(54) Title: METHOD OF DETECTING AND/OR QUANTIFYING PENTOSAN POLYSULFATE SODIUM

(57) Abstract: The invention refers to a method of detecting and/or quantifying a pentosan polysulfate sodium (PPS), its metabolites and/or derivatives in a biological sample. The method comprises several steps, wherein the PPS is deesterified, treated with an enzyme preferably an enzyme of a glycosidase hydrolase (GH) family and the reaction products are measured using liquid chromatography (LC) - mass spectroscopy, capillary electrophoresis and laser induced fluorescent detection, or capillary electrophoresis followed by mass spectrometry. The invention further comprises a kit for detecting and/or quantifying PPS, its metabolites and/or derivatives.
Method of detecting and/or quantifying pentosan polysulfate sodium

The present invention refers to a method of detecting and/or quantifying pentosan polysulfate sodium (PPS), its metabolites and/or its derivatives in a biological sample. Further the invention relates to a kit for performing such method.

Technical background

Numerous sulfated polysaccharides or oligosaccharides are suitable for prophylaxis and/or treatment of diseases such as cancer, viral diseases, painful bladder syndrome, Creutzfeldt-Jakob disease, mucopolysaccharidoses, sickle cell anemia, osteoarthritis etc. Poly- or oligosaccharides consist of the same or different monomer units selected from the group consisting of xylose, arabinose, rhamnose, fucose, glucose, mannose, galactose, fructose, aminoglucose, galactosamine, mannosamine, glucuronic acid, galacturonic acid, mannnuronic acid, carrageenan, PI-88, pentosan polysulfate sodium (PPS), fucoidan, laminaran, alginate, lentinan, pluran, xylan, dextran, heparin, keratin sulfate, chondroitin-4- and -6-sulfate, dermatan sulfate, heparin sulfate, hyaluronic acid, teichuronic acid, partial hydrolysates of starch, cellulose, glycogen, chitin or pectin. The poly- or oligosaccharides are of synthetic or natural origin, e.g., produced by plants or microorganisms.

Pentosan polysulfate sodium (PPS), which is chemically known as β-D-Xylan, (1-4), 2,3-bis (hydrogen sulfate) sodium salt having a molecular range of 4000-6000 Dalton shown in Fig. 1, is a semi-synthetic, polysulfated oligosaccharide comprising a mixture of multiply charged anionic polysaccharides. PPS is produced by chemical sulfation of polysaccharides (e.g., xylan) obtained from woody plants, for example beechwood trees. The resulting product typically contains approximately 15-17% sulfur in the form of approximately 1.5-1.9 covalently bound sulfate groups per sugar residue in a mixture of polydisperse polymeric molecules estimated to be approximately 4,000-10,000 in molecular weight. PPS consists of sulfated, linear polysaccharides of about 12 to 30 1-4 conjugated beta-D-xylopyranose units (Mr = approx. 4,000 - approx. 10,000), which has a D-glucuronic acid at approximately every tenth unit.
PPS is very heterogeneous regarding the length of the xylose polymer backbone (Xyₙ) and the distribution of 4-O-methyl glucuronic acid (MeGlcA) side chains (WO 2014/114723 A1). Statistically about 1/10 of xyloses are α 1-2 linked with MeGlcA; this glycosidic bond is chemically highly stable. Additionally, PPS is modified in a biological sample such as blood or urine for example by partial deesterification such as desulfation and/or depolymerization, which additionally increase the heterogeneity of PPS, its metabolites and/or its derivatives in a sample.

PPS like other esterified, for example sulfated poly- or oligosaccharides is most commonly used as an oral formulation to treat interstitial cystitis (also known as painful bladder syndrome) in humans and as an injectable drug to treat osteoarthritis in companion animals (Fuller, Ghosh et al., "Plasma and synovial fluid concentrations of calcium pentosan polysulfate achieved in the horse following intramuscular injection," Equine Veterinary Journal (2002)). PPS can also be used as an anticoagulant, preventing the formation of blood clots. It has further been used for treatment of hematomas, hemorrhoids, frostbites, burns, and multiparameter illnesses such as thrombosis and atherosclerosis.

While uses of PPS and other esterified poly- or oligonucleotides are becoming more widespread, a fundamental problem persists as attempts to detect and quantify PPS by mass spectrometry directly or in the ion pairing mode fail, particularly due to uncontrolled decomposition of the highly charged molecule in vacuo.

Binding assays using radiolabelled PPS for example or antibodies which are based on the interaction of PPS with polycations likewise fail as the interaction is highly dependent on the chain length of the PPS and its degree of sulfation. This performance of the assay is further influenced by cross reactivity with natural glucosaminoglycans (GAG) in the sample.

Liquid chromatography-mass spectrometry (LC-MS-MS) is an established method to quantify for example heparin, chondroitin sulfate, or dermatan sulfate by the determination of characteristic disaccharides after degradation of these natural GAG by specific enzymes like chondroitinases or heparin lyases. Also a method for the diagnosis of Pompe’s disease has been developed, based on the measurement of a characteristic tetrasaccharide Glc₄. However, the methods are not transferable to PPS, as it is not composed of disaccharide building blocks like natural GAG and the MeGlcA side chains are not regularly distributed. No enzymes are available, which are able to degrade or fragment the highly sulfated PPS. Chemical hydrolysis cleaves sulfate ester bonds as well as O-glycoside bonds between xyloses. However, xylose monosaccharides are not characteristic and suitable for the detection or quantification of PPS and/or its metabolites as xylose monosaccharide
may be resorbed from food directly or as a degradation product of intestinal bacteria from soluble fibre. Obviously, there is no way to obtain uniform and characteristic oligosaccharides, which are accessible to LC-MS-MS detection.

Moreover, protocols for selective desulfation of heparin exist but these require the formation of a quaternary ammonium salt of heparin and the transfer to the solvent DMSO which is not applicable to biological samples (Inoue and Nagasawa, Carbohydrate Research, Vol. 46, 1976, 87-95).

Hence, it is important to investigate a method which allows detecting and/or quantifying an esterified poly- and/or oligosaccharide such as PPS in a sample to control the amount of poly- and/or oligosaccharide such as PPS, its metabolites and/or its derivatives in a patient treated with any poly- and/or oligosaccharide such as PPS.

Summary of the invention

The present invention provides the first method for reliably detecting and/or quantifying an esterified for example sulfated poly- and/or oligosaccharide, e.g., PPS, its metabolites and/or derivatives in a sample. In a first step, the poly- and/or oligosaccharide such as PPS is selectively deesterified without significant depolymerization of the poly- or oligosaccharide. In a next step, the sample, i.e., the deesterified for example desulfated sugar backbone is incubated with an enzyme such as a glycoside hydrolase (GH) which is specifically degrading the backbone of the poly- and/or oligosaccharide of interest for example where the restriction site is determined by side chains such as MeGlcA side chains in PPS to obtain a characteristic oligo- or monosaccharide. In this step one or more enzymes are added to the sample.

After a separation step such as a centrifugation or ultrafiltration step to remove high molecular weight material, the filtrate is subjected to the LC-MS-MS and the deesterified poly- and/or oligosaccharide such as PPS, its metabolite and/or derivative is detected and/or quantified based on the products of the enzymatically degraded sugar backbone(s). An internal standard is added to the LC-MS-MS for the detection and/or quantification of the products of the enzymatic degradation which is a molecule chemically similar to the product resulting from the enzymatic degradation.

Further, the invention refers to a kit for detecting and/or quantifying a natural or chemically modified for example esterified poly- or oligosaccharide, e.g., PPS according to the method of the present invention comprising one or more compounds for reducing the protein content of the
sample, for deesterifying the esterified poly- or oligosaccharide, its metabolite and/or derivative, a compound to neutralize the reaction mixture, and/or one or more enzymes for degradation of the deesterified sugar backbone. Optionally the kit comprises materials or devices for the various filtration steps, and/or internal standards for quantification and reference standards.

**Brief description of the figures**

Fig. 1a depicts the chemical structure of pentosan polysulfate sodium (PPS) and Fig. 1b shows a specific embodiment of PPS, wherein the sulfate group next to the glucuronic acid of the poly- or oligosaccharide is replaced by an acetyl group, wherein the ratio of sulfate to acetyl group is 6:4 to 4:6.

Fig. 2a to 2f show further sulfated poly- and oligosaccharides such as PI-88, dextran sulfate, fucoidan, carrageenan, chitin and chitosan.

Fig. 3a to 3d depict the cleavage sites of different glycoside hydrolases which are GH10 xylanase, GH11 xylanase, and GH30 xylanase, and β-xylanase. The arrows indicate the positions where the enzyme is active in the sugar backbone, the crossed arrow indicates, where it is not active. The β-xylanase is cutting monomer by monomer starting from the non-reducing end of the sugar backbone up to the xylose bearing a side chain.

Fig. 4a to 4c presents different products of the enzymatic digestion of the sugar backbones with GH xylanase, e.g., the combination of GH30 xylanase and the β-xylanase, the combination of GH30 and GH10 xylanases, and the combination of GH30 and GH11 xylanases. Final products are xylose as well as xylose oligomers.

Fig. 5a to 5e depicts examples of internal standards for LC-MS-MS such as 3-O-acetyl-2-O-(4-O-methyl-α-D-glucopyranuronosyl)-6-D-xylopyranosyl-1,4-D-xylose, hexenuronic acid replacing MeGlcA or an acetylated derivative. In general, useful internal standards are derivatives of the diagnostic oligosaccharide like methyl- or acetylated aldehydes or amines obtained by reductive amination and/or introduction of a double bond (Fig. 5b). Even commercially available natural or modified oligosaccharides may be suitable as internal standards like acarbose or raffinose (Fig. 5d and 5e).
Fig. 6 shows different steps of the method according to the present invention, wherein some are essential and some are optional.

**Detailed description of the invention**

The present invention focus on a method for the detection and/or quantification of pentosan polysulfate sodium (PPS) in a biological sample such as plasma, serum, blood, urine or any other body fluid or tissue extract. However, the method is not limited to the detection and/or quantification of PPS, but it is likewise applicable for the detection and/or quantification of any other esterified poly- or oligosaccharide. Examples for such esterified poly- or oligosaccharides (Fig. 2) are PI-88, a mixture of highly sulfated, monophosphorylated mannose oligosaccharides for example derived from the extracellular phosphomannan of the yeast *Pichia* (Rosenthal et al., Ann Oncol., 2002, May; 13(5): 770-776); dextran sulfate, a complex, branched glucan (polysaccharide made of many glucose molecules) composed of chains of varying lengths (from 3 to 2000 kDa), the straight chain consists of α-1,6 glycosidic linkages between glucose molecules, while branches begin from α-1,3 linkages; fucoidan, a fucose-containing sulfated polysaccharide derived from brown seaweeds, crude extracts of which are commercially available as nutritional supplements (Kwak, Mar. Drugs, 2014 Jan. 28; 12(2): 851-870); carrageenan, a high-molecular-weight polysaccharides made up of repeating galactose units and 3,6 anhydrogalactose (3,6-AG), both sulfated and nonsulfated, the units are joined by alternating α-1,3 and β-1,4 glycosidic linkages (Jiao et al., Mar. Drugs 2011, 9, 196-223); chitin and chitosan, a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). Chitosan is for example made by treating shrimp and other crustacean shells with an alkali sodium hydroxide. Numerous sulfated poly- and/or oligosaccharides are used in the medical field and have for example antiproliferative, antiangiogenic, and/or anticancer properties (for example fucoidan) or show antithrombotic (antiplatelet) effects, to reduce blood viscosity, and function as a volume expander in hypovolaemia (for example carrageenans or dextran).

As already mentioned, PPS is very heterogenous and the present invention comprises any embodiments of PPS for example PPS comprising an acetyl group instead of a sulfate next to the glucuronic acid of the poly- or oligosaccharide (Fig. 1b), wherein the ratio of sulfate to acetyl group is for example 6:4 to 4:6 and the degree of sulfation is about 80 to about 100 %, 85 to 95 %, more than 90 %, more than 95 %, or more than 97 %.
An esterified poly- or oligosaccharide such as PPS is for example a sulfated poly- or oligosaccharide, and deesterifying a poly- or oligosaccharide such as PPS means for example desulfating the poly- or oligonucleotide such as PPS for example in a biological sample.

Polysaccharides are polymeric carbohydrate molecules composed of long chains of monosaccharide units bound together by glycosidic linkages and give on hydrolysis the constituent monosaccharides or oligosaccharides. An oligosaccharide is a carbohydrate polymer containing a small number (typically three to nine) of monosaccharides, which are the most basic units of carbohydrates.

The detection and/or quantification of esterified poly- or oligosaccharides in a biological sample follows the general principle shown in the present invention for example based on the detection and/or quantification of PPS, its metabolites and/or its derivatives. The method of detecting and/or quantifying a pentosan polysulfate sodium (PPS), its metabolites, and/or its derivatives in a biological sample comprises for example the following steps:

a) adding a base to a biological sample for alkaline deesterifying of PPS, its metabolite, and/or its derivatives at a basic pH,
b) adding a glycoside hydrolase (GH) to the deesterified sample, digesting the sample with the GH and filtering the sample with an ultrafiltration filter, or filtering the deesterified sample with an ultrafiltration filter, adding a GH to the sample on the filter, digesting the sample with the GH and filtering the sample again with the ultrafiltration filter, and
c) subjecting the filtrate of b) to liquid chromatography (LC)-mass spectroscopy (MS), LC-MS-MS, comprising one or more internal standards.

The method may comprise any additional centrifugation or filtration step such as an ultrafiltration step for example to isolate the products of the enzymatic digestion of step b) and/or to remove side products such as protein and/or oligopeptide which are for example produced in step a). For example, the method of the present invention may comprise an additional step following step a), wherein the deesterified sample is heated at neutral pH, or ammonium sulfate is added to the desulfated sample and the sample is centrifuged for removing residues. The method may comprise alternatively or additionally a further optional step following step a) to stop the deesterifying for example desulfating step by neutralization of the sample. The method may optionally comprise any other reaction to stop the deesterification for example desulfation.
The flow diagram of Fig. 6 shows an example of the different method steps of the present invention, wherein not all steps are essential, but optional, and the steps can be combined in different order, i.e., the numbering of steps described in the following is not definite or binding:

A sample (e.g., fresh or thawed) is used in the present method and in an optional first step cells and/or debris are removed for example by membrane filtration using a filter having a pore size of e.g., about 0.2 – 0.8, about 0.3 – 0.7, about 0.4 – 0.6, about 0.5 or about 0.45 μm.

Once the base is added to the sample for deesterifying the esterified poly- and/or oligosaccharide such as PPS, an optional second step follows, wherein proteins such as oligopeptides are removed which arise during the alkaline hydrolysis of the sugar esters for example by cleaving peptide bonds between amino acids. The proteins are removed for example by adding of proteinase K or passing the sample over an immune affinity matrix for removing for example albumin, immunoglobulin, and/or transferrin.

In an optional third step amino acids and short oligopeptides which arise for example during the proteinase K digestion of the second step are separated from the oligosaccharides for example by ultrafiltration using a filter having a cut-off MW of 5 to 10 kDa.

In a forth step the ester groups of the poly- and/or oligosaccharides such as PPS are separated from the poly- and/or oligosaccharides during the deesterification step, once the base is added, at alkaline pH. The sample is incubated for example with 0.3 M NaOH and/or KOH for 30 min. at 95 °C, or the sample is lyophilized and reconstituted with water. The esters separated from the poly- and/or oligosaccharides are sulfate-, phosphate-, and/or acetyl-esters, sulfate and/or acetyl-groups of amino sugars.

In a fifth step the remaining sugar backbones of the poly- and/or oligosaccharides, whereof the esters have been separated in step four, are digested with one or more enzymes for example specific glycoside hydrolases (GH) such as GH10 xylanases, GH11 xylanase, GH30 xylanase and/or β-xylanase.

In a sixth step one or more internal standards are added to the sample depending on the number of oligosaccharide products which are expected to be determined, i.e., detected and/or quantified. In addition, blanks and reference standards with or without internal standard are prepared.

In an optional seventh step high molecular weight products are removed from the sample by ultrafiltration having a cut-off MW of 5 to 10 kDa for example to avoid that these products clog the LC unit or interfere with mass spectrometry.

In an eighth step the sample is subjected to LC-MS-MS to detect and/or quantify the oligosaccharide products and thus, the esterified poly- and/or oligosaccharide in the sample. Alternatively, the oligosaccharide products in the sample are detected and/or quantified by reductive amination of the oligosaccharides with an acidic fluorescent dye such as APTS and analysed by CE-LIF (capillary electrophoresis and laser induced fluorescent detection). In this case internal standards may be
omitted or must have a reducible end group. In another alternative, the sample is analysed in CE-MS-MS (capillary electrophoresis followed by mass spectrometry).

In an embodiment of the present invention the pH is adjusted to 13 with a base such as NaOH or KOH and the sample is lyophilized to deesterify the esterified poly- and/or oligosaccharides such as PPS.

In general (e.g., in step a)) the pH of the sample is adjusted to 7 to 14, 8 to 12, 9 to 10, or to 8, 9, 10, 11, 12, or 13 for alkaline esterification such as desulfation. The pH is adjusted by adding a base, which is for example a hydroxide of an alkali or an alkaline earth metal such as NaOH, KOH, Ca(OH)\(_2\), etc., or a mixture of bases. The concentration of the base added to the sample is for example about 0.1 to 1 M, about 0.2 to 0.9 M, about 0.3 to 0.8 M, about 0.4 to 0.7 M, about 0.6 to 0.7 M, or about 0.1, about 0.2, about 0.3, about 0.5, about 0.6, about 0.8 or about 1 M.

For desulfating the poly- and/or oligosaccharides such as PPS, its metabolite and/or its derivative the sample is heated to about 50 to 95 °C, about 60 to 85°C, or about 70 to 80 °C, about 95 °C or is even boiled at 100 °C for about 5 to 45 min., about 10 to 40 min., about 15 to 20 min., or about 5, about 10, about 15, about 20, about 30, about 45 or about 60 min. after adding the base.

In some embodiments the sample was incubated with 0.3 or 0.5 M NaOH and/or KOH for 30 min. at 95 or 100 °C.

The deesterification of the poly- and/or oligosaccharide such as PPS is stopped for example by cooling the sample, e.g., on ice, to ambient temperature and/or by neutralizing the pH of the sample for example by adding an acid.

When the esterified poly- and/or oligosaccharide such as PPS and/or its metabolites and/or its derivatives are deesterified such as desulfated, the sugar backbone, e.g. a deesterified glucuronoxylan, remains, which is specifically degraded by addition of an enzyme such as a glycoside hydrolase [GH; e.g., Pollet et al., Critical Review in Biotechnology, 2010, 30(3): 176-191; Juturu and Wu, Appl. Biochem. Biotechnol. (2014) 174: 81-92], wherein one or more enzymes are added to the sample at the same time or successively. GH (also called glycosidases or glycosyl hydrolases) assist in the hydrolysis of glycosidic bonds in complex sugars. A classification of GHs was developed based on similarities of the catalytic domain sequences and GHs are listed in the Carbohydrate-Active enZYmes (CAZy) database. Xylanases have been classified in this database in different families such as 5, 8, 10,
11, 16, 26, 30, 43 and 62. Families 16 and 62 contain for example bifunctional enzymes which possess two distinct catalytic domains (Flint et al., 1993; Hernandez et al., 2001), like for family 43. In family 26, xylanases act on β-(1,3) bonds instead of β-(1,4). Several xylanases from GH5 subfamilies have been reassigned to family 30 (St. John et al., 2010). β-(1,4)-acting xylanases with a unique catalytic domain are for example characteristic for families 5, 8, 10, 11, and 30.

The GH families such as GH10, GH11 (Paes et al., Biotechnology Advance 30, (2012), 564-592) or GH30 (also known as former GH5), comprise different enzymes, wherein GH10 comprises for example a xylanase, an endo-1,3-beta xylanase, and a cellobiohydrolase, respectively. The families further comprise for example GH10 xylanase, GH11 xylanase, GH30 xylanase, e.g., of *Erwinia chrysanthemi*, or a β-xylanosidase. These enzymes may by be added to the sample in combination, e.g., GH10 xylanase and GH11 xylanase, GH10 xylanase and GH30 xylanase, GH11 xylanase and GH30 xylanase, or GH10 xylanase, GH11 xylanase, and/or GH30 xylanase and the β-xylanosidase. In some embodiments the combination comprises an exo-xylanase which degrades xylose from the non-reducing end, whereas the β-xylanosidase degrades the xylose from the reducing end.

The specific restriction patterns (cleavage sites) of the above mentioned GH xylanases and the β-xylanosidase are shown in Fig. 3. Depending on the enzyme or combination of enzymes used for the enzymatic digestion of the sugar backbone, e.g., in step b), different products (oligopeptide products) are obtained in the sample such as xylobiose, xylotriose and/or xylotetraose as shown in Fig. 4, e.g., the combination of GH30 xylanase and the β-xylanosidase as well as the combination of GH30 and GH10 xylanases results in a xylobiose, and the combination of GH30 and GH11 xylanases results in a xylotriose. This shows that depending on the selection and combination of available enzymes, uniform and characteristic oligosaccharide products are received. The xylobiose, the xylotriose and/or the xylotetraose is with or without a side chain such as MeGlcA. These products are detected and/or quantified for example in the LC-MS-MS, which requires internal standards for the detection and/or quantification of the oligosaccharide products of the enzymatic digestion for example with GH xylanase and/or β-xylanosidase. Alternatively, the products are detected and/or quantified by CE-LIF or CE-MS-MS.

The internal standard molecule is chemically similar to the product of the enzymatic digestion of the esterified poly- or oligosaccharide such as PPS, i.e., for example chemically similar to xylobiose, xylotriose and/or xylotetraose as shown in Fig. 4, regarding liquid chromatography (LC) but distinguishable by mass spectroscopy (MS). In an embodiment these oligosaccharides are chemically
modified for example by dehydrating 4-O-methyl glucuronic acid (MeGlcA) to hexenuronic acid, forming an acetal with methanol or ethanol at the reducing end of the oligosaccharide product or introduction of an amino group and/or a double bond by reductive amination. The oligosaccharide for producing the internal standard is for example isolated from the sample, i.e., an oligosaccharide product, chemically modified and added to the LC-MS-MS, or the oligosaccharides for the production of the internal standard are produced in parallel to the production of the esterified poly- or oligosaccharide such as PPS and are isolated in an intermediate state(e.g., Fig. 5e). Fig. 5 shows some examples of internal standards for the LC-MS-MS, which are for example based on a branched xylolobiose as shown in Fig. 4a. For example useful internal standards are derivatives of the diagnostic oligosaccharide like methyl- or acetylacetals or amines obtained by reductive amination. Also commercially available natural or modified oligosaccharides are suitable as internal standards for example acarbose or raffinose (Fig. 5d and 5e).

The present invention further refers to a kit for the detection and/or quantification of PPS, its metabolite and/or its derivatives, or any other esterified, e.g., sulfated, poly- and/or oligosaccharide, its metabolite and/or derivative based on the method of the present invention. The kit comprises for example a base for the deesterifying, e.g., desulfating, reaction of PPS, a neutralizing acid, one or more enzymes such as a GH xylanases and optionally an ultrafiltration filter. Further the kit optionally contains internal standards and reference standards. The kit is used for the preparation of a biological sample to be measured for example in a LC-MS-MS, CE-LIF or CE-MS-MS.
Claims

1. Method of detecting and/or quantifying a pentosan polysulfate sodium (PPS) and/or its metabolites in a biological sample comprising the following steps:
   a) adding a base to the sample for alkaline deesterifying of PPS and/or its metabolites at a basic pH,
   b) adding a glycoside hydrolase (GH) to the desulfated sample, digesting the sample with the GH and filtering the sample with an ultrafiltration filter, or filtering the deesterified sample with an ultrafiltration filter, adding a GH to the sample on the filter, digesting the sample with the GH and filtering the sample again with the ultrafiltration filter, and
   c) subjecting the filtrate of b) to liquid chromatography(LC)-mass spectroscopy (MS) (LC-MS-MS), comprising one or more internal standards, capillary electrophoresis and laser induced fluorescent detection (CE-LIF), or capillary electrophoresis followed by mass spectrometry (CE-MS-MS).

2. Method according to claim 1 comprising an additional step following step a), wherein the deesterified sample is heated at neutral pH, or ammonium sulfate is added to the deesterified sample and the sample is centrifuged for removing residues.

3. Method according to claim 1 or 2, wherein the base is NaOH or KOH.

4. Method according to any one of claims 1 to 3, wherein the pH is 7 to 14, 8 to 12, 9 to 10, or 13.

5. Method according to any one of claims 1 to 4, wherein the sample of step a) is boiled for 5 to 45 min., 10 to 40 min., 15 to 20 min., or 30 min. after adding the base.

6. Method according to claim 5, wherein the deesterifying of step a) is stopped by cooling the sample to ambient temperature and/or neutralizing the pH of the sample.

7. Method according to any one of claims 1 to 6, wherein the GH is GH10 xylanase, GH11 xylanase, GH30 xylanase, and/or a β-xylanase or any combination thereof.
8. Method according to claim 7, wherein the combination is selected from the group consisting of GH10 xylanase and GH11 xylanase, GH10 xylanase and GH30 xylanase, GH11 xylanase and GH30 xylanase, and GH30 xylanase and the β-xylosidase.

9. Method according to any one of claims 1 to 8, wherein the internal standard is an oligosaccharide which is chemically modified.

10. Method according to claim 9, wherein the oligosaccharide is a xylobiose, a xylotriose, a xylotetraose, raffinose and/or acarbose.

11. Method according to claim 9 or 10, wherein the oligosaccharide is modified with methanol or ethanol forming an acetal or by reductive amination introducing an amino group.

12. Method according to any one of claims 1 to 11, wherein the biological sample is selected from the group consisting of plasma, serum, blood, urine, body fluid and tissue extract.

13. Kit for preparing a biological sample for detecting and/or quantifying PPS and/or its metabolite in a sample according to the method of one of claims 1 to 12 comprising a base for the deesterifying reaction of PPS, one or more enzymes such a GH xylanase and a filter such as an ultrafiltration filter.

14. The kit of claim 13 further comprising an internal standard and/or a reference standard.
Fig. 1a Chemical structure of PPS

Fig. 1b Specific embodiment of PPS

\[ R = \text{SO}_3\text{Na}, \]
\[ G = \text{SO}_3\text{Na} \text{ or CO-CH}_3 \text{ (acetate)} \]

Fig. 2 Esterified poly- and oligosaccharides

a) PI-88

\[ R = \text{SO}_3\text{Na} \text{ or H} \]
\[ n = 0 - 4 \]
b) Dextran sulfate

\[
\begin{align*}
\alpha-1,6 & + \alpha-1,3 \\
\end{align*}
\]

\( R = \text{SO}_3\text{Na or H} \)

c) Fucoidan
d) Carrageenan

\[
\text{Carrageenan C}
\]

of D-galactose of (3,6)-anhydro-D-galactose

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e) Chitin

\[
\text{Chitin}
\]

f) Chitosan

\[
\text{Chitosan}
\]
Fig. 3 Cleavage sites of different glycoside hydrolases (GH) and a beta-xylosidase

a) GH10 xylanase

b) GH11 xylanase

c) GH30 xylanase

d) Beta-xylosidase
Fig. 4 Products of the enzymatic digestion

a) Xylobiose

![Chemical structure of xylobiose](image)

or

![Alternative chemical structure of xylobiose](image)

2-O-{4-O-methyl-α-D-glucopyranuronosyl}-6-D-xylpyranosyl-1,4-D-xylose

b) Xylotriose

![Chemical structure of xylotriose](image)

or

![Alternative chemical structure of xylotriose](image)

1,4-β-D-glucopyranosyl-[2-O-{4-O-methyl-α-D-glucopyranuronosyl}-β-D-xylpyranosyl]-1,4-D-xylose

c) Xylotetraose

![Chemical structure of xylotetraose](image)
1,4-β-D-glucopyranosyl-[2-O-(4-O-methyl-α-D-glucopyranuronosyl)-β-D-xylopyranosyl]-1,4-β-D-xylopyranosyl-1,4-D-xylose,

**Fig. 5** Internal standards for LC-MS-MS

a) 3-O-acetyl-2-O-(4-O-methyl-α-D-glucopyranuronosyl)-1,4-D-xylopyranosyl-1,4-D-xylose

or

b) 2-O-(hexenuronosyl)-6-D-xylopyranosyl-1,4-D-xylose

c) Ethylacetal

d) Introduction of an amino group
or

Acarbose

e) Raffinose (O-D-Galaktopyranosyl-(1α-6)-O-D-Glucopyranosyl-(1α-2β)-O-D-Fructofuranosid)
Fig. 6 Flow diagram presenting embodiments of the present invention

1. Fresh or thawed biological sample
2. Remove cells and debris by filtration
3. Remove proteins
4. Remove low molecular weight substances
5. Remove ester groups from polysaccharides at basic pH
6. Digest target polysaccharide with specific glycoside hydrolases
7. Add internal standard
8. Remove high molecular weight substances
9. Separate oligosaccharides by liquid chromatography

Apply mass spectroscopy to separate, fragment, identify and quantify oligosaccharide specific for PPS compared to internal standard

Calculate concentration of PPS and metabolites in the sample
**A. CLASSIFICATION OF SUBJECT MATTER**

INV. A61K31/737 C12N9/24 C08B37/00

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)

A61K C12N 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 practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBASE, COMPENDEX, FSTA, INSPEC

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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<th>Category*</th>
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<th>Relevant to claim No.</th>
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Further documents are listed in the continuation of Box C. See patent family annex.

**Date of the actual completion of the international search**

7 June 2016

**Date of mailing of the international search report**

24/06/2016

Name and mailing address of the ISA/ European Patent Office, P.B. 5018 Patentlaan 2 NL - 2280 HV Rijswijk Tel: (+31-70) 340-3040, Fax: (+31-70) 340-3016

Authorized officer

Gall-Truchot, A
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<td>WO 90/06954 A1 (UNIV SYDNEY [AU]) 28 June 1990 (1990-06-28) the whole document abstract page 4, line 13 - page 5, line 10 page 5, line 28 - page 6, line 6 claims 8, 13-17, 21-22 claims 27, 30-31, 34, 37-38</td>
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<td>YUAN Q ET AL: &quot;PILOT-PLANT PRODUCTION OF XYLO-OLIGOSACCHARIDES FROM CORNCOB BY STEAMING, ANZYMATIC HYDROLYSIS AND NANOFLTRATION&quot;, JOURNAL OF CHEMICAL TECHNOLOGY AND BIOTECHNOLOGY, JOHN WILEY &amp; SONS LTD, UNITED KINGDOM, vol. 79, no. 10, 1 October 2004 (2004-10-01), pages 1073-1079, XP001235875, ISSN: 0268-2575 the whole document abstract 2.3 Extraction and purification of xylo-oligosaccharides (pages 1074-1075) 2.4.2 Xylo-oligosaccharides assays -----</td>
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| X        | CN 103 254 625 A (QINGDAO YONGZHUO PLASTIC PROD) 21 August 2013 (2013-08-21) the whole document English abstract: The invention provides a manufacturing process for a modified bamboo fiber used for nylon. According to the invention, a bamboo cane is minced and then soaked in sodium hydroxide; then cellulose and xylanase are added for enzymatic hydrolysis; filtration is carried out after complete enzymatic hydrolysis; and an obtained filter residue is soaked in a glutaraldehyde solution, and the modified bamboo fiber is obtained after soaking. Addition of the modified bamboo fiber into nylon enables toughness, wear resistance and anti-aging performance of the nylon to be improved and an application scope of the nylon to be broadened. ----- -/--
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<td>WO 2013/002708 A1 (STORA ENSO OYJ [FI]; GAROFF NIKLAS [SE]; DANIELSSON CARL-OLA [SE]; EKM) 3 January 2013 (2013-01-03) the whole document abstract example 1, on page 10</td>
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