

## Replacing DMF with DMSO in the Eco GlycoWorks™ RapiFluor-MS™ Labeled N-Glycan Sample Preparation

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### Abstract

N,N-dimethylformamide (DMF) presents a significantly higher risk versus dimethyl sulfoxide (DMSO) to its users and the environment as highlighted by the 2023 European Chemical Agency of the European Union restrictions on the use of DMF. As a result, the effective substitution of DMSO for DMF as a cosolvent in both the RapiFluor-MS labeling reaction and final sample dilution steps of the rapid GlycoWorks N-Glycan analysis procedure is described. Results are presented for the N-Glycan profiles of a monoclonal antibody with low levels of sialylation, and for bovine fetuin which possesses an extensive array of high antennarity and multiply sialylated N-linked oligosaccharides. In addition, some of the considerations when using DMSO in the GlycoWorks procedure are highlighted.

### Benefits

A safer and more environmentally friendly GlycoWorks rapid N-Glycan sample preparation procedure using DMSO in place of DMF.

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## Introduction

The Waters™ GlycoWorks *RapiFluor*-MS N-Glycan labeling kit (e. g. User Guide [715004903EN < https://www.waters.com/webassets/cms/support/docs/715004903en.pdf >](#) ), provides a rapid and facile method capable of labeling released N-glycans for sensitive fluorescence and MS detection (Figure 1). The procedure consists of the rapid PNGase F release of N-glycans followed by labeling of the resulting glycosylamines with *RapiFluor*-MS (RFMS). The bulk of the byproducts of the reaction are then removed from the RFMS-labeled N-glycans with a quantitative HILIC-SPE cleanup procedure. The procedure in its entirety takes one to two hours, depending on the number of samples, using either a manual or automated procedure, and can be adapted to a wide range of samples.<sup>1-3</sup>

In the initial version of the procedure, released in 2015, it was observed that N,N-dimethylformamide (DMF) provided benefit as a sample cosolvent for RFMS-labeled N-glycans in an acetonitrile:water mixture. As a result, DMF was used as both a cosolvent in the final sample and anhydrous DMF was used for the solubilization of the RFMS label. Solubilization of RFMS with anhydrous solvent is critical as water can react with RFMS.

However, due to a better understanding of the potential deleterious impact of DMF on both the analyst and the environment as exemplified by the restrictions on the use of DMF in December of 2023 by the European Chemical Agency of the European Union ([echa.europa.eu](#)), a version of the GlycoWorks procedure using DMSO as a one-to-one replacement for DMF was evaluated. This summary will focus on the pertinent comparisons of the GlycoWorks procedure when using anhydrous DMF or DMSO during the RFMS labeling step and reagent-grade DMF or DMSO as a cosolvent for the RFMS-labeled and HILIC SPE-purified N-glycans.

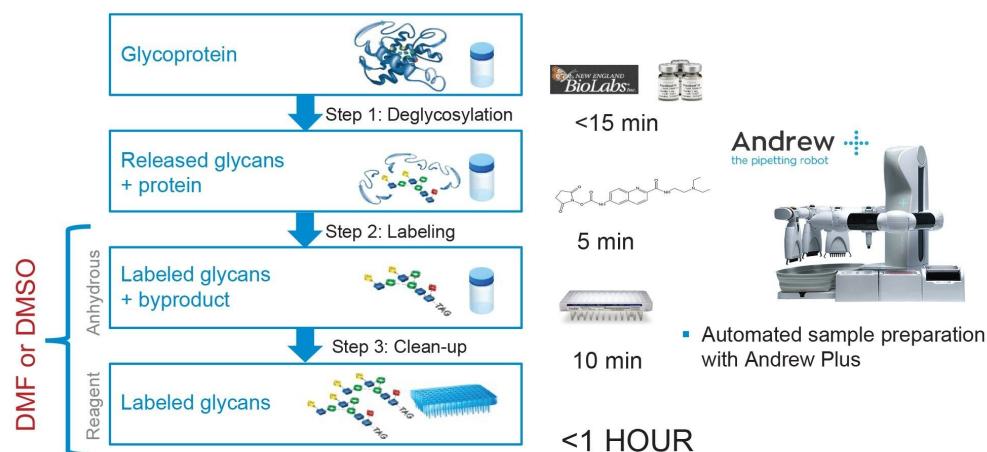


Figure 1. The Waters GlycoWorks RapiFluor-MS protocol is highlighted. DMF or DMSO is required as a cosolvent for the RFMS labeling step (Step 2) and as part of the sample diluent after SPE clean-up (Step 3). Step 2 requires anhydrous solvent while Step 3 does not require anhydrous solvent.

## Experimental

The GlycoWorks sample preparations and analyses were based on the procedures outlined in the User Guides 720005470EN <<https://www.waters.com/webassets/cms/library/docs/720005470en.pdf>> and 715004903EN <<https://www.waters.com/webassets/cms/support/docs/715004903en.pdf>> . Additional relevant details are provided with the presented data.

## Results and Discussion

### RFMS Labeling and Labeled N-Glycan Sample Dilution

There are three considerations during the RFMS labeling step. Primarily, the yield of the labeling procedure should be maximized and should not speciate between different glycoforms. In addition, over-labeling, or the

generation of N-glycans with more than a single label should be kept to a minimum. Previous work has demonstrated the veracity of the N-glycan profiles obtained with the GlycoWorks RFMS procedure using anhydrous DMF to solubilize RFMS,<sup>1</sup> and comparable labeling results were obtained using anhydrous DMSO in place of DMF (Figure 2).<sup>1</sup> In this study anhydrous DMSO or DMF was used to solubilize the RFMS reagent for the analysis of a monoclonal antibody (NISTmab™) and bovine fetuin mixture. This glycoprotein mixture was used to cover a broad range of N-glycans ranging from low antennarity neutral forms that are more abundant in the mAb to the high antennarity highly sialylated forms present on fetuin. Notably, the samples compared in Figure 2 were not cleaned up by HILIC-SPE prior to HILIC analysis to more clearly ascertain any differences in the labeling step.

The use of DMSO in the labeling procedure also produced comparably low levels of over-labeled N-glycans (Figure 3). This was demonstrated by the LC-MS analysis of the singly and doubly labeled predominant FA2 glycoform generated from the Intact mAb Standard (p/n: [186006552 < https://www.waters.com/nextgen/global/shop/standards--reagents/186006552-intact-mab-mass-check-standard.html>](https://www.waters.com/nextgen/global/shop/standards--reagents/186006552-intact-mab-mass-check-standard.html) -1). These samples were cleaned up by HILIC-SPE and the resulting aqueous samples were diluted with a solution containing the corresponding cosolvent prior to HILIC analysis (refer to User Guide 715004903EN) to maximize LCMS detection of trace-level doubly labeled FA2 glycans.

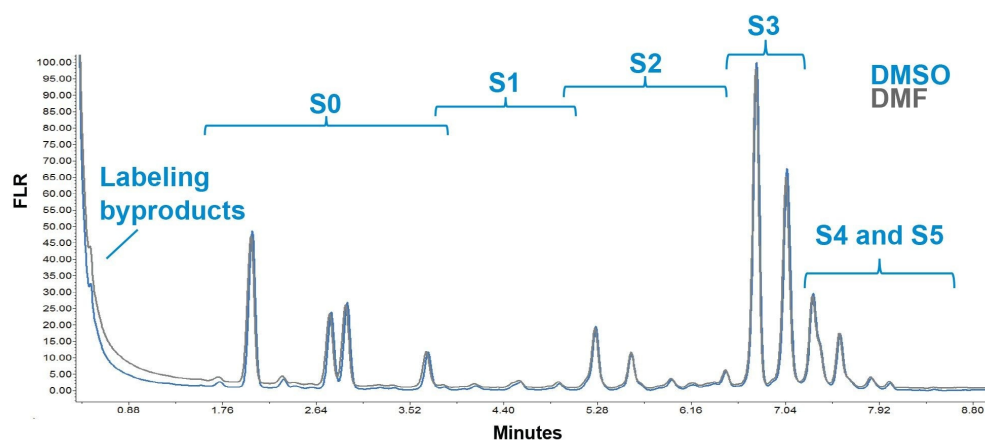


Figure 2. Shown are the HILIC-FLR N-glycan profiles, ranging from 0 to 5 sialic acids (S0-S5) per glycan, of a mixture of NISTmab (NIST RM 8671) and bovine fetuin (Sigma-Aldrich PN:F3004) at approximately 1 mg/mL each. Samples were denatured with RapiGest™, de-N-glycosylated with PNGase F, and labeled with RFMS that was solubilized in DMF or DMSO, as per User Guide 720005470EN. Direct HILIC analysis of 1  $\mu$ L of the crude preparation was performed using an ACQUITY™ Premier UPLC™ Glycan BEH™ Amide, 130 Å, 1.7  $\mu$ m, 2.1 x 50 mm (p/n: 186004740) column at 1.0 mL/min with a 10-minute analytical gradient. Additional conditions are listed in User Guide 715004903EN.

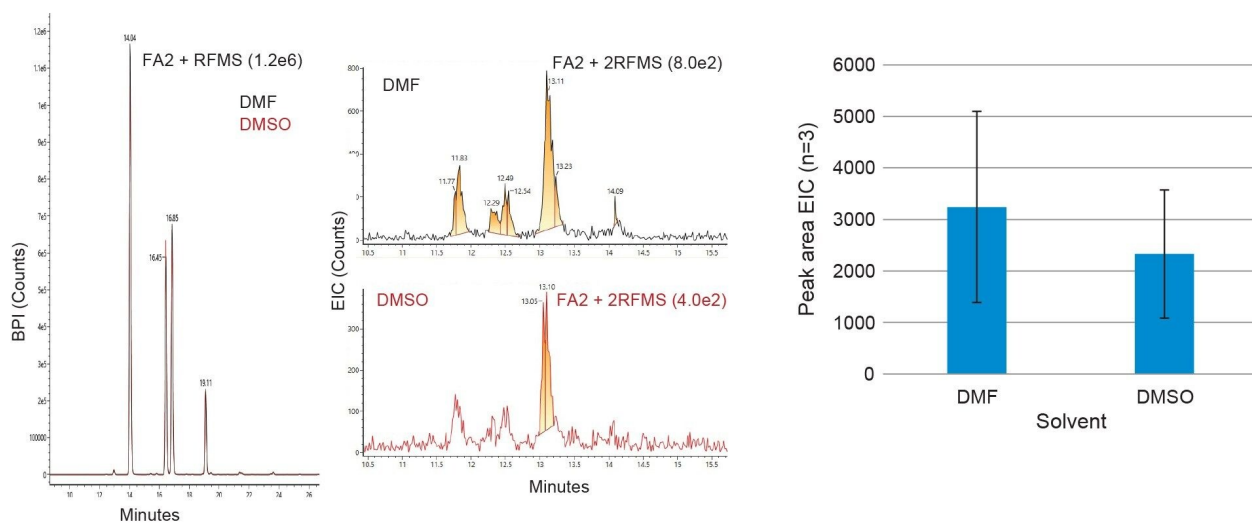


Figure 3. The HILIC-MS (Vion™ Q-ToF) analysis of Waters Intact mAb Standard, (p/n: 186006552-1) using either DMF or DMSO for both N-glycan RFMS labeling and final sample dilution steps to determine the extent of RFMS over-labeling. Over-labeling was determined based on the extracted ion intensity (EIC) of doubly labeled FA2 ( $m/z$  1044.023). Separation was performed using an ACQUITY Premier UPLC Glycan BEH Amide, 130 Å, 1.7 μm, 2.1 x 150 mm (p/n: 186009524) at 0.4 mL/min with a 35 minute analytical gradient. Additional conditions are provided in User Guide 715004903EN

## RFMS-Labeled N-Glycan Sample Stability and Loading

Following the HILIC SPE clean-up step (refer to User Guides 720005470EN and 715004903EN) a 90 μL sample in aqueous buffer is produced. To maximize the load of this sample onto an amide HILIC analytical column the sample is diluted with 310 μL of a 32:68 (v:v) mixture of DMF:ACN. Here DMF is used as a cosolvent to improve sample stability. However, it is recommended that the sample be re-mixed prior to analysis after 24 hours (User Guide 715004903EN).

It was observed that DMSO is an effective replacement for DMF in the sample dilution step, however, samples diluted with a DMSO:ACN mixture were slightly more prone to strong-solvent effects when loaded onto the amide HILIC analytical column. For this study, the RapiFluor-MS labeled N-Glycans from the mixture of NISTmAb and bovine fetuin used in the labeling study described previously, were purified by HILIC SPE, and diluted with a solution containing the corresponding cosolvent prior to HILIC analysis. As shown in Figure 4, comparable HILIC

profiles were obtained when using either DMF or DMSO in both the labeling and sample dilution procedures for a broad range of N-glycans. In addition, the relative abundances of three selected glycoforms covering neutral (FA2), tri-sialylated (A3G3S3), and tetra-sialylated (A3S1G3S3) N-glycans were quantitatively consistent.

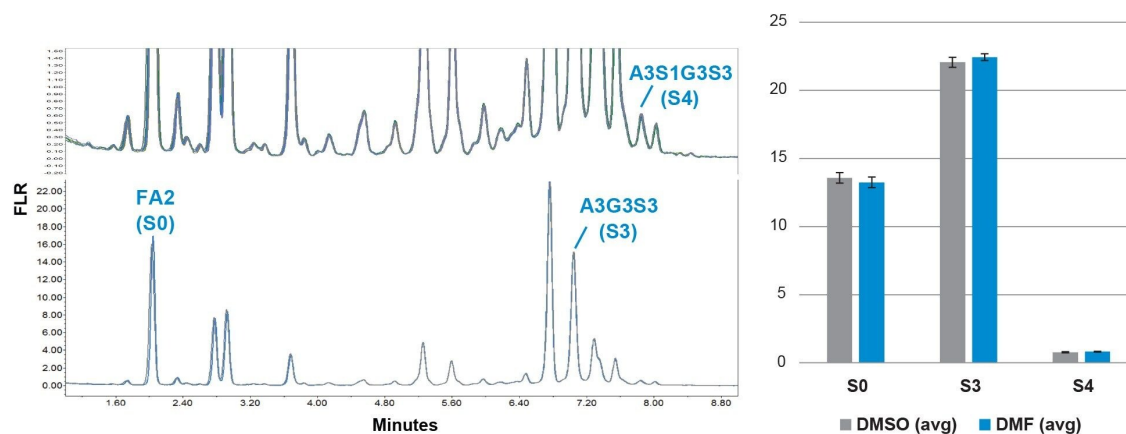


Figure 4. Shown are the HILIC-FLR N-glycan profiles and relative abundances ( $n=3$ , error bars are 1 SD) for three selected glycoforms for a mixture of NISTmab (NIST RM8671) and bovine fetuin. Samples were prepared as per User Guide 720005470EN using DMF or DMS to solubilize RFMS and as a component in the SPE purified sample diluent. Injection volume was 4  $\mu$ L. See Figure 2 for additional experimental details.

Comparable stabilities of the DMF and DMSO diluted samples over a period of 24 hours at 6 °C were also observed (Figure 5). However, while not significantly impacting the quantification it was observed that the neutral N-glycans using either cosolvent in the diluent exhibited a strong-solvent effect on the HILIC analytical column after 24 hours. Strong-solvent effects in HILIC separations, which result in band-broadening of weakly retained analytes, are due to disruption of the aqueous layer on the packed particle surface and are exacerbated by greater injection volumes and increased sample matrix polarity. This specific observation is likely the result of a 4  $\mu$ L injection volume on a low bed volume (2.1 X50 mm) amide HILIC column in combination with selective evaporative losses of the non-polar ACN component from the samples.

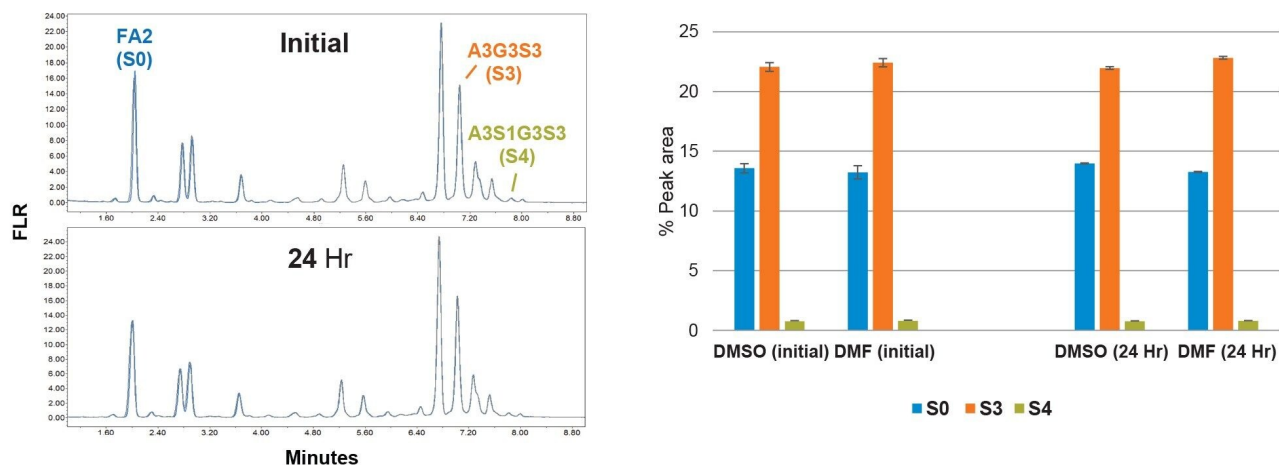


Figure 5. Shown are the initial and 24-hour sample stability timepoints at 6 °C for the HILIC-FLR N-glycan profiles and selected relative abundances ( $n=2$ , error bars are range) of a mixture of NISTmab and bovine fetuin. Samples were prepared and analyzed as described in Figure 4. Refer to text for discussion on the change in neutral glycan (FA2) peak shape.

It was also observed that strong-solvent effects were slightly greater for samples with DMSO in the diluent versus DMF (Figure 5). This may be the result of DMSO being slightly more polar with a dielectric constant of 47.2 versus 38.3 for DMF, and the high level of cosolvent in the sample (25% v/v). The impact of this difference is seen clearly in Figure 6, where injection volumes greater than 5  $\mu\text{L}$  resulted in more pronounced peak shouldering of the neutral glycoforms diluted with DMSO. The onset of strong-solvent effects will occur at larger injection volumes or as column volume decreases. While in most cases the use of DMSO in the sample diluent will not be problematic, HILIC method injection volumes may need to be reduced appropriately.



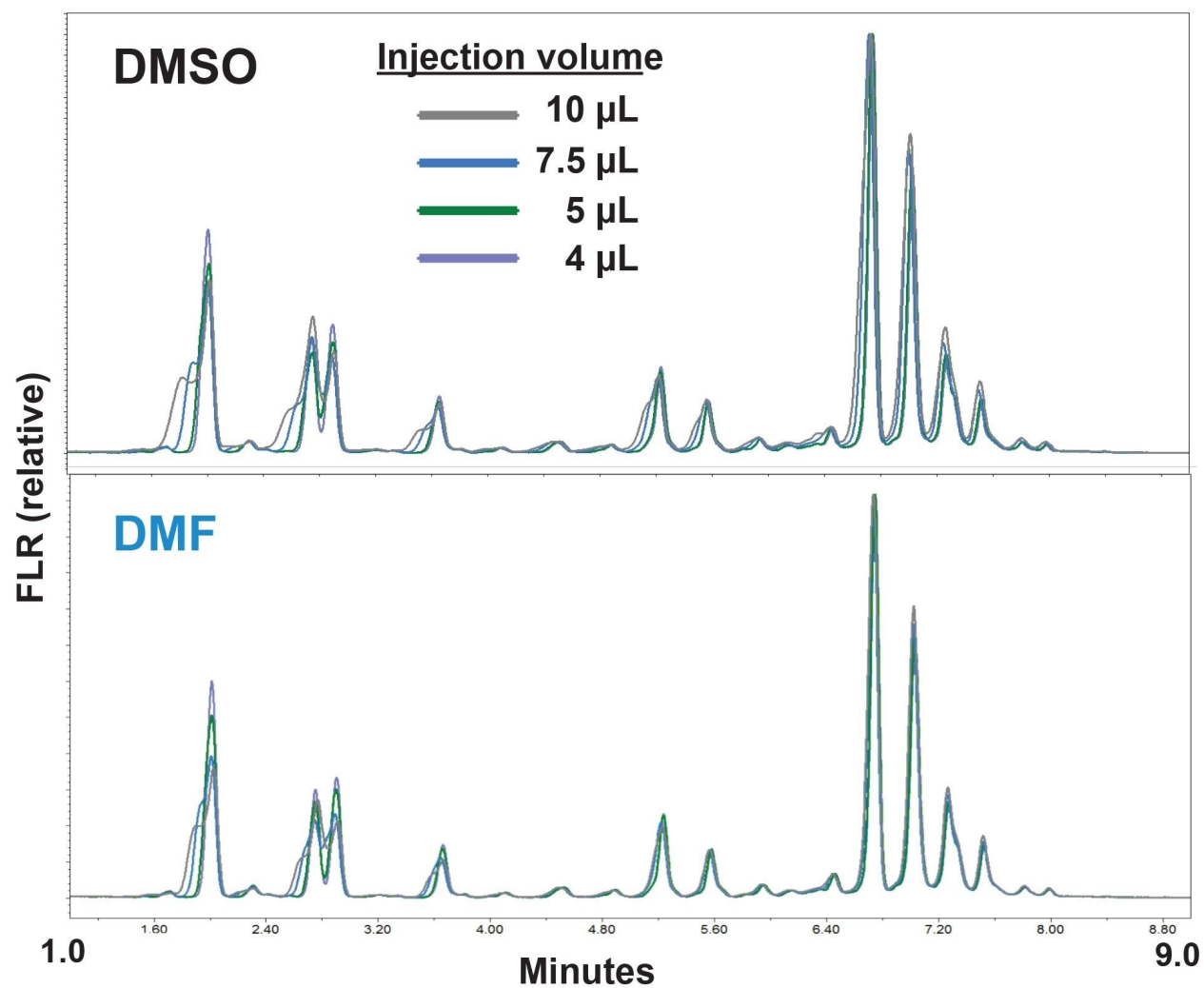


Figure 6. Shown are the strong-solvent effects for a range of injection volumes for the samples analyzed in Figures 4 and 5. Band broadening and peak splitting are slightly more pronounced in the DMSO-diluted samples.

## Conclusion

DMSO has been found to be an effective “1-for-1” replacement for DMF as a solvent and co-solvent in the

Rapifluor-MS N-Glycan GlycoWorks procedure. This work was prompted by an increased understanding of the deleterious impact that DMF can have on lab safety and on the environment as demonstrated by increased restrictions on the use DMF.

Comparable solubilization of RapiFluor-MS with anhydrous DMSO and subsequent RFMS labeling was demonstrated. DMSO, was also found to be effective as a component in the sample diluent used for RFMS-labeled and HILIC SPE purified N-glycans. However, maximum injection volumes for amide HILIC analysis are slightly reduced for DMSO versus DMF diluted samples. As a result, injection volumes when using DMSO as a diluent component may need to be reduced appropriately.

As further demonstration of the reliability of the RFMS GlycoWorks methodology when using DMSO in place of DMF, an automated procedure was successfully developed using DMSO to both solubilize RFMS and as a component in the HILIC sample diluent.<sup>4</sup> This procedure incorporates a diafiltration step to buffer exchange samples in incompatible matrices and concentrate samples prior to executing the RapiFluor-MS GlycoWorks procedure.

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## References

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720008157, December 2023



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