Method for determining the composition of the sugar moiety of a sugar containing compound

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(54) **Title**: METHOD FOR DETERMINING THE COMPOSITION OF THE SUGAR MOIETY OF A SUGAR CONTAINING COMPOUND

(57) **Abstract**: The present invention relates to methods of labeling sugar moieties of sugar containing compounds including glycopeptides. The compounds presented in the present invention facilitate reliable detection of sugar moieties of sugar containing compounds by a combination of spectroscopy methods. The invention further relates to a reference library comprising fragmentation patterns for *in silico* identification of compounds. In addition the present invention relates to a kit of parts comprising the compounds of the present invention *inter alia*.

Method for determining the composition of the sugar moiety of a sugar containing compound

Field of invention

The present invention relates to methods for determining the composition of the sugar moiety of a sugar containing compound. The inventors have identified derivatization agents for sensitive identification and characterization of sugar moieties of sugar containing compounds using chromatography in combination with fluorescence spectrometry and/or mass spectrometry *inter alia*. The invention further relates to mass spectrometry libraries and kits for determining the composition of the sugar moiety of a sugar containing compound using the methods described herein.

Background of invention

Post-translational modifications (PTM's) are important for life. Protein glycosylation is one type of PTM, which is acknowledged as one of the major post-translational modifications. N-glycosylation is the most abundant PTM and N-glycans are present on 30% of all known proteins. If a protein is glycosylated it is known as a glycoprotein.

The glycan structure has significant effects on protein folding, conformation, distribution, stability and activity of the protein. In one example a missing or incorrect glycan pattern may result in protein degradation instead of protein exportation from the cell (Cacan & Verbert, 1999). The protein glycoforms influence glycoprotein activity or toxicity (Goh et al., 2014).

Many therapeutic proteins are glycoproteins. Two of the major challenges of glycoprotein production are 1) controlling the glycan pattern and 2) obtaining a homogeneous population of a therapeutic glycoprotein during batch fermentation. Currently the glycan pattern and glycan homogeneity of a therapeutic glycoprotein are near impossible to predict and may be influenced by a multitude of factors including fermentation conditions, process parameters, changes in cell growth and/or cell density. Consequently comprehensive glycosylation profiling before, during, and after batch processing is crucial to obtain a pharmaceutical grade glycoprotein product of therapeutic glycoproteins. In addition the confirmation and the proportion of individual glycans is important for drug approval by public authorities such as FDA.

Most of the current screening methods for detection and characterization of glycans are based on retention time and fluorescence detection using a separation based on liquid chromatography (LC). This fluorescence-based analysis yields a too inadequate characterization of the glycan pattern to allow unequivocal identification. Combined LC-fluorescence detection coupled with mass spectrometry (MS) is more definitive, but common derivatization agents are either poorly fluorescent or do not ionize well in the MS; none of the existing derivatization agents yields both good fluorescent sensitivity and a good MS sensitivity.

The current industry standard method using 2-aminobenzamide derivatisation (2-AB) does not perform well in MS (Townsend, Lipniunas, Bigge, Ventom, & Parekh, 1996). Other methods using TMR, FITC etc. do not separate properly in HPLC systems and are thus unsuitable for LC-MS.

As a consequence the current derivatization agents are inadequate for the complete characterization glycan pattern analysis.

Summary of invention

The inventors of the present invention have identified derivatization agents for sensitive identification and characterization of glycans using chromatography means combined with mass spectrometry and/or fluorescence spectrometry. Some advantages of the compounds of the present invention over standard derivatization agents are:

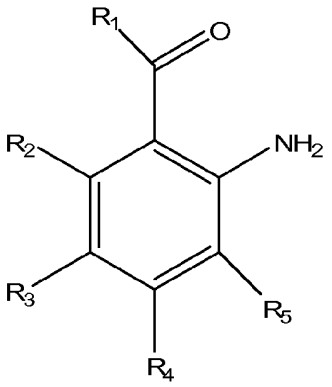
- The compounds of the present invention maintain good fluorescence spectrometry properties while yielding high mass spectrometry sensitivity.
- The compounds of the present invention ionize better, which gives a higher ion current and better MS sensitivity.
- A higher ion current also means that MS/MS can be used for rapid and reliable identification.
- With the improved MS signal even small glycan moieties can be accurately identified with MS and/or MS/MS.
- High MS sensitivity allows multiplexing (e.g. by isotope labeling) and thereby increases the analysis through-put and improves quantification.
- The compounds of the present invention with superior MS signal could work as a drop-in substitute compounds in labs which currently using 2-AB (2-Aminobenzamide) or 2-AA (2-Aminobenzamide) on a routine basis.

Thus in a main aspect the present invention concerns a method for determining the composition of the sugar moiety of a sugar containing compound, said method comprising the steps of:

a) providing a sugar containing compound,

b) optionally liberating the sugar moiety from the sugar containing compound,

c) reacting the sugar moiety with a compound having the general formula (I):



Formula (I)

wherein

R_1 is -OH or $-NH_2$;

R_2, R_3, R_4, and R_5 are individually selected from the group consisting of -H, halogen, C_{1-10} alkyl, C_{1-10} haloalkyl, C_{1-10} aminoalkyl, C_{1-10} nitroalkyl, C_{1-10} hydroxyalkyl, C_{1-10} alkenyl, C_{1-10} haloalkenyl, C_{1-10} aminoalkenyl, C_{1-10} nitroalkenyl, C_{1-10} hydroxyalkenyl, C_{1-10} alkynyl, C_{1-10} haloalkynyl, C_{1-10} aminoalkynyl, C_{1-10} nitroalkynyl, and C_{1-10} hydroxyalkynyl,

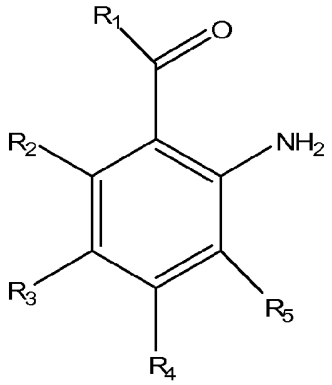
thus rendering a labeled sugar moiety,

and wherein the compound of formula (I) is not 2-AB (2-aminobenzamide), 2-AA (2-aminobenzamide), 2-amino-5-bromobenzoic acid or 2-amino-4-chlorobenzoic acid;

d) determining the structure of said labeled sugar moiety using analysis means.

To facilitate reliable, rapid, and convenient identification of sugar moieties the present invention further provides a mass spectrometry reference library comprising the fragmentation pattern of sugar moieties labeled with compounds of the present invention. Thus in one aspect the present invention concerns a reference library

comprising spectra of a sugar moiety labeled with a compound having the general formula (I),



Formula (I)

wherein

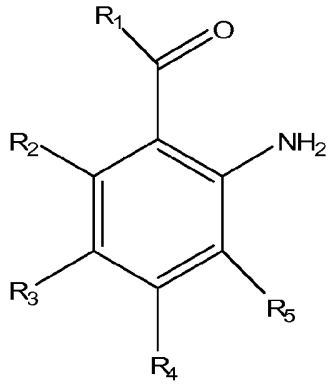
R_1 is -OH or -NH_2;

R_2, R_3, R_4, and R_5 are individually selected from the group consisting of -H, halogen, C_{1-10} alkyl, C_{1-10} haloalkyl, C_{1-10} aminoalkyl, C_{1-10} nitroalkyl, C_{1-10} hydroxyalkyl, C_{1-10} alkenyl, C_{1-10} haloalkenyl, C_{1-10} aminoalkenyl, C_{1-10} nitroalkenyl, C_{1-10} hydroxyalkenyl, C_{1-10} alkynyl, C_{1-10} haloalkynyl, C_{1-10} aminoalkynyl, C_{1-10} nitroalkynyl, and C_{1-10} hydroxyalkynyl,

and wherein the compound of formula (I) is not 2-AB (2-aminobenzamide), 2-AA (2-aminobenzamide), 2-amino-5-bromobenzoic acid or 2-amino-4-chlorobenzoic acid.

A kit comprising compounds as described herein and a mass spectrometry reference library may be the preferred way to distribute the present invention commercially. Thus in an aspect the present invention concerns a kit of parts comprising

a. a composition comprising a compound represented by formula (I) as described herein,



Formula (I)

wherein

R_1 is -OH or -NH_2;

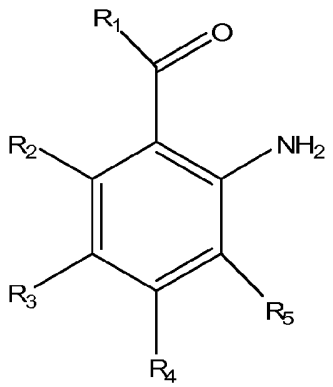
R_2, R_3, R_4, and R_5 are individually selected from the group consisting of -H, halogen, C_{1-10} alkyl, C_{1-10} haloalkyl, C_{1-10} aminoalkyl, C_{1-10} nitroalkyl, C_{1-10} hydroxyalkyl, C_{1-10} alkenyl, C_{1-10} haloalkenyl, C_{1-10} aminoalkenyl, C_{1-10} nitroalkenyl, C_{1-10} hydroxyalkenyl, C_{1-10} alkynyl, C_{1-10} haloalkynyl, C_{1-10} aminoalkynyl, C_{1-10} nitroalkynyl, and C_{1-10} hydroxyalkynyl,

and wherein the compound of formula (I) is not 2-AB (2-aminobenzamide), 2-AA (2-aminobenzamide), 2-amino-5-bromobenzoic acid or 2-amino-4-chlorobenzoic acid,

b. a reference library as described herein, and

c. instructions on how to label a sugar moiety of a sugar containing compound according to the method of any one of the preceding claims.

Additionally the present invention relates to the use of the compounds described in the present invention for derivatization of a sugar moiety of a sugar containing compound. Thus an aspect of the present invention concerns the use of a compound having the general formula (I) for derivation of a sugar moiety of a sugar containing compound as described herein:



Formula (I)

a)

wherein

R_1 is -OH or -NH_2;

R_2, R_3, R_4, and R_5 are individually selected from the group consisting of -H, halogen, C_{1-10} alkyl, C_{1-10} haloalkyl, C_{1-10} aminoalkyl, C_{1-10} nitroalkyl, C_{1-10} hydroxyalkyl, C_{1-10} alkenyl, C_{1-10} haloalkenyl, C_{1-10} aminoalkenyl, C_{1-10} nitroalkenyl, C_{1-10} hydroxyalkenyl, C_{1-10} alkynyl, C_{1-10} haloalkynyl, C_{1-10} aminoalkynyl, C_{1-10} nitroalkynyl, and C_{1-10} hydroxyalkynyl,

and wherein the compound of formula (I) is not 2-AB (2-aminobenzamide), 2-AA (2-aminobenzamide), 2-amino-5-bromobenzoic acid or 2-amino-4-chlorobenzoic acid.

Description of the Drawings

Figure 1: Fluorescence and MS data of 4TMB (2-amino-4-(trifluoromethyl)benzoic acid) and the reference 2-AB.

A: Fluorescence (FLR) data showing the pattern of labeled mannose sugars released from the glycoprotein RNase B. It shows how the 4TMB signal shows the same pattern of sugars as 2-AB. Solid line: 4TMB dashed line: 2-AB.

B: MS data clearly shows that signal from 4TMB labeled Man-5 sugar is higher than the reference compound 2-AB (950000 for 4TMB and 128000 for 2AB). Solid line: 4TMB dashed line: 2-AB. All is on same scale.

Figure 2: The reference compound - FLR and MS data of the 2-AB labeled Man-5 sugar from RNase B

XIC: MS peak corresponding to 2-AB labeled Man-5 sugar. Area under the peak: 127757 signal to noise:22

FLR: Fluorescence chromatogram of 2-AB labeled mannose sugars. Selected peak is Man-5. Area under the peak: 447737240. Signal to noise:489

Figure 3: FLR and MS data of the 4TMB (2-amino-4-(trifluoromethyl)benzoic acid) labeled Man-5 sugar from RNase B XIC: MS peak corresponding to 4TMB labeled Man-5 sugar. Area under the peak: 954817 with signal to noise:208

FLR: Fluorescence chromatogram of 4TMB labeled mannose sugars. Selected peak is Man-5. Area under the peak: 21668418 with signal to noise:345

Figure 4: FLR and MS data of the 4Me (2-amino-4-methylbenzoic acid) labeled Man-5 sugar from RNase B

XIC: MS peak corresponding to 4Me labeled Man-5 sugar. Area under the peak: 196883 with signal to noise: 37

FLR: Fluorescence chromatogram of 4Me labeled mannose sugars. Selected peak is Man-5. Area under the peak: 78475847 with signal to noise:539.

Figure 5: FLR and MS data of the A5F (2-amino-5-fluorobenzoic acid) labeled Man-5 sugar from RNase B

XIC: MS peak corresponding to A5F labeled Man-5 sugar. Area under the peak: 109521 with signal to noise:19

FLR: Fluorescence chromatogram of A5F labeled mannose sugars. Selected peak is Man-5. Area under the peak: 37107254 with signal to noise:332

Figure 6: FLR and MS data of the 5Cl (2-amino-5-chlorobenzoic acid) labeled Man-5 sugar from RNase B

XIC: MS peak corresponding to 5Cl labeled Man-5 sugar. Area under the peak: 73933 with signal to noise:11

FLR: Fluorescence chromatogram of 5Cl labeled mannose sugars. Selected peak is Man-5. Area under the peak: 27434449 with signal to noise:197

Figure 7: FLR and MS data of the A4F (2-amino-5-fluorobenzoic acid) labeled Man-5 sugar from RNase B

XIC: MS peak corresponding to A4F labeled Man-5 sugar. Area under the peak: 79927 with signal to noise: 15

FLR: Fluorescence chromatogram of A4F labeled mannose sugars. Selected peak is Man-5. Area under the peak: 12275305 with signal to noise:138

Figure 8: MS data of the 2-AB labeled bovine fetuin.

Figure 9: MS data of the 2-AA labeled bovine fetuin.

Figure 10: MS data of the 4TMB (2-amino-4-(trifluoromethyl)benzoic acid) labeled bovine fetuin.

Detailed description of the invention

The inventors of the present invention have identified derivatization agents that allow rapid and reliable screening of glycan moieties using chromatography combined with mass spectrometry and/or fluorescence spectrometry. Some advantages of the compounds of the present invention over standard assays using 2-AB or 2-AA are:

- The compounds of the present invention maintain good fluorescence spectrometry properties while yielding great mass spectrometry sensitivity.

- The compounds of the present invention ionize better, which gives a higher ion current and better MS sensitivity.
- A higher ion current also means that MS/MS can be used for rapid and reliable identification.
- With the improved MS signal even small glycan moieties can be accurately identified with MS and/or MS/MS.
- High MS sensitivity allows multiplexing (e.g. by isotope labeling) and thereby increases the analysis through-put and improves quantification.
- The compounds of the present invention with superior MS signal could work as drop-in substitute compounds in labs which currently using 2-AB or 2-AA on a routine basis.

Definitions

The term "free sugar" as used herein refers to all monosaccharaides and disaccharides. Free sugars may be added to foods by the manufacturer, cook, or consumer, plus sugars naturally or be present in honey, syrups, and fruit juices.

The term "liberated enzymatically" as used herein refers to an enzymatic hydrolysis and/or cleavage of a glycan moiety covalently attached to the side chains of the amino acid residue of a peptide. The glycan moiety may be N-linked, O-linked, or C-linked.

The term "liberated chemically" as used herein refers to a chemical hydrolysis and/or cleavage of a glycan moiety covalently attached to the side chains of the amino acid residue of a peptide. The glycan moiety may be N-linked, O-linked, or C-linked.

The term "liberated" as used herein refers to a hydrolysis and/or cleavage of a glycan moiety covalently attached to the side chains of the amino acid residue of a peptide. The glycan moiety may be N-linked, O-linked, or C-linked.

The term "degree of polymerization" as used herein refers to the number of monomeric units in a macromolecule an oligomer molecule, a block, or a chain e.g. the number of glycan molecules in a glycan moiety including all side chains. Degree of polymerization and glucose units is used interchangeably herein.

The term "retention time index" as used herein refers to a measure of the retention of a solute relative to the retention of a set of standards with known properties.

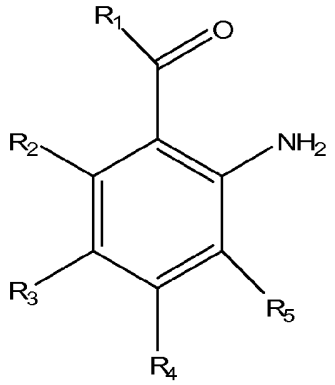
Sugar derivatization agents

Thus in a main aspect the present invention concerns a method for determining the composition of the sugar moiety of a sugar containing compound, said method comprising the steps of:

a) providing a sugar containing compound,

b) optionally liberating the sugar moiety from the sugar containing compound,

c) reacting the sugar moiety with a compound having the general formula (I):



Formula (I)

wherein

R_1 is -OH or $-NH_2$;

R_2, R_3, R_4, and R_5 are individually selected from the group consisting of -H, halogen, C_{1-10} alkyl, C_{1-10} haloalkyl, C_{1-10} aminoalkyl, C_{1-10} nitroalkyl, C_{1-10} hydroxyalkyl, C_{1-10} alkenyl, C_{1-10} haloalkenyl, C_{1-10} aminoalkenyl, C_{1-10} nitroalkenyl, C_{1-10} hydroxyalkenyl, C_{1-10} alkynyl, C_{1-10} haloalkynyl, C_{1-10} aminoalkynyl, C_{1-10} nitroalkynyl, and C_{1-10} hydroxyalkynyl,

thus rendering a labeled sugar moiety,

and wherein the compound of formula (I) is not 2-AB (2-aminobenzamide), 2-AA (2-aminobenzamide), 2-amino-5-bromobenzoic acid or 2-amino-4-chlorobenzoic acid;

d) determining the structure of said labeled sugar moiety using analysis means.

In an embodiment :

R_1 is OH or NH_2;

R_2, R_3, R_4, and R_5 are individually selected from the group consisting of

-H, -F, -Cl, -OH, $-NH_2$,

$-CH_3$, $-CH_2CH_3$, $-CH_2CH_2CH_3$, $-CH_2CH_2CH_2CH_3$

$-CH_2F$, $-CHF_2$, $-CF_3$,

$-CH_2Cl$, $-CHCl_2$, $-CCl_3$,

$-CH_2OH$, $-CH_2NH_2$,

$-CH_2CH_2F$, $-CH_2CHF_2$, $-CH_2CF_3$,

$-CH_2CH_2Cl$, $-CH_2CHCl_2$, $-CH_2CCl_3$,

$-CH_2CH_2OH$, and $-CH_2CH_2NH_2$.

In an embodiment:

R_1 of formula (I) is -OH;

R_2 of formula (I) is -H, -F, -Cl, $-CH_3$, $-CF_3$;

R_3 of formula (I) is -H, -F, -Cl, -OH;

R_4 of formula (I) is -H, -F, -Cl, $-CH_3$, $-CF_3$;

R_5 of formula (I) is -H, -F, -Cl, -OH, $-CH_3$, $-CF_3$.

In an embodiment:

R_1 of formula (I) is -OH;

R_2 of formula (I) is -H;

R_3 of formula (I) is -H, -F, -Cl;

R_4 of formula (I) is -H, $-CH_3$, $-CF_3$, -F;

R_5 of formula (I) is -H.

In one embodiment, at least one of R_2, R_3, R_4 and R_5 is selected from the group consisting of $-CH_3$ or a halogen. In some embodiments, at least one of R_4 and R_5 is selected from the group consisting of $-CH_3$ or a halogen and R_2 and R_3 are –H. In one embodiment, the halogen is selected from –Cl or –F, preferably the halogen is –F. Thus in some embodiments the method comprises reacting the sugar moiety with the compound of general formula (I) above, where the compound is a methylated or a halogenated 2-amino-benzoic acid. By 'halogenated' is understood that the compound is substituted in at least one position with at least one halogen. In some embodiments, the substituent is a trifluoromethyl ($-CF_3$).

In particular embodiments, the methylation or the halogenation is on position 4 or 5 and all the other positions of the benzene ring are hydrogen atoms. In particular embodiments, the halogen is selected from chlorine and fluorine. In one embodiment, the halogen is fluorine.

The inventors of the present invention have tested numerous compounds with respect to their FLR and MS signals (table 1). As can be seen in table 1 the following compounds have been tested by the inventors: 2-amino-4-(trifluoromethyl)benzoic acid, 2-amino-6-fluorobenzoic acid, 2-amino-4-methylbenzoic acid, 2-amino-6-methylbenzoic acid, 2-amino-5-(trifluoromethyl)benzoic acid, 2-amino-4-chlorobenzoic acid, 2-amino-6-(trifluoromethyl)benzoic acid, 3-hydroxyanthranilic acid, 5-hydroxyanthranilic acid, 2-amino-3-methylbenzoic acid, 2-amino-3-fluorobenzoic acid, 2-amino-4-fluorobenzoic acid, 2-amino-5-fluorobenzoic acid, 2-amino-3-chlorobenzoic acid, 2-amino-5-chlorobenzoic acid, 2-amino-6-chlorobenzoic acid, 2-amino-3-(trifluoromethyl)benzoic acid, 2-amino-4,6-difluorobenzoic acid, 2-amino-6-fluoro-3-methylbenzoic acid, and/or 2-amino-4-fluorobenzamide. Thus in an embodiment of the method as described herein above the compound of formula (I) is selected from the group consisting of 2-amino-4-(trifluoromethyl)benzoic acid, 2-amino-6-fluorobenzoic acid, 2-amino-4-methylbenzoic acid, 2-amino-6-methylbenzoic acid, 2-amino-5-(trifluoromethyl)benzoic acid, 2-amino-6-(trifluoromethyl)benzoic acid, 3-hydroxyanthranilic acid, 5-hydroxyanthranilic acid, 2-amino-3-methylbenzoic acid, 2-amino-3-fluorobenzoic acid, 2-amino-4-fluorobenzoic acid, 2-amino-5-fluorobenzoic acid, 2-amino-3-chlorobenzoic acid, 2-amino-5-chlorobenzoic acid, 2-amino-6-chlorobenzoic acid, 2-amino-3-(trifluoromethyl)benzoic acid, 2-amino-4,6-difluorobenzoic acid, 2-amino-6-fluoro-3-methylbenzoic acid, and/or 2-amino-4-fluorobenzamide.

As demonstrated in table 1 and in the figures the compounds of 2-amino-4-(trifluoromethyl)benzoic acid, 2-amino-4-methylbenzoic acid, 2-amino-5-fluorobenzoic acid, 2-amino-5-chlorobenzoic acid, and 2-amino-4-fluorobenzoic acid performed very well with respect to the FLR and MS signal properties.

In a particular embodiment the method is as described herein above the compound of formula (I) is selected from the group consisting of 2-amino-4-(trifluoromethyl)benzoic acid, 2-amino-4-methylbenzoic acid, 2-amino-5-fluorobenzoic acid, 2-amino-5-chlorobenzoic acid, 2-amino-4-fluorobenzoic acid.

In an embodiment the method is as described herein above, wherein the compound according to formula (I) is 2-amino-4-(trifluoromethyl)benzoic acid. In an embodiment the method is as described herein above, wherein the compound according to formula (I) is 2-amino-4-methylbenzoic acid. In an embodiment the method is as described herein above, wherein the compound according to formula (I) is 2-amino-5-fluorobenzoic acid. In an embodiment the method is as described herein above, wherein the compound according to formula (I) is 2-amino-5-chlorobenzoic acid. In an embodiment the method is as described herein above, wherein the compound according to formula (I) is 2-amino-4-fluorobenzoic acid.

Multiplexing

When conducting glycan profiling it is advantageous to limit the run-time of the analytical equipment such as analytical equipment comprising an UHPLC and/or HPLC. A shorter run-time results in higher throughput capacity and a more cost-efficient analysis. One way of obtaining shorter run-time is to analyze more than one sample such as two, such as three, such as four samples at the same time using the same analytical equipment. The present invention may solve this problem of analyzing several samples in parallel by labeling two or more samples using two or more compounds of the general formula (I) as described herein or a compound of the present invention in combination with any sugar labeling compound known in the art. Thus in an embodiment the step (c) of the method as described herein above, comprises reacting the sugar moiety with two or more different compounds having the general formula (I).

Another approach for analyzing more than one sample in parallel is to utilize different compound labels. Thus in an embodiment one of the atoms of the compound of formula (I) has been labeled. In an embodiment the labeled atom is an isotope of said atom. In a further embodiment the isotope is selected from the group comprising ^{12}C, ^{14}C, ^{1}H, ^{2}H, ^{3}H, ^{15}O, ^{16}O, ^{17}O, ^{18}O, ^{13}N, ^{14}N, ^{15}N,^{18}F, ^{19}F, ^{76}Br, ^{77}Br, ^{82}Br, ^{127}I, ^{35}Cl, and/or ^{37}Cl.

To facilitate separation of two or more samples being analyzed in parallel the sugar labeling of two or more samples is conducted separately. Thus in an embodiment step a) and/or step b) of the method described herein are separately conducted using at

least two sugar containing compounds as described herein and c) is separately conducted using at least two different compounds of the general formula (I).

Sugar moieties on sugar containing compounds

The present invention may be used to determine the composition of the sugar moiety of any sugar containing compound. In one embodiment the labeled sugar moiety as described herein has a degree of polymerization from 1 to 500 such as from 1 to 40, such as 1 to 30. In another embodiment the labeled sugar moiety has a solubility of between 1 to 500 g/l in water at 20°C and 1 atm. In an embodiment the sugar containing compound is a free sugar as defined herein.

One application of the present invention may be glycoprofiling of sugar moieties of glycopeptides from different origins. In an embodiment the sugar containing compound described herein comprises a glycopeptide, such as a glycopeptide originating from a heterologous expression host. In another embodiment the sugar containing compound is obtained from a biological sample. The sugar containing compound may be fetuin, such as bovine fetuin. The sugar containing compound may be RNase B, such as bovine Bovine Ribonuclease B.

In an embodiment the sugar containing compound comprises a pharmaceutical glycopeptide. In another embodiment the sugar containing compound is a pharmaceutical glycopeptide selected from the group comprising of mucoproteins (e.g. MUC1-20), proteoglycans, antibodies, Fc regions of antibodies, activin, inhibin, ADAM, Alpha 1-antichymotrypsin, Apolipoprotein H, CD70, Asialoglycoprotein, Avidin, B-cell activating factor, 4-1BB ligand, Cholesterylester transfer protein, Clusterin, Colony-stimulating factor, Hemopexin, Lactoferrin, Membrane glycoproteins, Myelin protein zero, Osteonectin, Protein C, Protein S, Serum amyloid P component, Sialoglycoprotein, CD43, Glycophorin, Glycophorin C, Thrombopoietin, Thyroglobulin, Thyroxine-binding proteins, Transcortin, Tumor necrosis factor alpha, Uteroglobin, and Vitronectin.

In a further embodiment the sugar moiety on the sugar containing compound is selected from the group comprising a N-linked glycan, O-linked glycan, a phospho-glycan, or a C-linked glycan, pentose, hexose, amino sugar, and/or a mixture hereof. In a preferred embodiment the sugar moiety on the sugar containing compound is

selected from the group comprising a N-linked glycan, O-linked glycan, C-linked glycan, and/or a combination hereof.

Liberating the sugar moiety of a sugar containing compounds

It may be desirable to liberate the sugar moieties from the sugar containing compound. In an embodiment the sugar moiety of the sugar containing compound is liberated chemically and/or enzymatically. In another embodiment the sugar moiety of the sugar containing compound is liberated chemically such as by hydrazinolysis or alkali/reducing conditions (β-elimination). In another embodiment the sugar moiety of the sugar containing compound is liberated enzymatically such as by use of PNGase F, PNgase A, Endoglycosidase H, Endoglycosidase Hf, Endoglycosidase F, Endoglycosidase D, Endoglycosidase S or O-Glycosidases.

Reducing and labeling the sugar moiety

In order to react the sugar moiety with a compound having the general formula (I) as described herein the sugar moiety may be reduced. In one embodiment the said sugar moiety is reduced, thus rendering a reduced sugar moiety prior to reacting with a compound of the general formula (I). In a further embodiment the reduction is conducted using reductive amination. In another embodiment the reduction is conducted using a reducing agent selected from the group consisting of sodium cyanoborohydride, triacetoxyborohydride, 4-amino-N-[2- (diethylamino)ethyl] benzamide, and 2-picoline borane or other suitable reducing agents. In a preferred embodiment the reductive amination is conducted using 2-picoline borane.

The reduction described above may be carried out in a solvent. In an embodiment the reductive is conducted in a solvent. In another embodiment the reductive amination is conducted in a solvent. In another embodiment the reductive amination is conducted in a solvent wherein said solvent is selected from the group consisting of water, DMSO, acetic acid, acetonitrile, and ethanol or other suitable solvents, or mixtures of one or more of the foregoing. In a preferred embodiment the reductive amination is conducted in a mixture of acetic acid and DMSO. In a more preferred embodiment the reductive amination is conducted in in a (10:1 to 100) mixture of acetic acid:DMSO. In a most preferred embodiment the reductive amination is conducted in in a (3:7) mixture of acetic acid:DMSO.

The temperature at which the sugar moiety is reacted with a compound having the general formula (I) affect the speed of the reaction. The hotter the faster the reaction goes. However sialic acid sugars starts to decompose around 80°C so it may be preferable to stay below this temperature. In an embodiment the step (c) of the present invention comprises reacting the sugar moiety described herein with a compound having the general formula (I) wherein the reaction is conducted at a temperature between 30 to 100°C for 15 minutes to 48 hours while being mixed. In another embodiment the step (c) of the present invention comprises reacting the sugar moiety described herein with a compound having the general formula (I), wherein the reaction is conducted at a temperature between 60°C to 70°C for 1 to 4 hours while being mixed. Longer and/or shorter reaction periods may also be applied as well as other temperature ranges.

Purification, separation and analysis of labeled sugar moieties

Very complex mixtures are often challenging to analyze since the analysis process often yields a very complex chromatogram. If the mixture is too complex peaks may overlap and render insufficient characterization. Thus it may be desirable to purify the sample prior to analysis. Purification of labeled sugars is known in the art. In one embodiment the labeled sugar moiety is purified prior to analysis.

Additionally it may be desirable to separate compounds to avoid overlapping signals. The purpose of separation is to yield an isolated compound *inter alia*. Methods for separating labeled sugar moieties are known in the art such as liquid chromatography, gas chromatography, and/or capillary electrophoresis. Thus in a further embodiment the method described herein further comprises a step of separating said labeled sugar moiety thus rendering an isolated labeled sugar moiety. In an embodiment the step of separating said labeled sugar moiety is conducted using chromatography and/or electrophoresis. In another embodiment the step of separating said labeled sugar moiety is conducted using liquid chromatographic separation. In a further embodiment the step of separating said labeled sugar moiety is conducted using high performance liquid chromatography. In yet an embodiment the step of separating said labeled sugar moiety is conducted using gas chromatography. In a further embodiment the step of separating said labeled moiety is conducted using capillary electrophoresis. Other appropriate methods for separation are known in the art and may be used in the present invention.

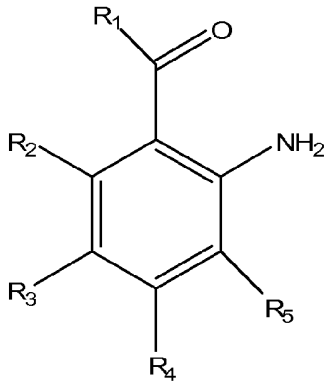
A multitude of analytical means may be used to determining the structure of the labeled sugar moiety using analysis means described in step d) of the present invention. In one embodiment step d) is conducted by optical spectrometry, mass spectrometry, dual mass spectrometry, and/or multi-stage mass spectrometry *inter alia*. In an embodiment the optical spectrometry is UV spectrometry. In another embodiment the optical spectrometry is fluorescence spectroscopy.

In a preferred embodiment the analysis means described herein is selected from the group consisting of high performance liquid chromatography fluorescence, ultra high performance liquid chromatography fluorescence, capillary electrophoresis fluorescence, high performance liquid chromatography mass spectrometry, high performance liquid chromatography tandem mass spectrometry, high performance liquid chromatography multi-stage mass spectrometry, ultra high performance liquid chromatography mass spectrometry, ultra high performance liquid chromatography tandem mass spectrometry, ultra high performance liquid chromatography multi-stage mass spectrometry, capillary electrophoresis mass spectrometry, capillary electrophoresis tandem mass spectrometry, and/or capillary electrophoresis multi-stage mass spectrometry.

Reference library

Two popular approaches for compound identification by mass spectrometry are 1) comparison to authentic standards, and 2) calculation of retention index and/or comparison of fragmentation pattern against a reference library (e.g. the NIST 14 Mass Spectral and Retention Index Library). Obtaining hundreds of different authentic standards may be cumbersome and expensive. On the other hand, a reference library comprising glycan moieties labeled with a compound having the general formula (I) glycan moiety as described herein would facilitate rapid and reliable identification of glycan moieties in complex solutions without the need for obtaining hundreds of authentic standards.

Therefore one aspect of the present invention concerns a reference library comprising a spectrum of a specific sugar moiety labeled with a compound having the general formula (I),



Formula (I)

wherein

R_1 is -OH or -NH_2;

R_2, R_3, R_4, and R_5 are individually selected from the group consisting of -H, halogen, C_{1-10} alkyl, C_{1-10} haloalkyl, C_{1-10} aminoalkyl, C_{1-10} nitroalkyl, C_{1-10} hydroxyalkyl, C_{1-10} alkenyl, C_{1-10} haloalkenyl, C_{1-10} aminoalkenyl, C_{1-10} nitroalkenyl, C_{1-10} hydroxyalkenyl, C_{1-10} alkynyl, C_{1-10} haloalkynyl, C_{1-10} aminoalkynyl, C_{1-10} nitroalkynyl, and C_{1-10} hydroxyalkynyl,

and wherein the compound of formula (I) is not 2-AB (2-aminobenzamide), 2-AA (2-aminobenzamide), 2-amino-5-bromobenzoic acid or 2-amino-4-chlorobenzoic acid.

Using mass spectrometry, tandem mass spectrometry and/or multi-stage mass spectrometry for generating a reference library ensures that the reference library described herein can be used for compound identification using mass spectrometry, tandem mass spectrometry and/or multi-stage mass spectrometry data. In an embodiment the reference library is for use in mass spectrometry, tandem mass spectrometry and/or multi-stage mass spectrometry.

Computer-readable data storage medium comprising a reference library

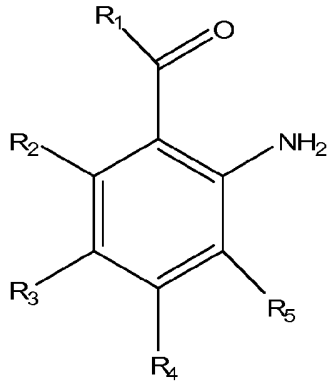
The reference library developed by means of the present invention may be distributed on a computer-readable data storage medium. The computer-readable data storage medium may include software which facilitates rapid comparison of target compound mass spectrometry fragmentation data with a reference library comprising mass spectrometry fragmentation data from authentic standards which have been labeled with a compound having the general formula (I) of the present invention. Thus an aspect of the present invention relates to data storage material comprising a reference

library of sugar moieties which have been labeled with the compound having the general formula (I), wherein the reference library comprises mass spectrometry, tandem mass spectrometry and/or multi-stage mass spectrometry fragmentation information of sugar moieties which have been labeled with a compound having the general formula (I) as described herein.

Kit of parts

In one aspect, the present invention concerns a kit of parts comprising a compound of the formula (I) described herein, a reference library described herein, and instruction for labeling a sugar moiety. In an aspect the present invention relates to a kit of parts, wherein the kit comprises:

a) a composition comprising a compound represented by formula (I),



Formula (I)

wherein

R_1 is -OH or $-NH_2$;

R_2, R_3, R_4, and R_5 are individually selected from the group consisting of -H, halogen, C_{1-10} alkyl, C_{1-10} haloalkyl, C_{1-10} aminoalkyl, C_{1-10} nitroalkyl, C_{1-10} hydroxyalkyl, C_{1-10} alkenyl, C_{1-10} haloalkenyl, C_{1-10} aminoalkenyl, C_{1-10} nitroalkenyl, C_{1-10} hydroxyalkenyl, C_{1-10} alkynyl, C_{1-10} haloalkynyl, C_{1-10} aminoalkynyl, C_{1-10} nitroalkynyl, and C_{1-10} hydroxyalkynyl,

and wherein the compound of formula (I) is not 2-AB (2-aminobenzamide), 2-AA (2-aminobenzamide), 2-amino-5-bromobenzoic acid or 2-amino-4-chlorobenzoic acid,

b) a reference library as described herein, and

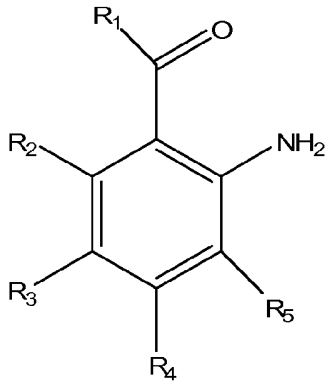
c) instructions on how to label a sugar moiety of a sugar containing compound.

The kit of parts may comprise other reagents to be used in labeling a sugar moiety. In an embodiment the kit described herein further comprises a reducing agent selected from the group consisting of sodium cyanoborohydride, triacetoxyborohydride, 4-amino-N-[2- (diethylamino)ethyl] benzamide, and 2-picoline borane or other suitable reducing agents known in the art.

To facilitate multiplexing as described herein the kit may further comprise a second compound represented by formula (I) described herein. In an embodiment the kit described herein further comprises a second compound represented by formula (I) described herein. In another embodiment the kit described herein further comprise a second compound represented by formula (I) described herein, wherein the second and the first compound represented by formula (I) are different. In another embodiment the kit further comprises any other sugar labeling compound.

Use for derivatization of a sugar moiety

Another aspect of the present invention relates to the use of a compound having the general formula (I) for derivatization of a sugar moiety of a sugar containing compound



Formula (I)

a)

wherein

R_1 is -OH or $-NH_2$;

R_2, R_3, R_4, and R_5 are individually selected from the group consisting of -H, halogen, C_{1-10} alkyl, C_{1-10} haloalkyl, C_{1-10} aminoalkyl, C_{1-10} nitroalkyl, C_{1-10} hydroxyalkyl, C_{1-10} alkenyl, C_{1-10} haloalkenyl, C_{1-10} aminoalkenyl, C_{1-10} nitroalkenyl, C_{1-10} hydroxyalkenyl, C_{1-10} alkynyl, C_{1-10} haloalkynyl, C_{1-10} aminoalkynyl, C_{1-10} nitroalkynyl, and C_{1-10} hydroxyalkynyl,

and wherein the compound of formula (I) is not 2-AB (2-aminobenzamide), 2-AA (2-aminobenzamide), 2-amino-5-bromobenzoic acid or 2-amino-4-chlorobenzoic acid.

Examples

Example 1:

Release of N-Glycans

Using Prozyme Kit or New England Biolabs Kit – 5 or 50 µg of Glycoprotein were incubated in 50min with PNGase F at 37°C according to manufacturer's description. After drying the sample was labeled.

Labeling of sugars

Oligosaccharide standards maltohexose and maltoheptose or released N-Glycan were labeled with the following procedure. At room temperature 9.6 mg of picoline borane (Sigma) is mixed with 56 µL DMSO and 24 µL acetic acid to make the reductive solution. 15 µL of this mixture is added to 1 mg of the labeling dye in question (2-Amino-3-methylbenzoic acid or 2-Amino-4-(trifluoromethyl)benzoic acid etc.). 5 µL of the finished labeling solution is added to an Eppendorf vial with 0.25 µg maltohexose and 0.5 µg maltoheptose (Carbosynth Ltd, UK) dried sugar or released N-Glycan. The vial is vortexed on high for 10 sec. then placed in a thermostated mixer for 2.5 hours at 65°C at 2000 rpm. After the reaction is done the labeled sugars are purified with Prozymes CU cleanup cartridges after the suppliers instructions. The final purified sugars in 25 µL water were diluted with 75 µL 100% Acetonitrile and then analyzed with LC-MS.

LC-MS

The LC-MS analysis were performed on a Ultimate 3000 (Thermo Fischer) HPLC coupled with a Velos iontrap MS (Thermo Fischer). Column was a Waters Glycan BEH 1.7 µm particle size 50 x 2.1 mm. LC-Gradient is A: 50mM Ammonium formate (Sigma), B: Acetonitrile (Merck Hypergrade LC-MS). Gradient starting at 16% A, 1 min. 16% A, 8 min. 24% A, 24min. 32% A, 25 min. 40% A, 25.5 min. 40% A, 26 min. 16% A, 30 min. 16% A. MS settings were 700mz to 2000mz window. Full scan, negative mode. Source fragmentation at 60V. Data collected in profile mode from 0 - 25 min.

LC-FLR

Analysis were performed on a Ultimate 3000 (Thermo Fischer) HPLC coupled with a RS Fluorescence dectector (Thermo Fischer). Lamp: Highpower, Filter: Exitation: 360nm Emission: 428nm. Detection sensitivity: 6.

MS data

From MS data on it is clear that signal from 2-amino-4-(trifluoromethyl)benzoic acid (4TMB) labelled Man-5 sugar is higher than the reference compound 2-AB (44737240 for 4TMB and 127757 for 2AB (figure 1).

Example 2: MS and FLR data.

Using the methods described in example 1 the following compounds were analyzed with respect to their LC-FLR and LC-MS properties (table 1). Data is area under the curve for 0.25 µg maltohexose (Carbosynth Ltd, UK) labelled with a range of derivatising agents.

Table 1. FLR and MS Signal intensities for derivatising agents

Name	FLR Signal	MS Signal
2-aminobenzoic acid	1,24E+07	8,28E+03
2-aminobenzamide	1,25E+07	6,24E+03
3-hydroxyanthranilic acid	0	0
5-hydroxyanthranilic acid	3,35E+06	3,99E+03
2-amino-3-methylbenzoic acid	4,27E+05	6,36E+02
2-amino-4-methylbenzoic acid	1,56E+07	1,09E+04
2-amino-6-methylbenzoic acid	2,88E+05	6,98E+03
2-amino-3-fluorobenzoic acid	5,47E+05	2,83E+03
2-amino-4-fluorobenzoic acid	4,06E+06	7,63E+03
2-amino-5-fluorobenzoic acid	6,52E+06	8,04E+03
2-amino-6-fluorobenzoic acid	1,26E+06	6,94E+03
2-amino-3-chlorobenzoic acid	0	0
2-amino-4-chlorobenzoic acid	3,91E+06	6,28E+03
2-amino-5-chlorobenzoic acid	6,03E+06	7,25E+03
2-amino-6-chlorobenzoic acid	0	0
2-amino-3-(trifluoromethyl)benzoic acid	0	0

2-amino-4-(trifluoromethyl)benzoic acid	5,50E+06	1,46E+04
2-amino-5-(trifluoromethyl)benzoic acid	2,79E+06	8,38E+03
2-amino-6-(trifluoromethyl)benzoic acid	4,47E+04	9,01E+03
2-amino-4,6-difluorobenzoic acid	1,41E+05	4,81E+03
2-amino-6-fluoro-3-methylbenzoic acid	0	0

Example 3: Reproducibility

Using the same conditions as in the above examples, MS signal was measured in 4 independent experiments. Data is area under the curve for Man6 from RNase B and shows that 2-Amino-4-(trifluoromethyl)benzoic acid (4TMB) gives a greater signal than 2-AB and 2-AA (table 2).

Table 2

	M6 Peak				Average	Standard deviation
4TMB	1419740,1	1488428	1335997	1403179,4	1411836,2	62597,395
2AB	450404,96	337070,4	400969	423928	403093,1	48428,678
2AA	571408,03	469371,8	561887,2	730658,54	583331,38	108465,21

Example 4: Glycan pattern analysis of bovine fetuine

Using the same conditions as in the above examples, bovine fetuin (5 µg) was labelled with 2-Amino-4-(trifluoromethyl)benzoic acid (4TMB), 2-AA or 2-AB. The resulting MS spectra are shown in figures 7, 8 and 9.

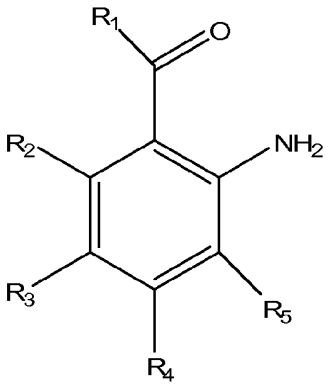
As shown in table 3, 4TMB gives a greater signal than 2-AB and 2-AA. This experiment shows that 4TMB can be used to analyse glycan pattern of a mixture of glycans.

Table 3

	Fetuin A3 peak
4TMB	31680,973
2AA	11218,723
2AB	7981,167

Claims

1. A method for determining the composition of the sugar moiety of a sugar containing compound, said method comprising the steps of:
   a) providing a sugar containing compound,
   b) optionally liberating the sugar moiety from the sugar containing compound,
   c) reacting the sugar moiety with a compound having the general formula (I):



Formula (I)

wherein

R_1 is -OH or $-NH_2$;

R_2, R_3, R_4, and R_5 are individually selected from the group consisting of -H, halogen, C_{1-10} alkyl, C_{1-10} haloalkyl, C_{1-10} aminoalkyl, C_{1-10} nitroalkyl, C_{1-10} hydroxyalkyl, C_{1-10} alkenyl, C_{1-10} haloalkenyl, C_{1-10} aminoalkenyl, C_{1-10} nitroalkenyl, C_{1-10} hydroxyalkenyl, C_{1-10} alkynyl, C_{1-10} haloalkynyl, C_{1-10} aminoalkynyl, C_{1-10} nitroalkynyl,and C_{1-10} hydroxyalkynyl, thus rendering a labeled sugar moiety

and wherein the compound of formula (I) is not 2-AB (2-aminobenzamide), 2-AA (2-aminobenzamide), 2-amino-5-bromobenzoic acid or 2-amino-4-chlorobenzoic acid;

   d) determining the structure of said labeled sugar moiety using analysis means.

2. The method according to claim 1, wherein:

   R_1 is -OH or $-NH_2$;

R_2, R_3, R_4, and R_5 are individually selected from the group consisting of
-H, -F, -Cl, -OH, $-NH_2$,
$-CH_3$, $-CH_2CH_3$, $-CH_2CH_2CH_3$, $-CH_2CH_2CH_2CH_3$
$-CH_2F$, $-CHF_2$, $-CF_3$,
$-CH_2Cl$, $-CHCl_2$, $-CCl_3$,
$-CH_2OH$, $-CH_2NH_2$,
$-CH_2CH_2F$, $-CH_2CHF_2$, CH_2CF_3,
$-CH_2CH_2Cl$, $-CH_2CHCl_2$, $-CH_2CCl_3$,
$-CH_2CH_2OH$, and $-CH_2CH_2NH_2$.

3. The method according to any one of the preceding claims, wherein:
R_1 of formula (I) is -OH;
R_2 of formula (I) is -H, -F, -Cl, $-CH_3$, $-CF_3$;
R_3 of formula (I) is -H, -F, -Cl, -OH;
R_4 of formula (I) is -H, -F, -Cl, $-CH_3$, $-CF_3$;
R_5 of formula (I) is -H, -F, -Cl, -OH, $-CH_3$, $-CF_3$.

4. The method according to any one of the preceding claims, wherein:
R_1 of formula (I) is -OH;
R_2 of formula (I) is -H;
R_3 of formula (I) is -H, -F, -Cl;
R_4 of formula (I) is -H, $-CH_3$, $-CF_3$, -F;
R_5 of formula (I) is -H.

5. The method according to any one of the preceding claims, wherein at least one of R_2, R_3, R_4 and R_5 is selected from the group consisting of $-CH_3$ or a halogen.

6. The method according to any one of the preceding claims, wherein at least one of R_4 and R_5 is selected from the group consisting of $-CH_3$ or a halogen and wherein R_2 and R_3 are –H.

7. The method according to any one of the preceding claims, wherein the halogen is selected from –Cl or –F, preferably the halogen is –F.

8. The method according to any one of the preceding claims, wherein the compound of formula (I) is selected from the group consisting of 2-amino-4-(trifluoromethyl)benzoic acid, 2-amino-6-fluorobenzoic acid, 2-amino-4-methylbenzoic acid, 2-amino-6-methylbenzoic acid, 2-amino-5-(trifluoromethyl)benzoic acid, 2-amino-6-(trifluoromethyl)benzoic acid, 3-hydroxyanthranilic acid, 5-hydroxyanthranilic acid, 2-amino-3-methylbenzoic acid, 2-amino-3-fluorobenzoic acid, 2-amino-4-fluorobenzoic acid, 2-amino-5-fluorobenzoic acid, 2-amino-3-chlorobenzoic acid, 2-amino-5-chlorobenzoic acid, 2-amino-6-chlorobenzoic acid, 2-amino-3-(trifluoromethyl)benzoic acid, 2-amino-4,6-difluorobenzoic acid, 2-amino-6-fluoro-3-methylbenzoic acid, and/or 2-amino-4-fluorobenzamide.

9. The method according to any one of the preceding claims, wherein the compound of formula (I) is selected from the group consisting of 2-Amino-4-(trifluoromethyl)benzoic acid, 2-Amino-4-methylbenzoic acid, 2-Amino-5-Fluorobenzoic acid, 2-Amino-5-Chlorobenzoic acid, and/or 2-Amino-4-Fluorobenzoic acid.

10. The method according to any one of the preceding claims, wherein the compound of formula (I) is 2-Amino-4-(trifluoromethyl)benzoic acid or 2-Amino-4-methylbenzoic acid.

11. The method according to any one of the preceding claims, wherein step (c) comprises reacting the sugar moiety with two or more different compounds of formula (I).

12. The method according to any one of the preceding claims wherein the labeled atom is an isotope of said atom.

13. The method according to any one of the preceding claims, wherein step a) and/or b) are separately conducted using at least two sugar containing compounds and c) is separately conducted using at least two different compounds of formula (I).

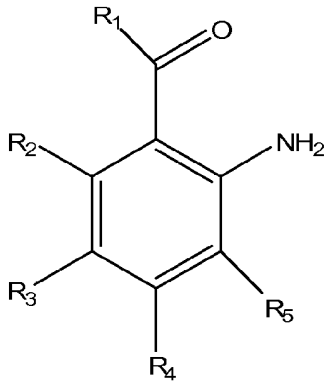
14. The method according to any one of the preceding claims, wherein said sugar moiety is reduced, thus rendering a reduced labeled sugar moiety.

15. The method according to any one of the preceding claims, wherein said reduction is conducted using reductive amination.

16. The method according to any one of the preceding claims, wherein said reduction is conducted using a reducing agent selected from the group consisting of sodium cyanoborohydride, triacetoxyborohydride, 4-amino-N-[2-(diethylamino)ethyl] benzamide, and 2-picoline borane or other suitable reducing agents.

17. The method according to any one of the preceding claims, further comprising a step of separating said labeled sugar moiety thus rendering an isolated labeled sugar moiety.

18. A reference library comprising a spectra of a specific sugar moiety labeled with a compound of formula (I),



Formula (I)

wherein

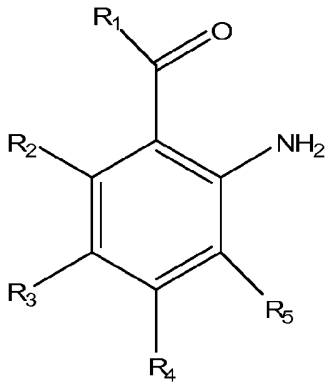
R_1 is OH or NH_2;

R_2, R_3, R_4, and R_5 are individually selected from the group consisting of H, halogen, C_{1-10} alkyl, C_{1-10} haloalkyl, C_{1-10} aminoalkyl, C_{1-10} nitroalkyl, C_{1-10} hydroxyalkyl, C_{1-10} alkenyl, C_{1-10} haloalkenyl, C_{1-10} aminoalkenyl, C_{1-10} nitroalkenyl, C_{1-10} hydroxyalkenyl, C_{1-10} alkynyl, C_{1-10} haloalkynyl, C_{1-10} aminoalkynyl, C_{1-10} nitroalkynyl, and C_{1-10} hydroxyalkynyl, and wherein the compound of formula (I) is not 2-AB (2-aminobenzamide), 2-AA (2-

aminobenzamide), 2-amino-5-bromobenzoic acid or 2-amino-4-chlorobenzoic acid.

19. A kit of parts comprising

a) a composition comprising a compound having the general formula (I),



Formula (I)

wherein

R_1 is OH or NH_2;

R_2, R_3, R_4, and R_5 are individually selected from the group consisting of H, halogen, C_{1-10} alkyl, C_{1-10} haloalkyl, C_{1-10} aminoalkyl, C_{1-10} nitroalkyl, C_{1-10} hydroxyalkyl, C_{1-10} alkenyl, C_{1-10} haloalkenyl, C_{1-10} aminoalkenyl, C_{1-10} nitroalkenyl, C_{1-10} hydroxyalkenyl, C_{1-10} alkynyl, C_{1-10} haloalkynyl,

b) C_{1-10} aminoalkynyl, C_{1-10} nitroalkynyl, and C_{1-10} hydroxyalkynyl, and wherein the compound of formula (I) is not 2-AB (2-aminobenzamide), 2-AA (2-aminobenzamide), 2-amino-5-bromobenzoic acid or 2-amino-4-chlorobenzoic acid,a reference library according to claim 18, and

c) instructions on how to label a sugar moiety of a sugar containing compound according to the method of any one of the preceding claims.

Figure 1:

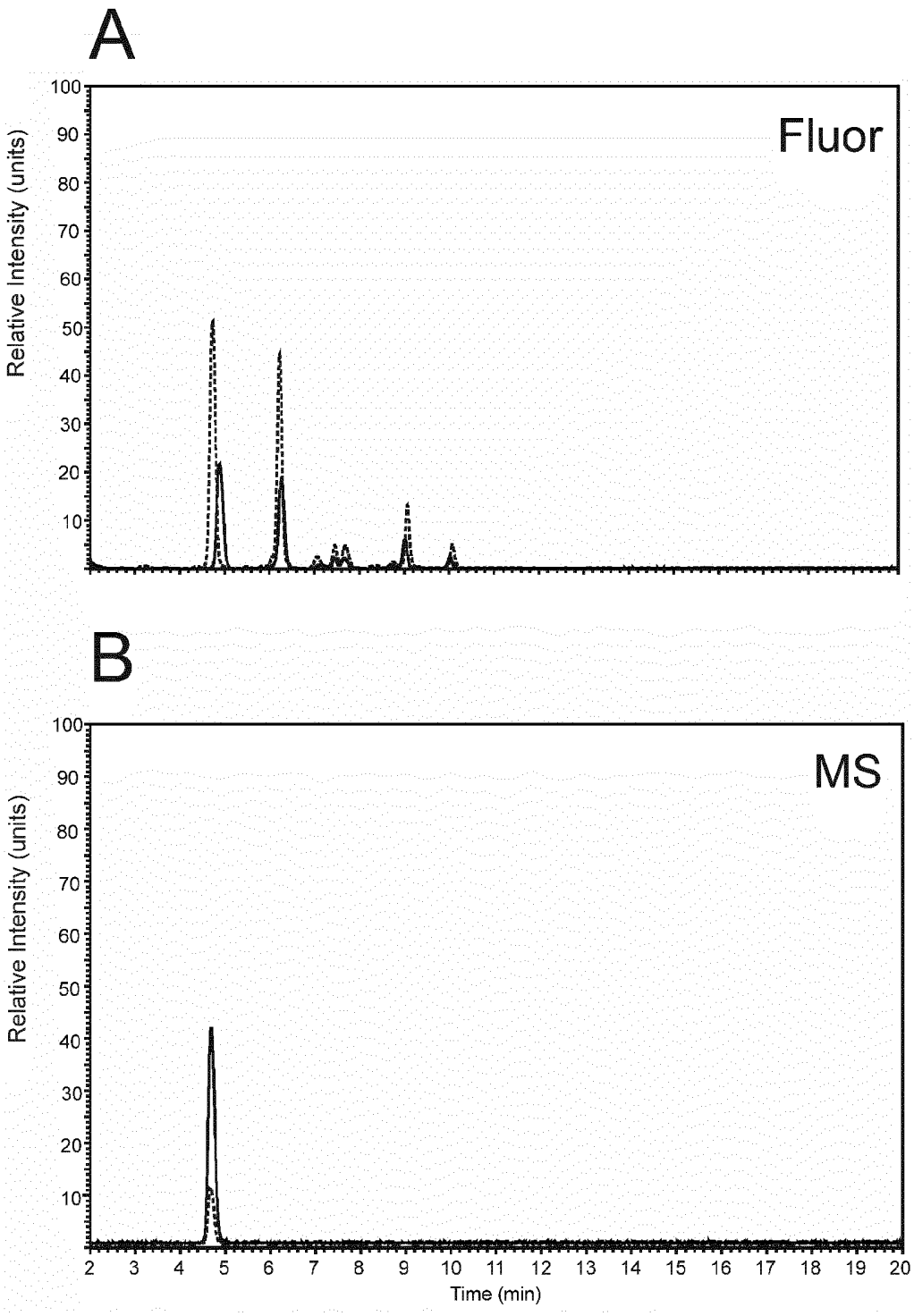

A
Fluor
Relative Intensity (units)
100
90
80
70
60
50
40
30
20
10
B
MS
Relative Intensity (units)
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Time (min)

Figure 2:

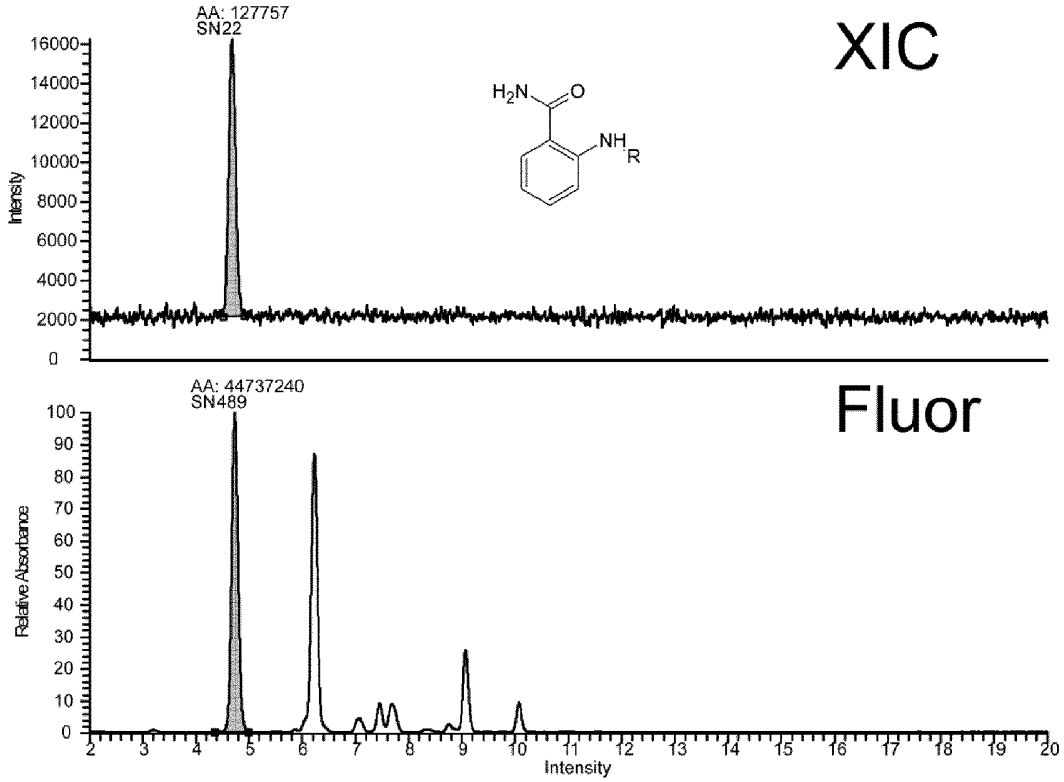


Figure 3:

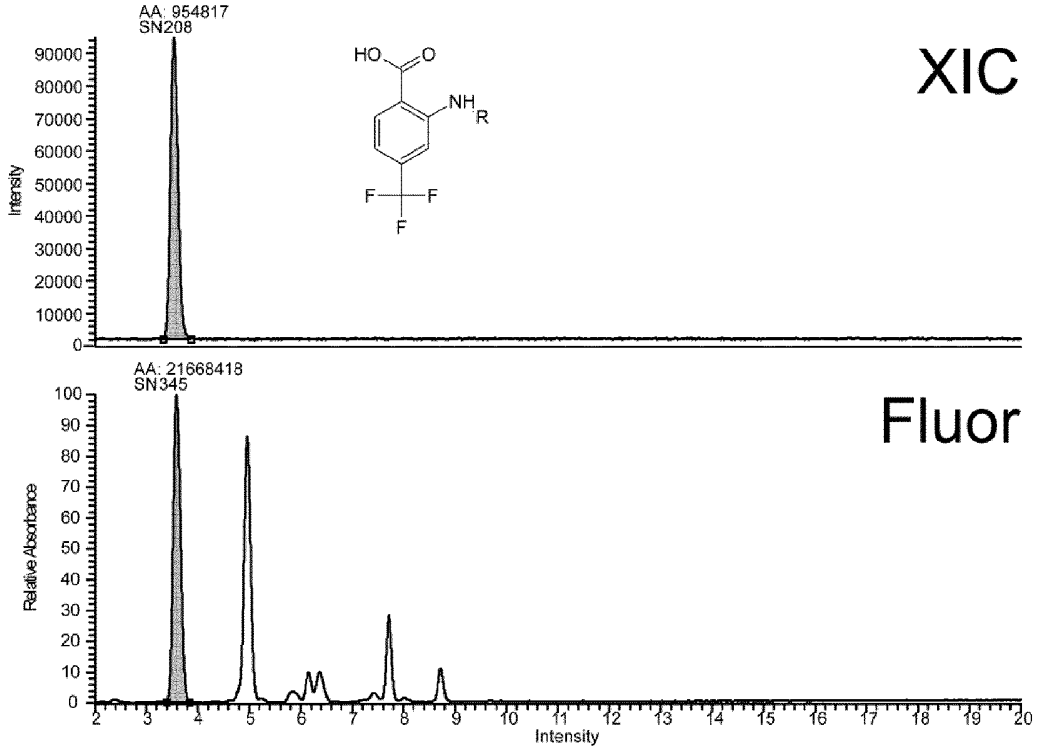


Figure 4:

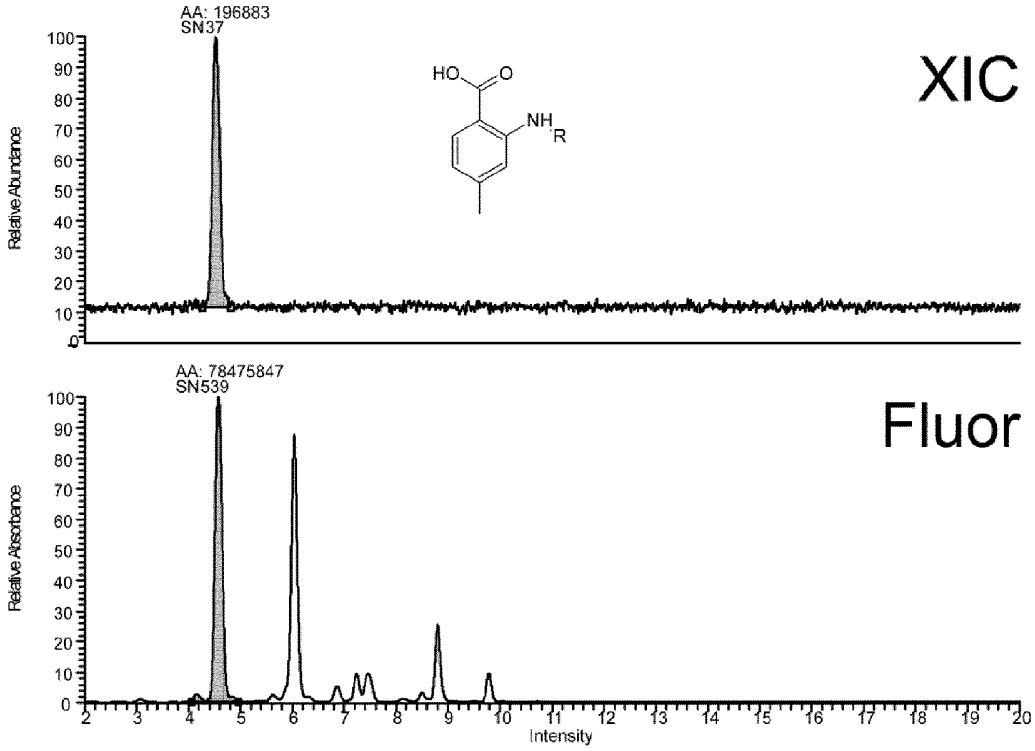


Figure 5

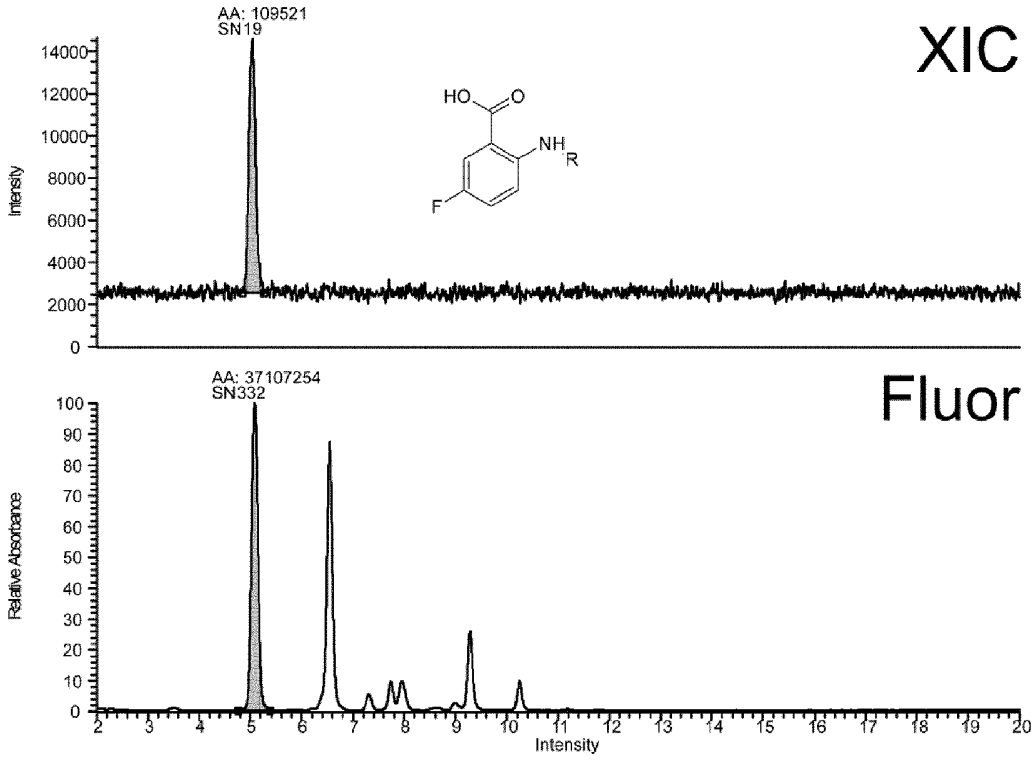


Figure 6:

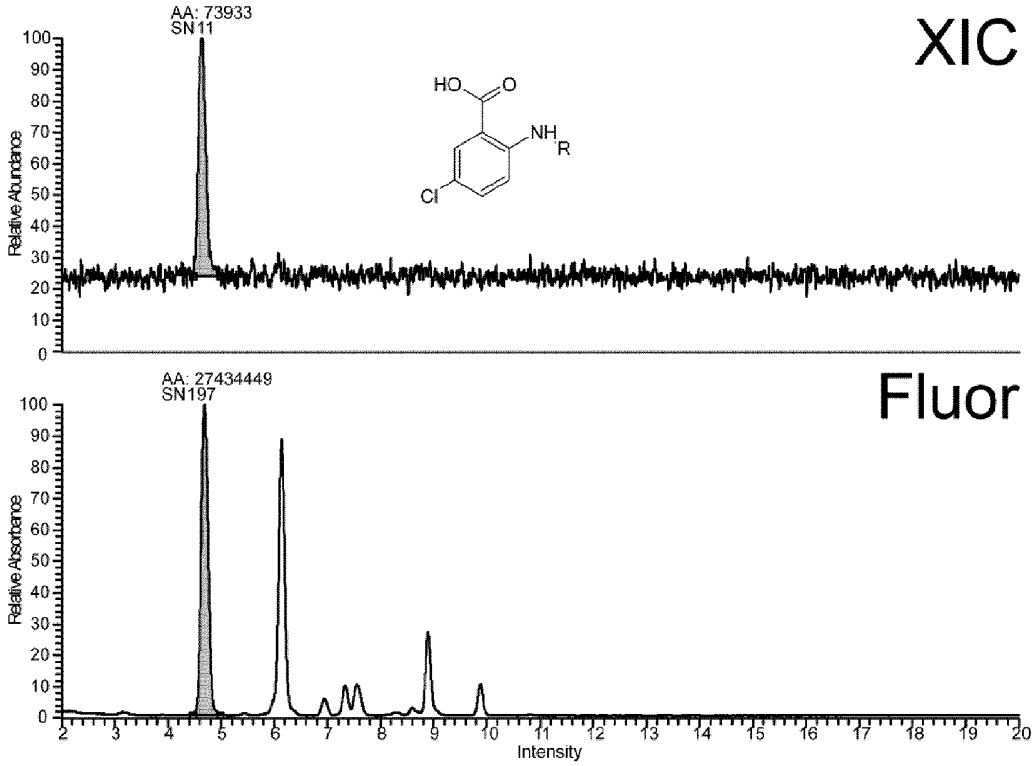


Figure 7:

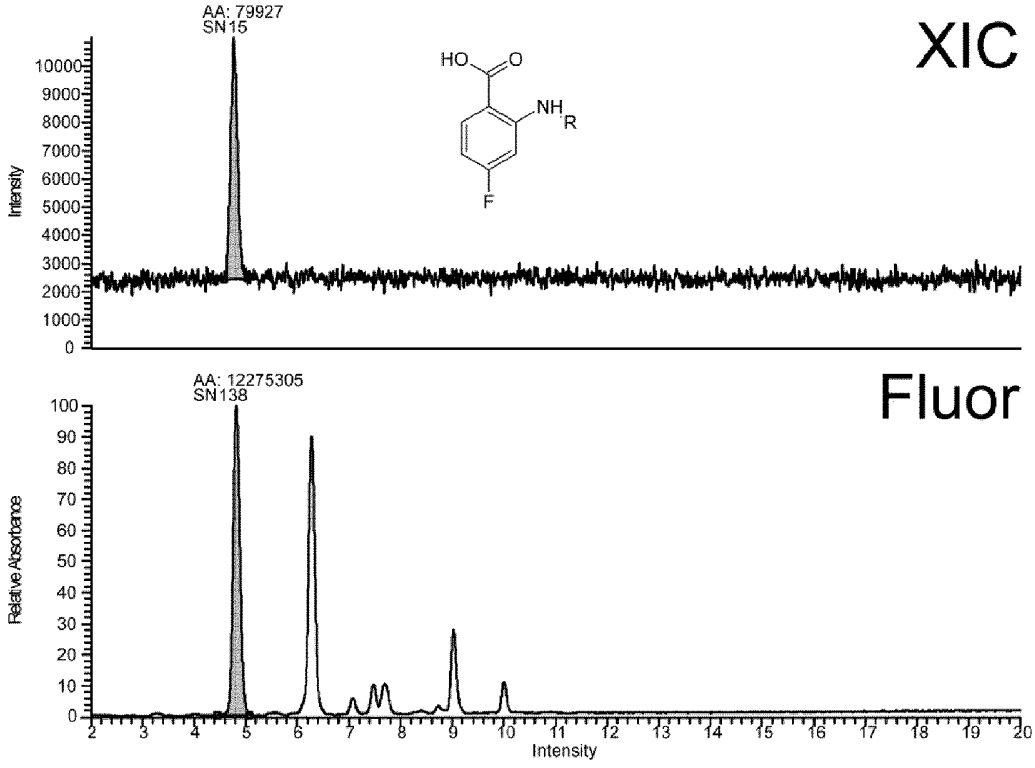
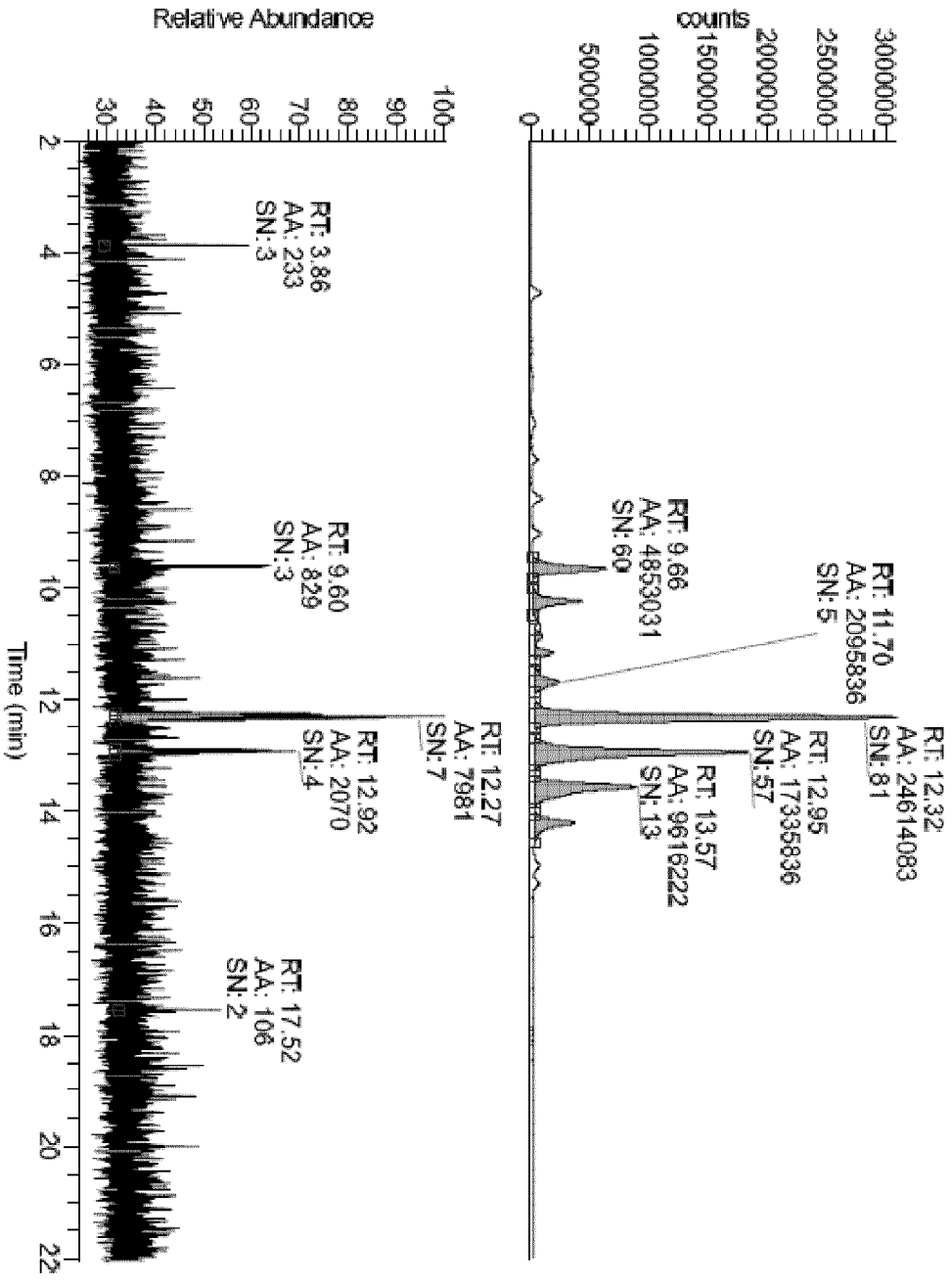


Figure 8:


Relative Abundance
counts
Time (min)
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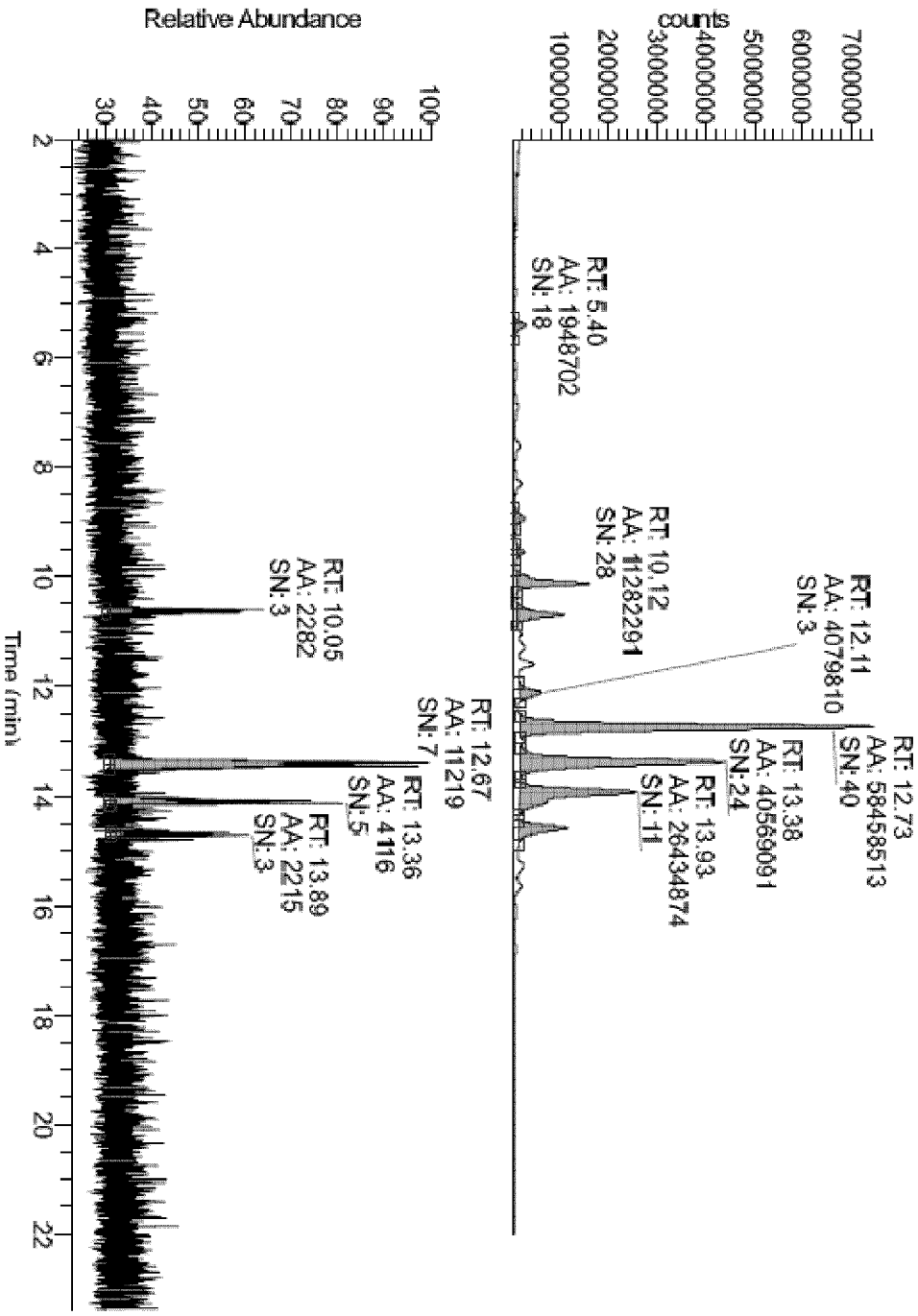
AA: 233
SN: 3
RT: 9.60
AA: 829
SN: 3
RT: 12.27
AA: 7981
SN: 7
RT: 12.92
AA: 2070
SN: 4
RT: 17.52
AA: 106
SN: 2
RT: 9.66
AA: 4853031
SN: 60
RT: 11.70
AA: 2095836
SN: 5
RT: 12.32
AA: 24614083
SN: 81
RT: 12.95
AA: 17335836
SN: 57
RT: 13.57
AA: 9616222
SN: 13

Figure 9:


Relative Abundance
counts
Time (min)
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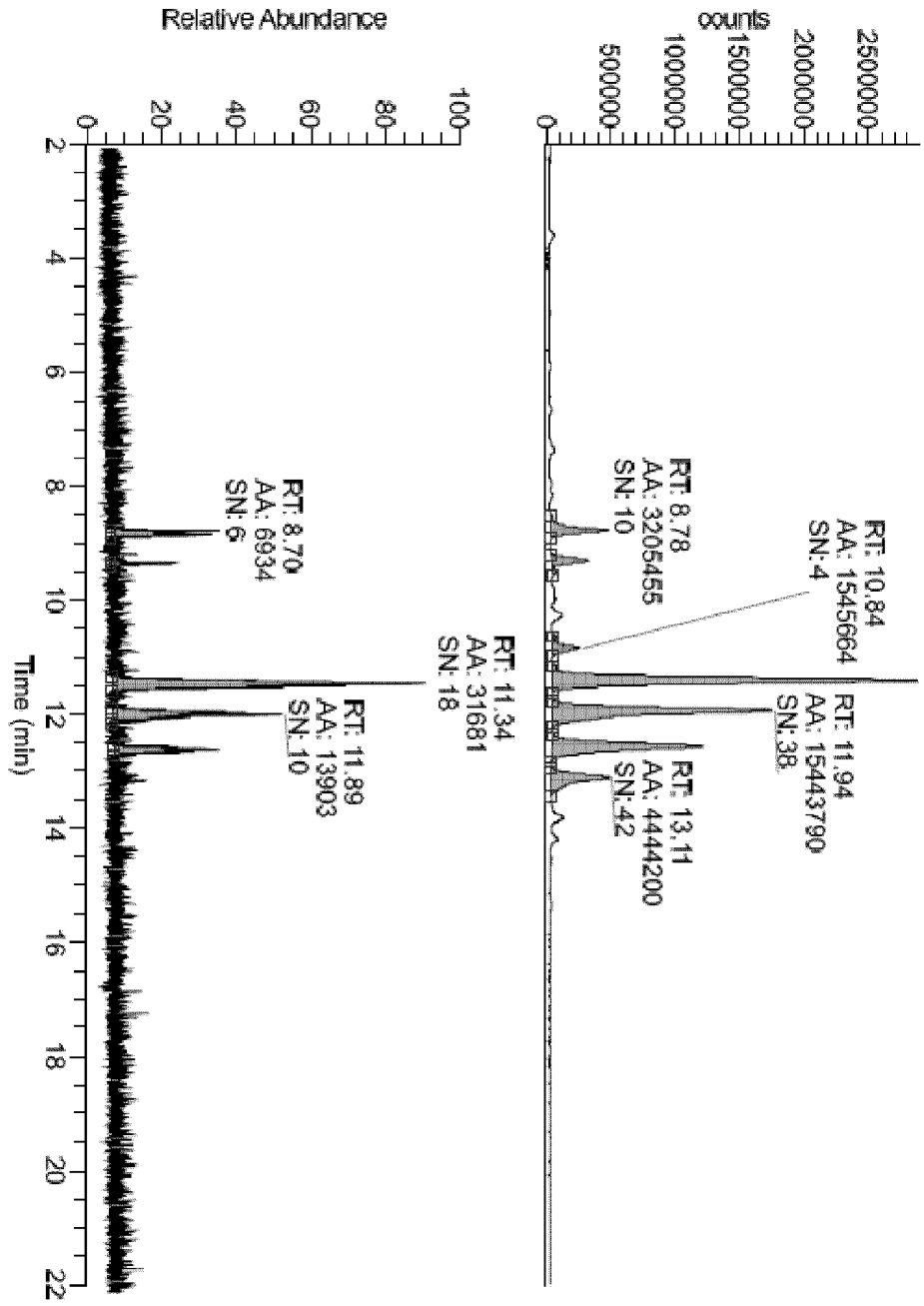
AA: 1948702
SN: 18
RT: 10.12
AA: 1128229
SN: 28
RT: 10.05
AA: 2282
SN: 3
RT: 12.11
AA: 4079810
SN: 3
RT: 12.67
AA: 11219
SN: 7
RT: 12.73
AA: 58458513
SN: 40
RT: 13.38
AA: 40569091
SN: 24
RT: 13.36
AA: 4116
SN: 5
RT: 13.93
AA: 26434874
SN: 11
RT: 13.89
AA: 2215
SN: 3

Figure 10:


Relative Abundance
counts
Time (min)
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AA: 6934
SN: 6
RT: 8.78
AA: 3205455
SN: 10
RT: 10.84
AA: 1545664
SN: 4
RT: 11.34
AA: 31681
SN: 18
RT: 11.89
AA: 13903
SN: 10
RT: 11.94
AA: 1544790
SN: 38
RT: 13.11
AA: 4444200
SN: 42

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/075889

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07H5/06 C07C229/56 C07C237/40 G01N33/00
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)
C07H C07C G01N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, CHEM ABS Data, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2008/128216 A1 (MOMENTA PHARMACEUTICALS INC [US]; PARSONS IAN CHRISTOPHER [US]; THIRUN) 23 October 2008 (2008-10-23) paragraph [0043] - paragraph [0045] paragraphs [0054], [0056], [0057], [0078], [0112] claims 3-5,10,11,17,51-55 the examples	1-17

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 December 2015	18/12/2015

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

3

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

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	R.REID TOWNSEND ET AL: "Multimode High-Performance Liquid Chromatography of Fluorescently Labeled Oligosaccharides from Glycoproteins", ANALYTICAL BIOCHEMISTRY, vol. 239, no. 2, 1 August 1996 (1996-08-01), pages 200-207, XP055108618, ISSN: 0003-2697, DOI: 10.1006/abio.1996.0315 cited in the application the whole document	1-19
A	BIGGE J C ET AL: "Nonselective and efficient fluorescent labelling of glycans using 2-amino benzamide and anthranilic acid", ANALYTICAL BIOCHEMISTRY, ACADEMIC PRESS INC, NEW YORK, vol. 230, no. 2, 1 January 1995 (1995-01-01), pages 229-238, XP002489980, ISSN: 0003-2697, DOI: 10.1006/ABIO.1995.1468 the whole document	1-19
X	WO 2014/053694 A1 (ORION CORP [FI]) 10 April 2014 (2014-04-10) examples 3, 15, 42, 43, 44, 46, 47, 53, 54, 55, 71, 72	19
A	ANUMULA ET AL: "Advances in fluorescence derivatization methods for high-performance liquid chromatographic analysis of glycoprotein carbohydrates", ANALYTICAL BIOCHEMISTRY, ACADEMIC PRESS INC, NEW YORK, vol. 350, no. 1, 1 March 2006 (2006-03-01) , pages 1-23, XP024942466, ISSN: 0003-2697, DOI: 10.1016/J.AB.2005.09.037 [retrieved on 2006-03-01] table 2 page 9 - page 10	1-19

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2015/075889

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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