



Internal Document

Determination of Acceptance Criteria for Fetuin System Suitability Standard in Sialic Acid Analysis

Document # Fetuin Specifications for Sialic Acid Analysis-v3.0.docx

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Introduction

This report summarises the data obtained for quantification of the sialic acid Neu5Ac from Ludger Fetuin system suitability standard [GCP-FET-50U] over 9 years (Jan 2015 to Jan 2024). This includes 94 independent analyses. The data from **Fetuin Specifications for Sialic acid Analysis v2.0** (47 independent analyses) has been combined with the data from the last 6 years (Jan 2018 to Jan 2024) (47 independent analyses) to provide updated pass criteria for the Ludger Fetuin system suitability standard.

In the document **Fetuin Specifications for Sialic acid Analysis v1.0** the acceptance criteria for analysis of Neu5Ac for fetuin at Ludger was set to be between 286 and 439 nmol/mg protein (average value 362 +/- 2 standard deviations). These data were obtained from the analysis of 44 independent measurements at Ludger laboratories over 5 years.

As of 18 Jan 2018, in the document **Fetuin Specifications for Sialic acid Analysis v2.0** the acceptance criteria for analysis of Neu5Ac for fetuin at Ludger was changed to be between 252 and 377 nmol/mg protein (average value 314 +/- 20%). These data were obtained from 47 independent analyses at Ludger laboratories over 3 years (2015, 2016 and 2017). A change in the preparation of the Neu5Ac standard (CM-NEUAC-01) on 12 Jan 2015 increased the accuracy of the nmol value for the standard. Thus an update in the acceptance values for measuring nmol of Neu5Ac per mg of Fetuin protein was calculated from this change in January 2015.

Methods

Reversed phase (RP) HPLC on a LudgerSepR1 **[LS-R1-4.6x150]** column is used to separate the DMB (1,2-diamino-4,5-methylenedioxybenzene) labelled sialic acids. Quantitative standards Neu5Ac **[CM-NEUAC-01]** and Neu5Gc **[GC-NEUGC-01]** are used to provide a standard curve to quantify the sialic acids in the samples that are analysed in parallel. A sialic acid reference panel (SRP) is also used to assess system suitability.

Sialic acids are released and labelled using a LudgerTag™ DMB sialic acid labelling kit **[LT-KDMB-A1]**. The sialic acids are released from the glycans by mild acid hydrolysis by incubation in 2M acetic acid for 2 hours at 80°C. One fifth of the released sialic acids are then labelled using DMB alongside the Neu5Ac and Neu5Gc quantitative standards and a SRP standard following **SOP-00178-LT-KDMB-A1-Sialic-Acid-Release-and- Labelling** (as described in the Ludger product document **lt-kdmb-a1-guide**),

Sialic acids are released from triplicate Fetuin positive controls **[GCP-FET-50U]** which is 50µg of glycoprotein (typically 34µg protein as measured by bicinchoninic acid (BCA) protein assay). 20% of the hydrolysed samples are DMB labelled for analysis by RP-HPLC.

For the RP-HPLC analysis of DMB labelled sialic acids a **LudgerSep-R1 column 4.6 x 150 mm** column **[LS-R1-4.6x150]** is run with solvents: (A) acetonitrile: methanol: water (9:7:84 by volume) and (B) acetonitrile

RP-HPLC analysis of the DMB labelled sialic acids is performed on a **LudgerSep-R1 column 4.6 x 150 mm** column **[LS-R1-4.6x150]** at 30°C on a Waters 2795 HPLC with 2475 fluorescence detector ($\lambda_{ex} = 373$ nm, $\lambda_{em} = 448$ nm), controlled by Empower software following the methods presented in Ludger product guides **[lt-kdmb-a1-guide** and **LS-R1-Guide]**.

Results

A range of data for the amount of Neu5Ac in nmoles/mg protein for the System Suitability Standard Fetuin (GCP-FET-50U) collected over 9 years (Jan 2015 to Jan 2024). An average value of 322 nmol of Neu5Ac per mg of Fetuin protein was obtained. A CV of up to 20% is deemed acceptable for the analysis of DMB labelled sialic acids. Therefore the updated acceptance range is between 257 and 386 nmol/mg protein.

During this period nine analyses were failed due to the fetuin result being out of specification.

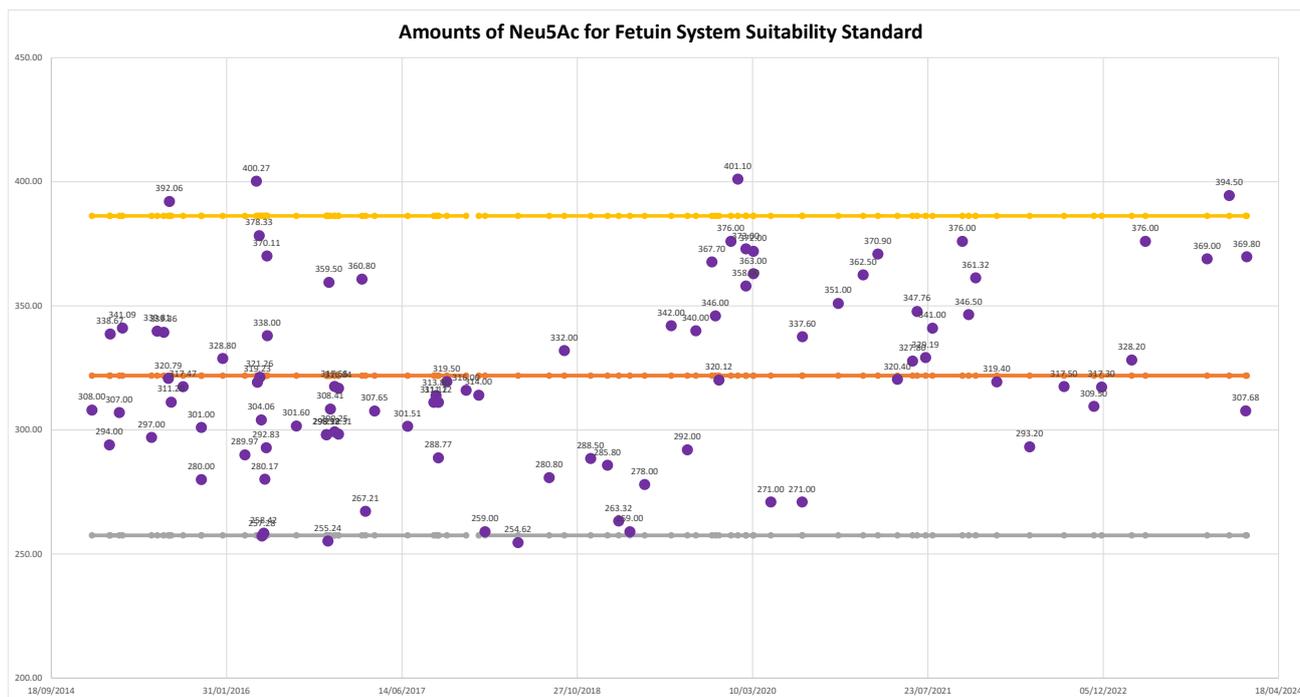


Figure 1. Plot of data

Average value = 322; Standard deviation = 36.5; CV = 11.3 %

Range for +/- 20% = 257 to 386 nmoles/mg protein.

Orange line – average 322 nmol/mg

Yellow line – Average +20%

Grey line – Average -20%

Conclusions

As of 21 May 2024, the acceptance range for the system suitability standard GCP-FET-50U used in DMB labelled sialic acid analysis:

257 to 386 nmoles of Neu5Ac per mg of protein.

Average value: 322 nmoles of Neu5Ac per mg of protein

Standard deviation: 36.5

CV: 11.3 %

Previous data from **Fetuin Specifications for Sialic acid Analysis v2.0** (report 18th January 2018, data recorded between January 2015 and December 2017)

252 to 377 nmoles of Neu5Ac per mg of protein.

Average value: 321 nmoles of Neu5Ac per mg of protein

Standard deviation: 32

CV: 10 %

Report Sign Off

I confirm that this report is an accurate account of the work undertaken.

Dr Richard Gardner (Head of Production)

Signature:  _____

Date: 22 May 2024

Dr Radoslaw Kozak (Head of Glycoprofiling)

Signature:  _____

Date: 22 May 2024

Dr Daniel Spencer (Head of Development and QC)

Signature:  _____

Date: 22 May 2024