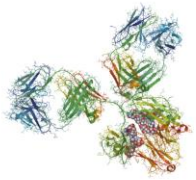

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Project GP-0057.

Validation of Sialic Acid Assay for Fetuin Glycoprotein.

DMB-Kit-Validation-Report-GP-0057-v1.0.doc

4th April 2017

Ludger Ltd

Culham Science Centre, Oxford OX14 3EB, United Kingdom

Tel: +44 870 085 7011, Fax: +44 870 163 4620

Chief Executive: Dr Daryl Fernandes (Email: daryl.fernandes@ludger.com)

Head of Glycoprofiling: Dr Louise Royle (Email: louise.royle@ludger.com)

www.ludger.com

1. Contents

2. Summary

This report covers the validation of an assay used to determine the amounts of Neu5Ac and Neu5Gc in Fetuin (GCP-FET-50U). The validation protocol follows ICH guidelines Q2 (R1).

Sialic acids were released form triplicate aliquots of samples by hydrolysis in 2M acetic acid for 2 hours at 80°C then DMB labelled prior to analysis by LudgerSep-R1 HPLC. Quantitative standards were used to determine the amounts of Neu5Ac and Neu5Gc present in the samples.

A summary of the results from the validation is shown in table 1.

All criteria PASS for Neu5Ac and Neu5Gc following acid hydrolysis of the target amount of 50 µg of Fetuin glycoprotein.

The amounts of Neu5Ac and Neu5Gc have been shown to be stable when the DMB labelled samples are stored at in the dark at 10°C for up to 72 hr, provided that the calibration standards have been stored in the same conditions and are analysed at the same time.

Conclusion.

This assay is suitable for the identification and quantification of the sialic acids Neu5Ac and Neu5Gc in Fetuin glycoprotein.

Table 1. Summary

Analytical Run	Acceptance Criteria	Pass/Fail	Comment
Test I Specificity	Background components do not interfere	PASS	
	Retention times within +/- 0.1 min	PASS	
Test II Repeatability	CVs <5% for retention times	PASS	CVs <0.1%
	CVs < 20% for peak area	PASS	CVs<10%
Test III Intermediate Precision	CVs < 20% for nmol/mg from Operators 1 and 2	PASS	
Test IV Linearity and Range	Mean values of nmol/mg between 80 & 120% target with CV's < 20%	PASS	CVs<10% within target amount
	R^2 nmol vs µg starting material >0.95	PASS	
	R^2 nmol vs peak area for Neu5Ac and Neu5Gc Standards >0.99	PASS	
Test V LOD	LOD S/N ≥3.3 for **Neu5Ac**	PASS	852,871 µV*sec
Test VI LOQ	LOQ S/N ≥10 for **Neu5Ac**	PASS	911,304 µV*sec
Test V LOD	LOD S/N ≥3.3 **Neu5Gc**	PASS	41,993 µV*sec
Test VI LOQ	LOQ S/N ≥10 **Neu5Gc**	PASS	93,965 µV*sec
Test VII Robustness Different Columns	CVs <20% for nmol/mg	PASS	CVs<5%
Test VII Robustness Sample Stability	CVs <5% for retention times for Neu5Ac and Neu5Gc	PASS	CVs<1%
	CVs <20% for nmol/mg for Neu5Ac and Neu5Gc	PASS	CVs<3%, for up to 72hr at 10°C in dark
Test VIII Accuracy	CVs <5% for retention times	PASS	CVs<1%
	CVs <20% for peak area	PASS	CVs<10%

3. Introduction

The objective of this analytical method validation study is to show that the following Ludger SOPs are suitable for their intended use which is identification and quantification of the sialic acids Neu5Ac and Neu5Gc in glycoprotein samples. The glycoprotein Fetuin (from bovine serum) is used for this study as it has a range of sialylated N- and O-glycans.

Table 2. Method SOPs

SOP #	**SOP Version**
SOP-00178-LT-KDMB-A1-Sialic-Acid-Release-and-Labelling	v5.3
SOP-00242-Sialic-acid-HPLC-and-Data-Analysis	v2.0
SOP-00243-LudgerSepR1-30min-DMB	v2.0

Validation Characteristics

According to the ICH guideline Q2 (R1) (for Analytical Validation) the following validation characteristics (checked boxes) are to be included in this validation study:

- ☒ Specificity
- ☒ Accuracy
- ☒ Repeatability (Intra-assay precision)
- ☒ Intermediate precision
- ☒ Linearity
- ☒ Working range
- ☒ Detection limit
- ☒ Quantitation limit
- ☒ Robustness

In case of any deviation from the relevant ICH guidelines the reason has to be stated.

4. Samples

Fetuin glycoprotein is used throughout this validation study. A sialic acid reference panel (CM-SRP-01) is used as a system suitability standard (SSS) for the chromatography system; Neu5Ac and Neu5Gc sialic acid standards are used to compare the retention times of the sialic acids with those from the sample for accuracy. The Neu5Ac and Neu5Gc sialic acid standards are also used quantitatively to produce a standard curve for determination of the amounts of sialic acids in the samples.

Table 3. Samples, controls and standards

Sample	**Product Name**	**Lot Number**
Fetuin glycoprotein	GCP-FET-50U	# B6AS-09
Water	-	-
SRP	CM-SRP-01	# B69E-01
Neu5Ac Standard	CM-NEUAC-01	# B696-01
Neu5Gc Standard	CM-NEUGC-01	# B6A4-01

5. Methods

5.1 Sialic Acid Release and DMB labelling

Sialic acids are released by hydrolysis in 2M acetic acid for 2 hours at 80 °C. Released sialic acids are labelled using a Ludger Tag™ DMB sialic acid Labelling Kit **[LT-KDMB-A1]** complete with Neu5Ac and Neu5Gc quantitative standards plus a sialic acid reference panel **[CM-SRP-01]** system suitability standard following **SOP-00178- LT-KDMB-A1-Sialic-Acid-Release-and-Labelling.** 20 % of the hydrolysed sample is DMB labelled at 50 °C for 3 hours, then analysed as follows.

5.2 HPLC Analysis

DMB labelled sialic acids are analysed by LudgerSep-R1-HPLC following **SOP-00242-Sialic-acid-HPLC-and-Data-Analysis** using a **LudgerSep-R1 column 4.6 x 150 mm** column at 30°C on a Waters 2795 HPLC with a 2475 fluorescence detector (λex = 373 nm, λem = 448 nm), controlled by Empower software version 3, build 3471. The running methods detailed in **SOP-00243-LudgerSepR-30min-DMB-Sialic-Acid-Method** are used: Gradient conditions are: 0 to 19 min, 100% A; 19 to 19.5 min, 100 to 10% A; 19.5 to 23.5 min, 10% A; 23.5 to 24 min, 10 to 100% A; 24 to 30 min, 100% A; at a flow rate of 0.5 mL/min. Solvent A is acetonitrile: methanol: water (9:7:84 by volume); Solvent B is acetonitrile. Samples are diluted 1 in 10 with water before injection. The 1 nmol Neu5Ac and Neu5Gc quantitative standards are diluted with water to produce standard curves (Neu5Ac range of 0.001 to 1 nmol; Neu5Gc range of 0.0002 to 0.5 nmol). 10 µL were injected onto the HPLC.

5.3 Data Processing and Reporting.

Data was processed according to **SOP-00242-Sialic-acid-HPLC-and-Data-Analysis.** The amount of Neu5Ac and Neu5Gc per sample (nmol) is calculated by reference to the calibration curves (the full range of standard dilutions are run both before and after the samples to produce the standard curve). The standard curves are calculated as a linear fit through zero, with the peak areas reported as nmol per sample (taking any dilution into account) within Empower software. The standard curve is based on the values for the quantitative standards Neu5Ac and Neu5Gc (1 nmol). CVs are generally less than 20%. The certificate of authenticity (CofA) for the Neu5Ac and Neu5Gc standards within the DMB labelling kit detail the amounts of the 1 nmol standards to two decimal places (e.g. for Neu5Ac Lot # B696-01the amount was 1.05 nmol, and NeuGc Lot # B6A4-01 the amount was 1.07 nmol). These conversion factors are used when converting the nmol amounts detected into nmol/mg protein.

The amount of Neu5Ac and Neu5Gc as nmol/mg protein is calculated with reference to having analysed 20% of the amount of protein in the original aliquot. For example if a 0.05 mg aliquot of protein is taken for hydrolysis, the mean amount of nmol is multiplied by 5 (because only 20% of the hydrolysed sample is analysed) and divided by 0.05 (the number of mg starting material).

For the Fetuin glycoprotein positive control 50 µg aliquots are analysed (of which only 34 µg is protein as measured by BCA analysis for Lot #B6AS-09). The Fetuin results are quoted in nmol per mg of protein to be

able to compare the data obtained in each work order. Published data [http://www.abrf.org/ABRFNews/1997/March1997/mar97monosach.html] reports the Neu5Ac content of Fetuin as 330-440 nmol/mg protein as measured across a number of different laboratories. Measurements at Ludger on product GCP-FET-50U (44 independent measurements over a period of 5 years) gave a mean value of 362 nmol/mg protein within the range of 286 to 439 nmol/mg protein (equivalent to ± two standard deviations). The acceptance criterion for sialic acid analysis is that the value for Fetuin should lie within this range. The Ludger BioQuant standard GPEP-A2G2S2 [which has been quantified by NMR (10 µg; 6.98 nmol sialic acid) was also run as a process positive control, with acceptance criteria of +/- 20% of the NMR value stated in the CofA.

All work will be performed at Ludger Ltd based at the Culham Science Centre, Abingdon, UK.

Table 4. List of releases, tests and operators

Release	Date Started	Operator	Test Performed	Data Analysed by	Data Checked by
A	4 Nov 16	Jenifer Hendel	I, VII (a), VIII	Louise Royle	Jenifer Hendel
B	13 Dec 16	Jenifer Hendel	II, III, IV, V, VI, VII (b)	Louise Royle	Jenifer Hendel
C	13 Mar 17	Indrani Harper	III	Louise Royle	Jenifer Hendel

6. Test Parameters and Results

6.1 Test Parameter I - Specificity

Procedure

According to the ICH-guidelines the specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. The components known to be present in the sample that could interfere with the analysis are formulation buffers.

Specificity is demonstrated by verifying that the components of the negative control do not interfere with any peaks and by the comparability of the sample and the control standard.

Description of the experiments

A comparison between water negative control and samples is carried out. To show the identity, the sialic acid profile of sample and the standards are compared.

Table 5. Sample description and procedure for specificity experiments

Sample	Description	Repetitions
Fetuin	Preparation and chromatography according to SOPs. Day 1, Operator 1	3 x release & labellings (3 HPLC runs)
Water	Preparation and chromatography according to SOPs. Day 1, Operator 1	3 x release & labellings (3 HPLC runs)
Neu5Ac Standard	Preparation and chromatography according to SOPs. Day 1, Operator 1	3 x labellings (3 HPLC runs)
Neu5Gc Standard	Preparation and chromatography according to SOPs. Day 1, Operator 1	3 x labellings (3 HPLC runs)

Reporting Criteria

- Chromatograms of each sample and controls.
- Retention times.

Acceptance criteria

- Buffer components do not interfere with sialic acid separation and determination.
- Retention times for Neu5Ac and Neu5Gc are comparable (within +/- 0.1 min) between samples, controls and sialic acid standards.

Results - Specificity

Figures 1 to 6 show chromatograms for all samples and controls. The water negative control does not contain any significant amounts of components which interfere with Neu5Ac and Neu5Gc separation and determination.

The retention times for samples, controls and Neu5Gc and Neu5Ac standards are listed in table 6. All retention times are comparable and fall within a 0.1 min range.

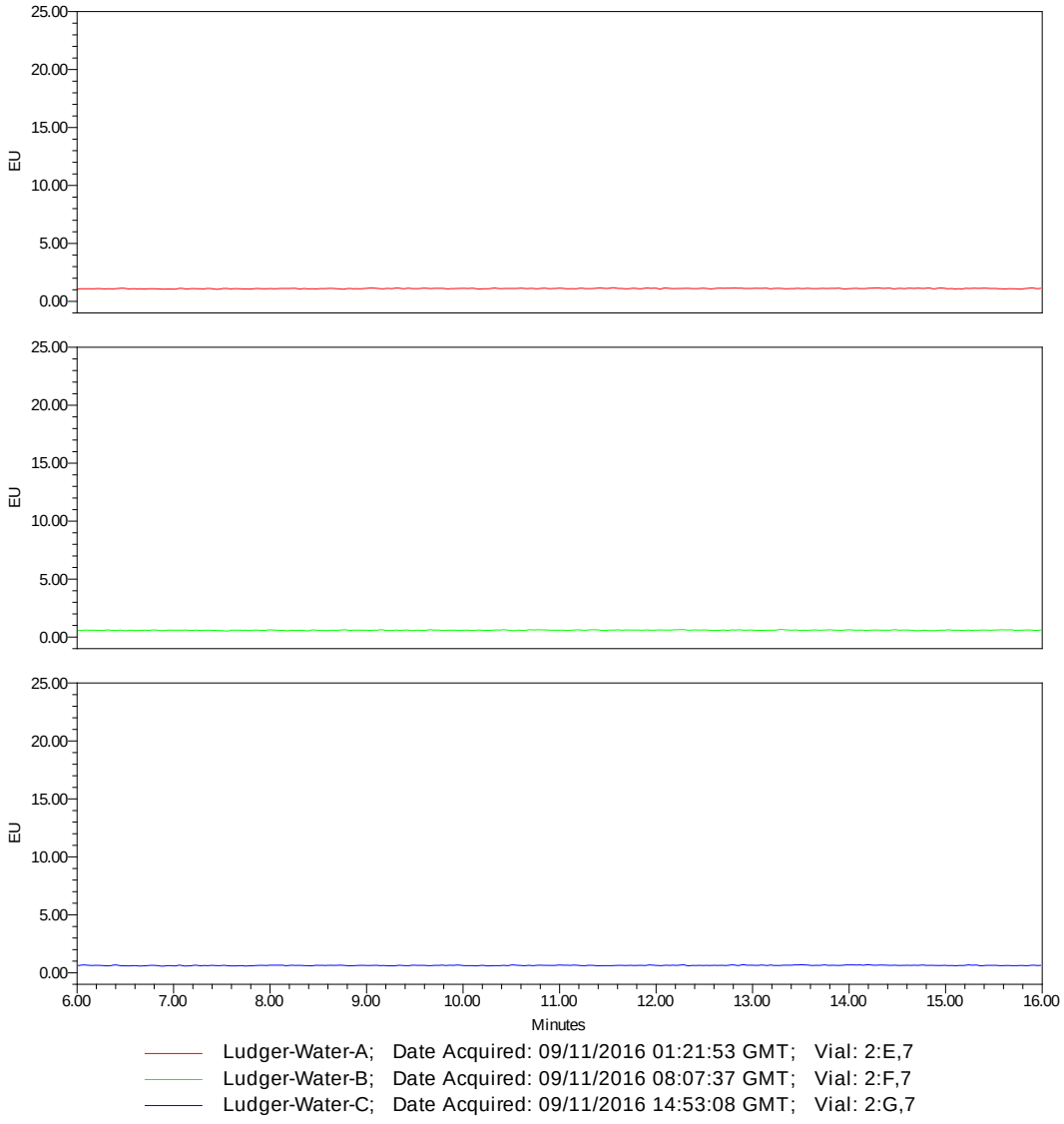


Figure 1. Chromatograms for Water negative control

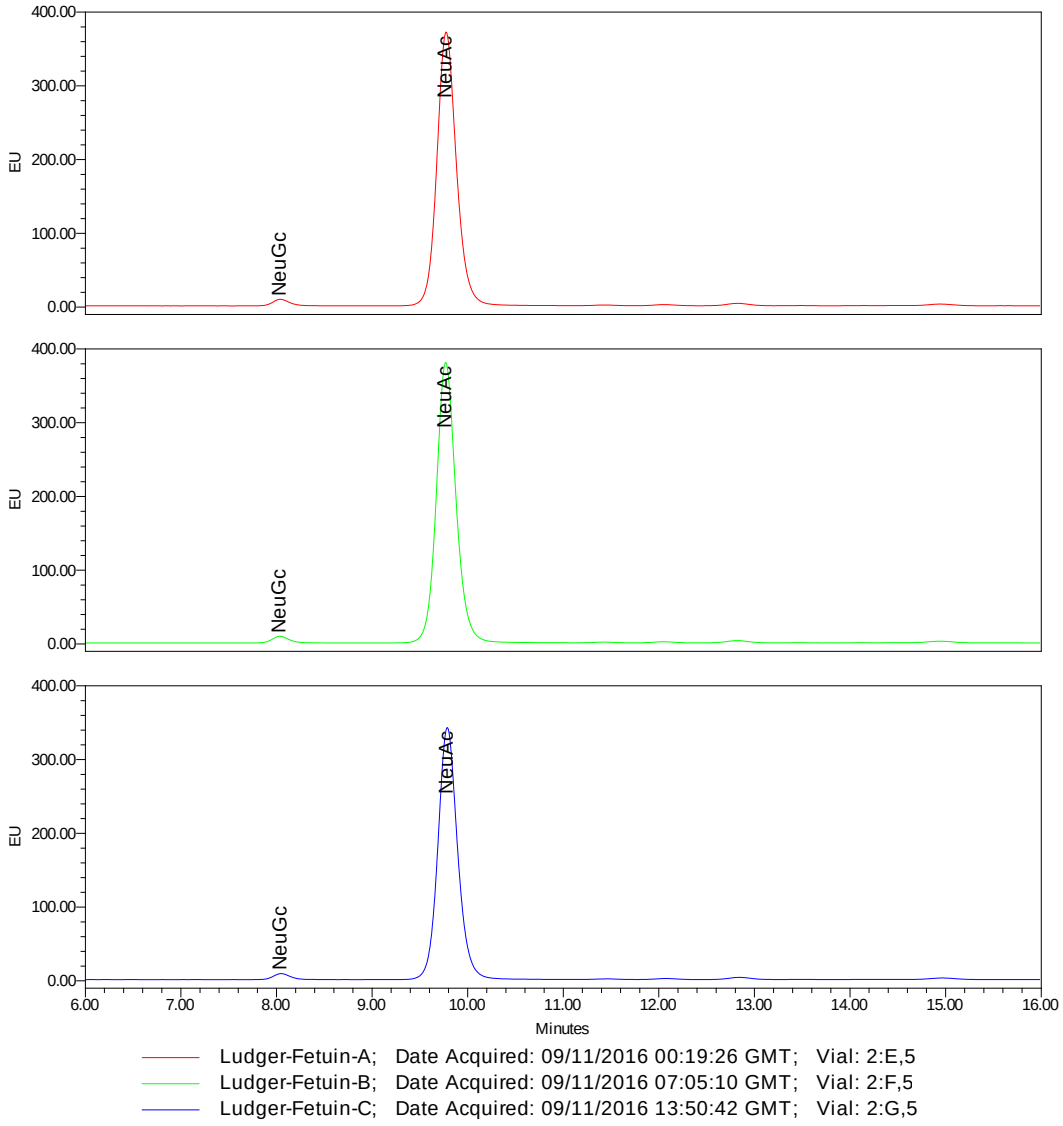


Figure 2. Chromatograms for Fetuin

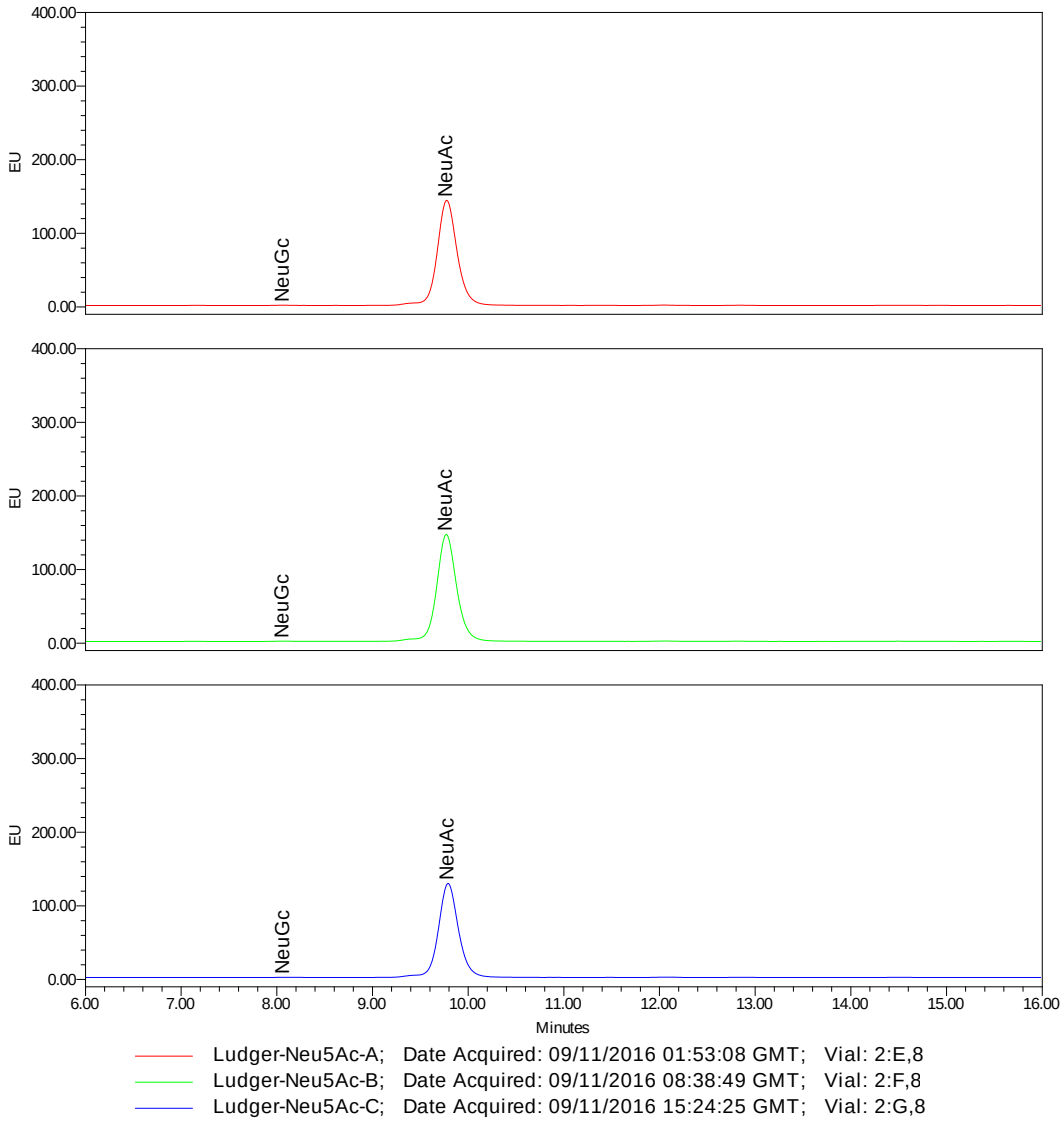


Figure 3. Chromatograms for Neu5Ac Standard (1 in 10 dilution)

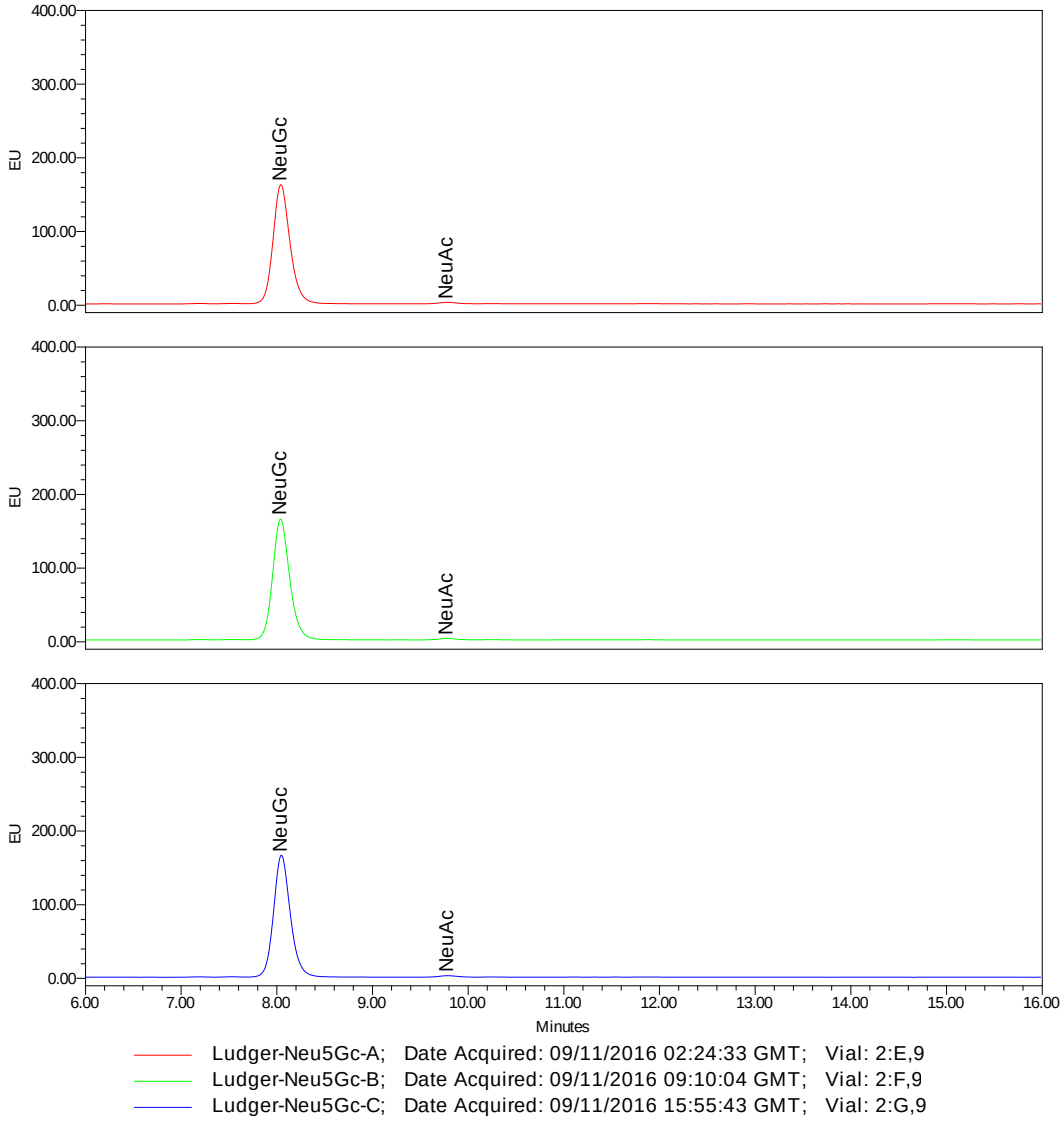


Figure 4. Chromatograms for Neu5Gc Standard (1 in 10 dilution)

Table 6. Table of retention times

		Retention time [min]	
Sample	**Replicate**	**Neu5Gc**	**Neu5Ac**
Fetuin	A	8.051	9.785
	B	8.047	9.780
	C	8.058	9.797
Neu5Gc Standard	A	8.052	-
	B	8.049	-
	C	8.058	-
Neu5Ac Standard	A	-	9.786
	B	-	9.782
	C	-	9.800
Retention times	**Minimum**	**8.047**	**9.770**
	Maximum	**8.058**	**9.800**
Range in Retention times		**0.011**	**0.020**

6.2 Test Parameter II – Repeatability (Precision)

Procedure

For the validation characteristic: repeatability, the following procedure will be used:

A minimum of 9 determinations covering the specified range for the procedure (e.g. 3 times per sample / 3 repetitions each).

Repeatability is determined using the glycoprotein sample Fetuin, which are prepared and analysed according to SOPs: day 1, one analyst (operator 1) using HPLC system 1 and column 1.

Table 7. Sample description and procedure for repeatability experiment

Sample	Description	Repetitions
Fetuin	Preparation and chromatography according to SOPs. Day 1, Operator 1	3 release & labellings (3x3 HPLC runs).

Reporting Criteria

- Report mean, standard deviation and CV for the area of Neu5Ac and Neu5Gc for each sample where n = 9.
- Report mean, standard deviation and CV for Retention time of Neu5Ac and Neu5Gc for each sample where n = 9.

Acceptance Criteria

- CV < 5% for retention times across all sample measurements.
- CV < 20% for peak areas for each sample.

Results - Repeatability

The data for area of Neu5Ac and Neu5Gc in Fetuin is shown in table 8. All CVs are less than 10%.

The data for retention times of Neu5Ac and Neu5Gc in Fetuin is shown in table 9. All CVs are less than 1%.

Table 8. Peak areas for Fetuin replicates

			Area[µV*sec]	
Sample	**Replicate**	**Injection**	**Neu5Gc**	**Neu5Ac**
Fetuin	A	1	1122133	48512156
		2	1078817	48564134
		3	1069473	48407174
	B	1	1112334	52655915
		2	1156973	52369577
		3	1140532	52240059
	C	1	1137383	54657520
		2	1132349	54134254
		3	1160695	54494528
Mean			**1123410**	**51781702**
STDEV			**31834**	**2619979**
CV%			**2.83**	**5.06**

Table 9. Retention times for Fetuin replicates

			Retention time[min]	
Sample	**Replicate**	**Injection**	**Neu5Gc**	**Neu5Ac**
Fetuin	A	1	8.088	9.836
		2	8.084	9.832
		3	8.085	9.831
	B	1	8.093	9.842
		2	8.093	9.842
		3	8.090	9.840
	C	1	8.103	9.854
		2	8.100	9.852
		3	8.100	9.852
Mean			**8.093**	**9.842**
STDEV			**0.007**	**0.009**
CV%			**0.08**	**0.09**

6.3 Test Parameter III - Intermediate Precision

Procedure

The following variations are included in the study:

☒ Different days

☒ Different analysts

The intermediate precision will be confirmed by analysing glycoprotein sample Fetuin which are prepared and run by two different operators on two different days in the same manner which is used in the repeatability study. The data generated under repeatability (precision) will be used to represent day 1 and operator 1.

Table 10. Sample description and procedure for intermediate precision experiment, different operators

Sample	Description	Repetitions
Fetuin	Preparation and chromatography according to SOPs. Day 1, Operator 1	3 release & labellings (3x3 HPLC runs).
Neu5Ac Standard	Preparation and chromatography according to SOPs. Day 1, Operator 1	1 x labelling (1 x 5 HPLC runs – standard curve)
Neu5Gc Standard	Preparation and chromatography according to SOPs. Day 1, Operator 1	1 x labelling (1 x 5 HPLC runs – standard curve)
Fetuin	Preparation and chromatography according to SOPs. Day 2, Operator 2	3 release & labellings (3x3 HPLC runs).
Neu5Ac Standard	Preparation and chromatography according to SOPs. Day 2, Operator 2	1 x labelling (1 x 5 HPLC runs – standard curve)
Neu5Gc Standard	Preparation and chromatography according to SOPs. Day 2, Operator 2	1 x labelling (1 x 5 HPLC runs – standard curve)

Reporting Criteria

- Report mean, standard deviation and CV for the nmol/mg protein of Neu5Ac and Neu5Gc where n = 9 for each sample from Operator 1, day 1.
- Report mean, standard deviation and CV for the nmol/mg protein of Neu5Ac and Neu5Gc where n = 9 for each sample from Operator 2, day 2.

Acceptance Criteria

- % CV < 20% for mean values of nmol of Neu5Ac and Neu5Gc per mg of protein.

Results – Intermediate Precision

The mean, standard deviation and CV for nmol of Neu5Ac and Neu5Gc from Operator 1, day 1 are reported in table 11, for Operator 2, day 2 are in table 12, and across both operators in table 13.

CV%'s are less than 10% for Neu5Ac and Neu5Gc across both operators.

Table 11. Amounts in nmol/mg protein for Fetuin replicates: Operator 1, Day 1

			nmol/mg protein	
Sample	**Replicate**	**Injection**	**Neu5Gc**	**Neu5Ac**
Fetuin	A	1	8.483	269.635
		2	8.194	269.921
		3	8.132	269.058
	B	1	8.417	292.251
		2	8.714	290.857
		3	8.605	290.144
	C	1	8.584	303.444
		2	8.551	300.565
		3	8.739	302.547
Mean			**8.49**	**287.60**
STDEV			**0.21**	**14.41**
CV%			**2.50**	**5.01**

Table 12. Amounts in nmol/mg protein for Fetuin replicates: Operator 2, Day 2

			nmol/mg protein	
Sample	**Replicate**	**Injection**	**Neu5Gc**	**Neu5Ac**
Fetuin	A	1	9.48	326.69
		2	9.60	325.11
		3	9.59	322.81
	B	1	9.12	315.73
		2	9.12	309.81
		3	8.95	305.37
	C	1	8.48	292.95
		2	8.57	292.49
		3	8.43	289.70
Mean			**9.04**	**308.96**
STDEV			**0.47**	**14.68**
CV%			**5.17**	**4.75**

Table 13. Amounts in nmol/mg protein for Fetuin replicates for operator 1 and 2

			nmol/mg protein	
Sample	**Replicate**	**Injection**	**Neu5Gc**	**Neu5Ac**
Operator 1 Fetuin	A	1	8.483	269.635
		2	8.194	269.921
		3	8.132	269.058
	B	1	8.417	292.251
		2	8.714	290.857
		3	8.605	290.144
	C	1	8.584	303.444
		2	8.551	300.565
		3	8.739	302.547
Operator 2 Fetuin	A	1	9.48	326.69
		2	9.60	325.11
		3	9.59	322.81
	B	1	9.12	315.73
		2	9.12	309.81
		3	8.95	305.37
	C	1	8.48	292.95
		2	8.57	292.49
		3	8.43	289.70
Mean			**8.76**	**298.28**
STDEV			**0.45**	**17.89**
CV%			**5.14**	**6.00**

6.4 Test Parameter IV – Linearity and Range

Procedure

A range of initial quantities of sample material are taken through the release and labelling procedure to confirm that starting with different amounts of material does not change the quantitation in nmol of Neu5Ac or Neu5Gc.

Table 14. Sample description and procedure for linearity experiment

Sample	Description	Repetitions
Fetuin	Preparation and chromatography according to SOPs Operator 1	3 release labelling,100% normal amount of sample (50 µg) (3 HPLC runs)
Fetuin	Preparation and chromatography according to SOPs Operator 1	3 release labelling, 50% normal amount of sample (25 µg), (3 HPLC runs)
Fetuin	Preparation and chromatography according to SOPs Operator 1	3 release labelling, 10% normal amount of sample (5 µg), (3 HPLC runs)
Fetuin	Preparation and chromatography according to SOPs Operator 1	3 release labelling, 200% normal amount of sample (100 µg), (3 HPLC runs)
Fetuin	Preparation and chromatography according to SOPs Operator 1	3 release labelling, 150% normal amount of sample (75 µg), (3 HPLC runs)
Neu5Ac Standard	Preparation and chromatography according to SOPs. Day 1, Operator 1	1 x labelling (1 x 5 HPLC runs – standard curve)
Neu5Gc Standard	Preparation and chromatography according to SOPs. Day 1, Operator 1	1 x labelling (1 x 5 HPLC runs – standard curve)

Reporting Criteria

- Report mean, standard deviation and CV for the nmol of Neu5Ac and Neu5Gc in samples.
- Plot of amount of sample taken per release versus nmol for Neu5Ac and Neu5Gc in samples.

Acceptance Criteria

- The mean values of nmol of Neu5Ac and Neu5Gc per mg of protein should be between 80 and 120% of target with CV's of <20%.
- R^2 >0.95 from a plot of nmol of Neu5Ac and Neu5Gc vs µg of protein starting material

Results – Linearity and Range for Samples

The mean, standard deviation and CV for nmol of Neu5Ac and Neu5Gc from the range of 5 to 100 µg of Fetuin glycoprotein (3.4 to 68 µg protein) is shown in table 15. The CV%s are all less than 10% for Neu5Ac and Neu5Gc .

Table 16 shows data for mean nmol/mg. The target amount was taken from the 50 µg of glycoprotein data. Data that falls within the acceptance range of 80 to 120% of the target is highlighted in green. The mean data for Neu5Ac falls within this for the whole range taken (5 to 100 µg glycoprotein). The data for Neu5Gc falls outside of the acceptance range at the 5 µg of glycoprotein level, but is within the acceptance range over the 25 to 100 µg of glycoprotein starting amounts.

Data for each individual measurement is shown in table 17 and plotted in figures 5 and 6. These figures show that the R^2 values for Neu5Ac and Neu5Gc are above 0.95 across the range of glycoprotein amounts tested.

Table 15. Data for nmol of the Fetuin from a range of 3.4 to 68 µg of protein starting amounts

CVs of less than 20% highlighted in green.

				nmol	
Amount of Fetuin Glycoprotein [µg]	**Amount of Fetuin Protein [µg]**	**% of normal amount**		**Neu5Gc**	**Neu5Ac**
100	**68**	**200**	**Mean**	**0.50655**	**18.10035**
			STDEV	0.03689	1.09544
			CV%	7.28	6.05
75	**51.0**	**150**	**Mean**	0.19945	14.06073
			STDEV	0.01133	0.89334
			CV%	5.68	6.35
50	**34**	**100**	**Mean**	**0.28882**	**9.80707**
			STDEV	0.00286	0.58559
			CV%	0.99	5.97
25	**17**	**50**	**Mean**	**0.14069**	**4.29696**
			STDEV	0.00221	0.06876
			CV%	1.57	1.60
5	**3.4**	**10**	**Mean**	**0.05782**	**0.92206**
			STDEV	0.00159	0.08480
			CV%	2.76	9.20

Table 16. The mean nmol/mg of monosaccharides for Fetuin from a range of 3.4 to 68 µg protein starting amounts

The target amount is taken from the 500 µg samples. Data that falls within the acceptance range of 80 to 120% of the target is highlighted in green.

Amount of Fetuin Glycoprotein [µg]	**Amount of Fetuin protein [µg]**	**Mean nmol/mg**	
		Neu5Gc	**Neu5Ac**
100.0	**68.0**	7.449	266.182
75.0	**51.0**	7.822	275.701
50.0	**34.0**	8.495	288.443
25.0	**17.0**	8.276	252.762
5.0	**3.4**	17.005	271.194

Acceptance Range		
120%	10.1936	346.1320
80%	6.7957	230.7547

Table 17. Data for nmol of sialic acids for Fetuin replicates across a range of 3.4 to 68 µg starting amounts.

				nmol	
Amount of Fetuin Glycoprotein [µg]	**Amount of Fetuin protein [µg]**	**% of normal amount**	**Replicate**	**Neu5Gc**	**Neu5Ac**
100	**68.0**	200	A	0.528	18.836
100	**68.0**	200	B	0.527	18.624
100	**68.0**	200	C	0.464	16.841
75	**51.0**	150	A	0.208	14.825
75	**51.0**	150	B	0.204	14.279
75	**51.0**	150	C	0.187	13.079
50	**34.0**	100	A	0.288	9.168
50	**34.0**	100	B	0.286	9.937
50	**34.0**	100	C	0.292	10.317
25	**17.0**	50	A	0.143	4.255
25	**17.0**	50	B	0.140	4.376
25	**17.0**	50	C	0.139	4.260
5	**3.4**	10	A	0.058	0.935
5	**3.4**	10	B	0.060	0.999
5	**3.4**	10	C	0.056	0.831
Linear Fit 5 to 100 µg glycoprotein			**R^2**	**0.987**	**0.990**
Linear Fit 25 to 100 µg glycoprotein			**R^2**	**0.980**	**0.984**

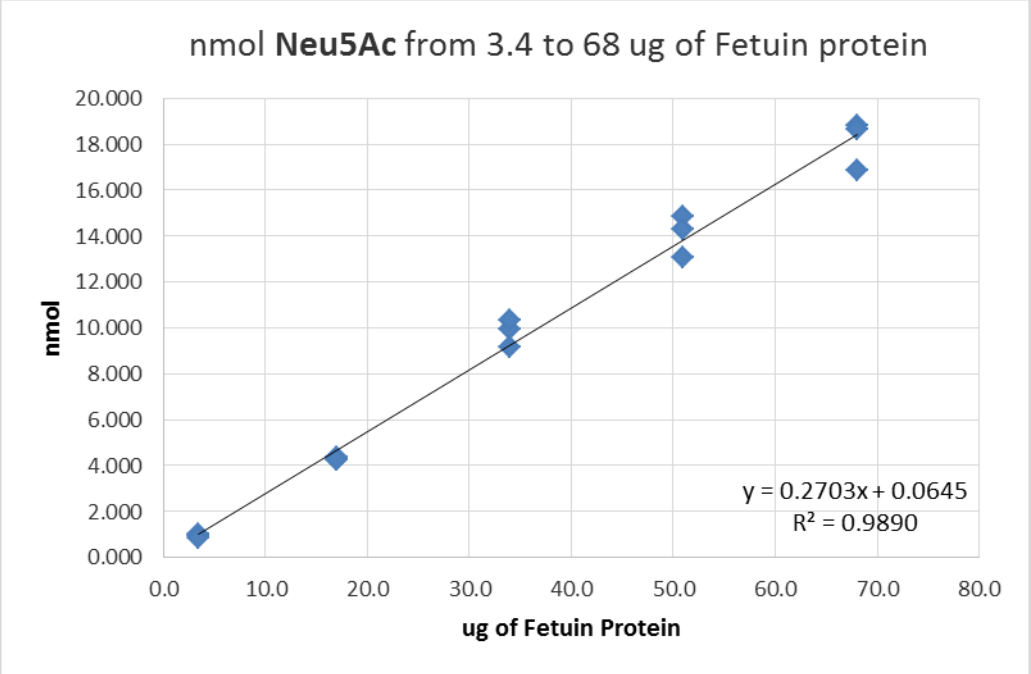


Figure 5. Plot of nmol for Neu5Ac vs amount of Fetuin protein taken through process (regression line is a linear fit not through zero)

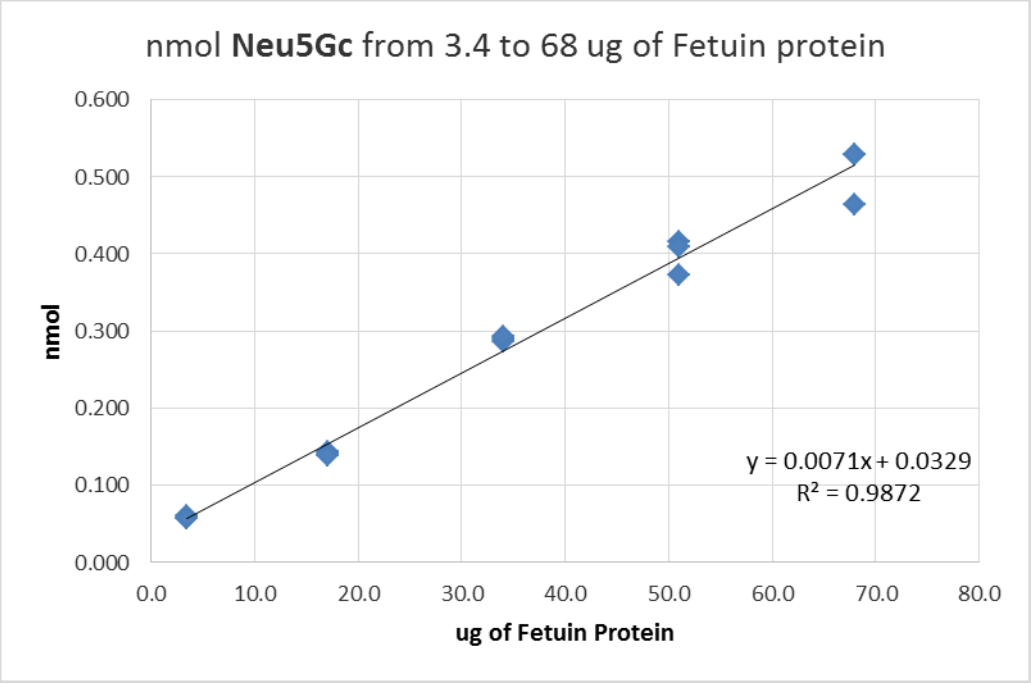


Figure 6. Plot of nmol for Neu5Gc vs amount of Fetuin protein taken through process (regression line is a linear fit not through zero)

Procedure

The linearity of DMB-sialic acid detection will be confirmed by a range of injection concentrations. This is to confirm that the relative percentage areas do not vary when different amounts of DMB labelled sialic acids are injected.

Table 18. Sample description and procedure for Linearity Experiment

Sample	Description	Repetitions
Neu5Ac Standard	Preparation and chromatography according to SOPs. Day 1, Operator 1	1 labelling (1 HPLC run at 8 dilutions in the range from undiluted to 1 in 5000 dilution in water)
Neu5Gc Standard	Preparation and chromatography according to SOPs. Day 1, Operator 1	1 labelling (1 HPLC run at 8 dilutions in the range from undiluted to 1 in 5000 dilution in water)

Reporting Criteria

- Plot of peak area vs amount in nmol of Neu5Ac or Neu5Gc.

Acceptance Criteria

- Determine working range where R^2 >0.99 from a plot of amount of Neu5Ac and Neu5Gc in nmol taken vs peak areas.

Results – Linearity and Range for Standards

The peak areas and amounts of Neu5Ac and Neu5Gc from the sialic acid standards are listed in table 19 and plotted in figures 7 and 8. The R^2 values are above 0.9999.

The working range of 0.0010 to 1 nmol is within the acceptance criteria for Neu5Ac.
The working range of 0.0002 to 0.5 nmol is within the acceptance criteria for Neu5Gc.

Table 19. Data for nmol of the sialic acid standards across a range of amounts

	Peak Area [uV*sec]	
nmol	**Neu5Ac**	**Neu5Gc**
0.0002		73192
0.0010	268162	248088
0.0100	2786370	2294659
0.0200	5463008	4492701
0.1000	27114971	23419560
0.2000	56413409	46856486
0.5000	139417649	118867221
1.0000	280996430	
Linear Fit	**0.99998**	**0.99996**

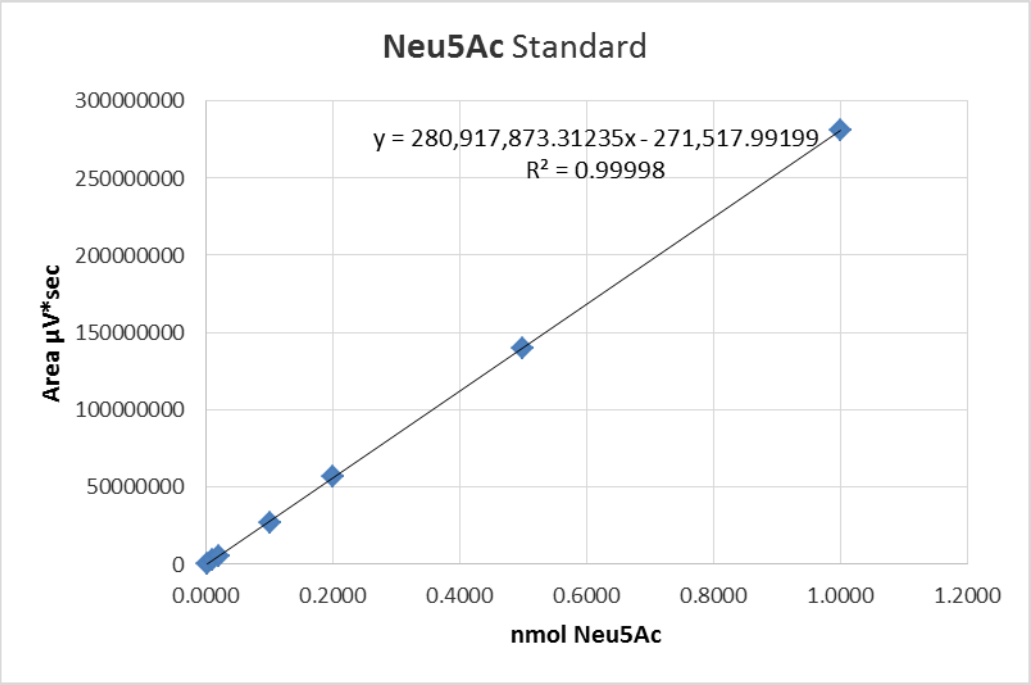


Figure 7. Plot of peak area vs amount for Neu5Ac Standard (regression line is a linear fit not through zero)

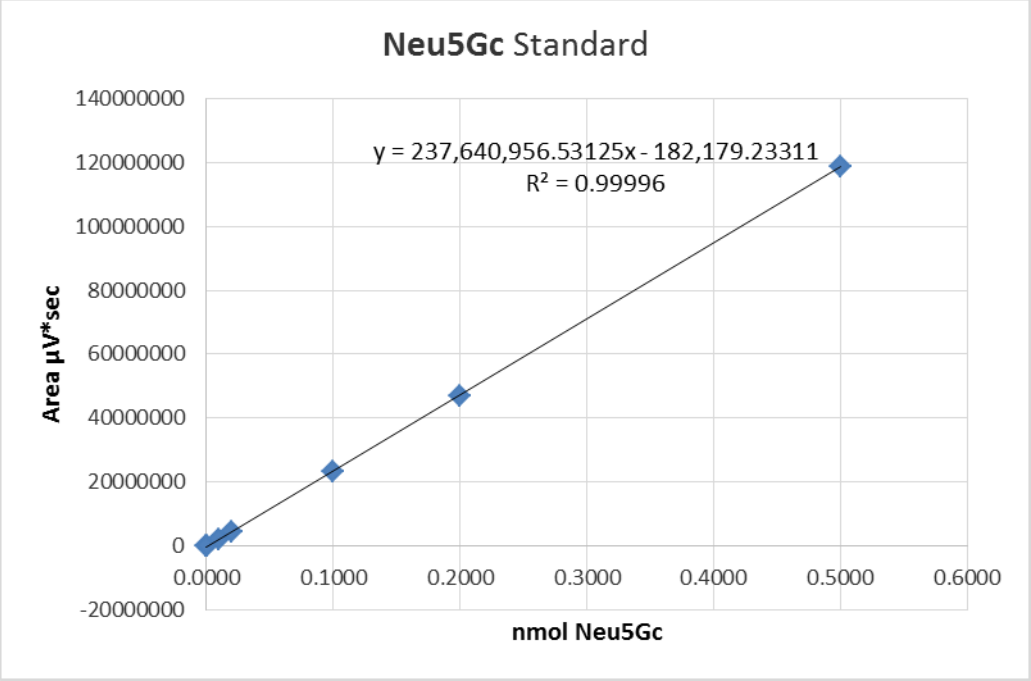


Figure 8. Plot of peak area vs amount for Neu5Gc Standard (regression line is a linear fit not through zero)

6.5 Test Parameter V and VI – Procedure Limit of Detection and Quantitation

Procedure

To determine the LOD and LOQ.

Table 20. Sample description and procedure for procedure limit of detection and quantitation

Sample	Description	Repetitions
Fetuin	No additional experiments – use data from Linearity and Range Study	No additional experiments – use data from Linearity and Range Study

Reporting Criteria

- Report LOD and LOQ as peak area.
- Report of %CV of LOQ.

Acceptance Criteria

- The response at LOD and LOQ must have a S/N ≥3.3 and ≥ 10.0, respectively.

Results – LOD and LOQ

The data on signal to noise is presented in table 21, and for peak areas in table 22. The baseline noise was measured over the range 20 to 22 min.

For Neu5Ac all samples in the range 5 to 100 µg give a signal to noise of above 10. A plot of S/N vs peak area (figure 9) gives a linear regression fit with R^2= 0.889, with the equation: Area = 8721.4 x S/N +824090. This equation gives the values for the LOD as 852,871 µV*sec and the LOQ as 911,304 µV*sec for Neu5Ac. The CVs on peak areas above the LOQ are less than 10.5% for Neu5Ac peak areas and in the range 6-33% on S/N.

For Neu5Gc samples in the range 5 to 100 µg give a signal to noise of above 10. A plot of S/N vs peak area (figure 10) gives a linear regression fit with R^2 of 0.878, with the equation: Area = 7757 x S/N +16395. This equation gives the values for the LOD as 41,993 µV*sec and the LOQ as 93,965 µV*sec for Neu5Gc. The CVs on peak areas above the LOQ are less than 10% for Neu5Gc peak areas and in the range 6-22% on S/N.

Both Neu5Ac and Neu5Gc show a linear relationship between sample amount and signal to noise and are present at levels above the LOQ across the whole range of amounts (5 to 100 µg).

Table 21. Signal to noise for Neu5Ac and Neu5Gc

S/N >10 highlighted in green = above LOQ.

Amount of Fetuin glycoprotein [ug]	Amount of Fetuin protein [µg]	Replicate	S/N					
			Neu5Ac			Neu5Gc		
			Replicates	Mean	CV%	Replicates	Mean	CV%
5	3.4	A	541.87	527.74	12.97	13.92	13.36	10.89
5	3.4	B	588.00			14.45		
5	3.4	C	453.34			11.70		
25	17.0	A	2780.94	2906.47	6.90	67.76	70.74	5.94
25	17.0	B	3137.78			75.55		
25	17.0	C	2800.69			68.92		
50	34.0	A	4408.43	6439.57	32.94	107.88	155.93	32.27
50	34.0	B	6269.13			151.67		
50	34.0	C	8641.16			208.23		
75	51.0	A	8609.96	8548.93	6.94	208.35	207.31	7.11
75	51.0	B	9109.61			221.50		
75	51.0	C	7927.22			192.07		
100	68.0	A	7359.86	9743.90	21.76	179.68	237.34	21.71
100	68.0	B	11417.51			278.90		
100	68.0	C	10454.32			253.44		

Table 22. Peak areas for Neu5Ac and Neu5Gc

CV <10% highlighted in green.

Amount of Fetuin glycoprotein [ug]	Amount of Fetuin protein [µg]	Replicate	Area [µV*sec]					
			Neu5Ac			Neu5Gc		
			Replicates	Mean	CV%	Replicates	Mean	CV%
5	3.4	A	4501529	4430526	10.23	101597	102882	6.85
5	3.4	B	4844161			110480		
5	3.4	C	3945888			96568		
25	17.0	A	22248386	22473149	1.64	480025	469215	2.08
25	17.0	B	22897324			466585		
25	17.0	C	22273737			461034		
50	34.0	A	48512156	51930864	6.03	1122133	1123950	1.12
50	34.0	B	52622915			1112334		
50	34.0	C	54657520			1137383		
75	51.0	A	78756328	74671394	6.40	1682503	1610534	6.22
75	51.0	B	75837359			1652963		
75	51.0	C	69420494			1496136		
100	68.0	A	100198463	96267763	6.08	2182079	2086386	7.81
100	68.0	B	99067724			2178879		
100	68.0	C	89537103			1898201		

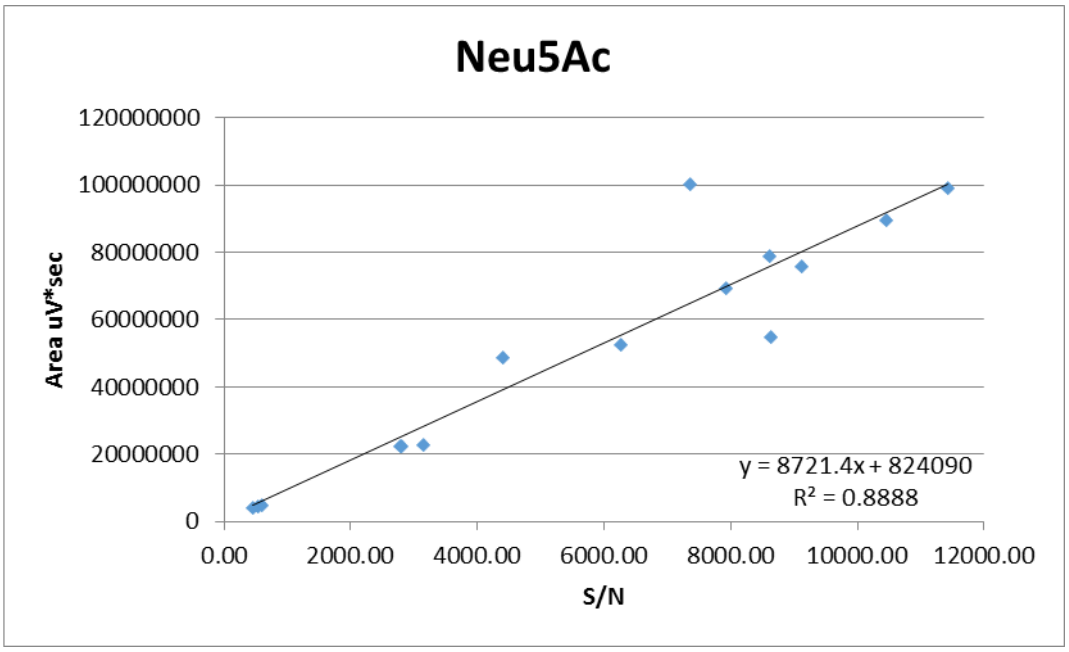


Figure 9. Plot of S/N vs peak area, linear fit not through zero for Neu5Ac

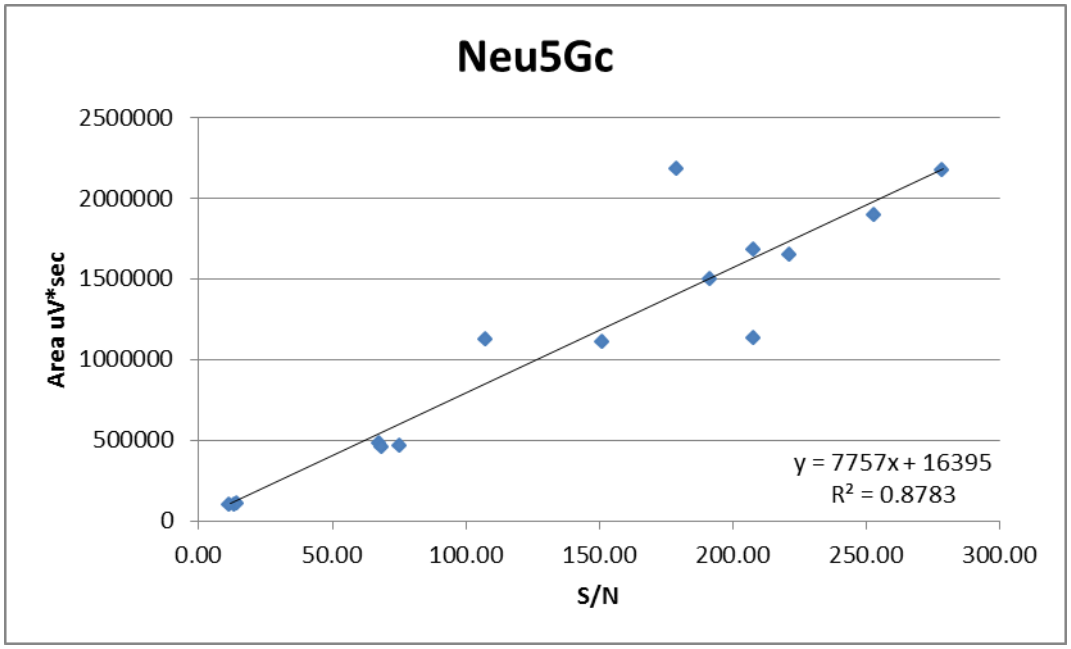


Figure 10. Plot of S/N vs peak area, linear fit not through zero for Neu5Gc

6.6 Test Parameter VII – Robustness

Different Columns

Procedure

To show the robustness of the method, the nmol/mg protein for Neu5Ac and Neu5Gc in the sample is compared on two different columns.

Table 23. Sample description and procedure for robustness: column experiment

Column	Sample	Description	Repetitions
1 (Batch #B43I-04)	Fetuin	Preparation and chromatography according to SOPs, Day 1, Operator 1	3 release labelling (3 HPLC runs)
1 (Batch #B43I-04)	Neu5Ac Standard		1 x labelling (1 x 5 HPLC runs – standard curve)
1 (Batch #B43I-04)	Neu5Gc Standard		1 x labelling (1 x 5 HPLC runs – standard curve)
2 (Batch # A97E-08)	Fetuin	Preparation and chromatography according to SOPs, Day 1, Operator 1	3 release labelling (3 HPLC runs)
2 (Batch # A97E-08)	Neu5Ac Standard		1 x labelling (1 x 5 HPLC runs – standard curve)
2 (Batch # A97E-08)	Neu5Gc Standard		1 x labelling (1 x 5 HPLC runs – standard curve)

Reporting Criteria

- Report mean, standard deviation and CV for the nmol/mg protein for Neu5Ac and Neu5Gc for each sample, where n = 6.

Acceptance Criteria

- The values for nmol/mg protein in each sample to be comparable for both columns with % CV < 20%.

Results – Robustness: Different columns

The nmol/mg protein data for analysis of Fetuin on two different columns is presented in table 24. All CVs are less than 5%.

Table 24. Sample Fetuin nmol/mg protein from analysis on 2 columns

			nmol/mg protein	
Sample	**Column**	**Injection**	**Neu5Gc**	**Neu5Ac**
Fetuin	1	1	7.97	401.82
		2	7.97	414.29
		3	7.28	374.93
	2	1	7.56	396.92
		2	7.97	410.50
		3	7.56	384.87
Mean			**7.72**	**397.22**
STDEV			**0.29**	**15.09**
CV%			**3.80**	**3.80**

Sample Stability

Procedure

Hold times of samples stored in the HPLC auto sampler will be investigated. Repeated HPLC runs from samples held in the HPLC auto sampler over three days will be analysed to determine possible sample degradation. The auto sampler temperature is set to 10 °C +/- 2°C.

Table 25. Sample description and procedure for sample stability experiment

Hold Time	Sample	Description	Repetitions
0, 24, 48 and 72 h	Fetuin	Preparation and chromatography according to SOPs, Operator 1, Day 1	1 release labelling (3x1 HPLC runs)
0, 24, 48 and 72 h	Neu5Ac Standard		1 x labelling (1 HPLC run)
0, 24, 48 and 72 h	Neu5Gc Standard		1 x labelling (1 HPLC run)

Reporting Criteria

- Overlay the chromatograms from each time point.
- Report mean, standard deviation and CV for the area and retention times of Neu5Ac and Neu5Gc in samples and standards.
- Define the acceptable time for sample storage based on the acceptance criteria.

Acceptance Criteria

When quoting the acceptable time for sample storage prior to injection:

- CV < 5% for retention times and peak areas.
- The values for nmol/mg protein in each sample to be comparable with % CV < 20%.

Results – Robustness: Sample Stability

An overlay of the chromatograms from the triplicate releases of Fetuin ran at 0, 24, 48 and 72 hours as shown in figure 11, and in figures 12 and 13 for the Neu5Ac and Neu5Gc standards. The data for retention time, peak area and nmol/mg are shown in tables 26 to 28 for Fetuin and tables 29 and 30 for Neu5Ac and Neu5Gc.
CVs are less than 1% for retention times and less than 5% for peak areas.
CVs for nmol/mg protein are less than 5% for Neu5Ac and Neu5Gc.
It is acceptable to store samples in the dark at 10°C for up to 72 hr, provided that the calibration standards have been stored in the same conditions and are analysed at the same time.

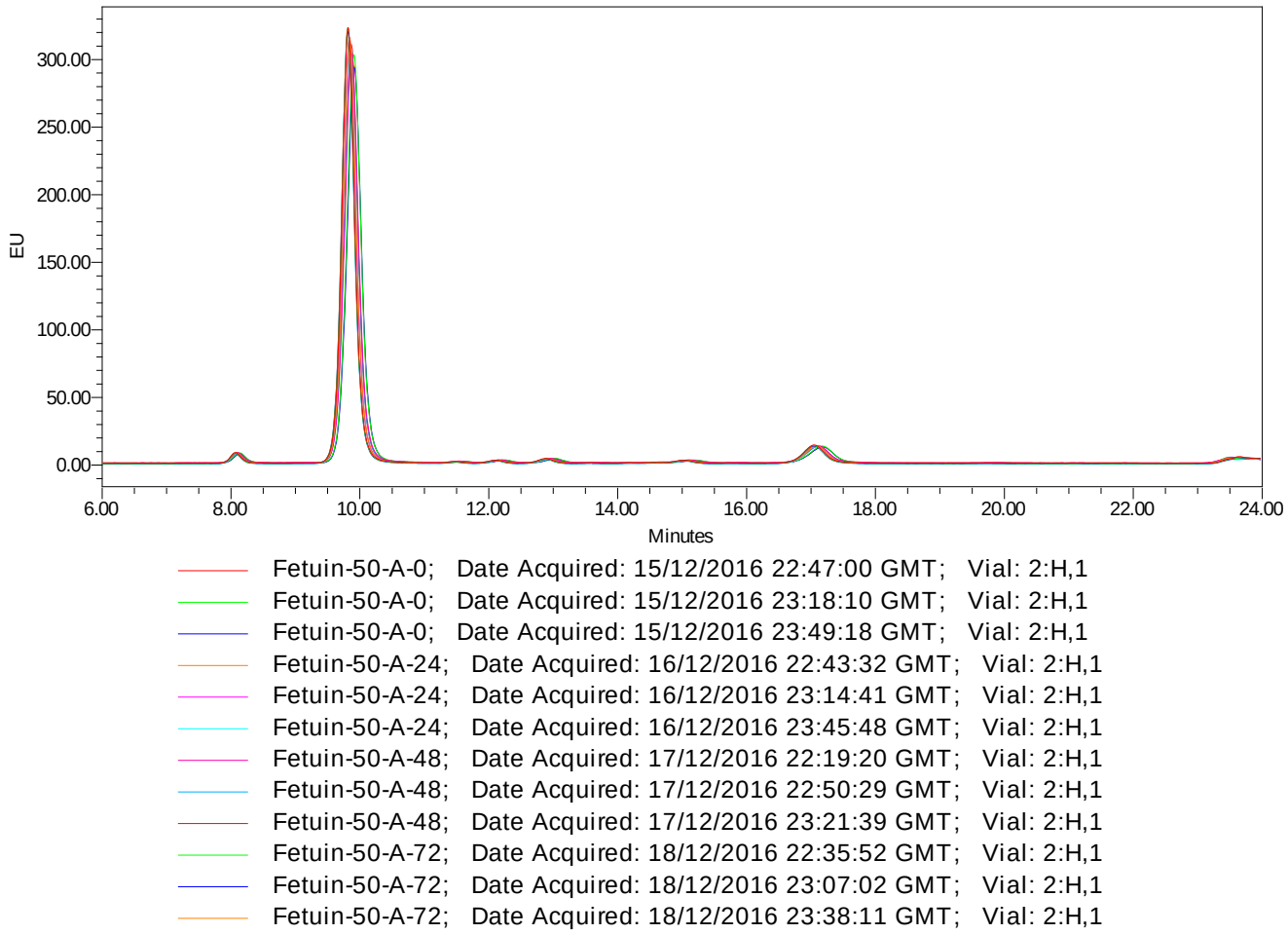


Figure 11. Overlay of Fetuin ran at 0, 24, 48 and 72 hours

Table 26. Retention times for Sample Fetuin ran at 0, 24, 48 and 72 hours

			Retention Times [min]	
Sample	**Hold Time**	**Injection**	**Neu5Gc**	**Neu5Ac**
Fetuin	0	1	8.088	9.836
		2	8.084	9.832
		3	8.085	9.243
	24h	1	8.106	9.861
		2	8.101	9.856
		3	8.104	9.859
	48h	1	8.127	9.889
		2	8.120	9.883
		3	8.122	9.884
	72h	1	8.151	9.926
		2	8.155	9.932
		3	8.155	9.932
Mean			**8.127**	**9.891**
STDEV			**0.022**	**0.031**
CV%			**0.3**	**0.3**

Table 27. Peak Areas for Sample Fetuin ran at 0, 24, 48 and 72 hours

			Peak Area [uV*sec]	
Sample	**Hold Time**	**Injection**	**Neu5Gc**	**Neu5Ac**
Fetuin	0	1	1122133	48512156
		2	1078817	48564134
		3	1069473	48407274
	24h	1	1022156	49008445
		2	1034036	49186165
		3	1069026	48842737
	48h	1	1041311	49240044
		2	1083316	49690642
		3	1053101	50247797
	72h	1	1058232	50032433
		2	1062369	48928324
		3	1011126	48905967
Mean			**1048297**	**49342506**
STDEV			**23165**	**521437**
CV%			**2.2**	**1.1**

Table 28. nmol/mg for Sample Fetuin ran at 0, 24, 48 and 72 hours

			nmol/mg protein	
Sample	**Hold Time**	**Injection**	**Neu5Gc**	**Neu5Ac**
Fetuin	0	1	8.483	269.635
		2	8.194	269.921
		3	8.132	269.058
	24h	1	8.059	267.823
		2	8.137	268.789
		3	8.366	266.922
	48h	1	7.912	265.744
		2	8.184	268.162
		3	7.988	271.153
	72h	1	8.416	268.721
		2	8.443	262.889
		3	8.111	262.771
Mean			**8.180**	**266.997**
STDEV			**0.190**	**2.780**
CV%			**2.3**	**1.0**

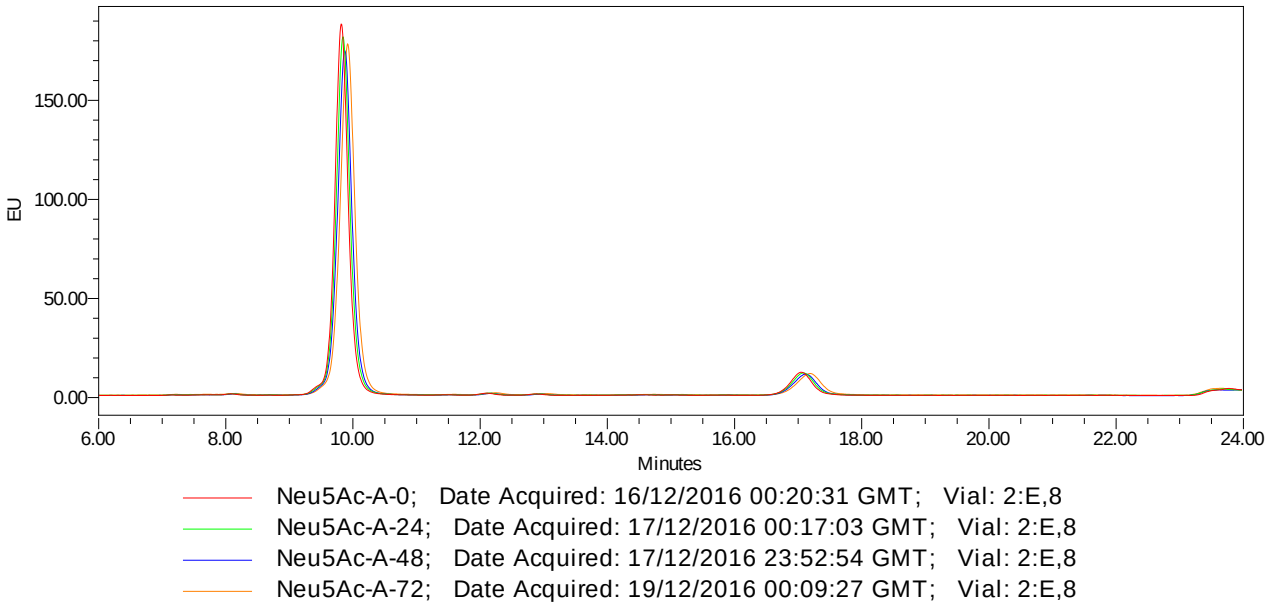


Figure 12. Overlay of Neu5Ac ran at 0, 24, 48 and 72 hours

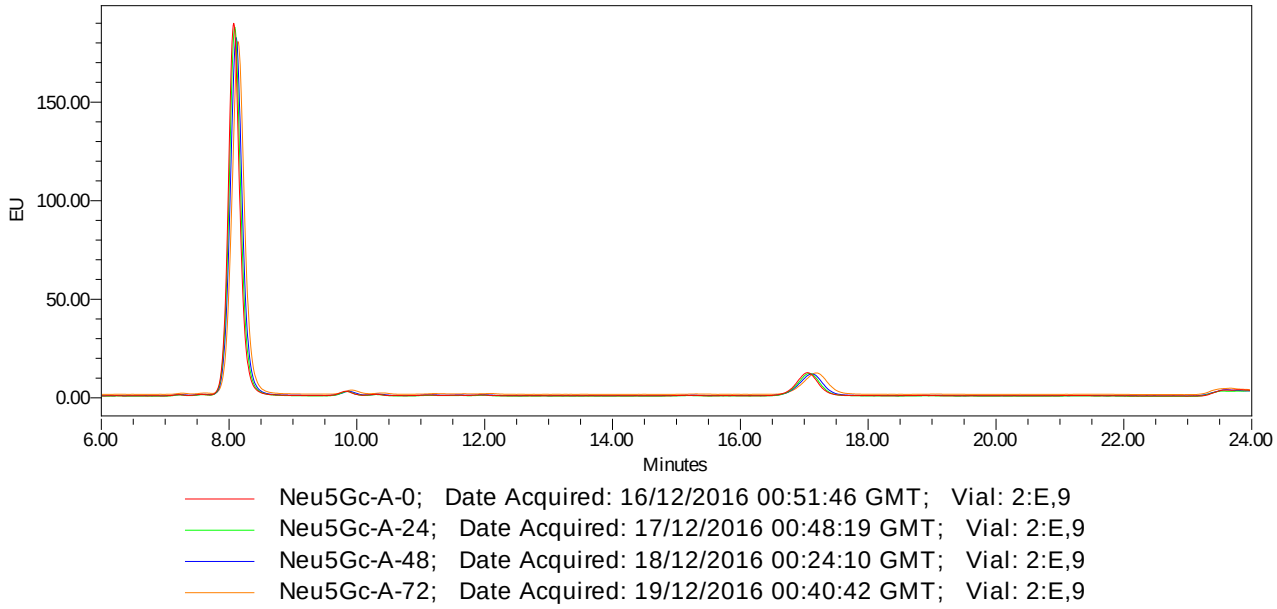


Figure 13. Overlay of Neu5Gc ran at 0, 24, 48 and 72 hours

Table 29. Retention times for Neu5Ac and Neu5Gc Standards ran at 0, 24, 48 and 72 hours

			Retention Times [min]	
Sample	**Hold Time**	**Injection**	**Neu5Gc**	**Neu5Ac**
Standards	0	1	8.092	9.836
	24h	1	8.108	9.863
	48h	1	8.129	9.891
	72h	1	8.160	9.935
Mean			**8.132**	**9.896**
STDEV			**0.026**	**0.036**
CV%			**0.3**	**0.4**

Table 30. Peak areas for Neu5Ac and Neu5Gc Standards ran at 0, 24, 48 and 72 hours

			Peak Area [uV*sec]	
Sample	**Hold Time**	**Injection**	**Neu5Gc**	**Neu5Ac**
Standards	0	1	24476722	28878927
	24h	1	25205915	29108882
	48h	1	25268116	28804055
	72h	1	25500328	30049183
Mean			**25324786**	**29320707**
STDEV			**155172**	**649029**
CV%			**0.6**	**2.2**

6.7 Test Parameter VIII - Accuracy

Procedure

Accuracy may be inferred from the linearity (parameter IV) and specificity (parameter I) results. Here we compare data from three lots of Neu5Ac and Neu5Gc standards for which the amounts are provided by the manufacturer based on quantitative NMR data.

Table 31. Sample description and procedure for accuracy experiments

Sample	Description	Repetitions
Neu5Ac Standard	Preparation and chromatography according to SOPs. Day 1, Operator 1	3 x labelling (3 HPLC runs)
Neu5Gc Standard	Preparation and chromatography according to SOPs. Day 1, Operator 1	3 x labelling (3 HPLC runs)

Reporting Criteria

- Report mean, standard deviation and CV for the peak areas and retention times for the three Neu5Ac and Neu5Gc.

Acceptance criteria

- CV < 5% for retention times.
- CV < 20% for peak areas.

Results – Accuracy

The data presented in tables 32 and 33 shows that the CVs are less than 1% for retention times and less than 10% for peak areas.

Table 32. Retention times for Sialic Acid replicates

		Retention Time [min]	
Sample	**Replicate**	**Neu5Gc**	**Neu5Ac**
Standards	A	8.078	9.786
	B	8.077	9.782
	C	8.081	9.800
Mean		**8.079**	**9.789**
STDEV		**0.002**	**0.009**
CV%		**0.0**	**0.1**

Table 33. Peak Areas for Sialic Acid replicates

		Peak Area [uV*sec]	
Sample	**Replicate**	**Neu5Gc**	**Neu5Ac**
Standards	A	43516	20806783
	B	42723	21176495
	C	40965	18742320
Mean		**42401**	**20241866**
STDEV		**1306**	**1311736**
CV%		**3.1**	**6.5**

7. Report Sign Off

Client / Sponsor: **Ludger Ltd**
Ludger Works Order: **GP-0057**

7.1 Operator Sign Off

Laboratory work — Indrani Harper (Technician)
Data Analysis
Data integrity — Checked by — Signature: I.Harper — Date: 13 Apr 2017

Dr. Jenifer Hendel (Senior Scientist)
Signature: _____ Date: 13 APR 17

Dr. Louise Royle (Head of Glycoprofiling)
Signature: L Royle Date: 13 APR 17

Report compiled — Dr. Louise Royle (Head of Glycoprofiling)
Signature: L Royle Date: 13 APR 17

7.2 Principal Investigator Sign Off

As Principal Investigator for this work I confirm that this report is an accurate account of the work undertaken.

Dr. Louise Royle (Head of Glycoprofiling)
Signature: L Royle Date: 13 APR 17

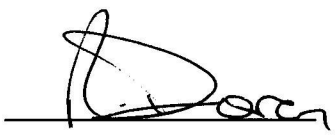
7.3 Data and Process Reliability Statement

Client / Sponsor: **Ludger Ltd**
Ludger Works Order: **GP-0057**

As part of the review and approval process, the study plan and processes were audited by Quality Assurance on the dates listed below. This report was also audited against its supporting raw data. Any changes that were required as a result of these audits have been addressed. It is therefore concluded that this report accurately reflects the agreed study plan, processes and the resulting raw data.

Study Plan Audit:	Date:	10 February 2017
Process Audit:	Date:	10 February 2017
Report Audit:	Date:	13 April 2017

Audited by: **name** Raymond Doran

Signature:  Date: 13 Apr 2017