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Highlights

- The biopharmaceutical industry needs high throughput N-glycosylation analysis tools.
- SWDMI supports rapid and large scale multicomponent analysis.
- The separation window covered the full separation of all sample components.
- Pressure and electrokinetic sample introduction mediated SWDMI is demonstrated.
- The method can be readily applied to large scale bioindustrial applications.

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Separation window dependent multiple injection (SWDMI) for large scale analysis of therapeutic antibody N-glycans

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Abbreviations: SWDMI - Separation Window Dependent Multiple Injection; CE - Capillary Electrophoresis; LIF – Laser Induced Fluorescence; APTS – 8-aminopyrene-1,3,6-trisulfonic acid; CESI – Capillary Electrophoresis Electrospray Ionization; TOF/MS – Time of Flight Mass Spectrometry

Keywords: high throughput analysis, N-glycans, biopharmaceuticals

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Abstract

There is a growing demand in the biopharmaceutical industry for large scale N-glycosylation analysis of biotherapeutics, especially monoclonal antibodies. To fulfill this high throughput analysis requirement with single column separation systems in most instances require finishing the entire analysis cycle including conditioning, injection and separation between sample injections. While in liquid chromatography it represents a challenge, multiple sample injection in capillary electrophoresis has already been demonstrated for one or two sample components by utilizing the concept of introducing sequential sample and buffer zones into the capillary tubing before the start of the separation process. It was also demonstrated in CE-MS mode, mostly to follow one sample component, identified by precise mass measurement. Here we introduce a novel multiple injection approach for rapid large scale capillary electrophoresis analysis of samples with biopharmaceutical interest supporting multicomponent optical detection with laser induced fluorescence. In Separation Window Dependent Multiple Injection (SWDMI) mode, the samples are consecutively injected in

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predefined time intervals, based on the window that covers the separation of all sample components. As a practical example, this newly developed SWDMI protocol was applied to rapid and large scale analysis of APTS labeled monoclonal antibody N-glycans using a short (20 cm effective length) capillary column. Full analysis of 96 samples (injected from a well plate) was obtained in 4 hours, in contrast to consecutive individual separation cycle processing of the same samples that required 12 hours.

1 Introduction

Rapid and large scale sample analysis in all omics fields has become one of the focus areas of the bioanalytical segment of pharmaceutical research and the means to expand biomedical knowledge in the post Human Genome Project era. It has provided new opportunities to elaborate basic scientific research that have triggered drug discovery projects ending up as marketed pharmaceutical products [1]. Current research endeavours to decipher and understand the structure function relationship of the carbohydrate moieties of therapeutic glycoproteins draw the attention on the importance of glycomics [2]. Recently, there is a growing demand in the biopharmaceutical industry for rapid N-glycosylation analysis of biotherapeutics, especially monoclonal antibodies during clone selection. Therefore, processes and/or techniques capable to provide significant improvements in separation throughput have great potential.

Introduced by Willams et al. [3] at the turn of the new millennium, Sequential Injection Before Analysis (SIBA) protocol significantly reduced analysis times without loss of resolution and increased the throughput for oligonucleotide analysis in capillary electrophoresis. Using the SIBA protocol, oligonucleotides were electrokinetically injected and the capillary was immersed into water to remove any residual oligonucleotide before dipped in the inlet and outlet electrophoresis reservoirs. After injection of five samples repeating the previous protocol, the separation process was started. The authors analyzed a single nucleotide for its n-1 nucleotide impurity with this approach, so the separation time window did not play a significant role in the process. Multiple injections of pharmaceutical compounds using capillary electrophoresis was reported by Veuthey and coworkers [4] by alternatively injecting sample and electrolyte zones into the capillary before applying the separation voltage. The authors optimized the length of the electrolyte zone for 2x longer that of the sample zone for better resolution. Another multiple injection procedure was developed by the same authors to reduce the analysis time per sample and was successfully applied to

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both UV and MS detection [5]. In order to reduce analysis time, multiple injections were performed by alternatively injecting one sample zone and one BGE zone into the capillary. Staub et al. [6] utilized a CE-UV-ESI-TOF/MS procedure for rapid identification and quantitation of insulin. Four samples were injected with background electrolyte zones in between containing the sample of interest and an internal standard. Another multiple-injection capillary zone electrophoresis procedure (MICZE) has been elaborated for the assay of salbutamol [7]. In this method, seven sample sets, each consisting of three of the same samples, were sequentially injected into the capillary and analyzed within a single run. This allowed a total of twenty-one sequential injections (six standards and fifteen samples), containing salbutamol and the injection marker oxprenolol. The injected sample plugs were separated by background electrolyte (BGE) plugs, through application of short-term voltage spikes over the capillary for different time periods. The samples in each set were segregated from each other by partial electrophoresis. After the final sample injection, the electrophoresis process was started for a time period corresponding to that in routine single-injection CZE.

Another interesting way to increase analysis throughput in capillary electrophoresis was the more recently introduced multisegment sample injection concept [8]. Using this approach, about one third of the total capillary length is usually loaded with repeated sample/spacer segments prior to conducting the actual CE separation. In other words, multisegment injection introduces the samples with BGE plugs in between and starts the separation once all samples are loaded. By the time when the separation starts, a significant portion of the capillary is filled up with multiple sample-spacer plugs, so the sample that was introduced first will observe a shorter separation distance that of the sample which was injected last and correspondingly in between. In multisegment injection mode only pressure injection methods have been reported and demonstrated to be feasible for metabolite analysis with MS detection. Hudson applied the method for CESI-MS analysis using long capillary columns (100 cm) with MS detection [9]. At the best of our knowledge, this approach has not been demonstrated for optical detection of multiple sample components.

To accommodate large scale multicomponent analysis in 96 well plate format with optical detection, our laboratory developed a novel Separation Window Dependent Multiple Injection (SWDMI) method. This approach does not require any spacers in between the loaded sample plugs and can be readily applied to shorter capillary tubings supporting optical (LIF in our case) detection of multiple sample components in an ultrafast fashion. The samples were

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serially injected one after another and the separation process immediately started after injecting the first sample.

2 Materials and methods

2.1 Chemicals: Immunoglobulin G (IgG) and sodium cyanoborohydride (1 M in THF) were from Sigma-Aldrich (St. Louis, MO). NEB Denaturing Buffer, 1x GlycoBuffer-2 and 1% NP-40 were from New England Biolabs (Ipswich, MA). PNGase F was from ProZyme (Hayward, CA). The 8-aminopyrene-1,3,6-trisulfonate (APTS) and NCHO Carbohydrate Separation Buffer were from SCIEX (Brea, CA). The Agencourt CleanSEQ magnetic beads were obtained from Beckman Coulter, (Indianapolis, IN).

2.2 Sample preparation: IgG samples were first denatured, followed by PNGase F digestion at 60^{o}C for 20 minutes. The released glycans were fluorescently labeled with APTS and our earlier described magnetic bead based purification protocol was applied both after glycan release and fluorophore labeling [10].

2.3 Capillary electrophoresis: In all capillary electrophoresis separations, a Beckman P/ACE MDQ system was used with laser induced fluorescent (LIF) detection, employing a 488 nm argon ion laser with a 520 nm emission filter. The NCHO separation capillary was 30 cm long (20 cm effective length, 50 um ID) and used with the NCHO gel buffer system (both from SCIEX). The applied separation voltage was 30 kV in reversed polarity mode (anode at the detection side) at 25^{o}C or 37^{o}C cartridge temperature, depending on the experiment. For injection either a one-step pressure injection was used: 1.2 psi for 0.15 min; or a three-step electrokinetic injection: water: 0.1 psi for 0.1 min followed by the sample: 2.3 kV for 0.1 min and a buffer push: 0.1 psi for 0.1 min.

3 Results and discussion

Separation Window Dependent Multiple Injection (SWDMI) utilized a serial injection concept during the electrophoresis process by periodically interrupting the separation to introduce consecutive samples with expectedly similar peak profile, e.g., IgG N-glycans during clone selection (Figure 1). Thus, the number of actual separation steps was equal to the number of samples injected in SWDMI.

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Separation window by definition was the migration time segment of the entire separation required to separate all sample components in one injection-separation cycle. Injection of each succeeding sample was implemented when the first peak of the previously injected sample was detected, considering the predetermined separation window. In SWDMI both pressure and electrokinetic injection methods were applicable. During the experiments, commercially available standard IgG was used since the conserved glycosylation pattern of this glycoprotein is well-characterized and regularly used for method validation and testing.

3.1 SWDMI by pressure injection

Figure 2 shows the CE-LIF analysis of four APTS labeled IgG N-glycan samples injected by pressure mediated Separation Window Dependent Multiple Injection mode into a 20 cm effective length capillary, represented by separation windows C1-C4. The separation was interrupted for each sample injection when the first peak of the mixture was detected, in this case the APTS peak at 1.28 min (depicted by the asterisk *). The separation was started by applying 30 kV (0.1 min ramp time) after the injection of the first sample for 0.15 min at 1.2 psi. Once the unreacted APTS peak was detected, the separation process was stopped and the second sample was injected by the same pressure parameters followed by immediate application of the separation voltage. The peaks corresponding to the IgG glycan structures of the first sample started to appear at 2.61 min in the first cycle (C1) regime and the separation window was 1.14 min long. Please note that the actual separation of the sample components was not interrupted by the consecutive injection processes while they passed the detection window, therefore the peak pattern was always adequately visualized. In this instance, due to the precise determination of the separation time window (1.14 min), the APTS peak of the second sample migrated rather close to the last peak of the first IgG sample, at 3.77 min. Since the second sample was injected in the middle of C1, the first cycle ended with the injection of the third sample that was injected in the middle of C2. The forth sample was injected at the time when the APTS peak of the second sample was detected in C3. To follow this concept, full analysis of four samples took in less than 10 minutes.

3.2 SWDMI by electrokinetic injection

Electrokinetic injection with the SWDMI process is required to analyze highly diluted samples or when high viscosity gel-buffer systems are used for the separation. This approach

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resulted in good signal to noise ratio, even from low concentration samples (Figure 3). As part of the electrokinetic injection process, a depleted ion zone (water injection) was electrokinetically introduced prior to the sample injection to support transient isotachophoresis based sample pre-concentration for dilute analytes. A pressure mediated buffer push was also applied after each sample injection to properly terminate the injection zones. The versatile user interface allowed making the method easily adjustable and adaptable for high throughput N-glycan analysis. The separation profile using electrokinetic injection mode of SWDMI is depicted in Figure 3. Please note that in case of electrokinetic injection, the two extra injection-related steps (water pre-injection, and post-injection buffer push) along with the associated tray movements made this approach somewhat more time consuming than pressure mediated SWDMI (288 min vs 240 min for 96 samples, respectively). Therefore, when sample concentration is not an issue and/or low viscosity separation matrix is used, pressure mediated SWDMI method is more advisable to achieve faster analysis.

3.3 Effect of the separation temperature

All of the above described multiple injection and separation methods were executed at 25°C capillary cartridge temperature. Further reduction of the overall analysis time with multiple injections was attempted by increasing the separation temperature to 37°C. Figure 4 compares the separations at 25°C (upper trace) and 37°C (lower trace) temperatures. Adequate separation of the sample peaks of interest (G0, G1, G1' and G2) was still maintained at higher temperature as shown by the lower trace in Figure 4 and the peak ratios in the profile remained the same. The overall separation time at the elevated temperature was approximately 230 minutes for 96 samples (including all injection and separation related tray movements) with the peak area reproducibility of 10.75% RSD and migration time reproducibility of 0.45% RSD.

4 Conclusions

Introduction of new generation protein therapeutics represents a growing current trend in the biopharmaceutical industry. More than half of these biotherapeutics are monoclonal antibodies and the analysis of their sugar moieties requires rapid and high-throughput separation methods. In the present work we demonstrated pressure and electrokinetic sample introduction mediated Separation Window Dependent Multiple Injection (SWDMI) for capillary electrophoresis analysis of the major IgG glycans using optical (LIF) detection. The separation window was determined as the migration time covering the actual separation of all

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sample components including the peak representing the remaining APTS dye opening the time window. Every consecutive injection was implemented when the APTS peak of the preceding injection reached the detection window. The effectivity of the approach was demonstrated by using a single capillary CE system for the analysis of 96 samples. The method can be readily applied to significantly shorten overall analysis times for large scale applications. In electrokinetic injection mode, the method required some extra time for additional tray movements and positioning. With pressure injection mode, this extra time was reduced; therefore, the cycle time was close to the actual separation time of the sample components. Further decrease in process timing was demonstrated by increasing the separation temperature from 25^{o}C to 37^{o}C, requiring approximately 230 minutes for 96 samples.

Acknowledgement

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Figure legends

Figure 1: Schematics of separation window dependent multiple injection (SWDMI). Consecutively injected sample plugs are labeled with different patterns.

Figure 2. CE-LIF analysis of APTS labeled IgG N-glycans using pressure mediated Separation Window Dependent Multiple Injection (SWDMI) method. C1-C4 represent the injection to injection cycle times. APTS peaks are labeled by the asterisk (*). The inset depicts the main IgG N-glycan peaks of interest, G0 -FA2; G1 - FA2(6)G1; G1' - FA2(3)G1; G2 - FA2G2. Conditions: Capillary: 30 cm (20 cm effective length) NCHO, 50 µm ID, N-linked Carbohydrate Separation Gel Buffer, E=30 kV, t=25°C. Injection: 1.2 psi for 0.15 min.

Figure 3. Electrokinetic sample introduction mediated Separation Window Dependent Multiple Injection for CE-LIF analysis of APTS labeled IgG N-glycans. The inset depicts the main IgG N-glycan peaks of interest. Separation conditions were the same as in Figure 2. Injection parameters: water: 0.1 psi for 0.1 min (forward); sample: 2.3 kV for 0.1 min (reversed polarity); buffer push: 0.1 psi for 0.1 min (forward).

Figure 4. Comparison of the separation of APTS labeled human IgG N-glycans at 25^{o}C (upper trace) and 37^{o}C (lower trace) utilizing pressure mediated Separation Window Dependent Multiple Injection.

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Figure 1

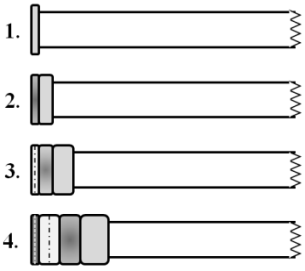

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Figure 2

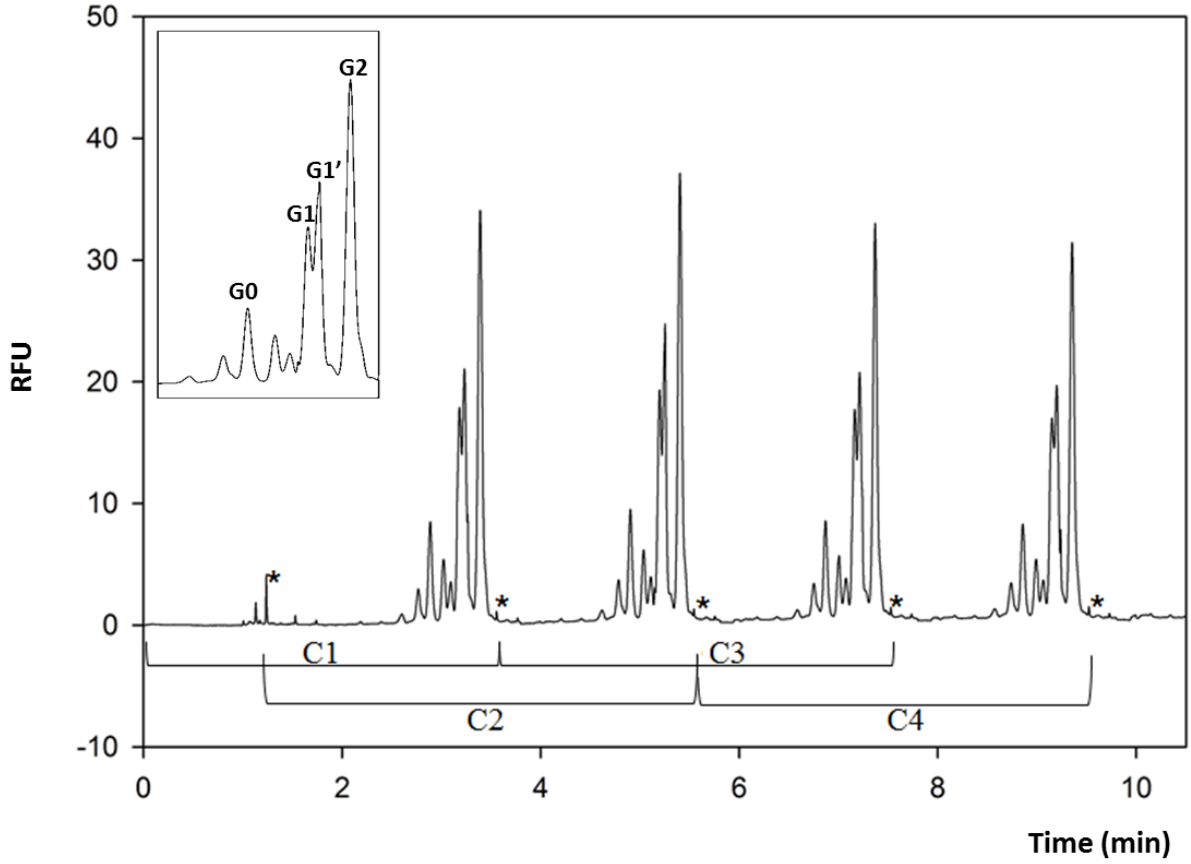

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Figure 3

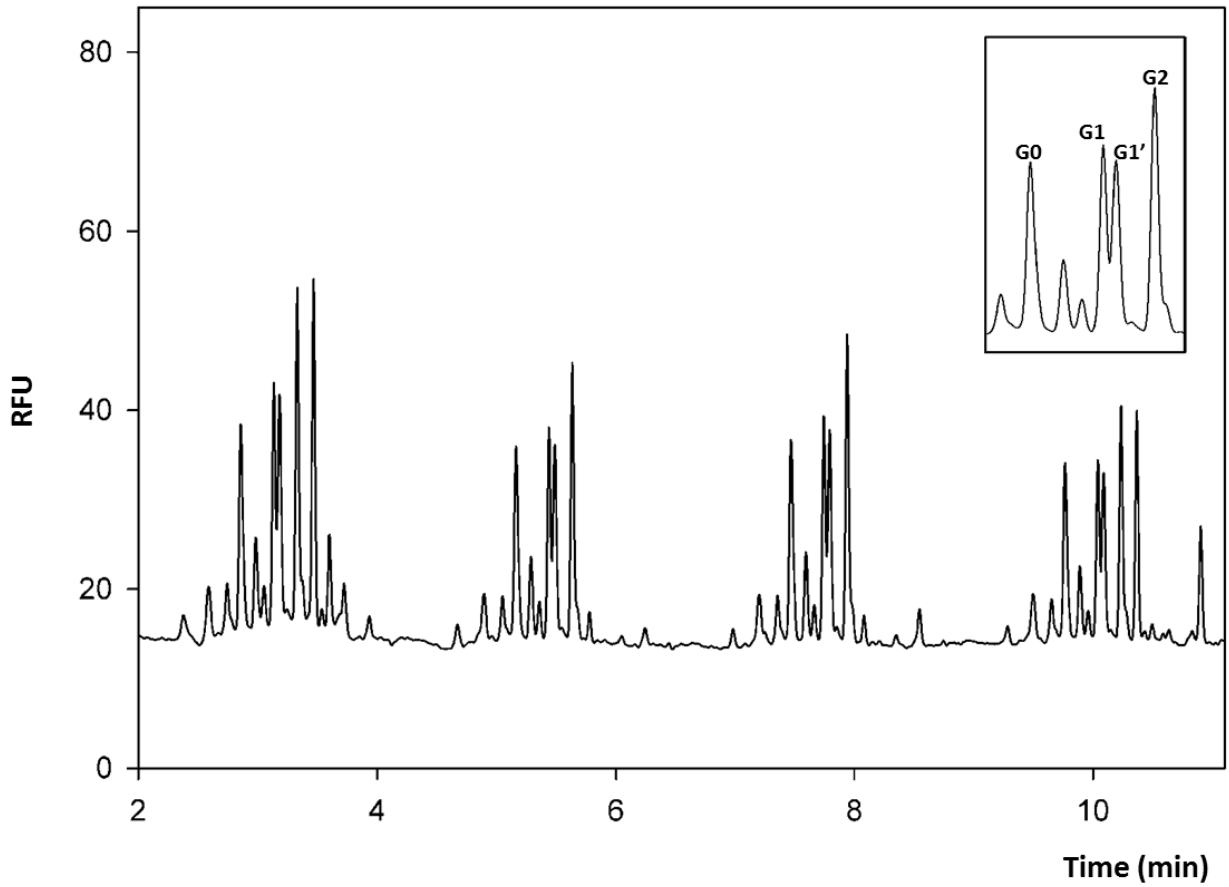

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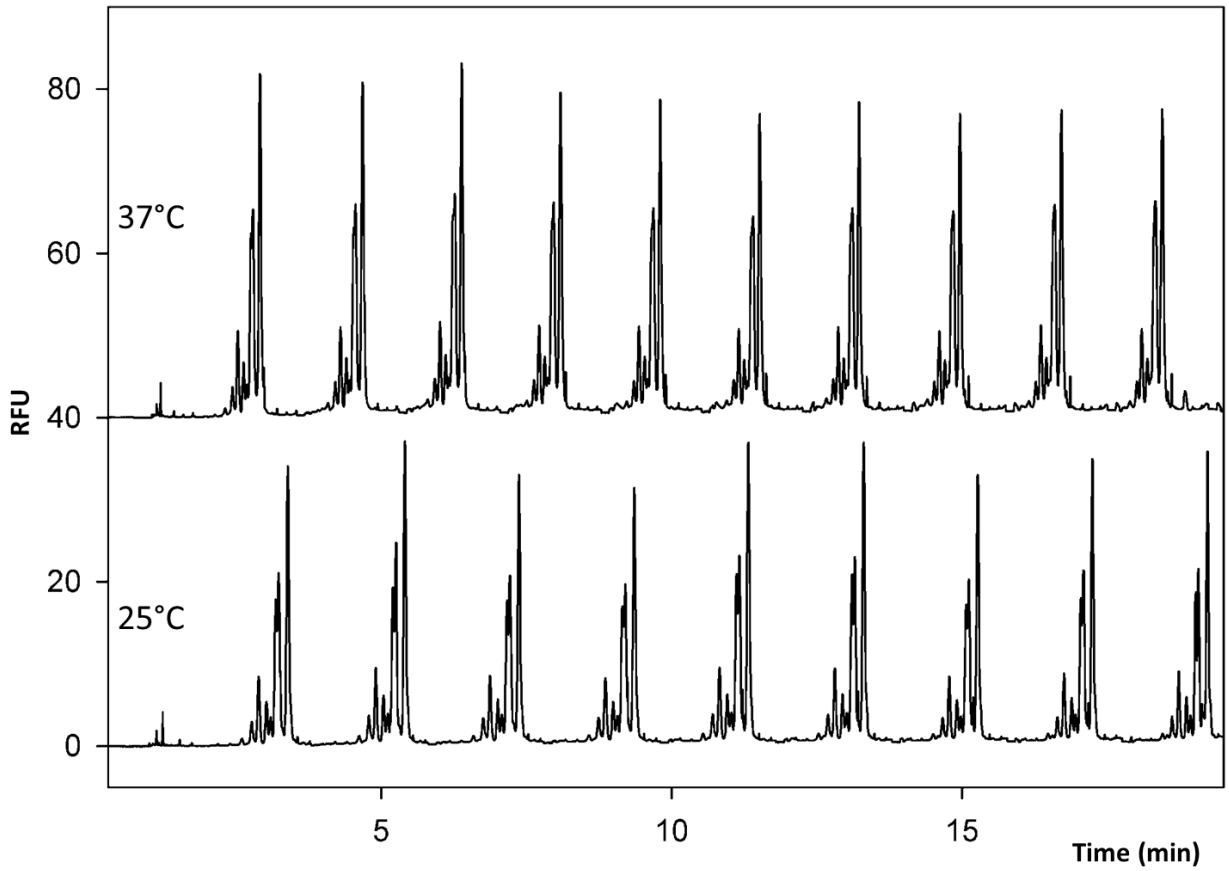
Figure 4



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Graphical Abstract: