

Analysis of Fluorophore Labeled N-glycans by the Multicapillary C100HT Biologics Analyzer and HILIC-UPLC: Emphasis on Neutral Structures

Glycoanalysis on the C100HT Biologics Analyzer

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INTRODUCTION

Deep N-glycomic analysis of therapeutic antibodies and recently developed new therapeutic glycoprotein modalities provides important critical quality attribute (CQA) information about the carbohydrate profiles of the products [1]. As it is well known, N-glycosylation changes affect some of the important biological functions of glycobiotherapeutics [2]. In the case of monoclonal antibodies and Fc fusion proteins, CQAs such as the effector function, anti-inflammatory properties and serum half-life can all be affected by glycosylation changes at the conserved Asn 297 site of the C_H2 domain of the heavy chain [3]. In addition, glycosylation analysis of any other possible site modifications, e.g., in the Fab part or the linked protein portion of Fc fusion proteins also represents a challenge [4].

The two most frequently used liquid phase separation methods for glycan analysis are UPLC and CE. In this technical note, first the separation of fluorophore labeled N-linked partitioned glycan

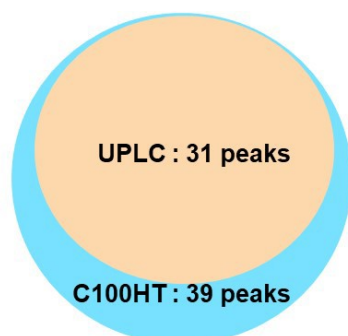


The C100HT Biologics Analyzer

libraries of high mannose, afucosyl biantennary and fucosyl biantennary types are compared using the multicapillary C100HT Biologics Analyzer and a HILIC-UPLC system. The N-glycans released from a high profile mAb therapeutics (Humira®) were also analyzed by both techniques and compared using industry standard sample preparation and separation methods.

Key Feature of C100HT Analyzer

- High throughput N-glycan analysis using the 12-capillary cartridge of the C100HT Biologics Analyzer is cc 50x faster than that of the same analysis by HILIC-UPLC.
- In the analysis of the three partitioned neutral glycan libraries in this study, 39 peaks were annotated by the multicapillary C100HT Biologics Analyzer, while the conventional HILIC-UPLC method (see under Methods) identified 31 features. The extra peaks identified by C100HT were the two of the Man 7 and two of the Man 8 positional isomers, as well as the A2G2BS2, A2G1B, A2G2S2 and A2 structures (Structural interpretation followed the nomenclature of Harvey et al [8].
- In the comparative analysis of the monoclonal antibody drug Humira®, both systems identified 9 features, 8 being identical. However, Man 6 was identified only by HILIC-UPLC and FA2[3]G1 was identified only using the C100HT system.



Number of Library Peaks Detected				
System	High Mannose	Afucosyl Biantennary	Fucosyl Biantennary	Total
C100HT	9	15	15	39
UPLC	5	13	13	31

Comparison between glycoanalyses accomplished on a C100HT Biologics Analyzer and a traditional HILIC-UPLC system

Methods

Chemicals: Reagents used to prepare the denaturation solution, digestion mixture, and labeling solution were provided in the C100HT chemistry kit (SCIEX, PN C13787). The partitioned N-glycan libraries of high mannose, afucosyl biantennary and fucosyl biantennary were purchased from Prozyme (Hayward, CA). The PNGase F was from Asparia Glycomics (San Sebastian, Spain). The therapeutic protein of Humira® was provided by the Medical School at University of Debrecen (Debrecen, Hungary). All other chemicals were from Sigma Aldrich (St. Louis, MO) at the highest purity grade.

Carbohydrate release and labeling: Release of the N-glycans from the mAb protein test item utilized PNGase F digestion following the procedure of the instructions of C100HT chemistry kit instructions. APTS labeling of the liberated carbohydrates was accomplished using the C100HT glycan kit with the reagent configuration optimized for the sample preparation and analysis of 1000 samples, based on the automation workflow of Szigeti et al. [5]. Briefly, 100 µg of glycoprotein sample was mixed with the magnetic beads and the denaturing solution, incubated at 60° C for 8 minutes and followed by PNGase F digestion at 60° C for 20 minutes. The released carbohydrates were captured by the magnetic beads under high organic solvent conditions and labeled with the addition of an APTS master mix at 60° C for 20 minutes. After the derivatization step the excess APTS was

removed by magnetic bead-mediated cleanup, repeated 3 times. The labeled glycans were eluted from the beads by 50 µL of water and immediately analyzed in the C100HT Biologics Analyzer. For the HILIC-UPLC experiments, glycan release and 2-AB labeling followed the earlier published method of Szekrenyes et al. [6].

Multicapillary gel electrophoresis: The 12-capillary C100HT Biologics Analyzer (SCIEX) was equipped with LED-induced fluorescence (LED-IF) detection with excitation and emission filters of 465 nm and 540 nm, respectively, enabling high sensitivity detection of 1-aminopyrene-3,6,8-trisulfonate (APTS) labeled N-glycans. The system was used with the C100HT cartridge, prefilled with the ultrahigh resolution separation gel matrix. All samples were electrokinetically injected by 2.0 kV for 2.0 seconds. The applied voltage during the separation was 6000 V.

HILIC-UPLC: Ultra-high performance liquid chromatography analyses of the 2-AB labeled N-glycans were performed on an AQUITY H-Class system (Waters, Milford, MA) equipped with a fluorescence detector (excitation 360 nm; emission filter at 428 nm) using a 2.1 x 150 mm HILIC BEH column (1.7 µm beads). The columns were fully equilibrated to the starting conditions (60°C; 78% of acetonitrile, 22% 100 mM of ammonium formate, pH 4.5) before each run. A 60-minute (including separation and column regeneration time) UHPLC-HILIC method was used in



Figure 1. Simultaneous C100HT analysis of the three APTS labeled neutral partitioned N-glycan libraries of high mannose, afucosyl biantennary and fucosyl biantennary (A-C and G-I) types, the oligosaccharide ladder (D and J) as well as the mAb test item (Humira®, F and L) N-glycans and the blank control (water injection, E and K) in duplicates to utilize all 12 channels of the cartridge.

gradient elution mode as specified in [6]. The Empower 2/3 software package was employed for data acquisition and analysis.

Results and Discussion

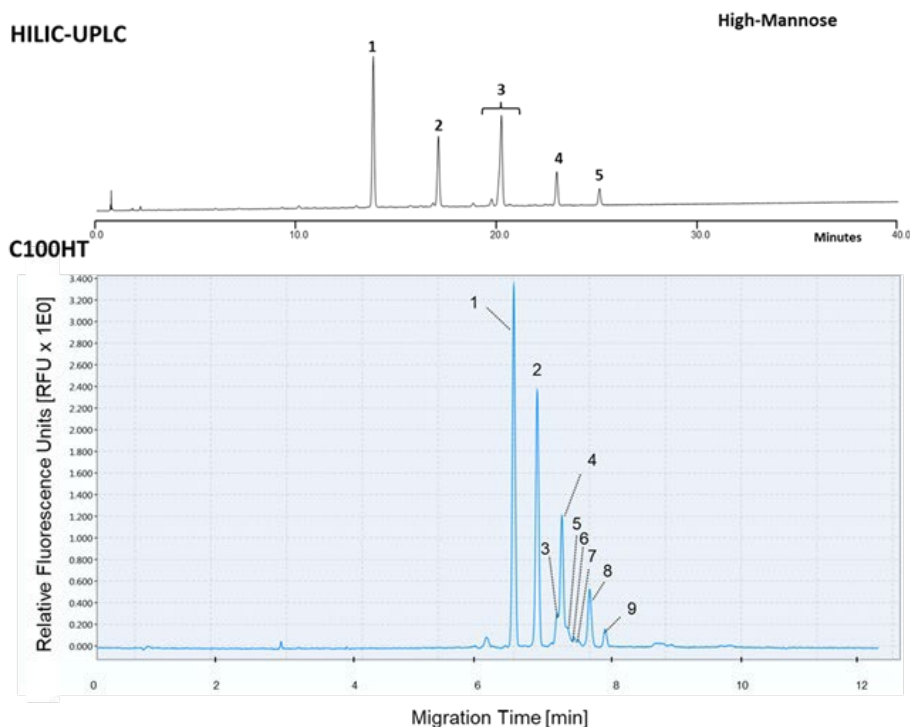
The C100HT Biologics Analyzer is an automated multicapillary gel electrophoresis platform capable of simultaneous analysis of up to 12 samples in 14-minute cycle times (including separation and capillary refill) with great reproducibility, thus, readily accommodates glycan screening of up to four 96 well plates per workday (8 h) [7]. All samples were simultaneously injected in duplicates including the APTS labeled three neutral partitioned N-glycan libraries along with the APTS labeled maltooligosaccharide ladder, a blank control (water) and the released and APTS labeled asparagine linked carbohydrates from the mAb test item (Humira®). Separation of all samples was completed in 10 minutes (Figure 1). Panels A-C and G-I depict the separation traces of the partitioned high mannose, afucosyl biantennary and fucosyl biantennary libraries, respectively. The separation of the maltooligosaccharide ladder and the electrophoretic trace Humira® N-linked glycans are shown in panels (D and J) and (F and L), respectively. Panels E and K depict the blank control water injection traces.

Fast and reproducible separation of all three partitioned libraries and the therapeutic protein test item were obtained, featuring

high resolution separation of all samples in 10 minutes (14 minute cycles) in duplicates, corresponding to the analysis of a full 96 well plate in approximately 2 hours. Since UPLC systems are not capable of simultaneous analysis of multiple samples, we can only show their individual separation traces in Figures 2-5 compared to their C100HT electropherogram counterparts.

Comparison of the analysis of 2-AB and APTS labeled partitioned high mannose N-glycan libraries by HILIC-UPLC and the multicapillary C100HT Biologics Analyzer is shown in the upper trace and lower traces of Figure 2, respectively. While both methods identified 5 major peaks, several additional features representing the positional isomers of the Man7 and Man8 structures were also identified in the C100HT separation trace, as shown in the table next to the electropherogram. The required separation time for the HILIC-UPLC system was 25 minutes in contrast to the multicapillary gel electrophoresis system, in which case the separation only required 8 minutes. Please note, that in addition to the faster separation time, the C100HT system was capable of simultaneously analyzing 12 samples.

Similar to as shown in Figure 2, two other partitioned neutral N-glycan libraries of afucosyl biantennary and fucosyl biantennary types were also analyzed using both techniques. Figure 3 shows the comparative analysis of the 2-AB and APTS labeled partitioned afucosyl biantennary N-glycan library by HILIC-UPLC



Sample	Sample Peak ID	Structure
APTS High Mannose Library	1	M5
	2	M6
	3	M7
	4	M8
	5	M9

Sample	Sample Peak ID	Structure	GU 25°C
APTS High Mannose Library	1	M5	6,331
	2	M6	7,137
	3	M7	7,828
	4	M7	8,009
	5	M7	8,181
	6	M8	8,293
	7	M8	8,452
	8	M8	8,988
	9	M9	9,535

Figure 2. Comparative analysis of 2-AB and APTS labeled high mannose N-glycan libraries by HILIC-UPLC (upper trace) and the C100HT Biologics Analyzer (lower trace).

(upper trace) and the C100HT Biologics Analyzer (lower trace). Here again, comparable separation performances were obtained, i.e. 15 identifiable features were separated by the C100HT Biologics Analyzer, while 13 by HILIC-UPLC. Most of the annotated peaks were the same with the two separation methods. The two extra peaks identified by the multicapillary gel electrophoresis system were A2G2BS2 (peak 13) and A2G1B (peak 22). Again, the required separation time for HILIC-UPLC was almost 30 minutes in contrast to the multicapillary gel electrophoresis system where the last peak showed up in less than 9 minutes. All identified glycans are listed in the tables next to the corresponding separation traces, using the annotation suggested by Harvey et al [8]. For the C100HT analysis, the capillary electrophoresis GU values are also listed for all structures.

Figure 4 compares the analysis of the fluorophore labeled fucosyl biantennary N-glycan library using the C100HT Biologics Analyzer and the HILIC-UPLC system. In this instance, 15 and 13 peaks were annotated by the multicapillary C100HT Biologics Analyzer and the HILIC-UPLC system, respectively. The two extra peaks identified by the C100HT Biologics Analyzer were the A2G2S2 (peak 27) and A2 (peak 31) glycans as shown in the Table next to the C100HT trace.

Finally, the analysis of the PNGase F released N-glycans from the monoclonal antibody therapeutic, Humira®, was compared using 2AB labeling for HILIC-UPLC and APTS derivatization for the C100HT Biologics Analyzer, shown in Figure 5. In this instance, both separation methods annotated 9 glycan structures as shown above the peaks in the corresponding separation traces. Out of the 9 peaks separated with both methods, 8 structures were the same and one was different as shown in the corresponding tables near the traces. Peak 8 was denoted as Man 6 in the HILIC-UPLC separation, while in the C100HT separation peak 8 was identified as FA2[3]G1, emphasizing the complementarity of the separation approaches. Again, HILIC-UPLC analysis required almost 30 minutes for each separation (not including column regeneration time) and the samples could be analyzed only consecutively, i.e., requiring more than a full day to process 12 samples using conventional methods. The C100HT Biologics Analyzer, on the other hand, needed only 10 min for the separation with the option of simultaneously analyzing 12 samples, i.e., enabling to process up to 4 x 96 well plates per workday.

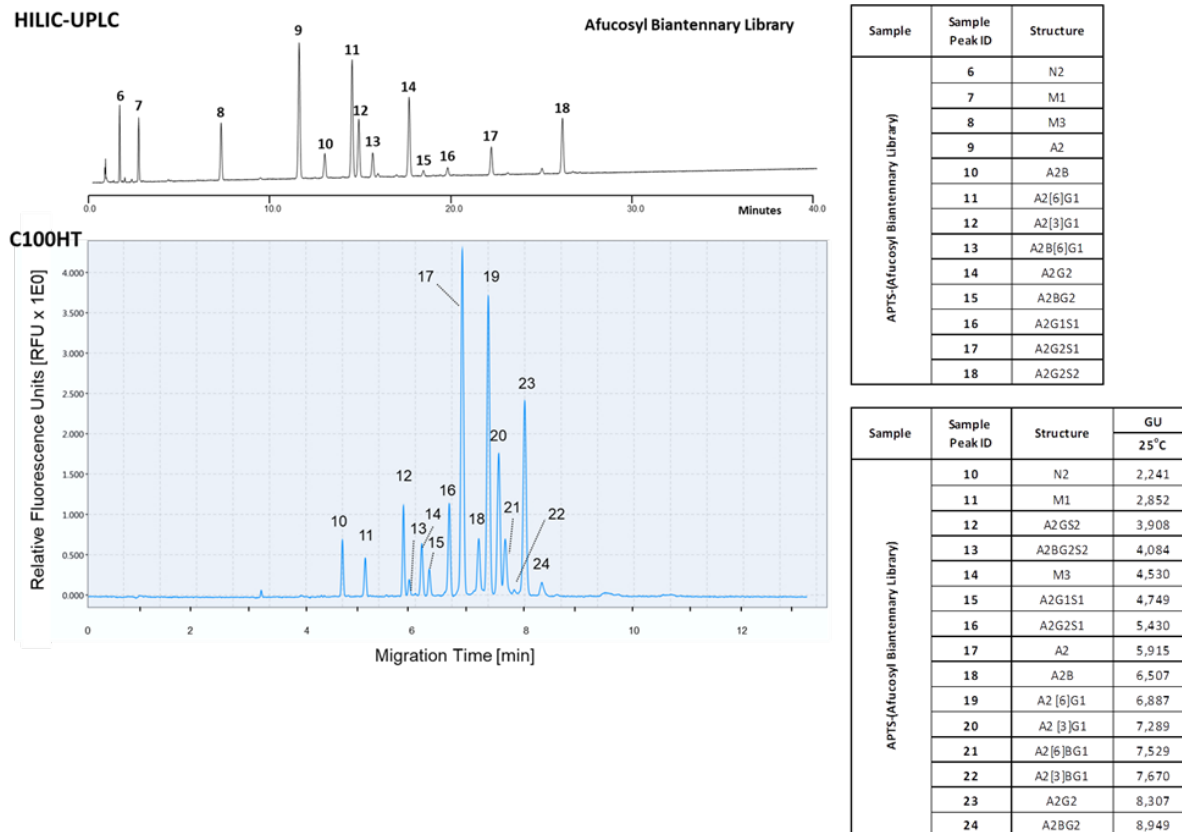


Figure 3. Comparative analysis of 2-AB and APTS labeled partitioned afucosyl biantennary N-glycan libraries by HILIC-UPLC (upper trace) and the C100HT Biologics Analyzer (lower trace). The tables at the right sides of both traces show the corresponding glycan structures using the Oxford nomenclature [8]. For the C100HT data the GU values are also listed.

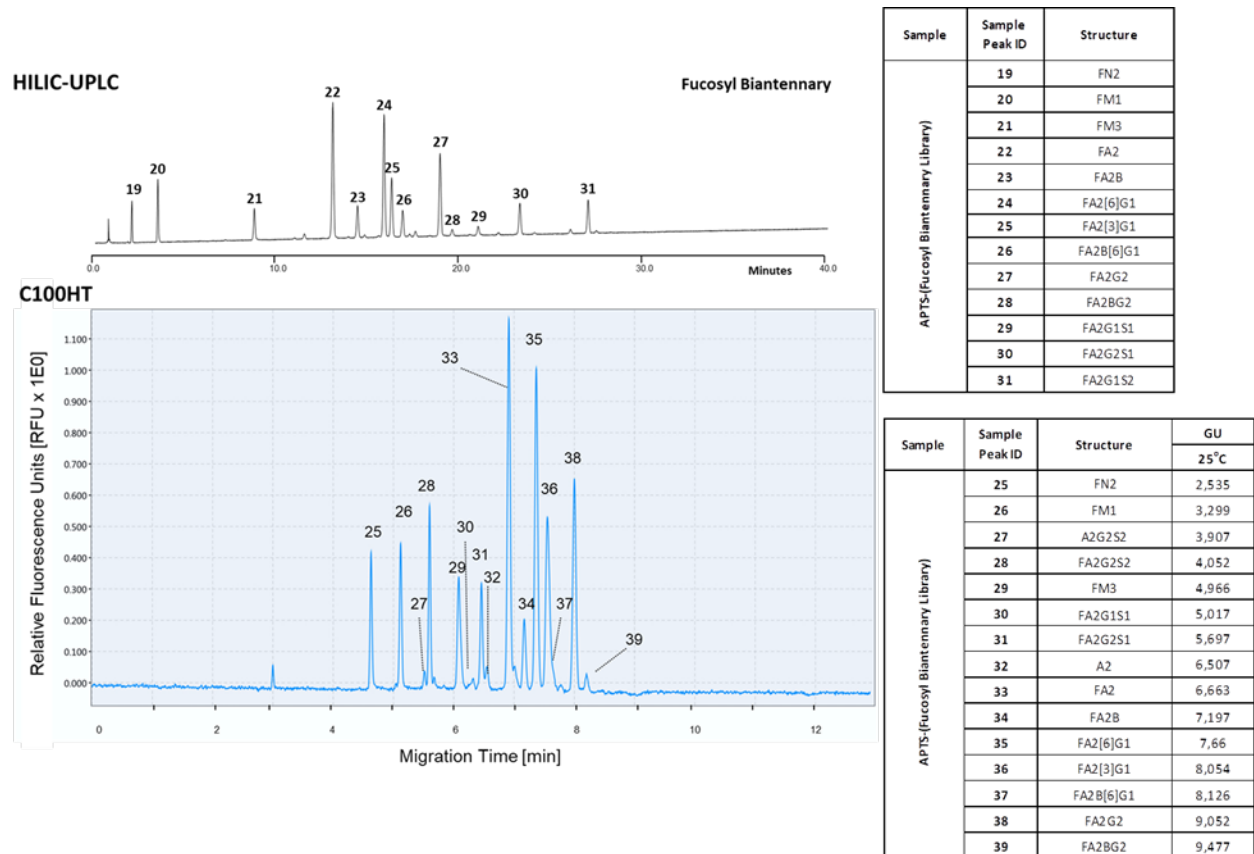


Figure 4. Comparative analysis of 2-AB and APTS labeled partitioned fucosyl biantennary N-glycan libraries by HILIC-UPLC (upper trace) and C100HT Biologics Analyzer (lower trace). The tables at the right sides of both traces show the corresponding glycan structures using the Oxford nomenclature [8]. For multicapillary electrophoresis the GU values are also listed.

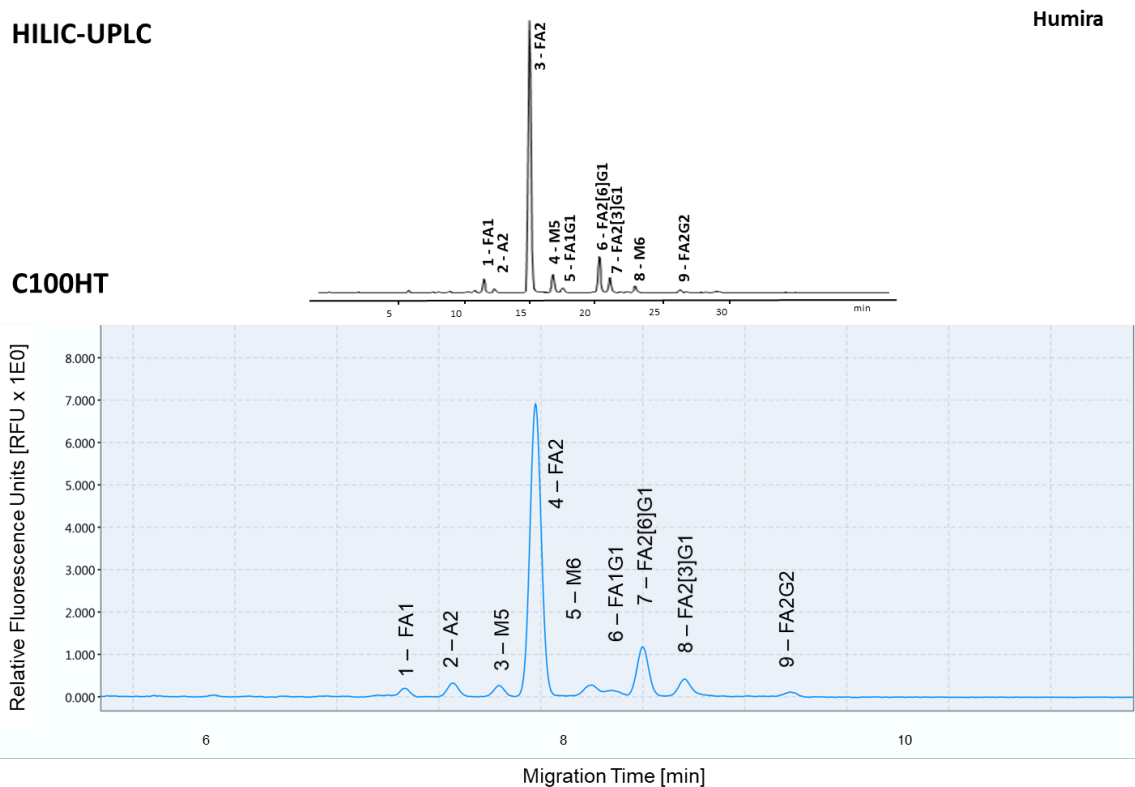


Figure 5. Comparative HILIC-UPLC and multicapillary C100HT analysis of PNGase F released and fluorescently labeled N-linked glycans of **Humira®**. The identified structures are shown above the peaks with their abbreviated structural interpretation.

Conclusions

- This technical note compares the separation performance both for analysis time and number of identified features of HILIC-UPLC and the C100HT Biologics Analyzer (multicapillary gel electrophoresis system), using standard industry conditions.
- First the fluorophore labeled partitioned neutral N-glycan libraries containing the most frequently occurring carbohydrate structures of biopharmaceutical interest, i.e., high mannose, afucosyl biantennary and fucosyl biantennary oligosaccharides were individually analyzed. 39 peaks were annotated by the multicapillary C100HT Biologics Analyzer, while the standard HILIC-UPLC system identified 31 features.
- The separation traces of the asparagine linked oligosaccharides released from Humira®, a high profile monoclonal antibody drug were also compared with the use of the multicapillary C100HT Biologics Analyzer using LED induced fluorescence detection and Hydrophilic Interaction Ultrahigh Performance Liquid Chromatography (HILIC-UPLC) with fluorescent detection, applying standard industry settings. Both methods identified a total of 9 features, with 8 glycans identical, but the Man 6 and FA2[3]G1 structures were detected only with HILIC-UPLC and C100HT system, respectively.
- In case of the C100HT Biologics Analyzer, the average separation time was approximately 10 minutes with the ability to simultaneously analyze up to 12 samples, i.e., offering an almost 50x faster approach than that of the same analysis by HILIC-UPLC, which require consecutive analysis of the individual samples.

Acknowledgment

The authors gratefully acknowledge the support of the National Research, Development and Innovation Office (NKFIH) (K 116263) grants of the Hungarian Government, the EFOP-3.6.3-VEKOP-16-2017-00009 co-financed by EU and the European Social Found.

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Document number: **RUO-MKT-02-9755-A**



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