# First steps in the development of a high throughput method for O-glycosylation analysis

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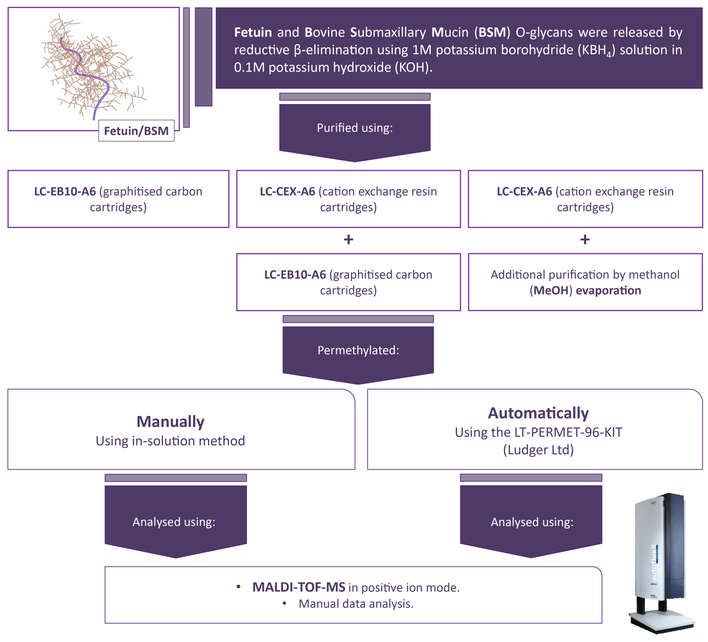
## Introduction

Colorectal cancer (CRC) is the second most common cancer in Europe, and is one of the most curable cancers when detected in its early stages. Population-based screenings are effective not only for early detection but also for prevention, but the current CRC screening methods either lack sensitivity and/or specificity, or cause discomfort and pain due to their invasive character. Although several CRC plasma biomarkers at DNA, protein as well as carbohydrate level have been proposed, they have not yet reached clinical application, with the exception of CEA (carcinoembryonic antigen) levels in the circulation which are of limited value.

Carbohydrates attached to proteins or lipids (known as glycans) are promising candidates as diagnostic and prognostic CRC biomarkers, since various studies have shown CRC-associated changes in glycosylation profiles, which were able to distinguish CRC from control tissue and plasma. Our project aims at the development and optimization of automated and validated, high throughput methods for analysis of colorectal cancer O-glycosylation.

An established approach for O-glycan analysis is based on their chemical release by reductive β-elimination and Matrix-assisted laser desorption/ ionization (MALDI)-time-of-flight (TOF) profiling. Here we present our progress in the development of a high throughput method for O-glycan analysis.




## Data and Conclusions

The additional purification step by MeOH evaporation, after the LC-CEX-A6 clean-up , allowed for more sensitive O-glycan analysis on both Fetuin and BSM samples. Notice how this procedure can help purifying the samples from borohydrates and/or salts, reducing non-specific signal, allowing for more O-glycan structures to be detected (marked in red) and thus resulting in a higher quality data analysis (fig.1,2).

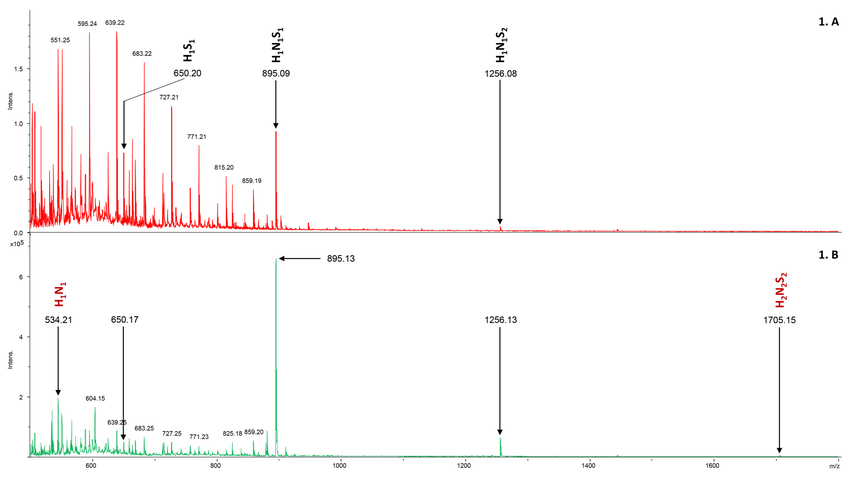



Figure 1: Released O-glycans from 200 µg of Fetuin (fig.1A, 1B) using 50 µL of 1M $KBH_4$ solution in 0.1M KOH. Samples treated with the additional MeOH purification step in fig.1B.

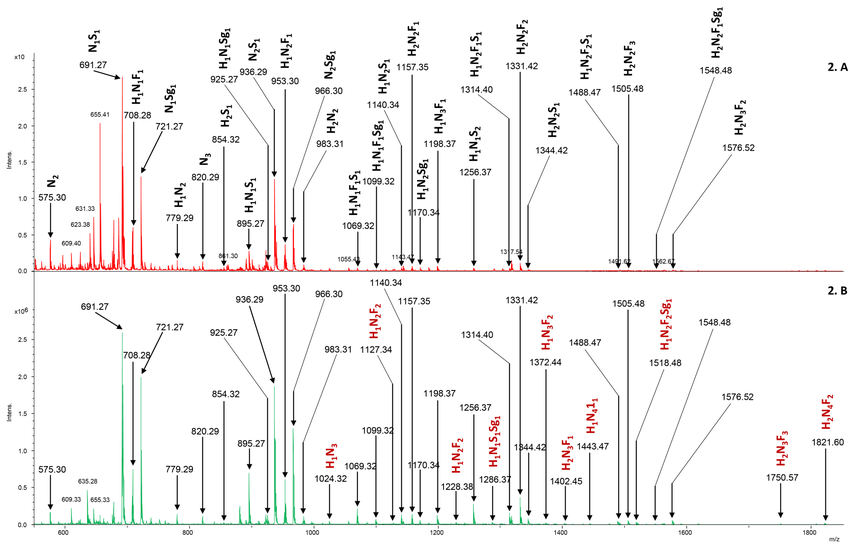



Figure 2: Released O-glycans from 200 µg of BSM (fig.2A, 2B) using 50 µL of 1M $KBH_4$ solution in 0.1M KOH. Samples treated with the additional MeOH purification step in fig.2B.

Among all the purification methods tested, the LC-CEX-A6 followed by an additional purification by MeOH evaporation resulted to be the most reliable system for obtaining O-glycans in a high yield. A comparison of the different purification methods tested on both Fetuin and BSM can be observed in figure 3 and 4.

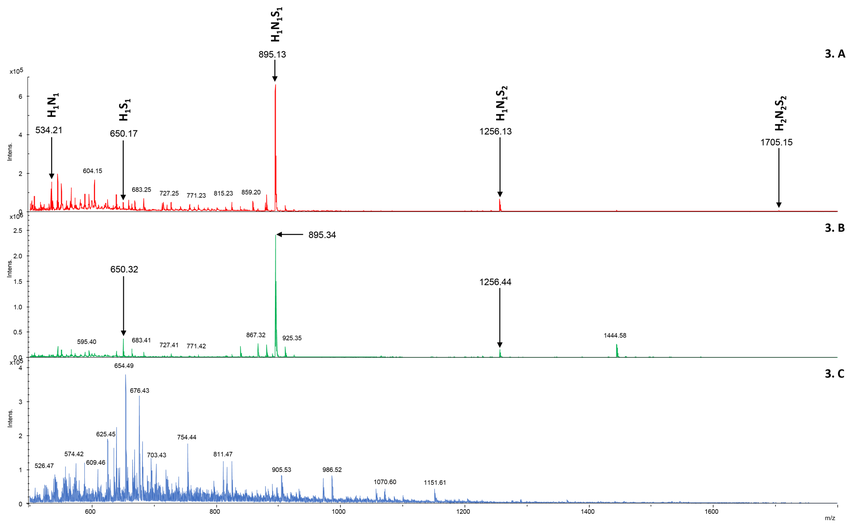



Figure 3: O-glycans released from 200 µg of Fetuin using 50 µL of 1M $KBH_4$ solution in 0.1M KOH.
**Fig.3.A.** LC-CEX-A6 followed by a MeOH evaporation step.
**Fig.3.B.** LC-CEX-A6 followed by LC-EB10-A6 purification.
**Fig.3.C.** LC-EB10-A6 purification.

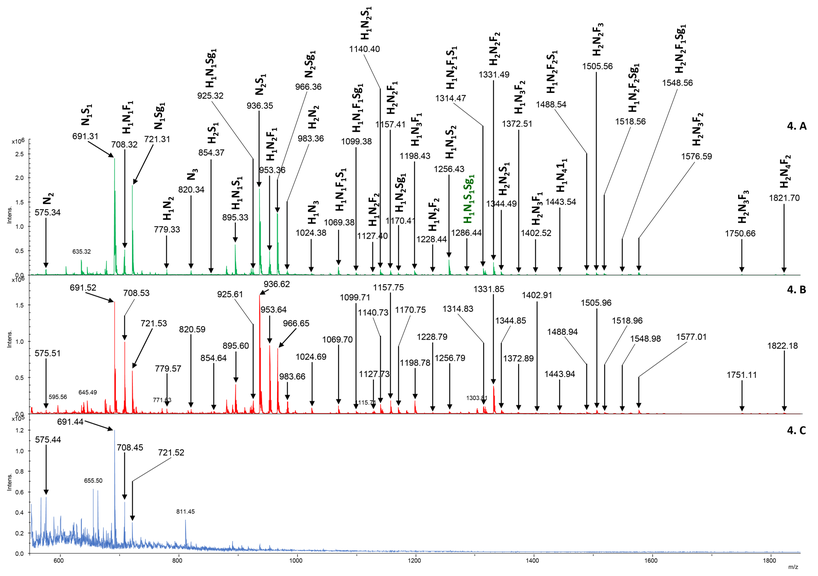



Figure 4: O-glycans released from 200 µg of BSM using 50 µL of 1M $KBH_4$ solution in 0.1M KOH.
**Fig.4.A.** LC-CEX-A6 followed by a MeOH evaporation step.
**Fig.4.B.** LC-CEX-A6 followed by LC-EB10-A6 purification.
**Fig.4.C.** LC-EB10-A6 purification.

Regarding the development of a high throughput method for O-glycan analysis we started testing the efficiency of our automated permethylation technique when compared to the manual procedure. The LT-PERMET-96-KIT (Ludger Ltd) microplate is suitable for high-throughput studies. Permethylation of released O-glycans have been performed prior to MALDI-TOF-MS analysis because it provides a number of advantages such as:

- improvement and enhancement of ionization efficiency of glycans
- stabilization of the labile sialic acid moieties
- the detection of both neutral and acidic glycans in positive ion mode

Figure 5 shows how the automated method results are comparable to the manual one.

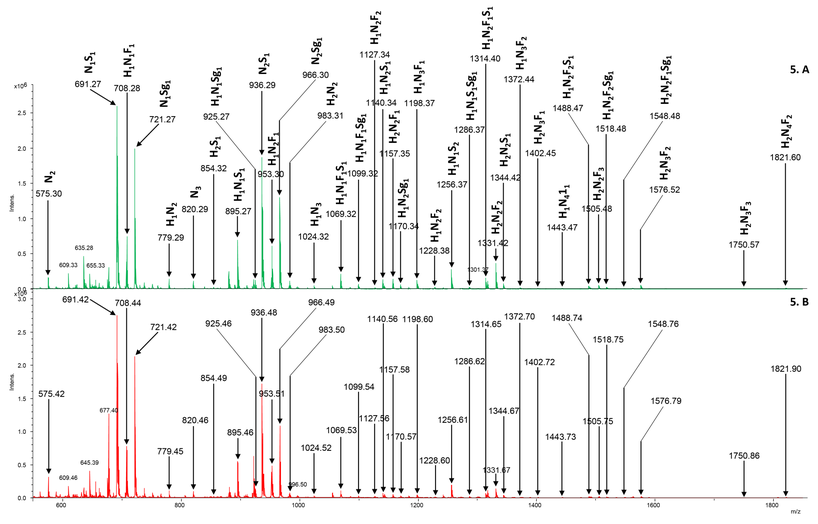



Figure 5: Released O-glycans from BSM, permethylated using both manual (fig.5A) and automated (fig.5B) technique. O-glycans were released from 200 µg of BSM using 50 µL of 1M $KBH_4$ solution in 0.1M KOH. An LC-CEX-A6 purification followed by an additional MeOH evaporation step was performed after the release.

O-glycans from different quantities of samples were also released from BSM in order to test the suitability of a high throughput release procedure for a 96 well plate format. Figure 6 shows that the results are comparable down to 50 µg of starting material.

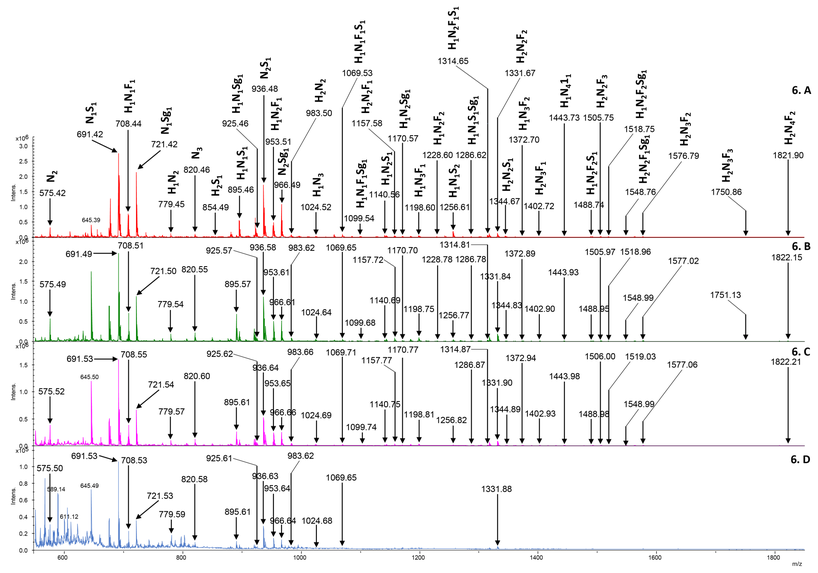



Figure 6: Released O-glycans from different quantities of BSM, permethylated with the automated procedure using the LT-PERMET-96-KIT. O-glycans were respectively released from 200 (fig.6A), 100 (fig.6B), 50 (fig.6C) and 20 (fig.6D) µg of BSM using 50 µL of 1M $KBH_4$ solution in 0.1M KOH. An LC-CEX-A6 purification followed by an additional MeOH evaporation step was performed after the release.

## Future Perspectives

The development of a high throughput method able to provide highly reproducible and quantitative analysis of colorectal cancer O-glycosylation is required in order to find improved diagnostic and prognostic biomarkers and pave the way for novel therapeutic targets.
Further research work is still to be made, but we feel we are in the right direction. With additional experiments already programmed, our aim is to suit the entire technique to high throughput sample analysis.

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