained 760 g of Purolite® PCR833H+ strong cationic ion exchanger resin. The cationic ion exchanger resin was washed with 50 Liter of a 200 mM $CaCl_2$ to exchange the H^+ ions by Ca^{2+} ions. Thus, the strong cationic exchanger resin was present in the Ca^{2+}-form.

The system was operated at 25°C with the following parameter settings: flow rate zone I was set to 30.00 ml/min, flow rate of zone II was set to 20.07 ml/min, the flow rate of zone IV was set to 17.04 ml/min. Feed was kept at 2.5 ml/min and eluent flow was set to 11.56 ml/min with a switching time of 17.92 min. As eluent water with 10 % (v/v) food grade ethanol was used.

Example 8: Obtaining lacto-*N*-tetraose in solid form by spray drying.

Fractions containing lacto-*N*-tetraose were concentrated under vacuum to obtain a lacto-*N*-tetraose concentration of 25 % (w/v). The solutions were then for sterile filtration and endotoxin removal passed through a 3 kDa filter (Pall Microza ultrafiltration hollow fiber module SEP-1013, Pall Corporation, Dreieich, Germany).

The thus-obtained sterile lacto-*N*-tetraose solution was then spray dried using a Mini Spray Dryer B-290 (Büchi Labortechnik GmbH, Essen, Germany). For the spray-drying of the lacto-*N*-tetraose, the solution was passed under pressure through the spray dryer nozzles with an outlet temperature between 120°C and 130°C and flow was adjusted to maintaining an exhaust temperature between 66°C to 67°C. Using these settings, a spray-dried powder with 7-9% moisture and a yield of 72% spray dried lacto-*N*-tetraose could be obtained. The moisture contents were determined by Karl-Fischer titration.

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Claims

5   1. Process for purification of a neutral human milk oligosaccharide in a
        continuous chromatography from a crude solution comprising the
        neutral human milk oligosaccharide and contaminants,

         wherein the crude solution comprising the neutral human milk oligo-
         saccharide and contaminants comprises or consists of a solution which
10   is selected from the group consisting of microbial fermentation ex-
         tract, biocatalysis reaction solution, chemical synthesis solution, and
         combinations thereof, wherein the purity of the neutral human milk
         oligosaccharide in the solution is <80 %,

         characterized in that the crude solution is applied to at least one puri-
15   fication step using simulated moving bed chromatography, wherein a
         purified solution comprising the neutral human milk oligosaccharide
         with a purity of ≥80 % is provided.

    2. Process according to claim 1, characterized in that the purity of neutral
        human milk oligosaccharide in the crude solution is ≤70 %, ≤60 %, ≤50
20   %, ≤40 %, ≤30 %, ≤20 %, ≤10 % or ≤5 % and/or the purified solution
        contains the neutral human milk oligosaccharide with a purity of ≥85
        %, preferably of ≥90 %.

    3. Process according to one of claims 1 or 2, characterized in that the at
        least one simulated moving bed chromatography has

25   i)   at least 4 columns, preferably at least 8 columns, more preferably
             at least 12 columns, wherein at least one column contains a weak
             or strong cation exchange resin, preferably a cation exchange
             resin in the H^+-form or Ca^{2+}-form; and/or

        ii) four zones I, II, III, IV with different flow rates;

30   iii) an eluent comprising or consisting of water, preferably ethanol
             and water, more preferably 5-15 vol.-% ethanol and 85-95 vol.-%
             water, most preferably 9-11 vol.-% ethanol and 89-91 vol.-% wa-

ter, wherein the eluent optionally further contains sulphuric acid, preferably ≤10 mM sulphuric acid or less; and/or

iv) an operating temperature of 15° to 60 °C, preferably 20° to 55 °C, more preferably 25° to 50 °C.

4. Process according to one of claims 1 to 3, characterized in that the purified solution is applied to at least one further purification step using simulated moving bed chromatography, wherein a purified solution comprising the neutral human milk oligosaccharide at a purity of ≥92 % is provided.

5. Process according to claim 4, characterized in that the further simulated moving bed chromatography has

i) at least 4 columns, preferably at least 8 columns, more preferably at least 12 columns, wherein at least one column contains a weak or strong cation exchange resin, preferably a cation exchange resin in the H^+-form or Ca^{2+}-form; and/or

ii) four zones I, II, III and IV with different flow rates;

iii) an eluent comprising or consisting of water, preferably ethanol and water, more preferably 5-15 vol.-% ethanol and 85-95 vol.-% water, most preferably 9-11 vol.-% ethanol and 89-91 vol.-% water, wherein the eluent optionally further contains sulphuric acid, preferably ≤10 mM sulphuric acid or less; and/or

iv) an operating temperature of 15° to 60 °C, preferably 20° to 55 °C, more preferably 25° to 50 °C.

6. Process according to one of claims 1 to 5, characterized in that the crude solution comprising the neutral human milk oligosaccharide and contaminants comprises or consists of a microbial fermentation extract, wherein the microbial fermentation extract is obtained in at least one step of microbial fermentation which is preferably followed by at least one step of

a) filtration of a solution, preferably the crude solution, to separate

soluble material from insoluble material after the microbial fermentation;

b) ion exchange chromatography, preferably cation exchange chromatography, more preferably cation exchange chromatography followed by anion exchange chromatography, of a solution, preferably of a solution obtained in step a);

c) concentration of a solution, preferably a solution obtained in step b), more preferably by evaporation of water and/or by nanofiltration, optionally by concentrating more than once;

d) dialysis of a solution, preferably of a solution obtained in step c), more preferably by electrodialysis and/or diafiltration, most preferably diafiltration with a nanofiltration membrane, optionally by dialysing more than once;

e) chromatography of a solution using simulated moving bed chromatography, preferably a solution obtained in step d);

f) filtration of a solution, preferably a solution obtained in step e), to separate neutral human milk oligosaccharide from coloured contaminants, more preferably by filtration through activated carbon; and

g) spray-drying a purified solution comprising the neutral human milk oligosaccharide, preferably a purified solution obtained in step f);

wherein most preferably, all steps a) to g) are implemented in succession.

7. Process according to one of claims 1 to 6, characterized in that before applying the solution to the at least one simulated moving bed chromatography step, the solution comprising the neutral human milk oligosaccharide is

i) filtered to remove any insoluble material, preferably filtered with activated carbon, charcoal and/or celite to remove any insoluble material and organic contaminants, more preferably filtered by cross-flow filtration, most preferably filtered by cross-flow filtration using a microfiltration membrane; and/or

ii)   applied to at least one purification step using cation and/or anion-exchange chromatography, preferably first at least one cation exchange chromatography step and then at least one anion exchange chromatography step.

8.   Process according to one of claims 1 to 7, characterized in that before applying the solution to at least one purification step using simulated moving bed chromatography or after a purification step using simulated moving bed chromatography, the solution comprising the neutral human milk oligosaccharide is electrodialysed and/or diafiltered, preferably diafiltered with a nanofiltration membrane, more preferably diafiltered with a nanofiltration membrane having a size exclusion limit of ≤20 Å, wherein most preferably the solution is dialysed to a conductivity of ≤15 mS/cm, preferably ≤10 mS/cm, more preferably ≤5 mS/cm.

9.   Process according to claim 8, characterized in that after dialysis, the solution comprising the neutral human milk oligosaccharide is concentrated

i)   to a concentration of ≥ 100 g/L, preferably ≥ 200 g/L, more preferably ≥ 300 g/L; and/or

ii)   employing a vacuum concentrator; and/or

iii)   by nanofiltration; and/or

iv)   at temperature of 4° to 50 °C, preferably 10° to 45 °C, optionally 20° to 40 °C or 30° to 35 °C.

10.   Process according to one of claims 1 to 9, characterized in that the purified solution is sterile filtered and/or subjected to endotoxin removal, preferably by filtration of the purified solution through a 3 kDa filter.

11.   Process according to one of claims 1 to 10, characterized in that the purified solution is spray-dried, particularly spray-dried at a the neutral human milk oligosaccharide concentration of 20-60 % (w/v), preferably 30-50 % (w/v), more preferably 35-45 % (w/v), a nozzle temperature of

110-150 °C, preferably 120-140 °C, more preferably 125-135 °C and/or an outlet temperature of 60-80 °C, preferably 65-70 °C.

12. Process according to one of claims 1 to 11, characterized in that the neutral human milk oligosaccharide is a neutral human milk oligosaccharide having more than 3 monosaccharide units, preferably a neutral human milk trisaccharide, tetrasaccharide, pentasaccharide or hexasaccharide, more preferably the neutral HMO is selected from the group consisting of 2'-fucosyllactose, 3-fucosyllactose, 2',3-difucosyllactose, lacto-*N*-triose II, lacto-*N*-tetraose, lacto-*N*-*neo*tetraose, lacto-*N*-fucopentaose I, lacto-*N*-*neo*fucopentaose I, lacto-*N*-fucopentaose II, lacto-*N*-fucopentaose III, lacto-*N*-fucopentaose V, lacto-*N*-*neo*fucopentaose V, lacto-*N*-difucohexaose I, lacto-*N*-difucohexaose II, 6'-galactosyllactose, 3'-galactosyllactose, lacto-*N*-hexaose, lacto-*N*-*neo*hexaose, *para*-lacto-*N*-hexaose, *para*-lacto-*N*-*neo*hexaose and difucosyl-lacto-*N*-*neo*hexaose, most preferably, the neutral HMO is 2'-fucosyllactose or lacto-*N*-tetraose.

13. Process according to one of claims 1 to 12, characterized in that the process for purification of a neutral human milk oligosaccharide in a continuous chromatography is a process for purification of a neutral human milk oligosaccharide in a continuous manner, wherein the neutral human milk oligosaccharide is preferably 2'-fucosyllactose or lacto-*N*-tetraose.

14. Neutral human milk oligosaccharide producible with the process according to one of claims 1 to 13, wherein the neutral human milk oligosaccharide is preferably spray-dried.

15. Neutral human milk oligosaccharide according to claim 14, wherein the neutral human milk oligosaccharide has

i) a solid granule form; and/or

ii) a glass transition temperature of 60 to 90 °C, preferably 62 to 88 °C, more preferably 64 to 86 °C, determined by differential scanning calorimetry; and/or

iii) a particle size of 5 to 500 μm, preferably 10 to 300 μm, determined by laser diffraction; and/or

iv) a mean particle size of 10 to 100 μm, preferably 20 to 90 μm, more preferably 30 to 80 μm, most preferably 40 to 70 μm, determined by laser diffraction; and/or

v) an amorphous state, preferably an amorphous state with no characteristic peaks of crystalline matter in X-ray powder diffraction; and/or

vi) a moisture content of ≤10%, preferably ≤ 8%, more preferably < 5%.

16. Neutral human milk oligosaccharide according to one of claims 14 or 15 for use in medicine, preferably for use in prophylaxis or therapy of a gastrointestinal disorder.

17. Neutral human milk oligosaccharide according to one of claims 14 to 16, characterized in that the neutral human milk oligosaccharide is a neutral human milk oligosaccharide having more than 3 monosaccharide units units, preferably a neutral human milk trisaccharide, tetrasaccharide, pentasaccharide or hexasaccharide, more preferably the neutral HMO is selected from the group consisting of 2'-fucosyllactose, 3-fucosyllactose, 2',3-difucosyllactose, lacto-*N*-triose II, lacto-*N*-tetraose, lacto-*N-neo*tetraose, lacto-*N*-fucopentaose I, lacto-*N-neo*fucopentaose I, lacto-*N*-fucopentaose II, lacto-*N*-fucopentaose III, lacto-*N*-fucopentaose V, lacto-*N-neo*fucopentaose V, lacto-*N*-difucohexaose I, lacto-*N*-difucohexaose II, 6'-galactosyllactose, 3'-galactosyllactose, lacto-*N*-hexaose, lacto-*N-neo*hexaose, *para*-lacto-*N*-hexaose, *para*-lacto-*N-neo*hexaose and difucosyl-lacto-*N-neo*hexaose, most preferably, the neutral HMO is 2´-fucosyllactose or lacto-*N*-tetraose.

18. Use of a neutral human milk oligosaccharide according to one of claims 14 to 17 as additive in food, preferably as additive in human food and/or pet food, more preferably as additive in human baby food.

Figure 1
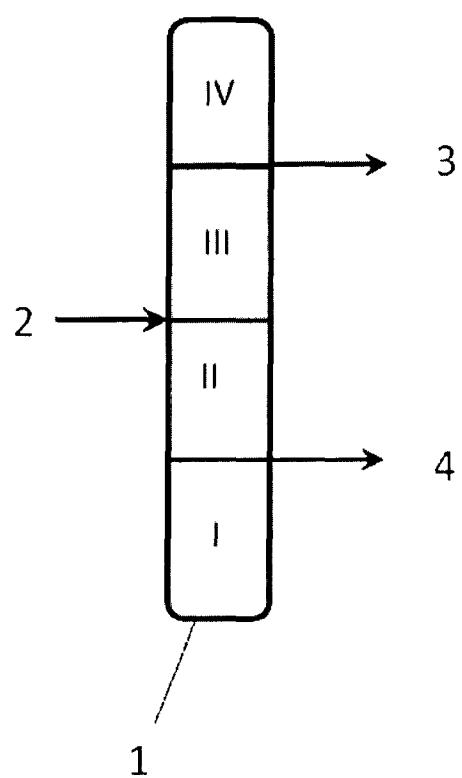
Figure 2
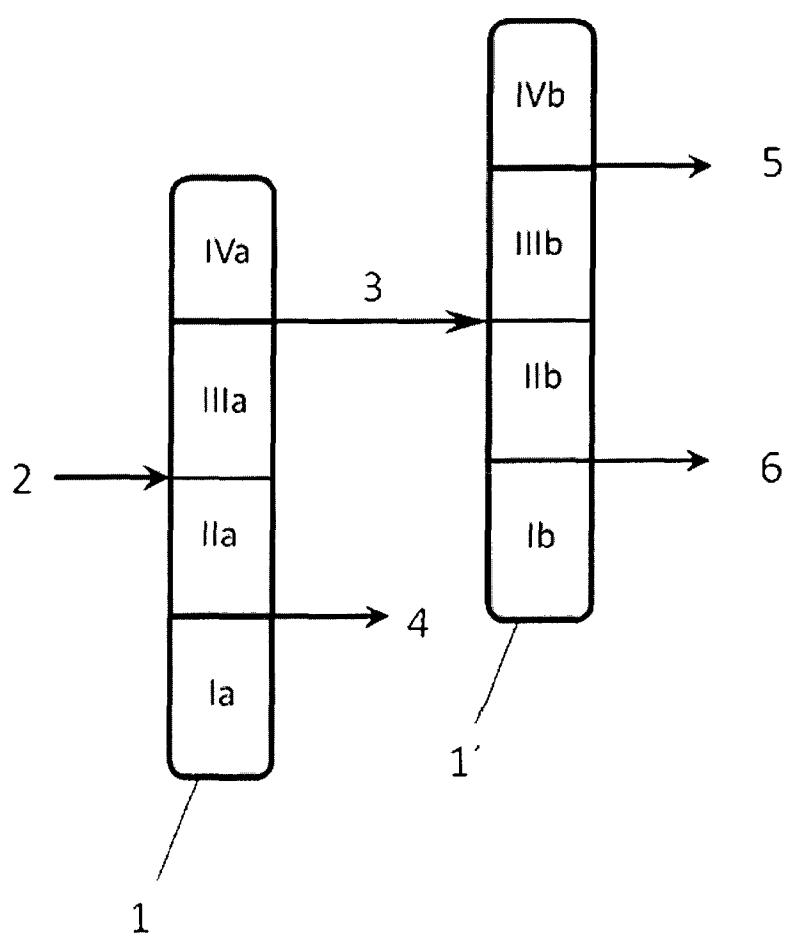
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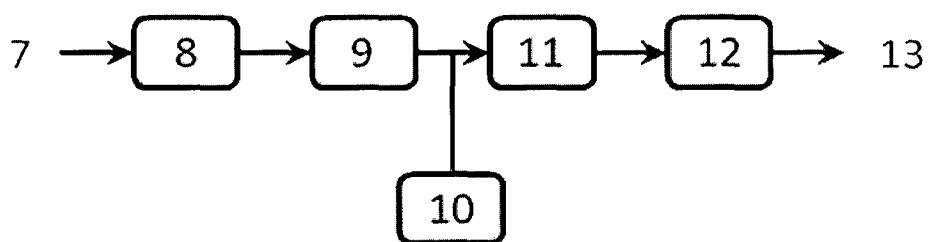
Figure 4
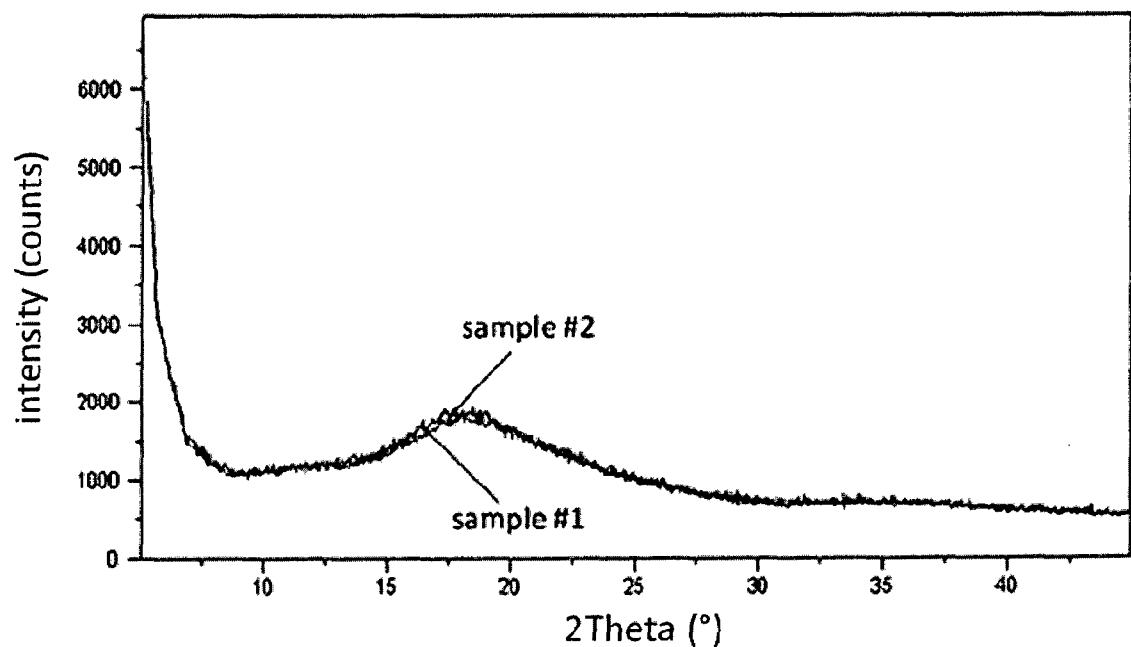
Figure 5

Figure 6

Figure 7

Flux versus VCF
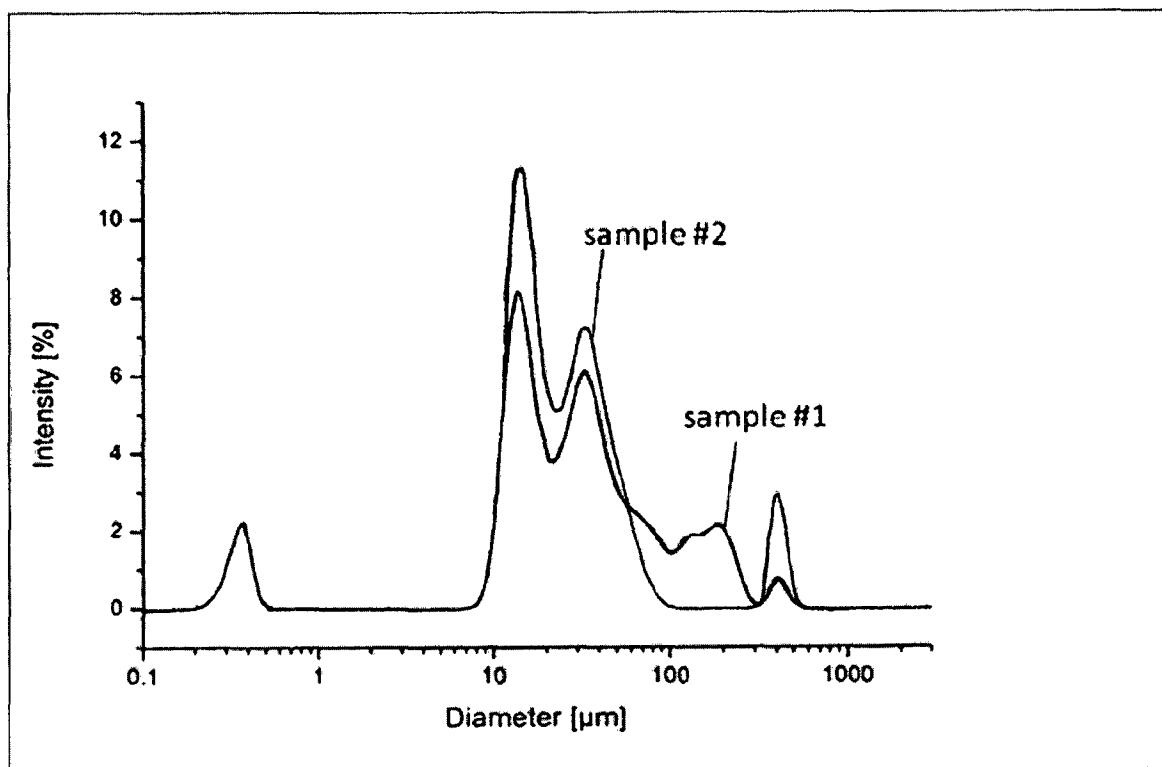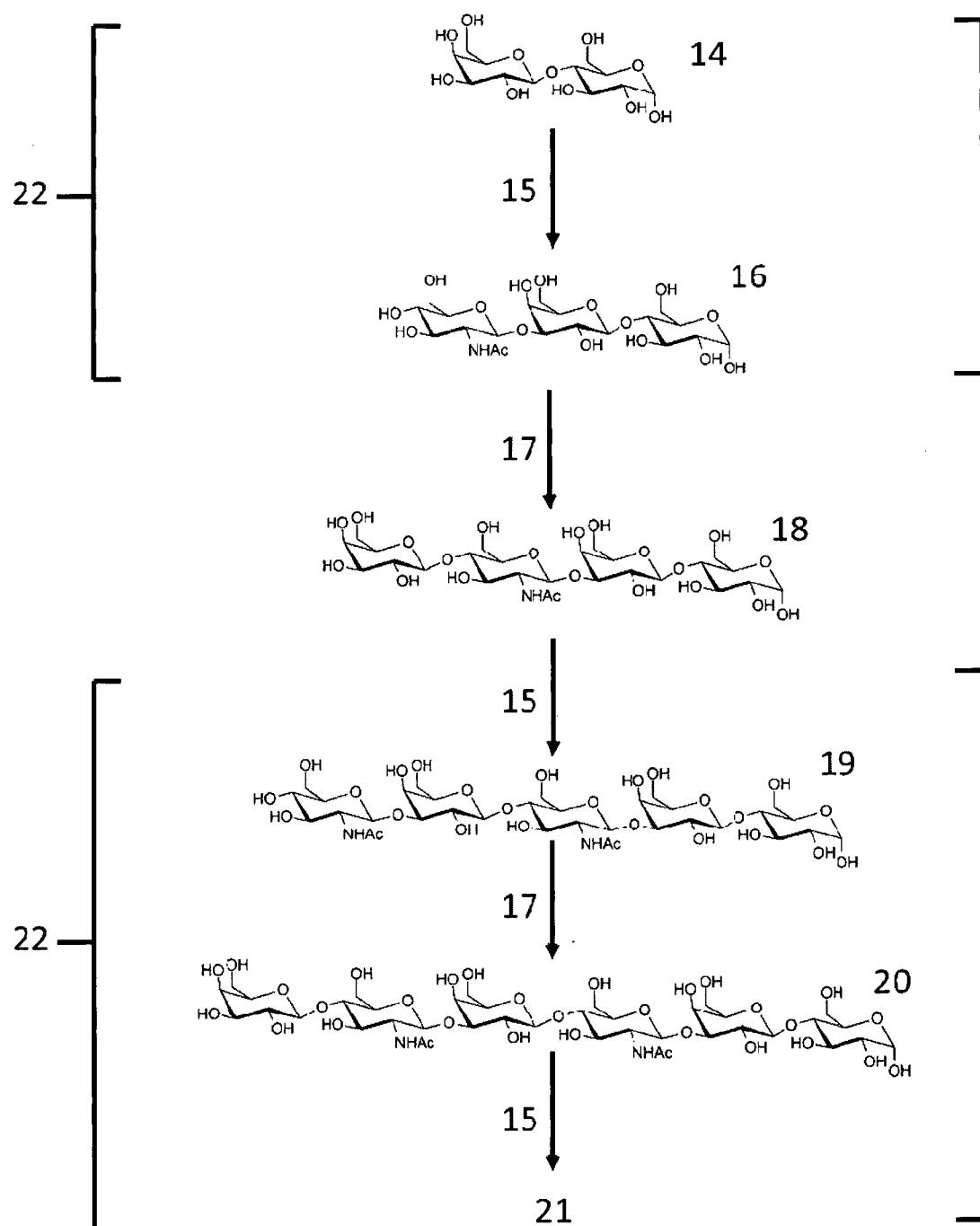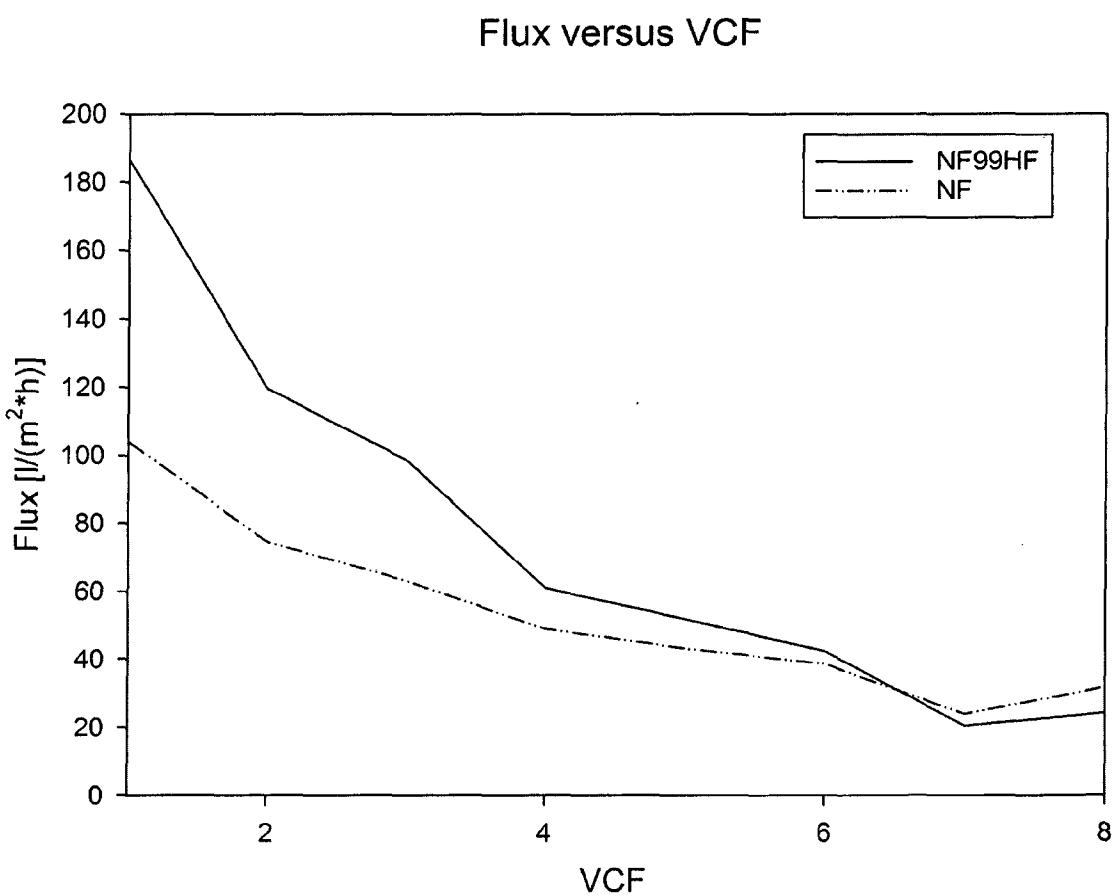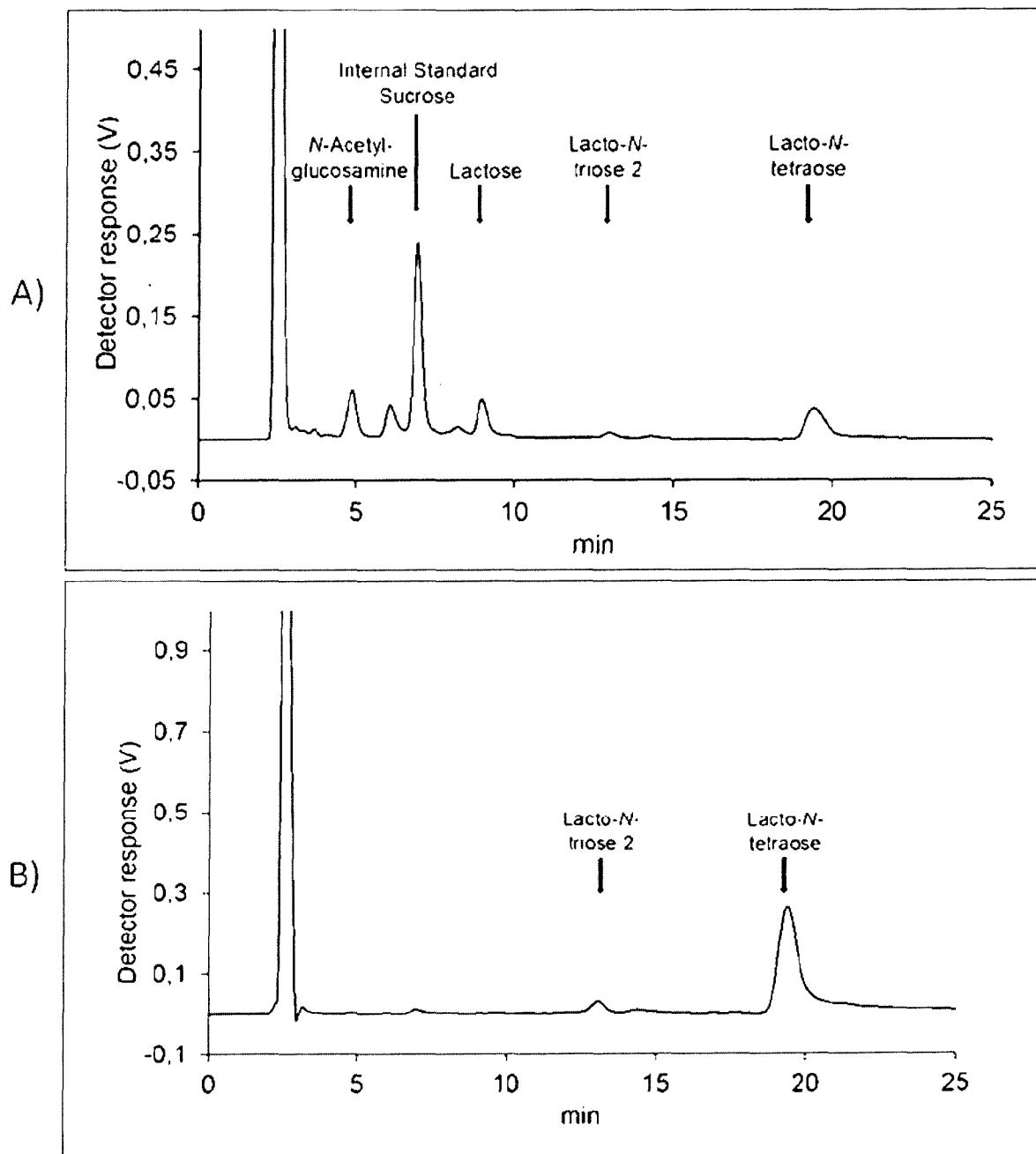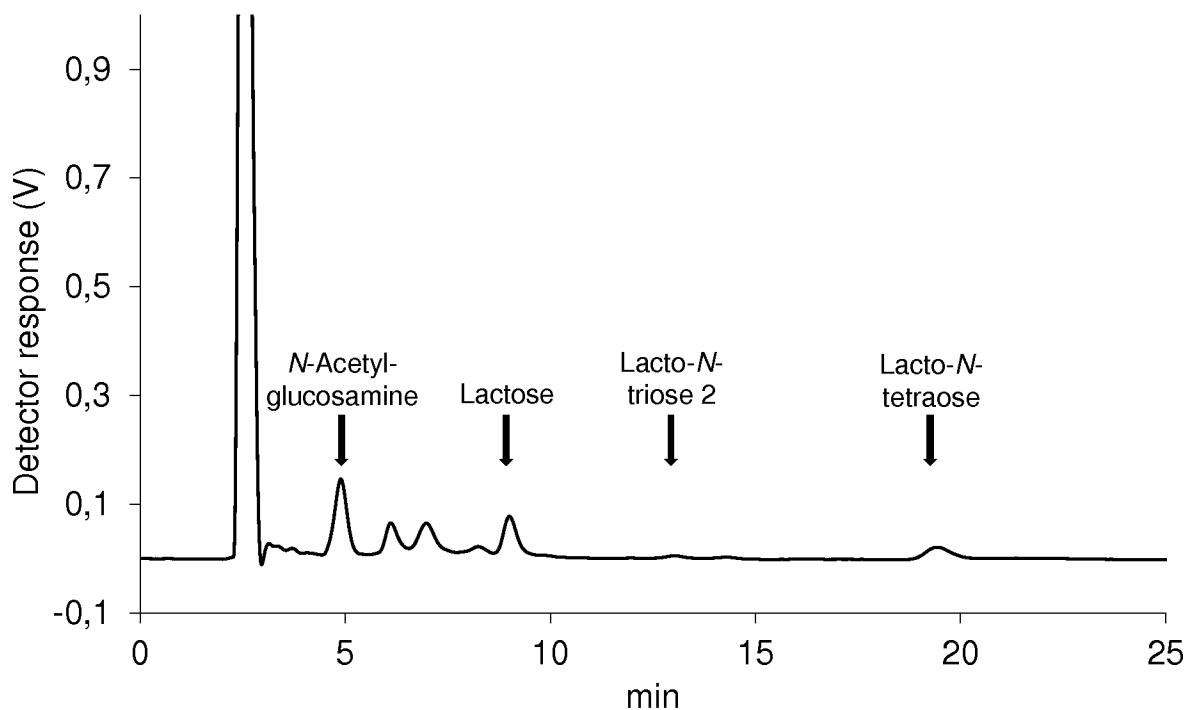
Figure 8

Figure 9

Figure 10

Figure 11

A)

B)
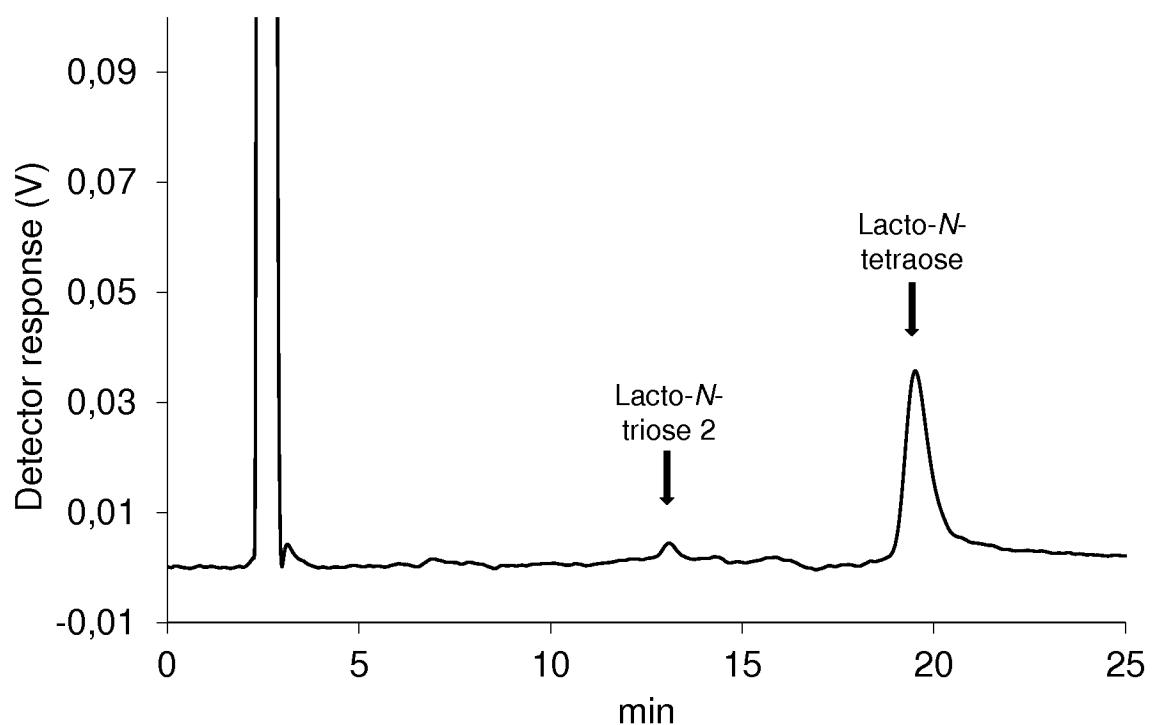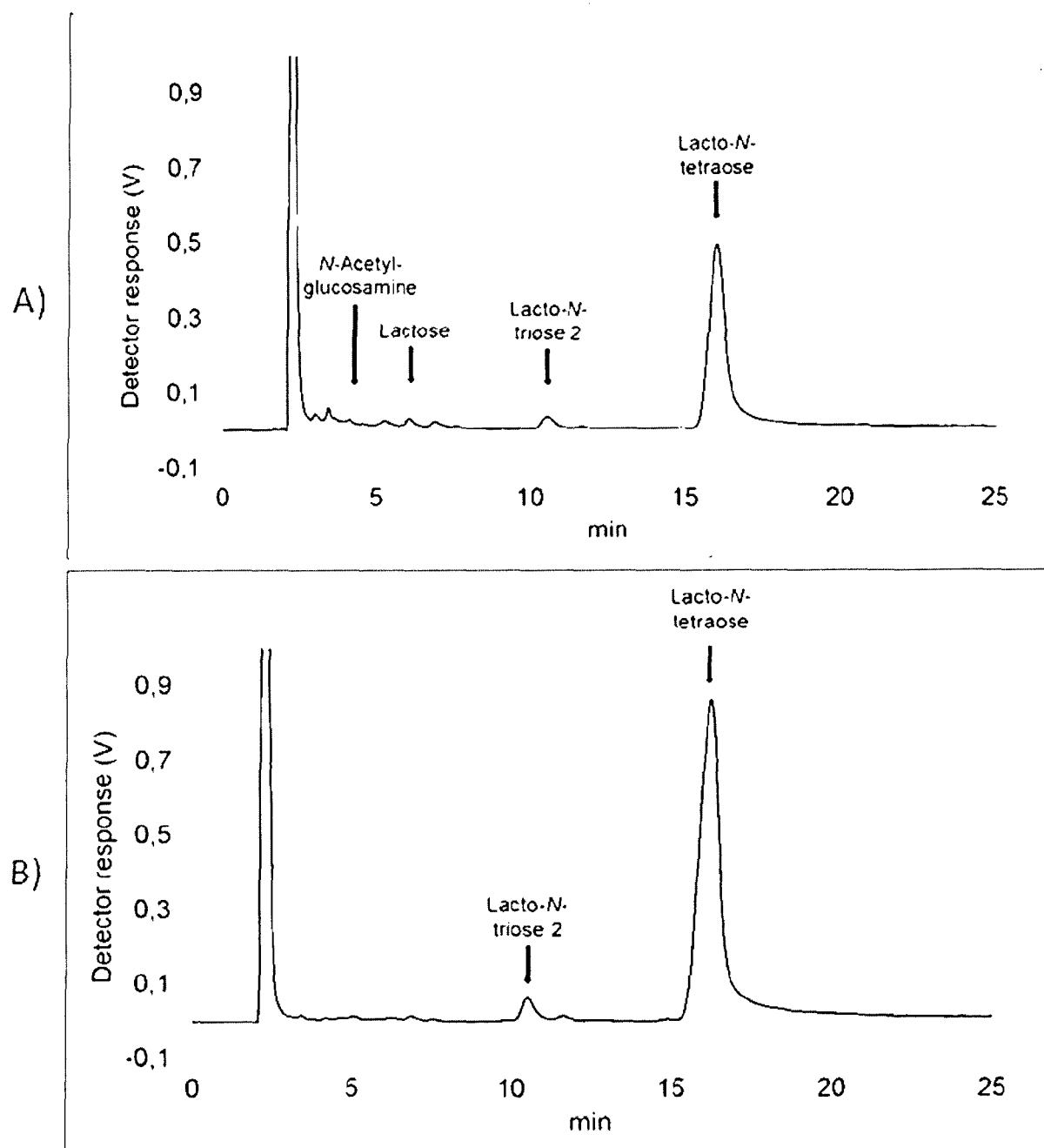
Figure 12

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07H3/06   A23L1/30   A61K31/702   A61P1/14
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07H A23L A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, CHEM ABS Data, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GEISSER A ET AL: "Separation of lactose from human milk oligosaccharides with simulated moving bed chromatography", JOURNAL OF CHROMATOGRAPHY, ELSEVIER SCIENCE PUBLISHERS B.V, NL, vol. 1092, no. 1, 21 October 2005 (2005-10-21), pages 17-23, XP027723567, ISSN: 0021-9673 [retrieved on 2005-10-21] the whole document ----- -/--	1-18

[X] Further documents are listed in the continuation of Box C.   [X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 December 2014	15/12/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Klein, Didier

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RICHARD KUHN ET AL: "KRISTALLISIERTE FUCOSIDO-LACTOSE", CHEMISCHE BERICHTE, VCH, vol. 89, no. 11, 1 November 1956 (1956-11-01), page 2513, XP009074773, ISSN: 0009-2940 the whole document -----	14-18
X	WO 2010/115935 A1 (GLYCOM AS [DK]; DEKANY GUYLA [AU]; BAJZA ISTVAN [HU]; BOUTET JULIEN [F) 14 October 2010 (2010-10-14) cited in the application the whole document -----	14-18
X	WO 2010/115934 A1 (GLYCOM AS [DK]; DEKANY GYULA [AU]; AGOSTON KAROLY [HU]; BAJZA ISTVAN []) 14 October 2010 (2010-10-14) the whole document -----	14-18
X	WO 2012/158517 A1 (GLYCOSYN LLC [US]; TRUSTEES BOSTON COLLEGE [US]; NEWBURG DAVID S [US];) 22 November 2012 (2012-11-22) the whole document -----	14-18

1

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Information on patent family members

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			CN	102459294	A	16-05-2012
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			EP	2417143	A1	15-02-2012
			EP	2417144	A1	15-02-2012
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