

# Ludger

## Internal Document

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

**Document # Fetuin Specifications for Sialic Acid Analysis-C4AM-04 v1.0.docx**

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## Introduction

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This report summarises the data obtained for quantification of the sialic acid Neu5Ac from Ludger Fetuin system suitability standard [GCP-FET-50U] Batch C4AM-04 over the last 9 months (February 2025 to October 2025). This includes 7 independent analyses.

As of the 21<sup>st</sup> May 2024, in the document **Fetuin Specifications for Sialic acid Analysis v3.0** the acceptance criteria for analysis of Neu5Ac for fetuin at Ludger was changed to be between 257 and 386 nmol/mg protein (average value 322 +/- 20%). These data were obtained from >90 independent analyses at Ludger laboratories over 9 years (2015 to 2024). This data was for a specific batch of fetuin that had not changed over the 9 years.

A new batch of fetuin [GCP-FET-50U] Batch C4AM-04 was manufactured in October 2024 and was first used as a system suitability standard in the analysis of Neu5Ac sialic acid using the Ludger DMB sialic acid analysis and labelling kit (LT-KDMB-A1) in February 2025. The protein concentration per 50 µg of fetuin glycoprotein was determined to be **29.5 µg**. The data showed that the levels of Neu5Ac sialic acid in this batch of fetuin (C4AM-04) were lower than the previous batches. This has been investigated and the outcome is a new set of pass criteria for this batch of the fetuin system suitability standard.

## Methods

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Reversed phase (RP) UPLC on a LudgerSepR1 [**LS-UR2-2.1x100**] 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-Labeling** (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 50 µg of glycoprotein (typically 29-35 µg 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-UPLC analysis of DMB labelled sialic acids a **LudgerSep-UR2 column 2.1 x 100 mm** column [**LS-UR2-2.1x100**] is run with solvents: (A) acetonitrile: methanol: water (9:7:84 by volume) and (B) acetonitrile

RP-UPLC analysis of the DMB labelled sialic acids is performed on a **LudgerSep-UR2 column 2.1 x 100 mm** column [**LS-UR2-2.1x100**] (using solvents: (A) acetonitrile: methanol: water (9:7:84 by volume) and (B) acetonitrile) at 30°C on a Waters Acquity HPLC with fluorescence detector ( $\lambda_{ex} = 373 \text{ nm}$ ,  $\lambda_{em} = 448 \text{ nm}$ ), controlled by Empower software following the methods presented in Ludger product guides [**lt-kdmb-a1-guide** and **LS-R1-Guide**].

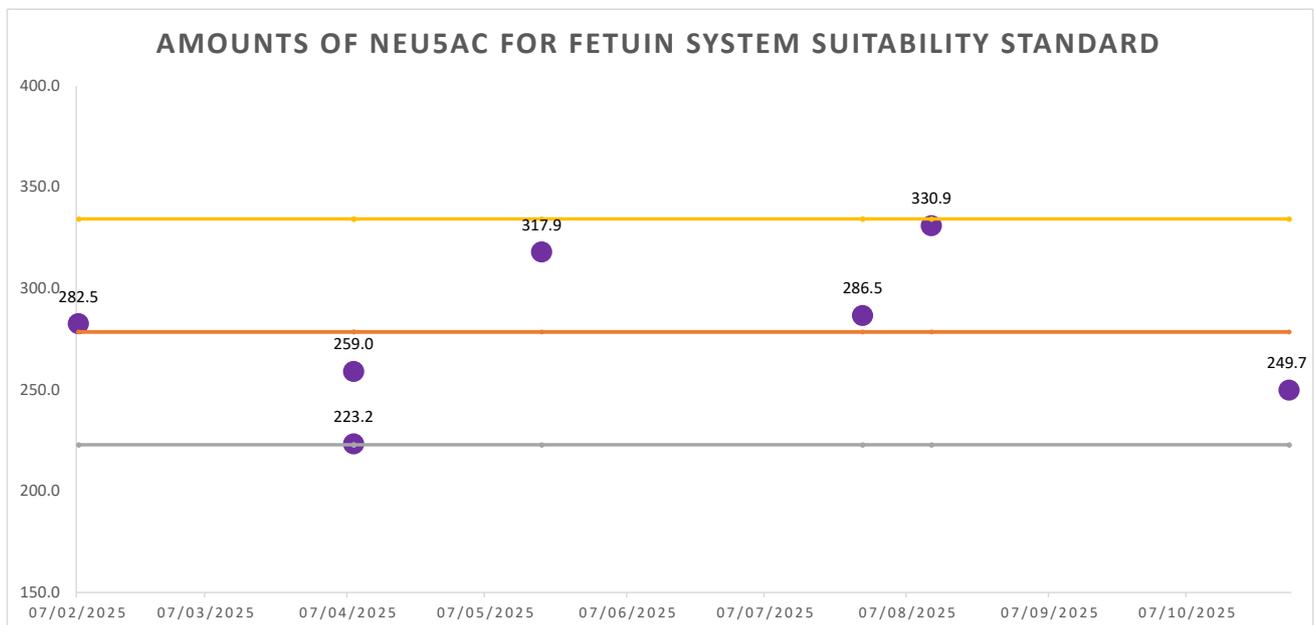
## Results

A range of data for the amount of Neu5Ac in nmoles/mg protein for the System Suitability Standard Fetuin (GCP-FET-50U) (C4AM-04), collected over 9 months (February 2025 to October 2025), is shown in Table 1. An average value of 278.5 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 acceptance range is between 222.8 and 334.2 nmol/mg protein.

Date	Work Order	DMB kit Batch	nmoles NeuAc/mg fetuin
07/02/2025	WP-8530	C527-01	282.5
08/04/2025	WP-8596	C548-01	259.0
08/04/2025	WP-8596	C548-01	223.2
19/05/2025	WG-50572	C548-01	317.9
28/07/2025	WP-8723	C57U-03	286.5
12/08/2025	WP-8735	C58B-01	330.9
29/10/2025	WP-8831	C5AR-01	249.7

**Table 1. List of data collected.**

A protein concentration of 29.5 µg per 50 µg of fetuin glycoprotein was used in the calculations to determine nmoles NeuAc per mg of fetuin.



**Figure 1. Plot of data**

Average value = 278.5; Standard deviation = 38.0; CV = 13.6 %

Range for +/- 20% = 222.8 to 334.2 nmoles/mg protein.

Orange line – average 278.5 nmol/mg

Yellow line – Average +20%

Grey line – Average -20%

## Conclusions

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The acceptance range for the system suitability standard GCP-FET-50U (C4AM-04) used in DMB labelled sialic acid analysis:

**222.8 to 334.2 nmoles** of Neu5Ac per mg of protein.

Average value: **278.5 nmoles** of Neu5Ac per mg of protein

Standard deviation: 37.96

CV: 13.63 %

## Report Sign Off

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

Dr Richard Gardner (Head of Production)

Signature: \_\_\_\_\_



Date: 30 October 2025