

High Efficiency Evaporative Fluorophore Labeling Of Glycans

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Introduction

As glycomics is currently becoming an important analytical challenge for the biopharma industry, newer and more efficient sample preparation methods are required. Albeit, numerous reductive amination based carbohydrate labeling protocols have been reported in the literature, the preferred way to conduct the reaction is in closed vials. In addition, published protocols apply comparable but not harmonized reaction conditions and reagent volumes for fluorescent labeling¹. Most of them suggest overnight reaction at 37°C^{2,3} or up to a couple of hours of reaction times at higher temperatures (50°C).⁴ The recently introduced rapid labeling approach of the Fast Glycan Sample Preparation and Analysis kit of SCIEX required only 20 minutes of reaction time at 60°C.⁵ This latter method utilized a novel evaporative labeling protocol to improve carbohydrate labeling efficiency with the idea of continuously increasing the concentration of the reagents during the tagging reaction⁶. This approach accommodated high labeling efficiency with reaching the optimal reagent concentrations for a wide range of glycan structures in complex mixtures during the reaction. Also importantly, the evaporative labeling process minimized sialylation loss, otherwise representing a major issue in reductive amination based carbohydrate tagging. In case of the use of minute amounts of dry samples to label, complete and uniform dispersion was obtained by supplementing the low volume labeling mixtures (several microliters) with the addition of extra low boiling point solvent (e.g., THF). It is important to note that evaporative labeling is fully automation-friendly, i.e., suitable for standard open 96 well plate format operation with the use of liquid handling robots.

Key Features of Evaporative Labeling

- Significant increase in labeling efficiency (up to 4x in total peak area)
- Decreased sialic acid loss
- Suitable for standard (open well) 96 well plate format operation
- Facilitates even dispensing of dry samples using highly evaporative non-reactive additives

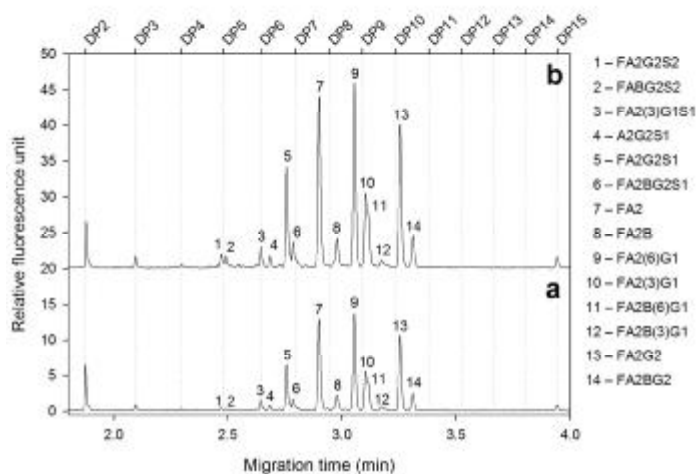


Figure 1. Comparison of conventional (trace **a**, closed vial method) and evaporative (trace **b**, open vial method) APTS labeling of PNGase F released hlgG1 glycans. Table 1 depicts the calculated total peak area values.

Methods

Sample Preparation

Human immunoglobulin G1 (hlgG1), sodium-cyanoborohydride (1M in THF), tetrahydrofuran, water (HPLC grade) and acetonitrile were obtained from Sigma Aldrich (St. Louis, MO, USA). Etanercept was kindly provided by the Medical School of the University of Debrecen (Debrecen, Hungary). The Fast Glycan Labeling and Analysis Kit, including the tagging dye of 8-aminopyrene-1,3,6-trisulfonic acid (APTS), HR-NCHO separation gel-buffer system and magnetic beads for excess dye removal were from SCIEX (Brea, CA, USA). The PNGase F enzyme was from Asparia Glycomics (San Sebastian, Spain).

Sample preparation started with the addition of the denaturation solution from the Fast Glycan Labeling and Analysis Kit (Part #B94499PTO) to 10 µl aqueous hlgG1 (test protein) and etanercept (target protein) solutions (10 mg/mL each) in a 200

uL PCR tube. The denaturation step proceeded for 8 minutes at 60°C, followed by the addition of PNGase F and the digestion buffer, and then incubation at 60°C for 20 minutes. In both instances with open vial cap, according to the kit protocol. The released N-linked carbohydrates were magnetic bead purified and mixed with the labeling reagent solution (SCIEX). The reaction mixtures were incubated in a heating block for 20 minutes at 60°C with closed (no evaporation) or open lid (evaporative labeling) vials without and with additional THF. After the labeling step, the samples were magnetic bead purified according to the Fast Glycan protocol (SCIEX) and analyzed by CE-LIF.

Analysis

A PA 800 Plus Pharmaceutical Analysis System (SCIEX, Brea, CA), equipped with a solid-state laser based fluorescence detector ($\lambda_{\text{ex}}=488 \text{ nm}/\lambda_{\text{em}}=520 \text{ nm}$) was used for all capillary electrophoresis analyses.

Capillary: EZ-CE™ cartridge (SCIEX) – 20 cm effective length (30 cm total, 50 μm I.D.) bare fused-silica capillary, part # A55625).

Separation matrix: HR-NCHO Separation Gel

Separation temperature: 30°C

Applied electric field (E): 1000 V/cm (U=30kV)

Injection: A pre-injection of water for 3.0 sec at 5.0 psi was followed by 1.0 kV/1.0 sec sample injection and 1.0 kV/1.0 sec bracketing standard injection.

Data processing:

For data acquisition and analysis the 32Karat, version 10.1 software package (SCIEX) was used.

Feature and Benefit

As depicted in Table 1, the effect of continuous evaporation on

Table 1. Comparison of total the peak area values and the sialoform subset of the hlgG1 N-glycan pool using the new evaporative (open vial) and the conventional (closed vial) reductive amination based fluorophore labeling.

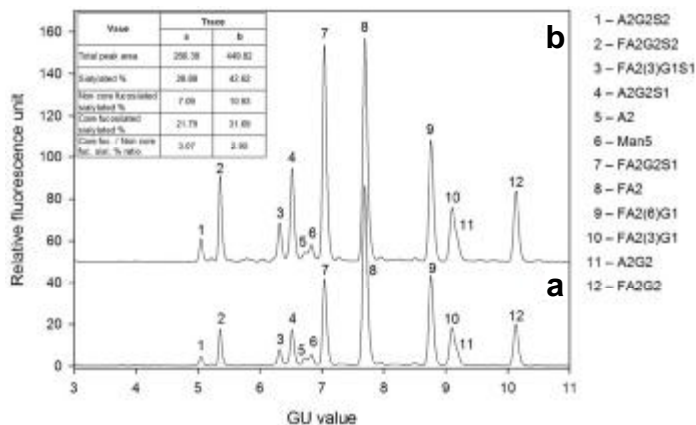
	Open vial (Evaporative labeling)	Closed vial
Total peak area	57.49 \pm 3.02	18.20 \pm 0.59
Sialoform subset, area	12.80 \pm 0.65	3.46 \pm 0.07
Sialoform subset, %	22.21 \pm 0.29	19.15 \pm 0.45

the reduction amination based fluorophore labeling reaction of sugars was conducted in an open vial format and compared to the conventionally used closed vial method. The reaction times were defined by reaching the complete drying time of the open vial method (20 minutes), and the same reaction conditions were used

for the closed-vial labeling method.

As apparent in Figure 1 and Table 1, with similar peak area of the bracketing standards, more than four times greater total peak areas were obtained with the use of the evaporative labeling protocol of the Fast Glycan kit. The peak area percentage of the sialylated glycan representing peaks (sialoform subset) were 19% using the closed-vial method, and 22% using the open-vial labeling approach, suggesting greater stability of the latter.

Evaporative labeling was also applied on tagging the PNGase F released N-glycans from a highprofile Fc fusion protein therapeutic of etanercept and compared to the closed lid based derivatization method. In this case, while the total peak area value doubled, a 13.75% increment was observed in percent values of the sialylated structures. The ratio of core and non-core fucosylated sialic acid containing glycans was also determined if there were any alterations caused by the evaporative labeling approach. As one can observe in the peak evaluation table inset in Figure 2, no significant differences were found using the two labeling protocols between the core fucosylated / non core fucosylated sialylated % ratio.



Conclusions

- A new, reductive amination based carbohydrate labeling method for CE-LIF analysis of glycans is presented
- Utilizing the open vial based fluorophore tagging approach resulted in significant increase in peak areas (up to 4x) compared to the traditional closed vial protocol
- Complete and uniform dispersion of minute amount of dry samples were obtained by using additional 10.0 µl tetrahydrofuran in the labeling mixture
- The new method is fully compatible with liquid handling robot based automation for 96 well sample-plate operation

Figure 2. Comparison of the conventional (trace a, closed vial) and evaporative (trace b, open vial) based labeling protocols for Etanercept. The inset depicts the actual labeling efficiency in both instances.

Table 2. Peak area comparison using the open vial labeling approach of dry sugar samples from hlgG samples with the addition of none (0 µl), 5 µl and 10 µl extra THF (total reaction volumes were 5, 10 and 15 µl, respectively).

Extra THF (µl)	Total peak area	Sialylated peak area subset
0	55.13 ± 1.47	12.22 ± 0.39
5.0	57.65 ± 1.90	12.85 ± 0.70
10.0	126.41 ± 1.18	26.94 ± 0.92

A comparative study was applied using the evaporative open vial labeling method with the addition of zero, 5.0 and 10.0 µl THF to the reaction mixtures (total reaction volumes were 5.0, 10.0 and 15.0 µl, respectively), to alleviate the challenging mechanical dispensing issue of dry samples with very low reagent volumes (≤5 µl microliters). This extra THF, added to the reaction mixture before the evaporative labeling step, accommodated uniform sample uptake by simple vortexing and more importantly, the same 20 minutes of reaction times could be used in all instances, due to the rapid evaporation of the low boiling point THF (66°C) from the reaction mixture in the open vial labeling format. The results are shown in Table 2, featuring more than doubled peak areas, when 10 µl of extra THF was added to the labeling solution to dissolve the sample (total reaction volume was 15 µl).

Acknowledgements

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