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An Alternate Approach for Sialic Acid Estimation in Glycoproteins by HPAEC-PAD method

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Agenda

- Introduction
- Measurement of Sialic Acid
- HPAEC-PAD
- Methodology
- Results and Discussion
- Conclusion

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Introduction

- N-acetylneuraminic acid (Neu5Ac) is the most common sialic acid determined in glycoproteins. N-glycolylneuraminic acid (Neu5Gc) is generally not produced in humans, hence its presence in the therapeutic agent can potentially generate an immune response.
- Hence the degree and identity of sialylation plays an important role in biopharmaceutical industries as it contributes to its efficacy, pharmacokinetics and potential immunogenicity of the therapeutic protein.

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Measurement of Sialic acid

- Sialic acids can be released from glycoproteins either by acid or enzyme treatment.
- The released sialic acid can be analyzed by several analytical methods like resorcinol, thiobarbituric acid, DMB fluorometric methods, etc. But these methods either lack sensitivity, or specificity in few cases and others require derivatization of samples.

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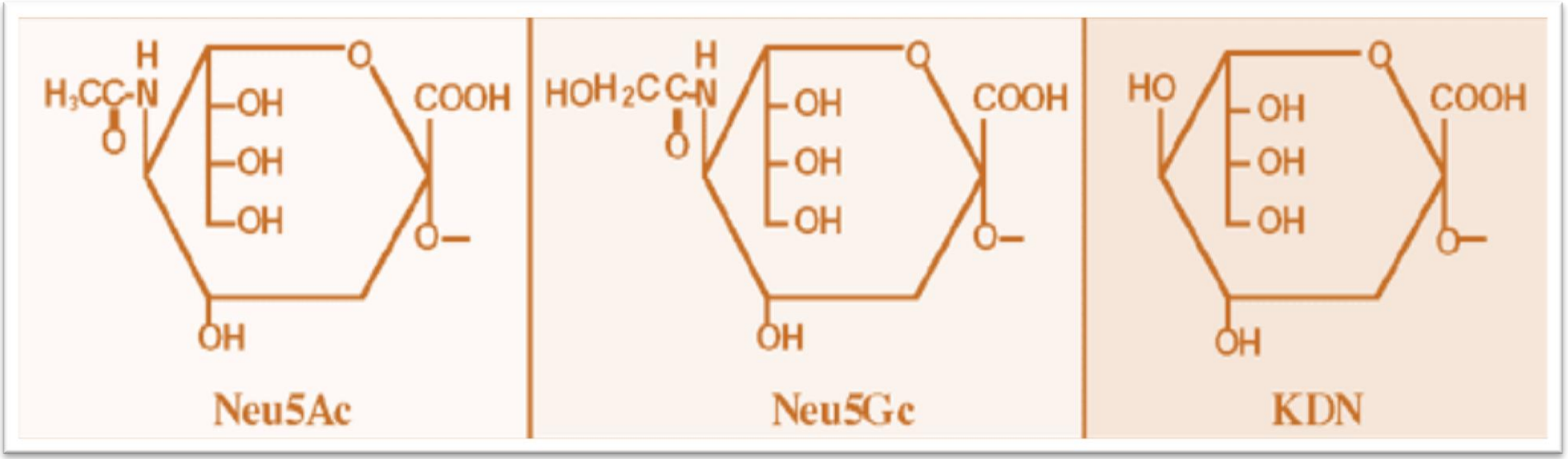
Measurement of Sialic acid

- The HPAEC-PAD method is a sensitive, specific method which does not require derivatization of sialic acid.
- 2-keto-3-deoxy-D-glycero-D-galacto-2 nonulosonic acid (KDN) is used as post hydrolysis Internal standard due to its structural similarities with Neu5Ac, Neu5Gc and to evaluate differences in sample handling, hydrolysis and electrode response.

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Structure of Sialic acid and Internal standard

Figure 1: Structures of Neu5Ac, Neu5Gc and KDN



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EPO – A Glycoprotein

- Erythropoietin (EPO) is a hematopoietic hormone produced in the adult kidney and fetal liver cells.
- It is a 39 kDa glycoprotein consisting of complex N- and O-oligosaccharide chains attached to a single polypeptide backbone that accounts for 40% of its molecular weight.
- The terminal site of glycans is occupied by sialic acid which increases the serum half life of the glycoprotein.
- Lack of terminal sialic acid on glycans results in high activity in *in vitro* bioassay but low *in vivo* activity.

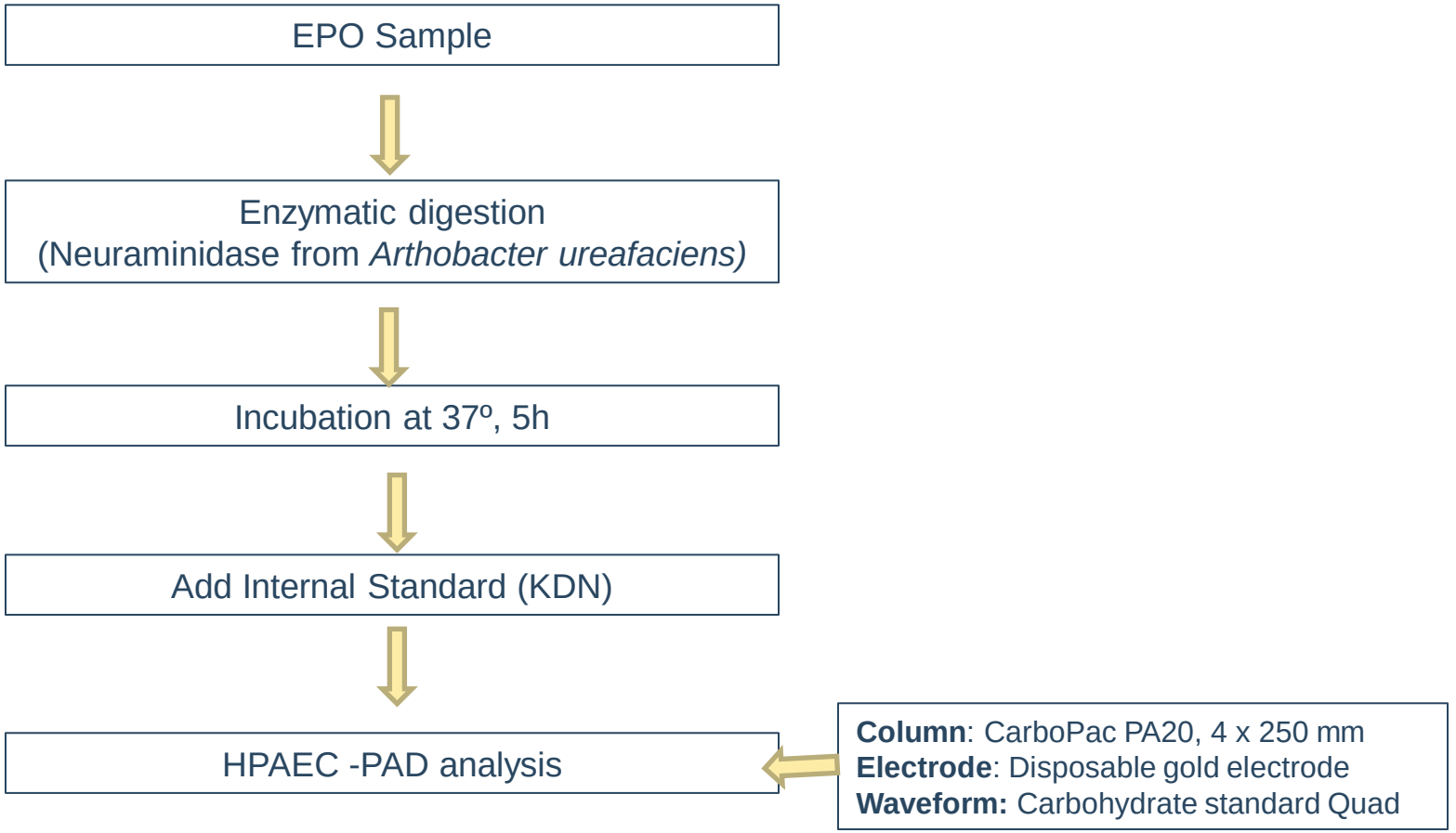
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Objective

The objective is to estimate the sialic acid (Neu5Ac and Neu5Gc) content using HPAEC-PAD as an alternate method to the calorimetric and fluorescent based methods.

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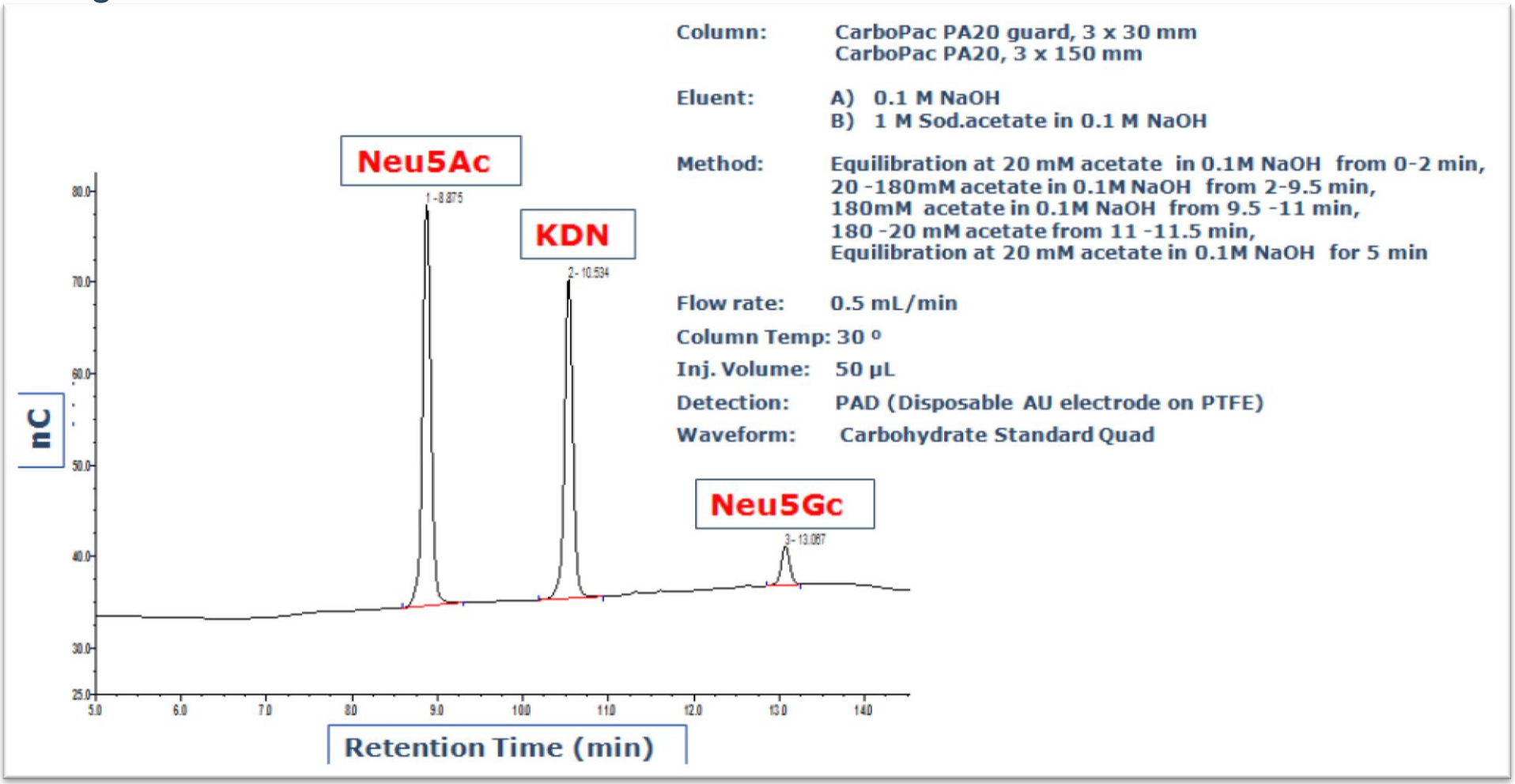
Method



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Method

Figure 2: HPAEC separation of Neu5Ac, Neu5Gc and KDN standards using CarboPac PA20 column



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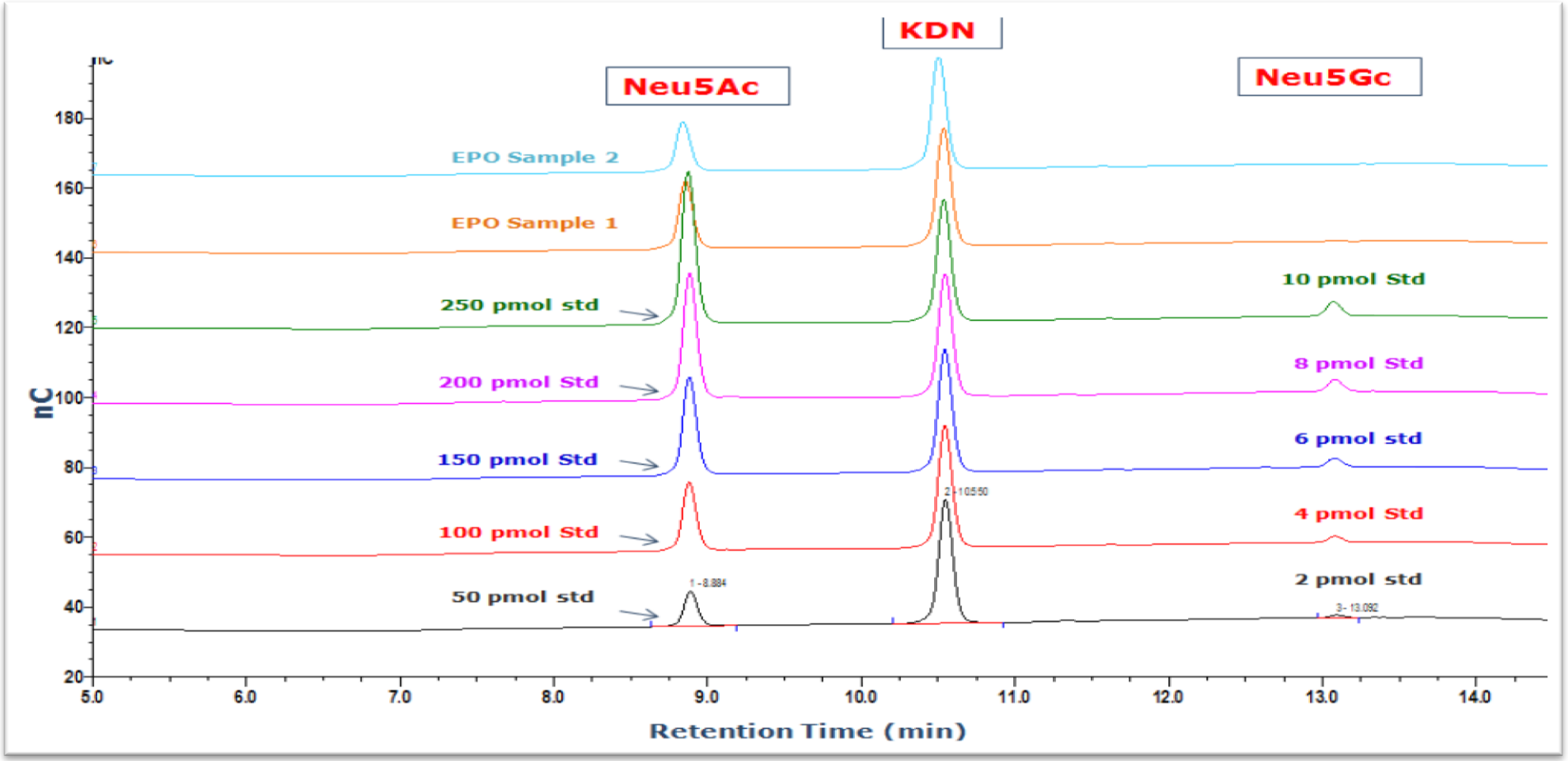
Results and Discussion

- Theoretically sialic acid per EPO protein varies from 9 to 14 residues.
- The response of standards is linear from 50 to 250 pmol for Neu5Ac and 2 to 10 pmol for Neu5Gc (r2 > 0.99) respectively.
- Estimated Neu5Ac content is used for calculating mole of sialic acid per mole of EPO protein.
- There is no detectable Neu5Gc content in both EPO sample as clearly seen in the given data.
- This method was further validated to determine its suitability for routine analysis of Neu5Ac and Neu5Gc in EPO samples

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Results and Discussion

Figure 3: HPAEC separation of Neu5Ac, Neu5Gc in standards and EPO samples. Internal standard KDN of 150 pmol conc. is spiked in both standards and samples



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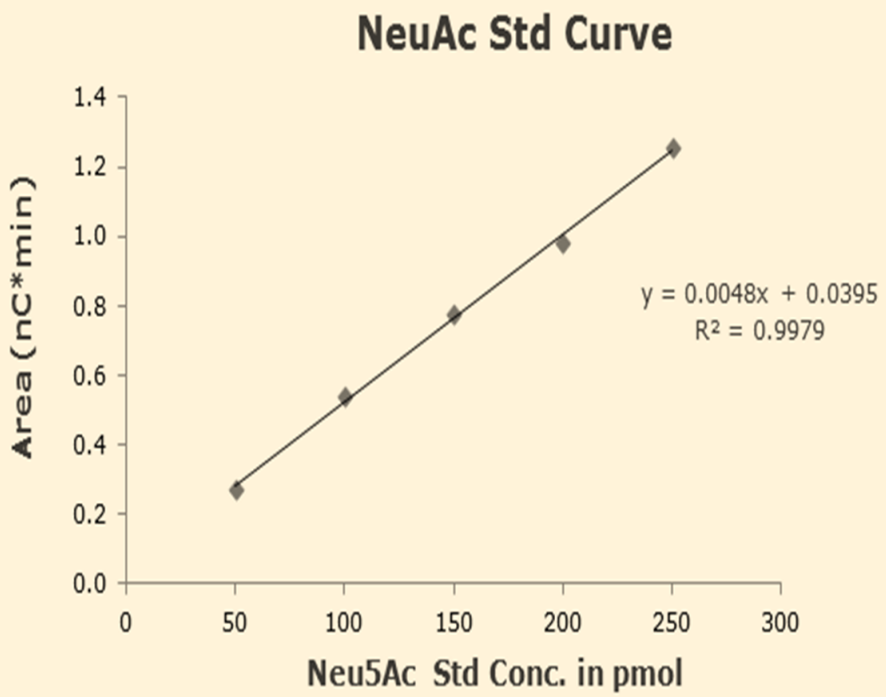
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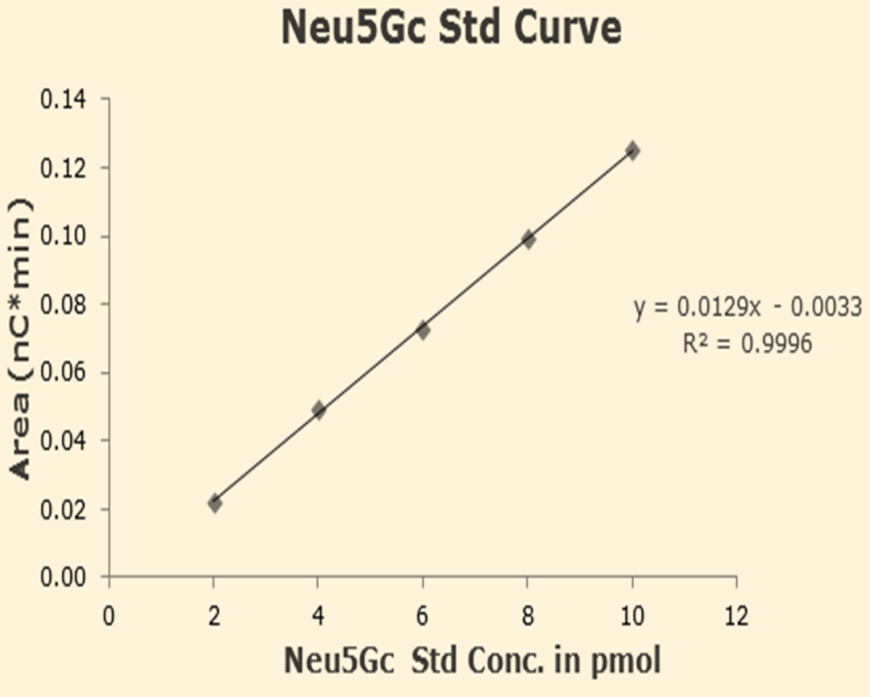
Results and Discussion

Figure 4: Sialic acid estimation in EPO using standard curve derived from corrected areas of Neu5Ac, Neu5Gc standards

Neu5Ac std conc. in pmol	Neu5Ac	KDN	Corrected Neu5Ac
	Area(nC*min)		
50	1.091	4.040	0.270
100	2.158	4.001	0.539
150	3.125	4.029	0.775
200	3.960	4.034	0.982
250	5.014	3.986	1.258

Neu5Gc std conc. in pmol	Neu5Gc	KDN	Corrected Neu5Gc
	Area(nC*min)		
2	0.089	4.040	0.022
4	0.198	4.001	0.049
6	0.294	4.029	0.073
8	0.401	4.034	0.099
10	0.501	3.986	0.126





Results and Discussion

Figure 4: Sialic acid estimation in EPO using standard curve derived from corrected areas of Neu5Ac, Neu5Gc standards

Sample	Area of Neu5Ac	Area of KDN	Corrected area of Neu5Ac	Estimated Neu5Ac content	Protein amount taken	Protein content considering MW of 30.6 kD	mol of Neu5Ac/mol of EPO ptn
	nC*min			pmol/inj	µg/inj	pmol/inj	
EPO Sample	1.891	4.047	0.467	111	0.3	9.8	11.4

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Conclusion

High-pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) method can be used for sialic acid estimation.

It has more advantages due to the following parameters:

- Requires less sample amount
- Sample preparation does not require any derivatization before analysis
- No interference from matrix components.
- Processed sample is stable at 15º up to 48 hrs.
- High sensitivity and specificity

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References

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Acknowledgement

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Questions

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Thank You

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