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(51) Int.Cl. 6 B01D 15/08, G01N 30/48, B01J 20/22
(30) 1998/01/29 (198 03 415.6) DE
(54) PROCEDE DE SEPARATION DE MELANGES DE SUBSTANCES AU MOYEN DE POLYSACCHARIDES
(54) SEPARATION OF SUBSTANCE MIXTURES USING POLYSACCHARIDES

(57) L’invention concerne un procédé de séparation, par chromatographie, de mélanges de substances au moyen de microparticules sphériques constituées de polysaccharides linéaires, insolubles dans l’eau, non modifiés chimiquement et physiquement.

(57) The invention relates to a method for separating substance mixtures by chromatography using spherical microparticles consisting of chemically and physically unmodified, water-insoluble, linear polysaccharides.
Abstract

Separation of substance mixtures using polysaccharides

The invention relates to a process for the chromatographic separation of substance mixtures using spherical microparticles of chemically and physically unmodified, water-insoluble, linear polysaccharides.
Description

Separation of substance mixtures using polysaccharides

The invention relates to a process for the separation of substance mixtures, in particular enantiomers, using spherical microparticles of chemically and physically unmodified, water-insoluble, linear polysaccharides as separation material.

Chromatography is an effective method for complete separation of components of a mixture, in particular for the separation of mixtures of similar compounds, for example of stereoisomers. Owing to the increasing interest in pure enantiomers for pharmaceutical and biochemical active substances and crop protection agents, chromatographic separation of enantiomeric compounds is of particular interest. For the purification of these compounds, improved processes are constantly being developed and better separation materials sought, in particular on the basis of the polysaccharides cellulose and starch, two readily accessible and inexpensive polymers containing chiral atoms.

Thus, the article Chromatographie 65 [Chromatography 65], LaborPraxis, 730-738 (1990), outlines the use of microcrystalline tribenzoylcellulose having particle sizes of from 10 to 20 µm compared with triacetylcellulose as sorbent for enantiomer separation in chromatography. Both substances can be used for analytical and preparative separations. They are accessible by derivatization of cellulose. Unmodified polymers are not used. The introduction of relatively small particle sizes of up to 5 µm in order to avoid dead volumes and compaction of the column, which occurs during operation, is described as desirable.
WO 95/05879 likewise deals with the separation of enantiomers by liquid chromatography. Particular attention is paid here to the mobile phase for improving the separation performance. As the stationary phase, use is made of carbamate derivatives of cellulose and amylose and ester derivatives of cellulose.

DE-A-43 17 139 describes a special process for the enantiomer separation of inhalation anesthetics by means of preparative gas chromatography. As the stationary phase, use is made of cyclodextrins derivatized by ester groups in polysiloxane solution on porous support material (Chromosorb). In order to obtain a suitable separation material, the cyclodextrins must be chemically modified, dissolved in polysiloxane and immobilized on a suitable support material.

US 5,403,898 employs polymeric siloxanes containing cyclodextrin derivatives as chiral stationary phase in analytical and preparative gas chromatography (GC), chromatography (LC). These peralkylcyclodextrins are chemically bonded to the polysiloxanes. The material used is thus obtained by chemical modification of cyclodextrins and subsequent chemical immobilization on polysiloxanes.

In US 5,302,633, in order to achieve insolubility of the chiral stationary phase, a vinyl derivative of a polysaccharide (for example cellulose) is first adsorbed on the surface of a porous support (for example silica gel), then polymerized. A second variant is the copolymerization of a porous support (for example modified silica gel) containing vinyl groups with a vinyl derivative of a polysaccharide. Here too, chemical modifications are necessary to obtain suitable separation material.
or chemical immobilization on the porous support. A chemical modification is thus necessary to obtain suitable separation material.

In DE-C 26 55 292 and DE-C 25 55 361, porous gels made from dextran derivatives are employed as separation media in electrophoretic separation materials. In order to achieve insolubility, dextran derivatives containing vinyl groups have to be crosslinked by free-radical polymerization.

Numerous polysaccharides are thus already known which are employed in chromatographic separation processes. However, they always have to be chemically and/or physically modified in order to achieve good separation performance, particle size distribution, solvent stability and solvent resistance. This is always associated with an increase in costs.

The term chemical and/or physical modifications is taken to mean, in particular, derivatization by the introduction of specific groups, covalent immobilization on a support material and subsequent chemical and/or physical crosslinking.

The object of the invention is therefore to provide a chromatography process by avoiding chemical and physical modification of the separation material, and the separation material as such.

This object is achieved by a process for the chromatographic separation of substance mixtures using spherical microparticles made from chemically and physically
unmodified, water-insoluble, linear polysaccharides (referred to below as separation material according to the invention) as separation material.

The term spherical microparticles is taken to mean microparticles having an approximately spherical shape. When describing a sphere by means of axes of equal length which are directed into space and start from a common origin and which
cal microparticles made from chemically and physically unmodified, water-insoluble, linear polysaccharides (referred to below as separation material according to the invention) as separation material.

The term spherical microparticles is taken to mean microparticles having an approximately spherical shape. When describing a sphere by means of axes of equal length which are directed into space and start from a common origin and which define the radius of the sphere in all spatial directions, a deviation of the axis lengths of from 1 to 40% is possible for the spherical microparticles. Spherical microparticles having deviations of up to 25%, particularly preferably up to 15%, are preferably obtained. The surface of the spherical microparticles can be compared macroscopically with that of a raspberry, where the depth of the "indents" or "recesses" is a maximum of 20% of the mean diameter of the spherical microparticles. Figures 1 to 4 show scanning electron microscope (SEM) micrographs (Camscan S-4) of the spherical microparticles used.

The avoidance of chemical and physical modification of the linear polysaccharides additionally allows high-cost process steps to be avoided and thus the economic efficiency of the chromatography process to be increased. A further advantage is the very good reproducibility of the chromatography process.

The term physical modifications is not taken to mean the conventional work-up steps, such as stirring, centrifugation, slurrying, etc.

Linear polysaccharides in accordance with the present invention can be polyglucans or other linear polysaccharides, such as pullulans, pectins, mannans or polyfructans. However, particular preference is given to poly(1,4-α-D-glucan).
The separation material according to the invention can be employed for the chromatographic separation of substance mixtures, particularly preferably stereoisomers, very particularly preferably enantiomers. Of particular interest is therefore the use of the separation material according to the invention in preparative and analytical chromatography, such as gas chromatography, preparative and analytical thin-layer chromatography and in particular high performance liquid chromatography (HPLC). Also of interest is use of the separation material according to the invention in gel permeation chromatography (GPC) for the separation of polymers of different molecular weights. An application which likewise falls within the scope of the invention is the removal of substances from solutions, emulsions or suspensions by specific or non-specific interactions with low-molecular-weight or polymeric compounds. However, ionic compounds, in particular metal cations, are also of interest for the application. Special effects can be achieved, in particular, in the removal of monosaccharides, disaccharides and oligosaccharides owing to the structural similarity to polysaccharides.

Also for reasons of biocompatibility of the separation material according to the invention, the invention likewise relates to the treatment of water, in particular waste water, the analysis of biological material, in particular blood and serum, or, for example, the separation or removal of genetic material (nucleic acids) or other biogenic compounds (for example oligonucleotides, peptides or proteins).

For the purposes of the invention, the term chromatography is taken to mean physical separation processes in which the substance separation takes place by partitioning and/or adsorption between a stationary phase and a mobile phase. For the purposes of this invention, a separation material according to the
as the stationary phase and combined with a liquid and/or gaseous mobile phase. The following are therefore regarded as particular embodiments: HPLC, GPC, GC, TLC and further or possible specifications of chromatographic processes or chromatography-like processes. In principle, any chromatographic process is accessible to the invention.

HPLC stands for high performance or high pressure liquid chromatography. In HPLC, very fine material (3-10 μm) is used, since the separation performance of a column increases with decreasing particle size of the stationary phase. The fine-particle nature of the separation materials requires high pressures (up to 400 bar).

In gel permeation chromatography (GPC), also known as gel filtration chromatography, which can also be operated as HPLC, the stationary phase consists of beads with a heteroporous swollen network whose pore size distribution varies over a number of orders of magnitude, so that fractionation takes place by molecular size, which allows rapid determination of the molecular size distribution of polymers.

Gas chromatography (GC) serves for the separation of substance mixtures which are in gas form or can be evaporated without decomposition, with a gaseous mobile phase. Gas-chromatographic analysis begins with application of a gas, a volatile liquid or a volatile solid to a thermostatted separating column. With the aid of the carrier gas (He, N₂ or H₂), the substances are transported through the column, where the chromatographic separation takes place.

Thin-layer chromatography (TLC) is a chromatographic method with a multi-step partitioning process, with the stationary phase (the separation material is referred
layer on a suitable support, for example glass, polyester or aluminum. The separation takes place on this layer by elution with an eluent (mobile phase).

The invention therefore relates to the separation material according to the invention as such containing spherical microparticles of chemically and physically unmodified, water-insoluble, linear polysaccharides and optionally further auxiliaries, additives and support materials.

The invention furthermore relates to the use of the separation material according to the invention and optionally further auxiliaries and additives for the removal or retention of substances in the sense of a filter medium or adsorbent (cf. Example 12).

Linear polysaccharides are polysaccharides which are built up from monosaccharides, disaccharides or other monomeric units in such a way that the monosaccharides, disaccharides or other monomeric units are always linked to one another in the same manner. Each basic unit defined in this way has precisely two linkages, in each case one to another monomer. The two basic units forming the beginning and end of the polysaccharide are excluded from this. These basic units have only one linkage to a further monomer. In the case of three linkages (covalent bonds), the term branch is used. Branches only occur to a minor extent, or not at all, and consequently they are not accessible to conventional analytical methods in the case of very small branching proportions.

Examples of preferred water-insoluble linear polysaccharides are linear poly-D-glucans, where the type of linkage is unimportant so long as linearity in the sense of the invention is present. Examples are poly-(1,4-alpha-D-glucan) and poly(1,3-beta-D-glucan), where
If the basic unit has three or more linkages, the term branching is used. The so-called degree of branching is given by the number of hydroxyl groups per 100 basic units which do not participate in the construction of the linear polymer backbone and which form the branches.

In accordance with the invention, the linear water-insoluble polysaccharides have a degree of branching of less than 8%, i.e. they have fewer than 8 branches per 100 basic units. The degree of branching is preferably less than 4% and in particular at most 1.5%.

If the water-insoluble linear polysaccharide is a polyglucan, for example poly(1,4-alpha-D-glucan), the degree of branching in the 6-position is less than 4%, preferably a maximum of 2% and in particular a maximum of 0.5%; and the degree of branching in the other positions which are not involved in the linear linkage, for example the 2- or 3-position in the case of the preferred poly(1,4-alpha-D-glucan) is preferably in each case a maximum of 2% and in particular a maximum of 1%.

Particular preference is given to polysaccharides, in particular poly-alpha-D-glucans, which have no branches or whose degree of branching is so small that it cannot be determined by conventional methods.

In accordance with the invention, the prefixes "alpha", "beta" or "D" relate exclusively to the linkages which form the polymer backbone and not to the branches.

The term nature-identical polysaccharides is taken to mean polymers which either are not naturally occurring in this form or only occur in a mixture with other compounds, which may also be other polymers. The
possible by processes which fall under the term biotechnological and genetic engineering processes, which is to be defined in its broadest sense. This is taken to mean firstly biotechnological processes or processes as defined below, but also those which give corresponding compounds through the use and modification of, for example, bacteria, fungi or algae. On the other hand, the term also covers, for example, polysaccharides obtained by applying biotechnological or genetic engineering processes to higher plants so that separation from the plants can take place. These plants include, in particular, potato, corn, cereals, cassava, rice and peas. However, other plants which produce polysaccharides are also included in the aspect according to the invention in this category.

For the purposes of the invention, preference is given to linear, water-insoluble polysaccharides which are prepared in a biotechnological, in particular a bio-catalytic/biotransformational or fermentative process. The term biotechnological processes covers all catalytic (= biotransformational processes, including processes which can only be carried out with enzymes or in combination with organisms which form intracellular or extracellular proteins which take on this task) or fermentative processes which can be carried out with naturally occurring or recombinant organisms.

For the purposes of this invention, the term linear polysaccharides prepared by biocatalysis (also biotransformation) means that the linear polysaccharide is prepared by catalytic reaction of monomeric basic units, such as oligomeric saccharides, for example monosaccharides and/or disaccharides, using a so-called biocatalyst, usually an enzyme, under suitable conditions for use for the reaction.

In the terminology of the invention, linear poly-
saccharides which can be obtained by fermentative processes using naturally occurring organisms, such as, for example, fungi, algae or bacteria, or using non-naturally occurring organisms, but natural organisms such as, for example, fungi, algae or bacteria, which have been modified with the aid of genetic engineering methods of general definition.

In addition, in order to achieve the effects described in the present invention, linear polysaccharides can also be obtained by treating non-linear polysaccharides containing branches with one or more enzymes in such a way that the branches are cleaved off from the polysaccharide backbone, giving linear polysaccharides after their removal. These enzymes are, for example, amylases, isoamylases, pullulanases or gluconohydrolases.

For the purposes of the present invention, the term "water-insoluble polysaccharides" is taken to mean compounds which, in accordance with the definition in the German Pharmacopoeia (DAB = Deutsches Arzneimittelbuch, Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart, Govi-Verlag, Frankfurt, Edition, 1987) corresponding to classes 4 to 7, come under the categories "sparingly soluble", "low solubility", "very low solubility" or "virtually insoluble" compounds.

In the case of the polysaccharides used in accordance with the invention, this means that at least 98% of the amount employed, in particular at least 99.5%, is insoluble in water under standard conditions (T = 25°C ± 20%, p = 101325 pascals ± 20%) (corresponding to classes 4 or 5).

For the present invention, preference is given to sparingly soluble to virtually insoluble compounds, in particular very low solubility to virtually insoluble
"Very low solubility" in accordance with class 6 can be illustrated by the following experimental description: one gram of the polyglucan/saccharide to be investigated is heated to 130°C under a pressure of 1 bar in 1 l of deionized water. The resultant solution only remains stable briefly for a few minutes. On cooling under standard conditions, the substance re-precipitates. After cooling to room temperature and separation by means of centrifugation, at least 66% of the amount employed can be recovered, taking into account experimental losses.

Production of uniform spherical microparticles

The poly(1,4-α-D-glucan) preferably employed can be prepared in various ways. A very advantageous method is described in WO 95/31553. The disclosure content of the specification is expressly incorporated herein by way of reference. In this method, poly(1,4-α-D-glucan) is prepared by means of a biocatalytic (biotransformational) process with the aid of amylase. Poly(1,4-α-D-glucan) can also be prepared by a biocatalytic process using polysaccharide synthases, starch synthases, glycol transferases, 1,4-α-D-glucan transfers, glycogen synthases or phosphorylases.

The patent application DE-A-197 37 481 describes the preparation of spherical microparticles of linear water-insoluble polysaccharides and their molecular weights and diameters. The above-mentioned application is incorporated herein by way of reference.

The spherical microparticles are produced by dissolving the water-insoluble, linear polysaccharide, in particular poly(1,4-α-D-glucan), in a solvent, particular preferably in DMSO, introducing the solution into a precipitation medium, preferably water, cooling the
and separating off the microparticles formed. Concomitant use of suitable additives allows modification of the properties of the particles, such as size, surface structure, porosity, etc., and of the way in which the process is carried out. Examples of suitable additives are surface-active substances, such as sodium dodecylsulfate or N-methylglucanoamide, and sugars, for example fructose, sucrose and glucose.

Properties of the spherical microparticles

The molecular weights $M_w$ of the linear polysaccharides used in accordance with the invention can vary in a broad range from $10^3$ g/mol to $10^7$ g/mol. For the preferred linear polysaccharide poly$(1,4$-$\alpha$-$D$-glucan), preference is given to molecular weights $M_w$ in the range from $10^4$ g/mol to $10^5$ g/mol, in particular from $2 \times 10^4$ g/mol to $5 \times 10^4$ g/mol. The molecular weight $M_w$ describes the weight average molecular weight and is determined by gel permeation chromatography compared with a calibration with a pullulan standard.

The particles can have mean diameters (number average) of from 1 nm to 100 μm, preferably from 100 nm to 10 μm, particularly preferably from 1 μm to 5 μm. The diameter distribution is distinguished by high uniformity.

The poly$(1,4$-$\alpha$-$D$-glucan) microparticles are distinguished by a specific surface area of from 1 m$^2$/g to 100 m$^2$/g, particularly preferably from 3 m$^2$/g to 10 m$^2$/g.

Experiments on the stability of the microparticles in various solvents show that a wide range of solvents is suitable for use in the chromatography process according to the invention. The solvents used, in the function of an eluent, are, for example, n-hexane,
acetone, acetonitrile, methanol, ethanol, n-heptane and water.

Chromatography methods

In the chromatography method, microparticles according to the invention made from linear polysaccharides, particularly preferably poly(1,4-α-D-glucan), are suspended, in particular, in a solvent, for example n-heptane, stirred up by means of an Ultraturrax and degassed in an ultrasound bath. The suspension is introduced into a column and packed uniformly and densely by repeated tapping of the column, repeated shaking in combination with brief treatment in an ultrasound bath, to give a packing with a low proportion of dead volumes. The separations are carried out in a state-of-the-art chromatography unit for HPLC. The sample concentration is from 2.0 to 0.025 mg/l of solvent, particularly preferably from 0.25 to 0.05 mg/l of solvent. The elution rate is from 2.0 to 0.25 ml/min, preferably from 1.0 to 0.4 ml/min, particularly preferably 0.5 ml/min. These figures can be varied depending on the solvent used. The pressures used are highly dependent on the polarity of the solvent. They are generally in the range from 30 bar to 200 bar. The following pressures, for example, were measured: from 138 to 159 bar for acetonitrile, about 100 bar for ethyl acetate, about 62 bar for t-butyl methyl ether and from 54 to 70 bar for n-heptane. An internal standard, for example 1,3,5-tri-tert-butylbenzene, can be used.

Description of the figures:
Fig. 1: Scanning electron photomicrograph (see Example 4) of the spherical microparticles used in a magnification of 5000x.
Fig. 2: Scanning electron photomicrograph (see Example 4) of the spherical microparticles used in a
Fig. 3: Scanning electron photomicrograph (see Example 4) of the spherical microparticles used in a magnification of 15,000×.

Fig. 4: Scanning electron photomicrograph (see Example 4) of the spherical microparticles used in a magnification of 20,000×.

Fig. 5: Agarose gel (detection with ethidium bromide 0.5 pg/ml at 256 nm) as described in Example 12, note caption.

Fig. 6: Agarose gel (detection with ethidium bromide 0.5 pg/ml at 256 nm) as described in Example 12, note caption.

The following examples explain the invention in greater detail without restricting the invention to these examples.
Examples:

Example 1

In-vitro preparation of a linear water-insoluble polysaccharide in a biocatalytic process using the example of poly(1,4-α-D-glucan) with amylolucrase

10 l of a 20% strength sucrose solution are introduced into a sterilized (steam sterilization) 15 l vessel. The enzyme extract containing amylolucrase is added in one portion. The enzyme activity in this experiment is 20 units (1 unit = 1 μmol of sucrose × min⁻¹ × mg of enzyme). The apparatus is provided with a precision glass stirrer, likewise sterilized. The vessel is closed, stored at 37°C and stirred. After a time of only a few hours, a white precipitate forms. The reaction is terminated after a period of 96 hours. The precipitate is filtered off and washed repeatedly with water in order to remove low-molecular-weight sugars. The residue remaining in the filter is dried at 40°C in a drying cabinet with application of a vacuum using a membrane pump (Vacuumbrand GmbH & Co., CVC 2). The weight is 651 g (yield 65%).

Example 2

Determination of the molecular weight of water-insoluble poly(1,4-α-D-glucan) from Example 1 synthesized using amylolucrase

2 mg of the poly(1,4-α-D-glucan) from Example 1 are dissolved in dimethyl sulfoxide (DMSO, p.a. from Riedel-de-Haen) at room temperature and filtered (2 μm filter). A portion of this solution is injected into a gel permeation chromatography column. The eluent used is DMSO. The signal intensity is measured by means of an RI detector and evaluated against pullulan standard (Polymer Standard Systems). The flow rate is 1.0 ml per minute. The measurement gives a number average molecular weight (Mn) of 7000 g/mol and a weight average
Example 3

Production of microparticles of poly(1,4-α-D-glucan)

a) 400 g of poly(1,4-α-D-glucan) are dissolved in 2 l of dimethyl sulfoxide (DMSO, p.a. from Riedel-de-Haen) at 60°C over the course of 1.5 hours. The mixture is then stirred at room temperature for one hour. The solution is added to 20 l of stirred bidistilled water via a dropping funnel over a period of 2 hours. The batch is stored at 4°C for 44 hours. A fine suspension forms. The particles are separated off by firstly decanting the supernatant. The sediment is slurried and centrifuged in small portions (RC5C ultracentrifuge: each 5 minutes at 5000 revolutions per minute). The solid residue is slurried with bidistilled water and re-centrifuged, and this process is repeated twice. The solids are collected, and the suspension of about 1000 ml is freeze-dried (Christ Delta 1-24 KD). 283 g of a white solid are isolated (yield 71%).

b) The collected supernatants are stored overnight at a temperature of 18°C. The work-up is carried out as described. A further 55 g of the white solid are isolated (yield 14%).

The total yield in this process is thus 85%.

Example 4

Investigation of the microparticles from Example 3 by electron microscopy

In order to characterize the particles, scanning electron microscope (SEM) micrographs are taken (Camscan S-4). Figures 1 to 4 show micrographs of the particles which show that they are spherical, very uniform particles with respect to shape, size and surface
Example 5

Investigations of the size distributions of the particles from Example 3

In order to characterize the size distribution of the particles from Example 3, investigations were carried out using a Mastersizer (Malvern Instruments). The investigation was carried out in Fraunhofer mode (evaluation: multi-modal, number) assuming a density of 1.080 g/cm$^3$ and a volume concentration in the range from 0.012% to 0.014%.

Table 1:
Characterization of the particle diameters from Example 3

<table>
<thead>
<tr>
<th>Examples</th>
<th>Diameter</th>
<th>Particle distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Examples</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>No.</td>
<td>dn$^2$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(μm)</td>
</tr>
<tr>
<td>3a</td>
<td>1.664</td>
<td>4.184</td>
</tr>
<tr>
<td>3b</td>
<td>0.945</td>
<td>2.345</td>
</tr>
</tbody>
</table>

$^1$ dn: number average diameter  
$^2$ dw: weight average diameter  
$^3$ dw/dn: particle diameter dispersity  
$^4$ d(10%): 10% of all particles have a smaller diameter than the value indicated  
$^5$ d(50%): 50% of all particles have a smaller diameter than the value indicated  
$^6$ d(90%): 90% of all particles have a smaller diameter than the value indicated

Example 6

Determination of the specific surface area of poly(1,4-α-D-glucan) (comparative example) and microparticles of poly(1,4-α-D-glucan) and potato starch (comparative
The measurements of the specific surface area are carried out using a Sorptomatic 1990 (Fisons Instruments). The evaluation is carried out using the default sorptomatic method. For the investigation, the samples are dried overnight at 80°C under reduced pressure (membrane pump from Vacuubrand GmbH & Co., CVC 2). The potato starch and the poly(1,4-α-D-glucan) obtained directly from biotransformation are ground in advance so that the weight of the particles is in the region smaller than 200 microns. A commercially available mill (Waring) is used for this purpose.

Table 2:

Characterization of the specific surface area of the microparticles of poly(1,4-α-D-glucan) (see Example 3)

<table>
<thead>
<tr>
<th>Example No.</th>
<th>Substance</th>
<th>Specific surface area $^2$/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Poly(1,4 α-D-glucan)</td>
<td>2.243</td>
</tr>
<tr>
<td>3</td>
<td>Microparticles of poly(1,4-α-D glucan)</td>
<td>4.527</td>
</tr>
<tr>
<td></td>
<td>Potato starch (Toffena, Südstärke)</td>
<td>1.280</td>
</tr>
</tbody>
</table>

Example 7:

Experiments on the stability of the microparticles in various solvents in order to determine the usable solvents.

In order to test the suitability of various solvents in the chromatographic application with microparticles of poly(1,4-α-D-glucan), the following procedure is used: 100 mg of particles from Example 3 are covered with in each case 3 ml of the solvent in a beaded rim bottle. The observation time is 24 hours. At hourly intervals, the particles are investigated for changes. For this purpose, the reaction vessel is swirled.
The results show that a wide variety of solvents can be used for the chromatographic use described here. The only exceptions are toluene, ethyl acetate and dimethyl sulfoxide. In individual cases not shown, the solvent must firstly be tested for its usability. The results of the experiments are shown in Table 3. The dielectric constant and the dipole moment and/or the Snyder polarity index have been given here as an indication of the polarity of the solvent.

Table 3:
Results of the investigations of the stability of microparticles of poly(1,4-α-D-glucan) (see Example 3) in various solvents

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Dielectric constant (e)</th>
<th>Dipole moment (D)</th>
<th>Polarity index*3</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Hexane</td>
<td>1.8865</td>
<td>-</td>
<td>0.0</td>
<td>No change</td>
</tr>
<tr>
<td>n-Heptane</td>
<td>1.9203</td>
<td>-</td>
<td>0.0</td>
<td>No change</td>
</tr>
<tr>
<td>Toluene</td>
<td>2.379</td>
<td>0.375</td>
<td>2.3</td>
<td>Transparent, slightly swollen</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>6.0814</td>
<td>1.79</td>
<td>-</td>
<td>Material, cloudy supernatant</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>8.93</td>
<td>1.60</td>
<td>3.4</td>
<td>Particles sticking, cloudy supernatant</td>
</tr>
<tr>
<td>Tetrahydrofuran</td>
<td>7.2</td>
<td>1.75</td>
<td>4.2</td>
<td>No change</td>
</tr>
<tr>
<td>1,4-Dioxane</td>
<td>2.2189</td>
<td>-</td>
<td>4.8</td>
<td>No change</td>
</tr>
<tr>
<td>Ethanol</td>
<td>25.3</td>
<td>1.69</td>
<td>5.2</td>
<td>No change</td>
</tr>
<tr>
<td>Acetone</td>
<td>21.01</td>
<td>2.68</td>
<td>5.4</td>
<td>No change</td>
</tr>
<tr>
<td>Acetone nitrile</td>
<td>36.64</td>
<td>3.924</td>
<td>6.3</td>
<td>No change</td>
</tr>
<tr>
<td>Methanol</td>
<td>33.0</td>
<td>1.7</td>
<td>6.6</td>
<td>No change</td>
</tr>
<tr>
<td>Dimethylformamide</td>
<td>38.25</td>
<td>3.62</td>
<td>-</td>
<td>No change</td>
</tr>
</tbody>
</table>
- 20 -

| Substance | 47.24 | 3.96 | Dissolved
|-----------|-------|------|-----------------
| Dimethyl sulfoxide | Water | 80.100 | 1.854 | 9.0 | No change

1. Dielectric constant in accordance with "Handbook of Chemistry and Physics, 77th edition"
2. Dipole moment in accordance with "Handbook of Chemistry and Physics, 77th edition"
3. Snyder polarity index from Merck, ChromBook

Example 8

10 Filling of a chromatography column with microparticles from Example 3a

5 g of the particles from Example 3a are suspended in about 60 ml of heptane. The suspension is briefly stirred up by means of an Ultraturrax (Ika) and subsequently degassed in an ultrasound bath (Sonorex RK510H). The suspension is then introduced with maximum flow into a column having the dimensions length 125 mm and diameter 4 mm (Hibar ready-made column from Merck AG). Repeated tapping on the column or repeated shaking of the column in combination with brief treatment in an ultrasound bath for about 1 minute ensure a dense and uniform packing of the separation material, giving packing with a low proportion of dead volumes.

Example 9

Separation of indole and 2-methylindole in heptane

The separations are carried out in a Lichograph chromatography unit from Merck. The individual components are the following: an L 6200 A gradient pump, an L-400 UV detector (measurement at 254 nm), a D-2500 chromato-integrator and a Hibar RT 125-4 ready-made separation column. The sample concentration is ideally from 0.05 to 0.1 mg/ml of solvent. 1,3,5-Tri-tert-butylbenzene (Fluka) can be used as internal standard.
The chromatography column is prepared with n-heptane (Aldrich for HPLC). The unit is run with n-heptane. The elution rate is 0.5 ml/min. The pressure is 54 bar. The substances to be separated are indole and 2-methylindole.

Table 4
Results of separation of indole and 2-methylindole in heptane

<table>
<thead>
<tr>
<th>Substance</th>
<th>Reaction time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,3,5-Tri-tert-butylbenzene</td>
<td>1.86</td>
</tr>
<tr>
<td>(standard)</td>
<td></td>
</tr>
<tr>
<td>Indole</td>
<td>9.12</td>
</tr>
<tr>
<td>Indole/2-methylindole</td>
<td>0.28/7.37</td>
</tr>
<tr>
<td>2-Methylindole</td>
<td>7.20</td>
</tr>
</tbody>
</table>

Example 10
Separation of indole and 2-naphthol in heptane

The experiment is carried out as described in Example 10.

Table 5:
Results of the separation of 2-naphthol and indole in heptane

<table>
<thead>
<tr>
<th>Substance</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Naphthol</td>
<td>2.24</td>
</tr>
<tr>
<td>2-Naphthol/indole</td>
<td>1.92/9.16</td>
</tr>
<tr>
<td>Indole</td>
<td>9.12</td>
</tr>
</tbody>
</table>

Example 11
Separation of an enantiomer mixture: racemic 1-(2-naphthyl)ethanol
The experiment is carried out analogously to Example 10. In this example, the pressure is about 70 bar. The concentration of the samples is 0.25 mg/ml of solvent.

Table 6:
Results of the separation of racemic 1-(2-naphthyl)-ethanol in heptane

<table>
<thead>
<tr>
<th>Substance</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,3,5-Tri-tert-butylbenzene (standard)</td>
<td>1.89</td>
</tr>
<tr>
<td>S-(-)-1-(2-Naphthyl)ethanol</td>
<td>32.03</td>
</tr>
<tr>
<td>1,3,5-Tri-tert-butylbenzene (standard)</td>
<td>1.87</td>
</tr>
<tr>
<td>R- (+)-1-(2-Naphthyl)ethanol</td>
<td>36.30</td>
</tr>
</tbody>
</table>

Example 12
Removal or retention of substances, use of the separation material according to the invention as filter medium

Disposable centrifuge filters (Schleicher & Schüll, for example Centrix, catalogue number 467012 (April 1996): "Filter 1" in Figs. 5 and 6) are equilibrated with 3 ml of a buffer 1 (see below) using 580 mg of the microparticles produced in accordance with Example 3 as filter material (if necessary centrifuge (2000 rpm)). 2 ml of an aqueous DNA plasmid solution (plasmid used: pBluescript II SK) with a concentration of 50 μg/ml are subsequently applied to the filter (if necessary centrifuge (5 min at 2000 rpm)). A reference run is carried out with ("Filter 2" in Figs. 5 and 6) Qiagen Midipräp (Hilden, Germany) and a further reference run is carried out with Qiagen Midipräp cartridges without Qiagen filter medium which are filled with the separation material.

In each case, 5 μl of eluent is provided with about
(marker) and applied to an agarose gel plate (60% of sucrose, 20 mM EDTA, 0.025% of bromophenol blue) with subsequent gel electrophoresis (Biorad, power supply, model 100/200). As reference for the estimation and classification of detected plasmids, a marker (MW 5000) is applied in track 1 and the DNA plasmid solution used is applied as reference. Fig. 5 shows that the sample DNA was retained by the separation material according to the invention (filter medium here).

5 ml of a second buffer (buffer 2) are subsequently applied to the column (filter) for elution and rapidly worked up by centrifugation (5 min at 2000 rpm).

The eluates (references as above) are applied to an agarose gel plate (60% of sucrose, 20 mM EDTA, 0.025% of bromophenol blue), and gel electrophoresis is subsequently carried out (Biorad, power supply, model 100/200) (see Fig. 6). As comparison, a marker (MW 5000) (track 1) and the DNA plasmid solution (track 7) were applied.

Fig. 6 shows that on use of the separation material according to the invention, the plasmid DNA employed is eluted from the column again. The comparison with commercially available filter media shows the quality of the filter process, which has at least the same quality.

Buffer description:

Buffer 1:  750 mM NaCl
           50 mM MOPS (3-[N-morpholino]propanesulfonic acid, pKₐ 7.2)
           15% of isopropanol
           0.15% of Triton X-100

Buffer 2:  1.25 M NaCl
           50 mM tris, tris·Clm, pH 8.5
           15% of isopropanol
Fig. 5:
From left to right:

Track 1: marker (Boehringer Mannheim DNA Molecular Weight Marker X)

Track 2: filter 2 without separation material (blank reference)

Track 3: filter 2 filled with separation material according to the invention

Track 4: filter 1: Qiagen cartridge with separation material according to the invention

Track 5: filter 1: Qiagen MidiPräp (Hilden, Germany) reference

Track 6: filter 2 with separation material

Track 7: pure DNA plasmid solution (commercially available plasmid pBluescript II SK)

Fig. 6:
From left to right:

Track 1: marker (Boehringer Mannheim DNA Molecular Weight Marker X)

Track 2: filter 2 without separation material (blank reference)

Track 3: filter 2 filled with separation material according to the invention

Track 4: filter 1: Qiagen cartridge with separation material according to the invention

Track 5: filter 1: Qiagen MidiPräp (Hilden, Germany) reference

Track 6: filter 2 with separation material

Track 7: pure DNA plasmid solution (commercially available plasmid pBluescript II SK)
Patent claims

1. A process for the chromatographic separation of substance mixtures using spherical microparticles of chemically and physically unmodified, water-insoluble, linear polysaccharides.

2. The process as claimed in claim 1, wherein the separations are carried out by column chromatography.

3. The process as claimed in claim 2, wherein the separations are carried out with the aid of HPLC (high-performance or high-pressure liquid chromatography).

4. The process as claimed in claim 1, wherein the separations are carried out by thin-layer chromatography.

5. The process as claimed in claim 1, wherein the separations are carried out with the aid of gel permeation chromatography.

6. The process as claimed in one of claims 1 to 5, wherein the substance mixtures are stereoisomers.

7. The process as claimed in claim 6, wherein the substance mixtures are enantiomers.

8. The process as claimed in one of claims 1 to 7, wherein the microparticles have a diameter of from 10 nm to 100 μm.

9. The process as claimed in claim 8, wherein the microparticles have a diameter of from 1 μm to 5 μm.
10. The process as claimed in one of claims 1 to 7, wherein the polysaccharides employed are poly(1,4-α-D-glucans).

11. The process as claimed in one of claims 1 to 7, wherein polysaccharides are employed which can be prepared by a biotechnological process.

12. The use of spherical microparticles of linear, water-insoluble polysaccharides for the chromatographic separation of substance mixtures.

13. The use of spherical microparticles of linear, water-insoluble polysaccharides as adsorbent or filter medium.

14. A separation material containing spherical microparticles of chemically and physically unmodified, water-insoluble, linear polysaccharides and, if desired, auxiliaries, additives and/or support materials.

15. A separation material as claimed in claim 14, wherein the microparticles have a specific surface area of from 3 m²/g to 10 m²/g.

16. A filter medium and/or adsorbent containing spherical microparticles of chemically and physically unmodified, water-insoluble, linear polysaccharides and, if desired, auxiliaries, additives and/or support materials.

17. The use of a separation material as claimed in one of claims 1 to 16 for the removal and, if desired, purification and isolation of biological material from liquids.
18. The use as claimed in claim 17, in which the biological material is a nucleic acid.
UNSCANNABLE ITEM

RECEIVED WITH THIS APPLICATION

(ITEM ON THE 10TH FLOOR ZONE 5 IN THE FILE PREPARATION SECTION)

DOCUMENT REÇU AVEC CETTE DEMANDE
NE POUVANT ÊTRE BALAYÉ

(DOCUMENT AU 10 IÈME ÉTAGE AIRE 5 DANS LA SECTION DE LA
PRÉPARATION DES DOSSIERS)