FOOD STANDARDS AGENCY

STANDARD OPERATING PROCEDURE (SOP) xxx

Version 1.0, October, 2009

STANDARD OPERATING PROCEDURE FOR DETERMINATION OF GLYCOSYLATION OF N-LINKED GLYCOPROTEINS BY MALDI-TOF MS

Prepared by J. T. Marsh, A. Lovegrove and P. Shewry, Rothamsted Research
Date October 2009

Approved by _____ **Date** _____

CONTENTS

1. HISTORY / BACKGROUND

1.1 Background

Methods were developed to identify and characterise *N*-linked glycans on plant proteins to determine whether proteins expressed in transgenic plants are inauthentically glycosylated. The method was evaluated in a range of crop species and found to be reproducible and robust.The method were then reduced to an SOP suitable for routine use in industry and by regulatory authorities.

1.2 Not applicable

2. PURPOSE

To provide a robust reproducible methodology for the analysis of *N*-linked glycans for routine analytical laboratory use. The method is applicable to purified glycoproteins as well as to crude extracts. It is also sufficiently sensitive to be applied at the single seed level. It may be applied to a range of glycoproteins by regulatory authorities and by industry with standard mass spectrometry equipment (MALDI-Tof).

3. SCOPE

The method allows comparison of *N*-glycans derived from a purified glycoprotein or a total protein extract from various plant species using a mass spectrometric based approach. The MALDI-TOF MS peak heights of different *N*-glycans are not a quantitative reflection of the composition of the different glycans but will be indicative of the *relative* levels.

4. DEFINITIONS AND ABBREVIATIONS

MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry

FWHM , Full Width at Half Maximum

5. PRINCIPLE OF THE METHOD

A purified glycoprotein or a plant extract is digested with a commercial protease into peptides and glycopeptides. *N*-glycans are then released from the glycopeptides with a commercial endoglucosidase . The free glycans are purified away from the peptides and per-methylated. MALDI-TOF MS is then conducted on the permethylated glycan

samples. The resultant glycoprofile obtained is then annotated as per-methylated glycans which have a known mass by reference to a glycan database. Experimental and control samples may then be compared in terms of their glycoprofile.

6. MATERIALS AND EQUIPMENT

6.1 Chemicals

The highest possible grade of reagent must be used for all steps in this SOP.

2,5-dihydroxybenzoic acid, Puriss. p.a. matrix substance for MALDI-MS: Fluka 85707, FW=154.12, store <4 °C, harmful.

Acetic acid glacial, HPLC grade: Fisher Scientific A/0406/PB15, FW=60.05, store <30 °C, corrosive and flammable.

Acetone, Analytical grade: Fisher Scientific A/0600/17, FW=58.08, store <30 °C, highly flammable and an irritant.

Acetonitrile, HPLC grade: Fisher Scientific A/0627/PB17, FW=41.05, store <30 °C, highly flammable and harmful.

Ammonium acetate, Analytical grade: Fisher Scientific A/3400/53, FW=77.08, store <30 °C.

Chloroform, Super purity solvent: Romil Pure Chemistry H135, FW=119.05, store <30 °C out of direct sunlight, harmful.

Dimethyl Sulphoxide, Super purity solvent: Romil Pure Chemistry H280, FW=78.13, irritant.

Dowex 50WX8-100 resin:Sigma-Aldrich 217492, irritant.

Ethanol absolute, Super purity solvent, Romil Pure Chemistry, H314, FW=46.07, store <30 C out of direct sunlight, highly flammable.

Formic acid, Puriss. p.a. for mass spectrometry: Fluka 94318, FW=46.03, corrosive.

Glass wool: Sigma-Aldrich 18421.

Hydrochloric acid Analytical grade: Fisher Scientific H/1200/PB17, FW=36.46, corrosive.

Trifluoroacetic acid Uvasol: Merck T-6508, FW=114.2, corrosive

Iodomethane, Purum: Sigma-Aldrich 67692, FW=141.94, store <30 °C out of direct sunlight, toxic and photosensitive.

Methanol, HPLC grade: Fisher Scientific M/4056/17, FW=32.04, store <30 °C, highly flammable and toxic.

Pepsin (lyophilized powder): Sigma P6887, FW=35 000, store at -20 °C.

PNGase : Roche 11642995001, FW=52 500, store at -20 °C

Sodium hydroxide (anhydrous) pellets, Analytical grade: Fisher Scientific S/4920/53, FW=40, corrosive.

ADH: Sigma A-7011, mol wt=141kDa, store at -20 °C

Trypsin: Sigma T-8642, mol wt=28.3kDa store at -20 °C. Harmful.

α-cyano-4-hydroxycinnamic acid: Sigma C02020. FW=189.2. Store at -20 °C. Irritant.

6.2 Water

Should be purified by reverse osmosis and have a resistance of 18.2 MΩ

6.3 Solutions, standards and reference materials

2,5-dihydroxybenzoic acid (DHB), 10 $mgml^{-1}$, in 50 (v/v) % methanol. Weigh 1 mg 2,5-dihydroxybenzoic acid, into a 1.5 ml micro tube. Add 1 ml of 50 (v/v) % methanol, to the solid. Vortex for 30 sec. Make fresh on day of use.

5% (v/v) Acetic acid. Measure 10 ml of HPLC-grade glacial acetic acid into a 50 ml glass measuring cylinder. Measure 190 ml of water into a 200 ml glass measuring cylinder. Add the water to a 250 ml glass bottle, followed by the acetic acid, mix on a stirrer for 30 seconds and store at room temperature.

5% (v/v) Formic acid. Measure 10 ml of formic acid into a 50 ml glass measuring cylinder. Measure 190 ml of water into a 200 ml glass measuring cylinder. Add the water to a 250 ml glass bottle followed by the acid and mix on a stirrer for 30 seconds. Store at room temperature.

35% (v/v) Acetonitrile. Measure 70 ml of 100% (v/v) acetonitrile into a 100 ml glass measuring cylinder. Measure 130 ml of water into a 200 ml glass measuring cylinder. Add the water to the acetonitrile in a 250 ml glass bottle, mix on a stirrer for 30 seconds. Store at room temperature.

50% (v/v) Acetonitrile. Measure 100 ml of 100% (v/v) acetonitrile into a 100 ml glass measuring cylinder. Measure 100 ml of water into a 100 ml glass measuring cylinder. Add the water to the acetonitrile in a 250 ml glass bottle, mix on a stirrer for 30 seconds. Store at room temperature.

50% (v/v) Methanol. Measure 100 ml of 100% (v/v) methanol into a 100 ml glass measuring cylinder. Measure 100 ml of water into a 100 ml glass measuring cylinder.

Add the water to the methanol in a 250 ml glass bottle, mix on a stirrer for 30 seconds.Store at room temperature.

100% (v/v) Acetonitrile. Decant 200 ml of 100% (v/v) acetonitrile into a 250 ml glass bottle. Store at room temperature.

100% (v/v) Methanol. Decant 200 ml of 100% (v/v) methanol into a 250 ml glass bottle. Store at room temperature.

100% (v/v) Ethanol. Decant 200 ml of 100% (v/v) ethanol into a 250 ml glass bottle. Store at room temperature.

50 mM ammonium acetate pH 5.0 ±0.1

4 M Hydrochloric acid

Pepsin solution, 1 mg ml^{-1}, in 5% (v/v) formic acid. Weigh 1 mg pepsin (lyophilised powder), into a 1.5 ml eppendorf. Add 1 ml of 5 % (v/v) formic acid, to the solid. Vortex for 30 sec. Store at -20 °C.

49.5% (v/v) acetonitrile, 49.5%(v/v) ethanol, 1% (v/v) trifluoroacetic acid. Pipette 4.95ml of acetonitrile into a glass bottle. Add 4.95ml of ethanol and 100υl of trifluoroacetic acid. Mix on a stirrer for 30 seconds and store at room temperature.

2mg/ml α-cyano-4-hydroxycinnamic acid in 49.5% (v/v) acetonitrile, 49.5%(v/v) ethanol, 1% (v/v) trifluoroacetic acid. Weigh 2mg α-cyano-4-hydroxycinnamic acid into a 1.5ml micro-tube. Add 1ml of 49.5% (v/v) acetonitrile, 49.5%(v/v) ethanol, 1% (v/v) trifluoroacetic acid, vortex for 20 sec, make fresh each time of use.

6.4 Commercial kits

Micro BCA Protein Assay Kit:Thermo Scientific-Pierce Protein Research Products, 23235.

Sep-Pak® Classic C18 Cartridges, Waters, WAT051910.

6.5 Plasticware

10 ml cylindrical test tubes without rim (use for protein estimation using the BCA assay), Aptaca 5005/S.

50 ml Centrifuge tube conical basesterile, Greiner Bio-one Limited 227261.

Cuvettes semi-micro disposable (for Micro BCA Protein assay), Fisher Scientific CXA-105-30K.

Micro tubes 1.5 ml, polypropylene, with attached polypropylene cap, Sarstedt 72.690.001. (commonly known as ‘eppendorfs’)

Nesco film

Pipette tips 0.1-10 µl: Starlab S1111-3800.

Pipette tips 1-200 µl: Starlab S1111-0006.

Pipette tips 100-1000 µl: Starlab S1111-2821.

6.6 Glassware

Borosilicate glass bottles, FAB-OFF-503R, Fisher Scientific.

50 ml Measuring cylinders, FB50107, Fisher Scientific.

100 ml Measuring cylinders, FB50109, Fisher Scientific.

150 mm Glass Pasteur Pipette, PIP4100, Scientific Laboratory Supplies.

Beaker squat form with graduations and spout borosilicate glass 800mL, FB33115, Fisher Scientific.

Glass syringe 10 ml: Fortuna-optima.

Pyrex glass centrifuge tubes 10 ml: 73785-10, Kimble.

Tubes are washed with 2 ml of acetone, dispensed by glass Pasteur pipette then vortexed for 30 sec and the acetone poured into a waste container. Acetone is evaporated from the tubes by placing the tubes in a fume hood overnight prior to use.

6.7 Equipment

-85°C Ultra low freezer: C66085, New Brunswick Scientific.

Balance: R180D, Sartorius Research.

Centrifuge: J25, Avanti.

Oven: Maxi-oven, Hybaid.

Freeze-dryer: Super-modulyo, Edwards.

Fridge-Freezer -20°C: CDAA751F, Beko.

Dry block heating system: QBDT, Grant Instruments (Cambridge) Ltd

MALDI-TOF MS: Micromass Manchester (now Waters Corporation)

Microcentrifuge: 5415D, Eppendorf.

Nitrogen gas: BOC gases.

Mortar and pestle (porcelain): MWA-250-050U/B, Fisher Scientific.

Pipettes (1ml, 200 µl, 20 µl and 1 µl): Gilson.

pH meter: Basic, Denver Instruments.

Spatula: 2351 Chattaway micro stainless steel 130mm x 4mm, FB65063, Fisher Scientific.

Single branch manifold with 4 PTFE taps: Glass solutions (Cambridge) Ltd

Spectrophotometer: 6715 UV/VIS spectrophotometer, Jenway.

Stirring hotplate: (Fisher Scientific)

Stirring rod: PTFE white 300mm x 8mm, FB58262, Fisher Scientific.

Target plates: Micromass (now Waters Corporation) Manchester.

Thermoblock: Falcon 15 ml, Eppendorf.

Thermomixer comfort: Eppendorf.

Vortex : Relax top, Heidolph.

Unstirred water bath: JB Aqua 2,Grant Instruments (Cambridge) Ltd.

7. PROCEDURES

A simplified flow chart is shown in figure 1.

Glycan Release

1. 28 µl of a 1 $mgml^{-1}$ pepsin solution (in 5 % (v/v) formic acid) is added to 100 µl of a protein sample (100 μgml^{-1} for purified glycoprotein; 1 $mgml^{-1}$ for total protein extract) in a 1.5 ml micro-tube. The sample/enzyme mixture is vortexed for 5 sec and then spun in a microcentrifuge (5415D, Eppendorf) at 10 000 x g for 30 sec at room temperature.

2. The sample/enzyme mixture is and then incubated at 37 °C for 24 hr (in a Maxi-oven, Hybaid).

3. Following incubation the pepsin is inactivated by boiling for 5 min at 100 °C, in a hot block (QBDT, Grant Instruments).

4. The sample is then spun down at 10 000 x g for 30 sec at room temperature. Nesco film is wrapped over the opened micro-tube and pierced with a needle. The sample is then frozen by placing in a -80 °C freezer for 1 hr. A freeze-dryer (Super-modulyo, Edwards) is pre-cooled, under vacuum, for 1 hr, before the frozen sample is inserted. The sample is lyophilized overnight.

5. The lyophilized sample is then re-suspended in 100 µl of 50 mM ammonium acetate, pH 5.0.

6. 4 µl of PNGase is added to the sample. The sample is then mixed and spun down in a microcentrifuge (5415D, Eppendorf) at 10 000 x g for 30 sec at room temperature.

7. The sample is then incubated at 37°C in an oven (Maxi-oven, Hybaid) for 16 hr.

8. Repeat step 4.

Peptide Removal

All subsequent steps should take place in glass tubes (Pyrex glass centrifuge tubes 10 ml, Kimble) and all solutions should be dispensed by glass Pasteur pipettes (150 mm Glass Pasteur Pipette, Scientific Laboratory Supplies). This is to prevent peaks occurring in the mass spectrum that originate from plasticware.

1. Re-suspend the lyophilized sample in 0.5 ml 5 % (v/v) acetic acid.

2. Rinse a 10ml glass syringe (Fortuna-optima) with 2x 5ml of methanol and eject the methanol into a waste container.

3. Using a glass Pasteur pipette, fill the tallest end of a Sep-Pak® Classic C18 Cartridge (Waters) with 100% (v/v) methanol.

4. Fill syringe with 5ml of 100% (v/v) methanol. Quickly attach the Sep-Pak® Cartridge to the filled syringe and push the liquid through the matrix. The cartridge is detached just before the last of the liquid enters the matrix.ie the matrix must not be allowed to dry.

5. Rinse a 10ml glass syringe with 10 ml of 5 % (v/v) acetic acid and eject the acetic acid into a waste container. Fill with 5 ml of 5 % (v/v) acetic acid.

6. To equilibrate the Sep-Pak matrix: Using a glass Pasteur, fill the tallest end of a Sep-Pak® Cartridge with 5 % (v/v) acetic acid.

7. Quickly attach the Sep-Pak® Cartridge to the filled syringe and push the liquid through the matrix. The cartridge is detached just before the last of the liquid enters the matrix.ie the matrix must not be allowed to dry.

8. Add the sample to the tallest end of a Sep-Pak cartridge using a glass Pasteur pipette.

9. Once the sample has entered the Sep-Pak, fill the Sep-Pak cartridge with 5 % (v/v) acetic acid (i.e. repeat step 6).

10. Fill the glass syringe with 3 ml of 5 % (v/v) acetic acid.

11. Quickly attach the Sep-Pak® Cartridge to the filled syringe and push the liquid through the matrix collecting the eluate in a 10 ml glass centrifuge tube (Kimble).

12. Wrap Nesco film over the opened tube and pierce with a needle. The sample is then frozen by placing it in a -80 °C freezer for 1 hr. A freeze-dryer (Super-modulyo, Edwards) is pre-cooled, under vacuum, for 1 hr, before the frozen sample is inserted. The sample is lyophilized overnight.

13. Re-suspend 20 g of Dowex 50WX8-100 resin in 150 ml 4M hydrochloric acid, in a glass beaker. Stir on a Fisher stirring hotplate (Fisher Scientific) magnetic stirrer, using a magnetic bar (Fisher Scientific) for 10 min, setting 4. Allow to settle for 3 min and then discard the hydrochloric acid by pouring into a waste container.

14. Repeat step 13 two times.

15. Re-suspend the resin in 150 ml water and stir as before, monitoring the pH value. Repeat this step until the pH is the same as the water supply.

16. Re-suspend the resin in 50 ml 5 % (v/v) acetic acid and stir as before.

17. Repeat step 16 two times.

18. Using a fine spatula (Fisher Scientific) gently push a small quantity of glass wool into Pasteur. The glass wool acts as a frit and should take up approximately 5 mm of the Pasteur pipette column. To this add 1 ml of the Dowex resin suspension (stirring as before), so that the Pasteur column is approximately one quarter full**.**

19. Wash/equilibrate the Dowex column by adding 4 ml of 5 % (v/v) acetic acid using a glass pipette. Allow the acetic acid to flow through under gravity.

20. Re-suspend the lyophilized sample in 0.5 ml 5 % (v/v) acetic acid.

21. As the last drop of wash solution passes into the column, place a 10 ml glass test-tube (Kimble) under the column to collect everything that passes through the resin after sample addition. Apply the sample to the column, using a glass pipette.

22. Add 4 ml of 5 % (v/v) acetic acid to the column. Collect this wash into the same glass test-tube.

23. Repeat step 12.

Glycan Treatment

The following process should be conducted in a fume hood.

1. Dispense five sodium hydroxide pellets into a pre-warmed (50 °C, Maxi-oven, Hybaid) mortar and pestle. Grind the pellets into a fine powder and add 3 ml of dimethyl sulphoxide (DMSO) using a glass Pasteur pipette.

2. Add 1 ml of the suspension to the lyophilized sample ***(To avoid excessive absorption of moisture from the atmosphere, the sample should only be removed from the freeze-drier at the last moment)*** using a glass Pasteur pipette.

3. Add 0.5 ml of iodomethane to the sample using a glass Pasteur pipette. Cap the tubes and vortex (Heidolph) for 5 sec at full speed.

4. Mix the samples for 20 min in a Thermomixer with a 15 ml tube Thermoblock (Eppendorf) at 750 rpm, room temperature.

5. Quench the reaction by addition of 1 ml of water to the sample using a glass Pasteur pipette. Immediately vortex for 5 sec full speed.

6. Add 2 ml of chloroform to the sample using a glass Pasteur pipette. Vortex the mixture for 15 sec and allow the mixture to settle into two layers over a period of 10 min.

7. Remove and discard the upper aqueous phase using a glass Pasteur pipette

8. Add 2 ml of water to the chloroform layer, vortex for 15 sec, and allow the mixture to settle into two layers over a period of 10 min as before.

9. Repeat step 7.

10. Repeat step 8.

11. Repeat step 7.

12. Dry the sample under a stream of nitrogen. (We use a glass manifold with 4 PTFE taps (Glass solutions) attached to a nitrogen supply, with glass Pasteur pipettes attached to the taps via silicon tubing).

13. Re-suspend the lyophilized sample in 0.2 ml 35 % (v/v) acetonitrile.

14. Rinse a 10ml glass syringe with 10 ml of water and eject the water into a waste container. Fill with 5 ml water.

15. Using a glass Pasteur pipette, fill the tallest end of a Sep-Pak® Classic C18 Cartridge (Waters) with water.

16. Quickly attach the Sep-Pak® Cartridge to the filled syringe and push the liquid through the matrix. The cartridge is detached just before the last of the liquid enters the matrix.ie the matrix must not be allowed to dry.

17. Rinse a 10ml glass syringe with 10 ml of 100 % (v/v) acetonitrile and eject the acetonitrile into a waste container. Fill with 5 ml of 100 % (v/v) acetonitrile.

18. Using a glass Pasteur, fill the tallest end of a Sep-Pak® Cartridge with 100 % (v/v) acetonitile.

19. Repeat step 16.

20. Rinse a 10ml glass syringe with 10 ml of 100 % (v/v) ethanol and eject the ethanol into a waste container. Fill with 5 ml of 100 % (v/v) ethanol.

21. Using a glass Pasteur, fill the tallest end of a Sep-Pak® Cartridge with 100 % (v/v) ethanol.

22. Repeat step 16.

23. Rinse a 10 ml glass syringe with 10 ml of water and eject the water into a waste container. Fill with 10 ml of water.

24. Using a glass Pasteur, fill the tallest end of a Sep-Pak® Cartridge with water.

25. Repeat step 16.

26. Add the sample to the tallest end of a Sep-Pak cartridge using a glass Pasteur pipette (Scientific Laboratory Supplies).

27. Fill with glass syringe 6 ml of water.

28. Repeat step 24.

29. Repeat step 16.

30. Rinse a 10ml glass syringe with 10 ml of 50 % (v/v) acetonitrile and eject the acetonitrile into a waste container. Fill with 2 ml of 50 % (v/v) acetonitrile.

31. Repeat step 18.

32. Quickly attach the Sep-Pak® Cartridge to the filled syringe and push the liquid through the matrix collecting the eluate in a 10 ml glass centrifuge tube (Kimble).

33. Wrap Nesco film over the opened tube and pierce with a needle. The sample is then frozen by placing in a -80 °C freezer for 1 hr. A freeze-dryer (Super-modulyo, Edwards) is pre-cooled, under vacuum, for 1 hr, before the frozen sample is inserted. The sample is lyophilized overnight.

MALDI

1. For glycan analysis: Re-suspend the lyophilized sample in 30 µl of 2,5-dihydroxybenzoic acid (10 $mgml^{-1}$ in 50 % (v/v) methanol).

2. The MALDI-Tof is tuned before each run using a tryptic digest of alcohol dehydrogenase (see below). The resolution of the MALDI is tuned, by adjustment of the pulse voltage, to give a value of 10,000 FWHM (Full Width at Half Maximum), for peptides in the mass range 1000-4000m/z, using the ADH digest at a concentration of 50fmol/ul on target. N.B. for calibration and tuning with the peptide-mixture the matrix used is 2mg/ml α-cyano-4-hydroxycinnamic acid in 49.5% (v/v) acetonitrile, 49.5%(v/v) ethanol, 1% (v/v) trifluoroacetic acid. Matrix and sample digest are mixed 1:1.

3. The MALDI-Tof mass spectrometer is calibrated before each run. Obviously calibration procedures are different for different Manufacturers's mass spectrometers, but briefly. A trypsin digest of ADH (alcohol dehydrogenase) is carried out as detailed in the mass spectrometer Manufacturer's manual (Water's). The ADH digest is then aliquoted and stored at -80^{o}C. The digest is spotted onto the MALDI target mixed 1:1with matrix, to give a 100fmol/ul concentration on target and allowed to air dry.

4. The appropriate calibration file (ADH.ref) is selected and the MALDI set to acquire 10 spectra between 800-3500 m/z using an appropriate laser energy. The calibration parameters are set at a 4th order polynominal and peaks are matched after automated data acquisition is complete. At least 6 peaks are matched out of 12 and the mean residues must be at a value of 10^{-3} + or - 0.006 or better. Only after this is completed are glycan samples to be spotted onto the target plate.

5. 1ul of each glycan sample is spotted onto the target plate, at least in duplicate, but preferably in triplicate, and allowed to air-dry at room temperature. The target plate is loaded into the MADLI-Tof MS and spectra acquired using a standard PMF (peptide mass fingerprinting) programme.

7.1 Sample preparation

7.1.1 Protein acetone precipitation

One volume of protein extract (total or purified) is precipitated by adding 5 volumes of ice-cold 100 % acetone (stored at – 20 °C) in a 50 ml centrifuge tube (Greiner Bio-one Ltd). The sample is then stored at – 20 °C for 24 h. The sample is centrifuged at 10000 g, for 15min, at 4 °C in a J25 centrifuge (Avanti). The supernatant is discarded and the protein pellet is dried at room temperature for 30 min.

7.1.2 BCA assay

The sample is re-suspended in 5 ml water and protein estimation is carried out using a Pierce BCA assay, according to the manufacturer's instructions. Bovine serum albumin (supplied with the kit) is used for standard curve measurement.

7.1.3 Resuspension

7.1.3.1 Total protein extracts

One volume of total plant protein extract, equivalent to 2 mgml^{-1}, is added to one volume of 10 % (v/v) formic acid, pH 3.0, giving a final concentration of 1 mgml^{-1}. The sample may now be frozen at – 20 °C or used immediately for peptide digestion.

7.1.3.2 Purified Glycoprotein

One volume of purified glycoprotein, equivalent to 200 µgml^{-1}, is added to one volume of 10 % (v/v) formic acid, pH 3.0, giving a final concentration of 100 µgml^{-1}

ie. 1:1. The sample may now be frozen at – 20 °C or used immediately for *N*-glycan release procedure.

7.2 Quality Assurance

N/A

8. CALCULATIONS AND DATA ANALYSIS

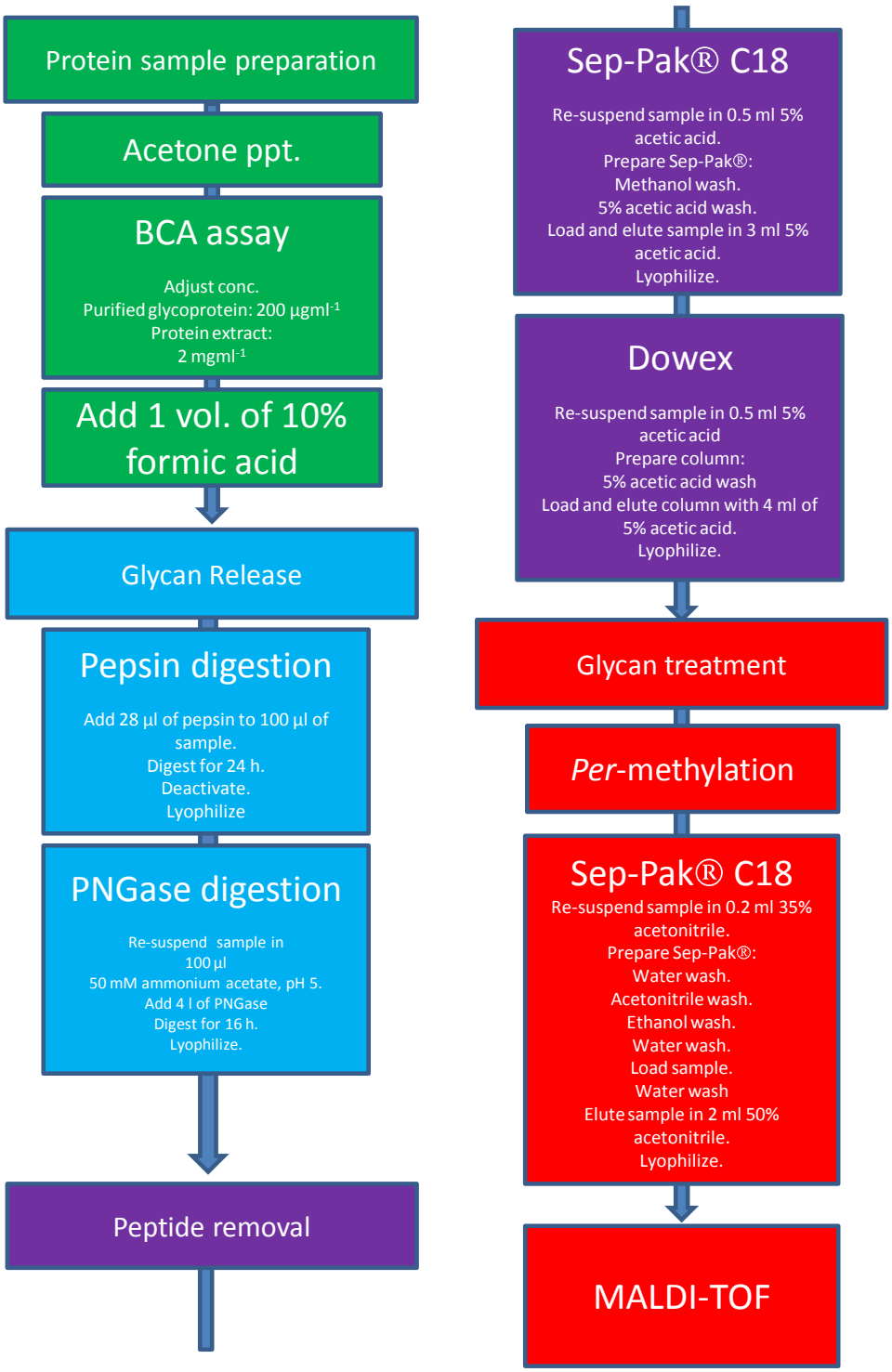
N/A

9. RELATED PROCEDURES

N/A

10. APPENDICES

Figure 1. Simplified flow chart of the procedure follows:



---------- END OF DOCUMENT ----------