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- (54) Title: **POLYSACCHARIDE OF ECHINACEA ANGUSTIFOLIA**
- (57) Abstract: The present invention relates to a polysaccharide of *Echinacea angustifolia* roots, having molecular weight of 1.3×10^5 Da and consisting of rhamnose, arabinose, galactose and galacturonic acid in ratio 0.5:2.5:1.75:10.25. The polysaccharide is recovered from spontaneous or cultivated *Echinacea angustifolia* roots by means of a process comprising the following steps: a) depriving the roots of non polysaccharidic constituents by extraction with solvent; b) extracting the polysaccharidic fraction from the roots as directly obtained from the preceding step; c) isolating the polysaccharide by chromatography of the polysaccharidic fraction. Due to its immune-stimulating properties, the polysaccharide can be used for the preparation of medicaments, food supplements¹ or nutraceutical compositions to administer whenever it is desirable to strengthen the immune system body defenses.
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POLYSACCHARIDE OF *ECHINACEA ANGUSTIFOLIA*

Field of the invention

The present invention relates to the field of polysaccharides, in particular to a polysaccharide of *Echinacea angustifolia* and to a process for the preparation thereof. The polysaccharide can be used in the treatment of pathological conditions in which it is desirable to strengthen the immune defenses.

Background of the invention

Echinacea is a plant which originates from North America and Mexico; its therapeutical properties were well known to native Americans, who used it for healing wounds. Due to the fact that *Echinacea* was deemed able to increase the resistance to infections, during the first years of the last century its use in the treatment of local and generalised infections became widespread. *Echinacea*, in particular *Echinacea angustifolia*, is nowadays highly recommended for the treatment of influenza syndromes and in particular for the treatment of cold, for healing wounds and for the treatment of mycosis.

The general action is apparently due to the aspecific stimulation of the immune system and to the sensitisation of germs and pathogens to chemotherapeutics and antibiotics. The cicatrizing properties seem ascribable to the capacity of stabilizing hyaluronic acids through of hyaluronidase inhibition exerted by one of the active principles contained in the plant, i.e. echinacoside, and to the massive macrophages activation induced by polysaccharides. In this way any foci of infection remain localised and accumulation of mucopolysaccharides and histoplastic material necessary for reparative processes is favoured.

As a large fraction of *Echinacea* polysaccharides consists of inulin, which cannot be deemed responsible for the above cited properties, there is

the need to identify the active polysaccharide and to provide an effective process for the extraction and purification thereof.

Detailed disclosure of the invention

Object of the present invention is a polysaccharide from *Echinacea angustifolia* roots (hereinafter referred to as "the polysaccharide"), having molecular weight of 1.3×10^5 Da and consisting of rhamnose, arabinose, galactose and galacturonic acid in 0.5:2.5:1.75:10.25 ratio. In the skeleton of the polysaccharide, straight and branched portions alternate, the straight portions consisting of partially acetylated (9%) and methylated (35%) galacturonic acid residues linked via α -(1-4) bond and the branched portions consisting of an alternation of galacturonic acid and rhamnose, to which side chains containing arabinose and galactose in 2.5:1.75 ratio are attached. The polysaccharide is characterized by ^1H -NMR, ^{13}C -NMR spectra and GCP profile as reported in figures 1-3.

The polysaccharide is recovered from roots of spontaneous or cultivated *Echinacea angustifolia* by means of a process comprising the following steps:

- a) removing non-polysaccharide components from the roots by extraction with solvent;
- b) extracting the polysaccharide fraction from the roots as directly obtained from the preceding step;
- c) isolating the polysaccharide by chromatography of the polysaccharide fraction.

The purpose of step a) is to remove the non-polysaccharide components of the roots, mainly echinacoside and analogues thereof, as well as the large group of alkylamides which are also characteristic components of *Echinacea angustifolia* (R. Bauer et al., *Phytochemistry* 28, 505, 1989; *Planta Med.* 55, 367, 1989). Accordingly, the roots are extracted with a solvent selected from acetone or an alcohol from one to three carbon atoms, optionally in admixture

with water, at a temperature ranging from 20°C to the boiling temperature, preferably under reflux. The water content of the solvent must not exceed 40% (v/v). Ethanol at a concentration between 80 and 95% (v/v) is the preferred solvent.

5 The purpose of step b) is to extract the mixture of the polysaccharide components from the roots. The extraction is carried out with water, acetone or an alcohol from one to three carbon atoms, in admixture with water, at a temperature ranging from 20°C to the boiling temperature of the solvent, preferably from 40 to 70°C. When mixtures of solvents are used, the water
10 content will be 60% or higher, preferably 85% (v/v). According to a preferred embodiment of the invention, the solvent is 15% (v/v) ethanol.

 Step c) provides the fractioning of the extract from step b) and the separation of the polysaccharide from other polar components of the extract. This step preferably consists in size-exclusion chromatography or ion
15 exchange chromatography.

 In the first case, the polysaccharide is purified based on its molecular dimensions: in fact the polysaccharide has a characteristic mass, which is different from that of all the other components. A suitably cross-linked resin can separate chemical species having different dimensions; processing the
20 extract from step b) through a resin of this type (for example Toyopearl HW-65S and Superdex[®]200HR) allows to purify the polysaccharide of the invention.

 The purification of the polysaccharide by ion exchange chromatography is based on the acidic character of the polysaccharide, due to the presence of
25 carboxy functions in the galacturonic acid units. Processing the extract from step b) through an anion exchange resin allows to retain only the polysaccharide and any other acidic components (which are present in small amounts) and to remove all the neutral or basic components. The resin is

washed with a saline or acidic aqueous solution to recover the molecules entrapped by the resin. The salts or the acids in the purified solution are thereafter removed by means of ultrafiltration dialysis. A sufficiently high cutoff (e.g. 10,000 or 100,000 Da) is selected to remove also the acidic impurities in the starting extract and retained by the anionic resin.

Preferred anion exchange resins are strong ion exchange resins, such as Diaion HPA 25 and Q Sepharose[®]Fast Flow.

The polysaccharide purified solution obtained according to one of the chromatographic processes described in step c) is then concentrated and dried under vacuum or freeze-dried. The polysaccharide is an ivory-colour powder.

Step c) can also comprise preliminary purification steps, useful for making the chromatography easier. Even though not indispensable, these steps allow to remove a first aliquot of impurities from the extract of step b) and to reduce the amount of resin.

Preliminary treatments are selected from:

c1) concentration of the extract from step b) under reduced pressure and subsequent purification by treatment with a mixture of water and acetone or water and an alcohol from one to three carbon atoms, preferably ethanol. The mixture will contain 50-70%, preferably 66.5%, of alcohol or acetone. According to a preferred embodiment of the invention, the residue resulting from concentration of the extract is dissolved at room temperature with three parts of water and diluted under stirring with 7 volumes of 95% ethanol. The precipitated fraction, which contains the polysaccharide, is collected by filtration, washed with ethanol at a concentration from 50 to 70% and subjected to chromatographic purification.

c2) treatment of the polysaccharide fraction from step b), or of the enriched fraction from step 1c), with water at room temperature. In this way the polysaccharide of the invention, which is highly soluble in water, is

separated from other sparingly soluble polysaccharides. The extract resulting from step b) (or the fraction enriched in polysaccharides from step c1)) is suspended in water at room temperature and stirred to promote dissolution. The insoluble residue is separated and the aqueous solution is subjected to chromatography.

c3) enzymatic treatment, useful for hydrolising inulin-like oligosaccharides and polysaccharides, which represent one of the main impurities of the extract from step b). The extract (or one of the partially purified products from steps c1) or c2)) is treated, in aqueous solution, with a catalytic amount of inulinase for 10-24 hours. The enzyme is then heat- or trypsin-inactivated and the carbohydrate fragments formed upon hydrolysis are removed by dialysis (tangential ultrafiltration with cutoff higher than 10,000 Da, preferably 100,000 Da). The retentate thus obtained is then subjected to chromatography.

c4) high cutoff ultrafiltration (cutoff higher than 10,000 Da, preferably 100,000 Da) to remove low molecular weight impurities. In this case, the extract from step b) (or one of the partially purified products from steps c1) or c2)) is dissolved in water, preferably 10 or 20 volumes, and dialysed. The retentate, which contains the polysaccharide of the invention, is then subjected to chromatography.

The polysaccharide of the invention showed immune-stimulating properties in mice, in particular proved able to stimulate T-lymphocytes activation and to counteract the effect of cyclosporin A, thus reducing the mortality due to *Candida albicans* infection. The polysaccharide of the invention can be therefore used for the preparation of medicaments, food supplements or nutraceutical compositions to be administered in situations in which an increase of the immune system body defenses is desirable.

The polysaccharide can be formulated according to conventional

techniques, for example according to those described in Remington's Pharmaceutical Sciences Handbook, XVII ed. Mack Pub., N.Y., U.S.A.

The present invention is hereinafter illustrated by means of some examples.

5 **EXAMPLES**

In all the following examples, the determination of the polysaccharide HPLC titre is carried out with a TosoHaas TSK-Gel G 5000 PWXL column eluted with water containing 0.5% of triethylamine in isocratic conditions at a flow rate of 0.5 ml/min. During the analysis, which lasts 30 minutes, the
10 column is kept at 50°C. The injection volume is 50 µl. An evaporative detector ELSD (Evaporative Light Scattering Detector) Sedex mod. 75 (S. E. D. E. R. E.) -whose nebulizer is kept at 60°C with gas pressure of 2.2 bars is coupled to the column.

Example 1

15 Extraction of *Echinacea angustifolia* roots (steps a) and b))

600 grams of ground roots of *Echinacea angustifolia* are extracted under reflux for four hours with 2.5 L of 90% (v/v) ethanol. The percolate is collected and further seven extractions with the same solvent (step a)) are carried out. The resulting extract is discarded. The roots are then extracted
20 seven times at 70°C with 15% (v/v) ethanol, each extraction lasting four hours. The combined percolates are filtered and concentrated under vacuum, affording 170 g of brown residue (whole polysaccharides extract, step b)).

Example 2

25 Recovery of the polysaccharide by pre-purification with solvents and anion exchange chromatography

170 g of the whole polysaccharides extract from step b) of example 1 are dissolved in 570 ml of water and 1.13 L of ethanol are then added. The mixture is stirred for about one hour, the precipitate is decanted for about 20

minutes, filtered, washed with 850 ml of 66.5% (v/v) ethanol and dried at 55°C under vacuum for 48 hours, affording 140 g of a hazel-color solid. The solid is taken up with 2.1 L of water and the resulting suspension is left under stirring for 20 minutes, then separated from the insoluble residue (which is discarded).

The aqueous solution, enriched in the polysaccharide of the invention, gives a dry residue of 38.7 g. The solution is loaded on a chromatographic column containing 0.9 L of Diaion HPA 25 resin conditioned with AcOH/AcNH₄ buffer at pH 6.1. The resin is washed with 5.4 L of AcOH/AcNH₄ buffer solution at pH 6.1, and the fraction containing the polysaccharide is eluted with 5.4 L of a 0.5 M AcNH₄ aqueous solution. The eluate is concentrated to 0.3 L under reduced pressure. The concentrated solution is subjected to dialysis by tangential ultrafiltration with 6 L of purified water, using a 10,000 Da cutoff spiral-wound membrane in polyethersulfone.

The retentate is recovered and concentrated under reduced pressure and the residue is heat-dried under vacuum, affording 7.1 g of an ivory-color powder (polysaccharide HPLC titre: 96%).

The polysaccharide has average molecular weight of 1.3×10^5 Da ($s=5365$), with $<M_n$ 14320 ($s=2180$), which is consistent with a determination by means of LALLS (Low Angle Laser Light Scattering) and consists of galacturonic acid, galactose, arabinose and rhamnose in 10.25:1.75:2.5:0.5 ratio (HPAEC analysis). The polysaccharide has characteristic ¹H-NMR, ¹³C-NMR spectra and GPC profile, as reported in figures 1-3 respectively.

Example 3

Recovery of the polysaccharide by ultrafiltration pre-purification and size-exclusion chromatography

170 g of the whole polysaccharides extract from step b) of example 1

are dissolved in 850 ml of purified water. The solution is dialysed by tangential ultrafiltration with 10 L of purified water, using a 50,000 Da cutoff spiral-wound membrane in polyethersulfone.

5 The retentate, which contains 110.5 g of purified extract, is recovered and loaded on a chromatographic column kept under medium pressure and containing 22 L of Toyopearl HW-65S resin conditioned with water. The elution is carried out with purified water, dividing the eluates in fractions 200 ml each. The fractions are GPC-analyzed and those containing sufficiently pure polysaccharide are pooled.

10 The pooled fractions are then concentrated under reduced pressure and the residue is heat-dried under vacuum, affording 5.7 g of ivory-color powder (polysaccharide HPLC titre: 95%).

Example 4

15 Recovery of the polysaccharide by enzymatic pre-purification and anion exchange chromatography

170 g of the whole polysaccharides extract from step b) of example 1 are dissolved in 3230 ml of purified water and 850 mg of enzyme inulinase are added. The mixture is left under gentle stirring at 40°C for 24 hours, then the enzyme is inactivated with 80 mg of trypsin, stirring for 2 hours at 36°C. The 20 solution is heated at 85°C for 2 hours and concentrated under reduced pressure to 1.7 L.

The concentrated solution is subjected to dialysis by tangential ultrafiltration with 17 L of purified water, using a 100,000 Da cutoff spiral-wound membrane in polyethersulfone. The retentate (which contains 11.5 g of 25 purified extract) is recovered and loaded on a chromatographic column containing 0.8 L of Diaion HPA 25 resin conditioned with AcOH/AcNH₄ buffer at pH 6.1. The resin is washed with 4.8 L of AcOH/AcNH₄ buffer solution at pH 6.1, and the fraction containing the polysaccharide is eluted

with 4.8 L of a 0.5 M AcNH₄ aqueous solution. The eluate is concentrated to 150 ml under reduced pressure and the concentrate is dialysed by tangential ultrafiltration with 1.5 L of purified water, using a 50,000 Da cutoff spiral-wound membrane in polyethersulfone.

5 The retentate is concentrated under reduced pressure and the residue is heat-dried under vacuum, obtaining 6.9 g ivory-color powder (polysaccharide HPLC titre: 97%).

BIOLOGICAL SECTION

Experiment 1

10 Test for the production of γ -interferon in T-lymphocytes (Zucca M. et al, New Microbiol. 1996, 19, 39-46)

 Murine T-lymphocytes obtained by separation of splenocytes on nylon-wool column, were cultured in 1640 RPMI medium with 4% of fetal calf serum in microtitre plates optionally pre-incubated with α -CD3 (anti-CD3
15 monoclonal antibody as cell function activator responsible for interferon production). The substances to test were added to the wells and after 48 hours γ -interferon released in the incubation medium was evaluated.

Table 1

TREATMENT	γ -Interferon pg/ml
Medium	4.5 $\pm$ 0.5
α -CD3	149.5 $\pm$ 25.0
α -CD3 + Polysaccharide 0.1 μ g/ml	410.0 $\pm$ 45.7
α -CD3 + Polysaccharide 1.0 μ g/ml	466.3 $\pm$ 71.8
α -CD3 + Polysaccharide 10.0 μ g/ml	690.5 $\pm$ 95.5

Experiment 2Effect on mortality induced by *Candida albicans* in mice

(Microbiology, 2000, 146,1881-9)

5 Yeasts were cultured in Sabouraud agarized medium and intravenously inoculated at a concentration of 2.9×10^5 in mice immune-suppressed with 1 mg/Kg ip of cyclosporin A (CsA). Mice were treated daily with 5 and 10 mg/Kg i.p. of the polysaccharide of the invention until death of all the control mice (untreated). The results were evaluated as survived animals in the treated groups.

10

Table 2

TREATMENT	% OF SURVIVED ANIMALS
<i>Candida albicans</i> (CA) + CsA	0
CA + CsA + polysaccharide 5 mg/kg	30
Ca + CsA + polysaccharide 10 mg/Kg	50

CLAIMS

1. Polysaccharide of *Echinacea angustifolia* roots having molecular weight of 1.3×10^5 Da and consisting of rhamnose, arabinose, galactose and galacturonic acid in 0.5:2.5:1.75:10.25 ratio.
5
2. Polysaccharide according to claim 1 characterized by a skeleton with alternate straight and branched portions, the straight portions consisting of partially acetylated and methylated galacturonic acid residues linked via α -(1-4) bond and the branched portions consisting of an alternation of galacturonic acid and rhamnose, to which side chains containing arabinose and galactose in 2.5:1.75 ratio are attached.
10
3. A process for the preparation of the polysaccharide of claims 1 or 2 comprising the following steps:
 - a) removing non-polysaccharide components from the roots extraction with solvent;
15
 - b) extracting with solvent the polysaccharide fraction from the roots as directly obtained from the preceding step;
 - c) isolating the polysaccharide by chromatography of the polysaccharide fraction.
- 20 4. A process according to claim 3 wherein the solvent used in step a) is acetone or an alcohol from one to three carbon atoms.
5. A process according to claim 4 wherein the solvent is in admixture with water.
6. A process according to claim 5 wherein the water content of the solvent is not higher than 40%.
25
7. A process according to any one of claims 4-6 wherein the solvent is ethanol with a concentration ranging from 80 to 95%.
8. A process according to any one of claims 4-7 wherein the extraction

temperature ranges from 20°C to the boiling temperature of the solvent.

9. A process according to claim 3 wherein the solvent used in step b) is water or a mixture of water and acetone or water and an alcohol from one to three carbon atoms.

5 10. A process according to claim 9 wherein the water content of the solvent mixture is 60% or higher.

11. A process according to any one of claims 9 or 10 wherein the solvent is 15% ethanol.

12. A process according to any one of claims 9-11 wherein the extraction is
10 carried out a temperature ranging from 20°C to the boiling temperature of the solvent.

13. A process according to claim 3 wherein the chromatography of step c) is a size-exclusion chromatography or an ion-exchange chromatography.

14. A process according to claim 3 wherein, before chromatography of step
15 c), the polysaccharide fraction from step b) is subjected to one or more preliminary purification treatments.

15. A process according to claim 14 wherein preliminary purification treatments are selected from:

c1) precipitation and isolation of a polysaccharides-enriched fraction
20 by dissolution in a mixture of water and 50-70% of acetone or water and 50-70% of an alcohol from one to three carbon atoms;

c2) removal of the insoluble polysaccharide fraction by treatment with water;

c3) treatment with inulinase;

25 c4) ultrafiltration with cutoff 10,000 Da or higher.

16. A process according to claim 15 wherein preliminary purification treatments c1) and c2) are carried out.

17. A process according to claim 15 wherein preliminary purification

treatment c3), optionally preceded by one or both purification treatments c1) and c2), is carried out.

18. A process according to claim 15 wherein preliminary purification treatment c4), optionally preceded by one or both purification treatments c1) and c2), is carried out.

19. Polysaccharide of *Echinacea angustifolia* obtainable with the process of any one of claims 3 - 18.

20. Polysaccharide of any one of claims 1, 2 and 19 for use as a medicament.

21. Pharmaceutical and nutraceutical compositions and food supplements containing the polysaccharide of any one of claims 1, 2 and 19.

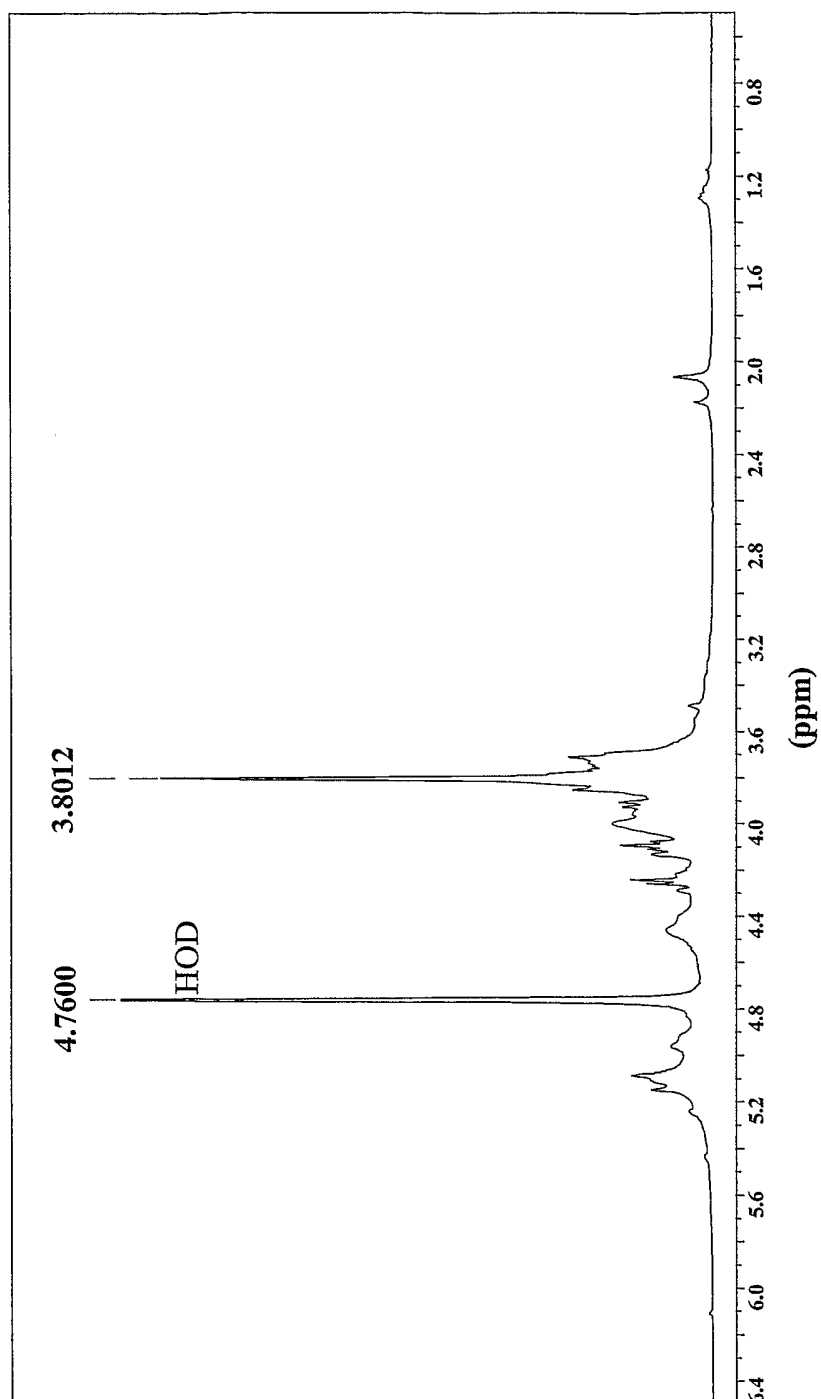


Figure 1: ^1H NMR spectrum of the polysaccharide 1

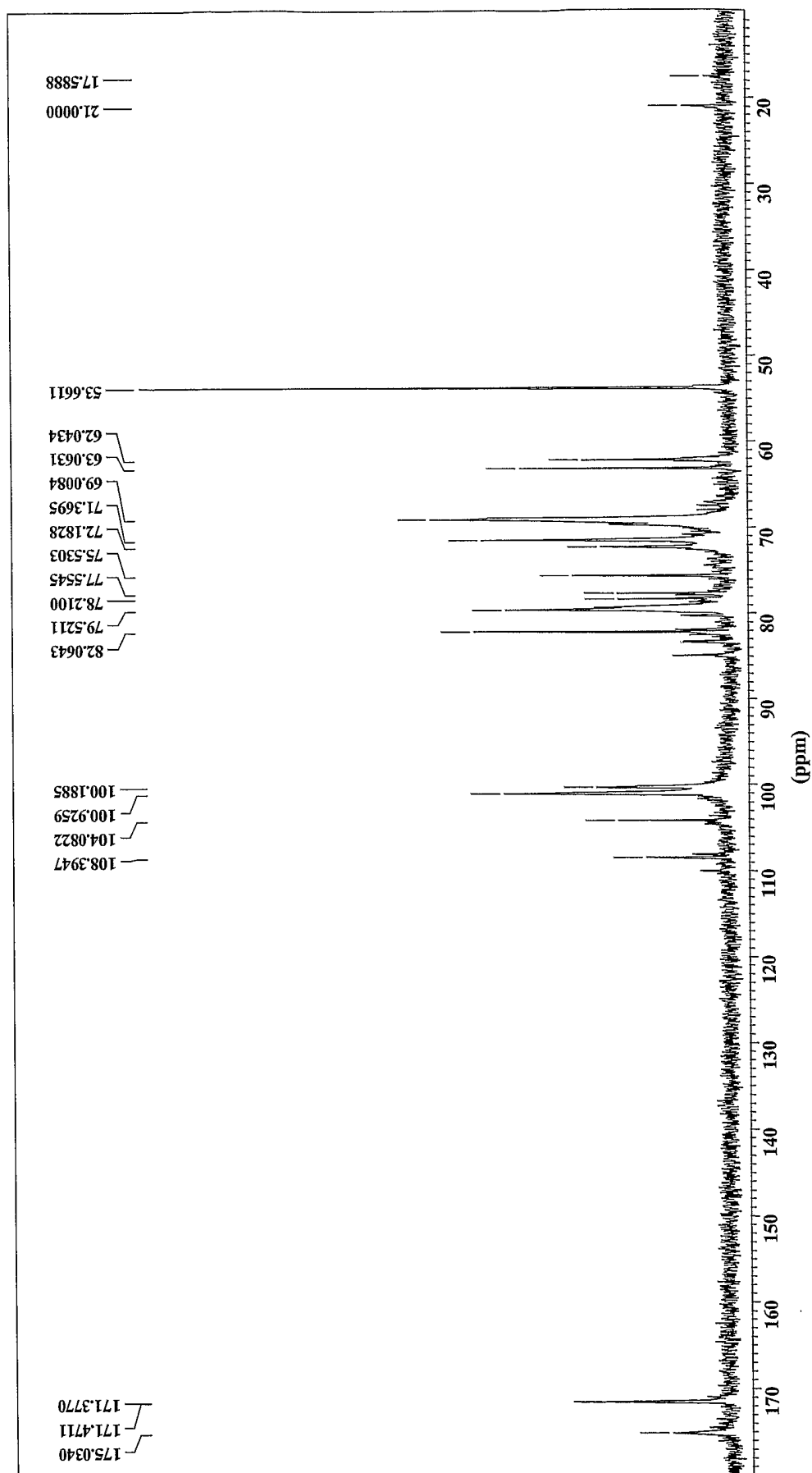


Figure 2: ^{13}C NMR spectrum of the polysaccharide 1

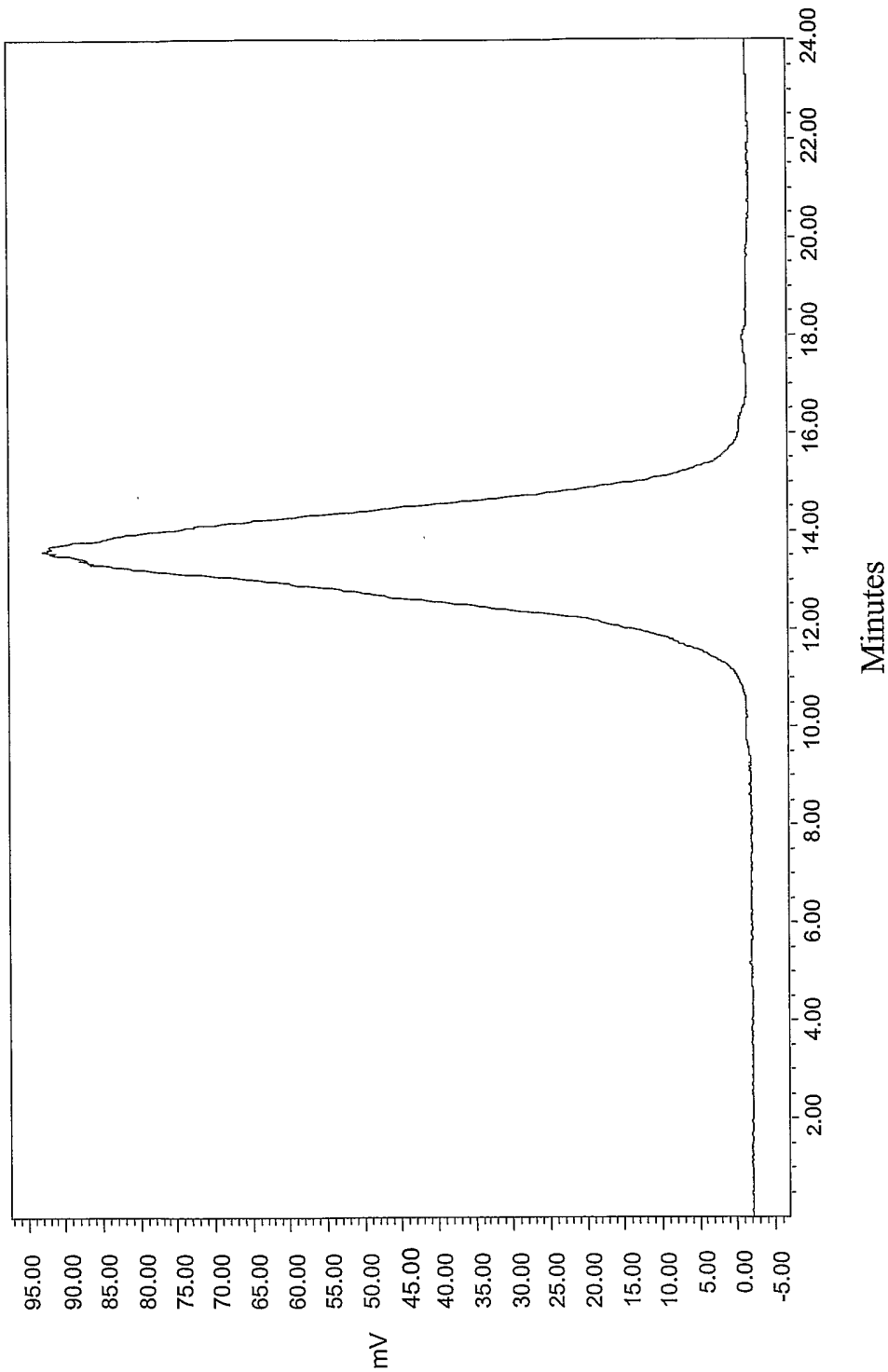


Figure 3: GPC profile of the polysaccharide 1

INTERNATIONAL SEARCH REPORT

International Application No

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A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C08B37/00 C08B37/18 A61K31/715

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)

IPC 7 C08B

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 practical, search terms used)

WPI Data, PAJ, CHEM ABS Data, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DE 32 17 214 A (WAGNER HILDEBERT) 10 November 1983 (1983-11-10) claim 2 page 7, line 6 - line 17 page 9, line 19 -page 10, line 3 page 10, line 18 -page 12, line 25	1-21
A	DE 950 674 C (GRUENENTHAL CHEMIE) 11 October 1956 (1956-10-11) the whole document --- -/--	1-21

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in 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 document but published on or after the international filing date

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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

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 03/06717

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Information on patent family members

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