

High Throughput Mass Spectrometry Analysis using Immediate Drop-On-Demand Technology (I.DOT) Coupled with an Open Port Sampling Interface (OPSI)

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Rationale: The sampling throughput of Immediate Drop-On-Demand Technology (I.DOT) coupled with an Open Port Sampling Interface (OPSI) is limited by software communication. To enable much-needed high throughput mass spectrometry (MS) analysis capabilities, a novel software was developed that allows for flexible sample selection from a 96-well plate and for maximized analysis throughput using I.DOT/OPSI-MS coupling.

Methods: Wells of a 96-well I.DOT plate were filled with propranolol solution and were used to test maximum sampling throughput strategies to minimize analysis time. Demonstration of chemical reaction monitoring was done using acid-catalyzed ring closure of 2,3-diaminonaphthalane (DAN) with nitrite to form 2,3-naphthotriazole (NAT). Analytes were detected in positive electrospray ionization mode using multiple reaction monitoring (MRM).

Results: A maximum throughput of 1.54 s/sample (7.41 min/96-well plate with three technical replicates) was achieved and it was limited by the peak width of the MS signal resulting in an occasional slight overlap between the peaks. Relative standard deviation was 10 ± 1 % with all tested sampling strategies. Chemical reaction monitoring of DAN to NAT using nitrite was successfully accomplished with 2 s/sample throughout showing almost complete transformation in 10 minutes with no signal overlap.

Conclusions: This work illustrates the development of a noncontact, automated I.DOT/OPSI-MS system with improved throughput achieved through an optimized software interface. Its achievable analysis time and precision makes it a viable approach for drug discovery and *in situ* reaction monitoring studies.

Keywords: high throughput sampling, open port sampling interface, Immediate Drop-On-Demand Technology

INTRODUCTION

The open port sampling interface (OPSI) is a robust and self-cleaning solid and liquid sample introduction system for subsequent ionization and mass spectrometry (MS) analysis.¹ The OPSI operates by dissolving or mixing solid and liquid analytes, respectively, into a continuous solvent stream which is subsequently electrosprayed and detected by mass spectrometry. This versatile interface has been shown to enable quick, manual analysis of unprocessed solid or liquid samples like plastics, ink pens, skin and vegetable oils.^{1,2} Later, automated sample introduction into the OPSI was demonstrated by using an autosampler,^{3,4} and droplet-on-demand devices using pressure-pulse,^{4,5} piezoelectric^{2,6} and acoustic⁷⁻¹⁶ dispensation processes. Furthermore, OPSI was also modified for automated solid phase microextraction (SPME) analysis.¹⁷⁻¹⁹

Analyzing samples from a 96-well plate was accomplished with sample-to-sample throughput of ~27-45 s and ~5-8 s using an autosampler^{3,4} and an Immediate Drop-On-Demand Technology (I.DOT) system,^{4,5} respectively. Highest sampling rate of 3 Hz and 10 Hz from the same well was demonstrated with a commercial device^{8,9} and in a proof-of-concept highly optimized laboratory settings,⁹ respectively, using acoustic droplet ejection. Of these couplings, the I.DOT/OPSI-MS system offers a compromise between ease-of-setup, flexibility, complexity, and throughput for well plate analysis. Recently we have demonstrated its use for high-throughput characterization of enzyme activity in which the original I.DOT hardware did not require any modification, only to use an OPSI mounted onto a 3D printed part that replaced the target plate in the device.⁴ In those experiments a software developed in-house enabled manipulation of the mouse pointer and the mouse click events to control the software provided by the vendor without any

user intervention. While the I.DOT/OPSI-MS system was fully automated, it was not an optimal solution as the in-house software had to wait on the vendor software for visual feedback on the status of distinct stages of the droplet generation process that ultimately limited throughput to 8 s/sample.⁴ In addition, the vendor software has an optimization algorithm built-in to deposit droplets from the source wells to target wells, but unfortunately at this moment the well order is not customizable if e.g., multiple distinct well groups are to be sampled consecutively.

Herein, we present a novel software interface to control the I.DOT for efficient OPSI-MS analysis. Sample analysis throughput and signal reproducibility are established via high-throughput sampling of propranolol from 96-well plates. Effect of sampling tactics on analysis time are evaluated using different sampling order of the wells to make the most efficient use of the I.DOT hardware. The ability of the system to monitor and to optimize chemical reactions is demonstrated by monitoring of the reactant and product of the acid-catalyzed ring closure of 2,3-diaminonaphthalene (DAN) with nitrite to form 2,3-naphthotriazole (NAT).

EXPERIMENTAL SECTION

Chemicals and Reagents. Solvents of acetonitrile (ACN) with 0.1% formic acid (FA) and water with 0.1% FA were obtained from Sigma-Aldrich (St. Louis, MO, USA). Propranolol, NaOH, cc. HCl, NaNO₂, DAN and NAT were also obtained from Sigma-Aldrich.

I.DOT/OPSI-MS System. Figure S1 illustrates the overall I.DOT/OPSI-MS combination as based on previous designs.⁵ Briefly, a 96-well plate I.DOT system (Dispendix GmbH, Stuttgart, Germany) was fitted with a 3D-printed OPSI mount in the target plate position (Figure S1A-1B). The OPSI was constructed using a 3-port PEEK tee with a 1.27 mm o.d. through hole (IDEX Health and Sciences, Lake Forest, IL, USA). A 0.889 mm (i.d.) × 1.067 mm (o.d.) stainless steel capillary (McMaster Carr, Elmhurst, IL, USA) was inserted in the middle port, with length to make the total OPSI height to 18.5 mm and aligned to face up toward the source plate of the system. A 50-cm-long 0.254 mm (i.d.) × 0.793 mm (o.d.) PEEK capillary (IDEX) was pulled through the middle port and through one of the side ports of the manifold. This capillary was set ~0.5 mm below the top of the stainless-steel outer capillary and connected on the other end to a modified ESI ion source (original ESI capillary was replaced with a 98-mm-long 0.203 mm (i.d.) × 0.406 mm (o.d.) stainless steel capillary) of a model 7500 triple quadrupole (SCIEX, Concord, Ontario, Canada) mass spectrometer (Figure S1C). The OPSI was operated in vortex mode using a 150 µL/min flow rate of 75/25/0.1 (v/v/v) ACN/water/FA that enabled fast transport characteristics in the I.DOT/OPSI-MS system. 40 µL of analytes were placed into the wells of the 96-well I.DOT plate. Propranolol, DAN and NAT were monitored in positive-ion electrospray mode using multiple reaction monitoring (MRM) of m/z 260.1 → 116.1, 159.1 → 115.1, and 170.1 → 115.1 using collision energies of 26, 35, and 35 eV, and dwell times of 100, 20, and 20 ms, respectively. Structure, m/z , and origin of major product ion for propranolol, DAN and NAT are shown in Scheme S1. Ionization spray voltage (IS) was 5500 V, inlet temperature was 300 °C, and GAS1 and GAS2 were 80 and 40, respectively, in all cases.

I.DOT-MS Coupler Software. Screenshots of the in-house developed software (ORNL I.DOT-MS Coupler v2.50) are shown in Figures S2-S5. The software was developed using Visual C# 2022 programming environment (Microsoft Corp., Redmond, WA, USA) and I.DOT SiLA application programming interface (API) (Dispendix GmbH). Two features are highlighted here, the first being the ability to specify samples and replicates for analysis with ease and flexibility. To achieve this, the software accepts start and end of sample ranges in row/column format (e.g. A1-B8), in well number format (e.g., 61-62, where wells 61 and 62 correspond to F1 and F2, respectively) and rectangular areas along with the direction of scanning can also be defined (e.g., RVF4-H7 represents samples in a rectangular ('R') area with left upper corner of F4 and bottom right corner of H7 where wells scanned along the columns vertically ('V')). In addition, these samples can be measured multiple times using different approaches (by sample, by group or all sequentially) and their order can also be reversed, see Supporting Information for details. The second important feature is the possibility to set a minimum wait time between sample droplets that enables baseline separation of the mass spectrometric peaks. Depending on the OPSI configuration, the typical width of a peak is 1.2-3 s^{4-6,20} whereas the I.DOT is capable of dispensing samples <1 s/sample from the same well.

RESULTS AND DISCUSSION

Achieving Maximum Throughput Using the I.DOT/OPSI-MS System. The I.DOT non-contact dispenser applies precisely timed pressure pulses to dispense a given sample volume from 96-well or 384-well source plates with microfluidic pores on the bottom of each well, with a 96-well system used herein.²¹ If droplets from a specific well

is required, e.g., B5, an 8-channel sealing mechanism is moved above the column containing the given well ('5' in this case) and the top of all 8 wells is sealed down with the sealing mechanism. Simultaneously, the OPSI is moved under the given well (B5 in this case). Once both the source plate and the OPSI are in place, a valve corresponding to the row ('B' in this case) is opened and closed at a frequency of 100 Hz (multiple times if needed) to deliver the given volume of sample into the OPSI. Due to this mechanism, the minimum time between dispensing liquids from two wells depends on the locations of these wells. The minimum sample-to-sample time when dispensing droplets from the same well, when dispensing from wells of the same column but different rows, and when dispensing from wells of the same row but different columns was 0.86 ± 0.07 s, $1.070 + (0.073 \times \Delta\text{row})$ s and $2.190 + (0.052 \times \Delta\text{column})$ s, respectively, where Δrow and Δcolumn are the number of rows or columns, respectively, that need to be moved (e.g., $\Delta\text{row} = 6$ when moving from row 5 to row 11). Note, that with the current implementation, moving the source plate and the OPSI into position cannot be separated from sample dispense, it is one integrated function in the API provided. Based on this data, the maximum sample-to-sample time moving between rows of the same column is $1.070 + (0.073 \times 11 \text{ rows}) = 1.873$ s. Changing between columns of the same row takes at least $2.190 + (0.052 \times 1 \text{ column}) = 2.242$ s.

These data suggested that for a given set of samples a strategy that requires the least column changes would result in maximum throughput. This was tested by measuring the total time needed by the I.DOT system to dispense 25 nL of 500 nM propranolol in triplicate from all 96 wells of a sample plate via three different strategies we term "well-by-well" (WbW), "row-by-row" (RbR), and "column-by-column" (CbC)

(Figures 1-3 and Table S1). The WbW strategy (Figure 3, black bars) begun at A1, moved right to A12, and repeated this pattern for subsequent rows B-H. Technical replicates were acquired by repeating this process a total of three times (Figure 1A). During the experiment column changes, the most time-consuming steps, were required 287 times between samples leading to a 10.8 min total analysis time that corresponds to ~2.25 s per sample (see Table S2 in the Supporting Information) and no overlap in the MS signal was observed (Figure 2A). The RbR strategy (Figure 3, grey bars) followed the well order of the WbW approach, but triplicate samples were collected for each well before moving to the next well (Figure 1B) resulting in a total analysis time of 6.43 min or ~1.34 s per sample. This represents a ~40% time saving from the WbW method due to cutting the number of column changes to about a third (95 vs 287). However, overlap in the MS signal was always observed when the same well was sampled consecutively (Figure 2D). Finally, the CbC strategy (Figure 3, white bars) began at A1, moved sequentially down to H1, and repeated this pattern for columns 2-12. Triplicate samples were collected for each well before moving to the next well (Figure 1C). This approach resulted in 4.85 min total analysis time or ~1.01 s per sample, representing a ~25% time saving compared to the RbR method due to further minimizing the number of column changes (11) while the rest of the 84 movements between samples required row changes which are less time-consuming. While this was the fastest method, overlap in the MS signal was always observed unless a column change occurred between samples (Figure 2G).

Based on data presented above, a minimum delay between samples (1.5 and 2 s, Figures 2 and 3) was implemented to avoid overlap between MS signals. MS signal was baseline resolved for times ≥ 2 s. Using WbW sampling (Figures 2B-C) the total analysis

time did not change as I.DOT movement well-to-well was >2 s. In contrast, the RbR approach resulted in total analysis times of 8.37 and 9.92 min for 1.5 and 2 s delays, respectively (Figures 2E-F), and 7.41 and 9.58 min for the CbC method using 1.5 and 2 s delays, respectively (Figures 2H-I). All peaks were base-line resolved at 2 s minimum sample-to-sample time, but some peaks were not separated when using 1.5 s. Peak analysis and calculation of areas corresponding to individual propranolol samples were accomplished using in-house developed software. Briefly, each droplet acquisition was integrated across the baseline-resolved start and end times of the signal transient using a peak finding algorithm. Start and end times were evaluated to ensure no overlap with adjacent droplet acquisition signals. In the case of overlapping signal transients (e.g., Figure 2D) the end time was adjusted to capture as much of the peak as possible until reaching the start time of the adjacent peak. In all experiments the relative standard deviation of peak areas was 10 ± 1 % indicating appropriate analytical reproducibility.

From these data, three important conclusions can be made. First, due to the internal mechanics of the specific droplet generator used here the order of the wells can have a substantial effect on the total analysis time. Second, setting a minimum sample-to-sample delay time is crucial for MS analysis to prevent signal overlap from well-to-well. In the current setup, 2 s always enabled baseline separation. And third, these experiments suggested that improving on MS signal separation by further optimizing the OPSI (e.g., greater flow rate and reduced travel distance as that was shown with acoustic droplet generation^{7-16,19}) would allow the I.DOT/OPSI approach to achieve ~1 s/sample throughput.

Reaction Monitoring and Reaction Parameter Optimization Using

I.DOT/OPSI-MS. The evaluation of reaction kinetics can be critical in the understanding of chemical processes and optimizing reaction conditions. The OPSI interface with acoustic dispensation has been used successfully to demonstrate both of these features in several cases.^{11,12,16} The current I.DOT/OPSI-MS system was also assessed in a similar scenario. A well-known quantification reaction of nitrite is shown in Scheme 1. This reaction is based on the use of 2,3-diaminonaphthalane (DAN) as derivatization reagent for nitrite (NO_2) in acidic medium to form fluorescent product 2,3-naphthotriazole (NAT).²² To start the reaction, 4 μL of 50 μM DAN in 0.6 M HCl solution was added to 40 μL of 1 μM of aqueous NaNO_2 solution (10/1 (c/c) DAN/ NaNO_2) in an I.DOT 96-well source plate and mixed. The mass spectrometer acquisition was started simultaneously ($t = 0$ min). The source plate was then moved into the I.DOT system and the software started resulting in the first samples being dispensed at ~ 0.4 min. The reaction was continuously monitored by ejecting droplets with a 2 s minimum delay time. Figure 4A-C show the calculated peak areas of NAT and DAN signals for the whole time period and observed MS signal traces of NAT and DAN at the start and end of the experiment as examples, respectively. The NAT curve plateaued at about 10-12 min indicating complete reaction.

After demonstrating that the I.DOT/OPSI-MS system can successfully follow the DAN/NAT reaction at 2 s/sample throughput, the reaction was monitored in four separate wells each having 10 μM NaNO_2 but different concentration of DAN (1 μM , 10 μM , 100 μM and 1 mM, Figure 5). Each well was sampled consecutively with a 5 s minimum delay time (20 s cycle time for the four wells). Using 1 mM DAN (empty circles, 100/1 (c/c) DAN/ NaNO_2) the NAT signal quickly plateaued in about 5 min in Figure 5A. Lowering

DAN to 100 μM (filled squares, 10/1 (c/c) DAN/ NaNO_2) nitrite is also completely consumed even if plateauing of NAT took about 10 min. Using 10 μM DAN (empty squares, 1/1 (c/c) DAN/ NaNO_2) the NAT signal plateaued but at a lower level indicating incomplete consumption of NO_2^- . As expected, incomplete production of NAT was even more obvious by using 1 μM DAN (filled circles, 1/10 (c/c) DAN/ NaNO_2) where only 10% of nitrite was expected to react with the available DAN. These experiments demonstrated that in the current case and out of the four DAN solutions a minimum of 100 μM DAN must be needed for complete consumption of nitrite. More importantly, this experiment demonstrated that the I.DOT/OPSI-MS system can be used for monitoring multiple reactions with appropriate sensitivity, relative quantitation, and throughput.

CONCLUSIONS

Here we have shown a novel and easy-to-use software interface for flexible sample selection and for maximized throughput employing an I.DOT/OPSI-MS system for high throughput chemical analysis. The system enabled 2 s/sample throughput from a 96-well plate with baseline separation in measured signals by MS. Analysis of a 96-well plate in triplicate took about 9.5 min while providing a relative standard deviation of peak areas of 10 ± 1 %. It was also shown that the system is capable of ~ 1 s/sample throughput, which is now limited by the width of signal transients of the OPSI-MS in the current setup. However, 3 Hz and 10 Hz sampling rates were already demonstrated using optimized OPSIs with high solvent flow rates coupled to acoustic sampling.^{8,9} The same OPSI configuration would allow 1 Hz sampling without overlap of the MS signal for droplets generated by the I.DOT system.

Reaction monitoring (2 s resolution) of acid-catalyzed ring closure of DAN with nitrite to form NAT was demonstrated by recording signals for the reactant and the product using the I.DOT/OPSI-MS system. Reaction condition optimization was demonstrated by mixing 10 μM NaNO_2 with 1 μM , 10 μM , 100 μM , and 1 mM DAN in four separate wells and sampling the wells consecutively every 5 s (20 s cycle time). Data showed that maximum product quantity was reached after about 10 min using a 10/1 (c/c) DAN/ NaNO_2 mixture indicating that nitrite is completely consumed and, thus, could be quantified. It also indicated that the reaction can be terminated at 10 min if reactions are executed offline.

Future work will focus on improving on the OPSI-MS coupling performance to achieve baseline-resolved MS signal at the ~ 1 s/sample maximum throughput provided by the I.DOT. Theoretically, the OPSI could also be 3D-printed similarly to Sosnowski et al.²³ for easier integration with the target plate holder and thus with the I.DOT. While its current speed and precision is satisfactory, further reducing the achievable analysis time would make it an even more viable approach for drug discovery and *in situ* reaction monitoring studies. In addition, a version of the I.DOT system supporting higher density 384-well source plates is commercially available and the presented software/hardware solution should be applicable for that system as well with very little modification.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at (link)

Operation of the in-house developed I.DOT Assistant software

Examples to Specify Samples for Analysis

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AUTHOR CONTRIBUTIONS

Vilmos Kertesz: Conceptualization, methodology, data curation, formal analysis, visualization, software, writing – original draft, investigation.

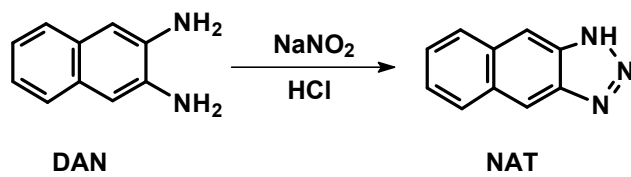
Dana L. Carper: Investigation, writing – review & editing.

John F. Cahill: Conceptualization, methodology, experimental design, formal analysis, writing – original draft, investigation, funding acquisition, project administration.

CONFLICTS OF INTEREST

The authors declare no competing financial interest.

FIGURES/SCHEMES



Scheme 1. Acid-catalyzed ring closure of 2,3-diaminonaphthalene (DAN) with nitrite to form 2,3-naphthotriazole (NAT).

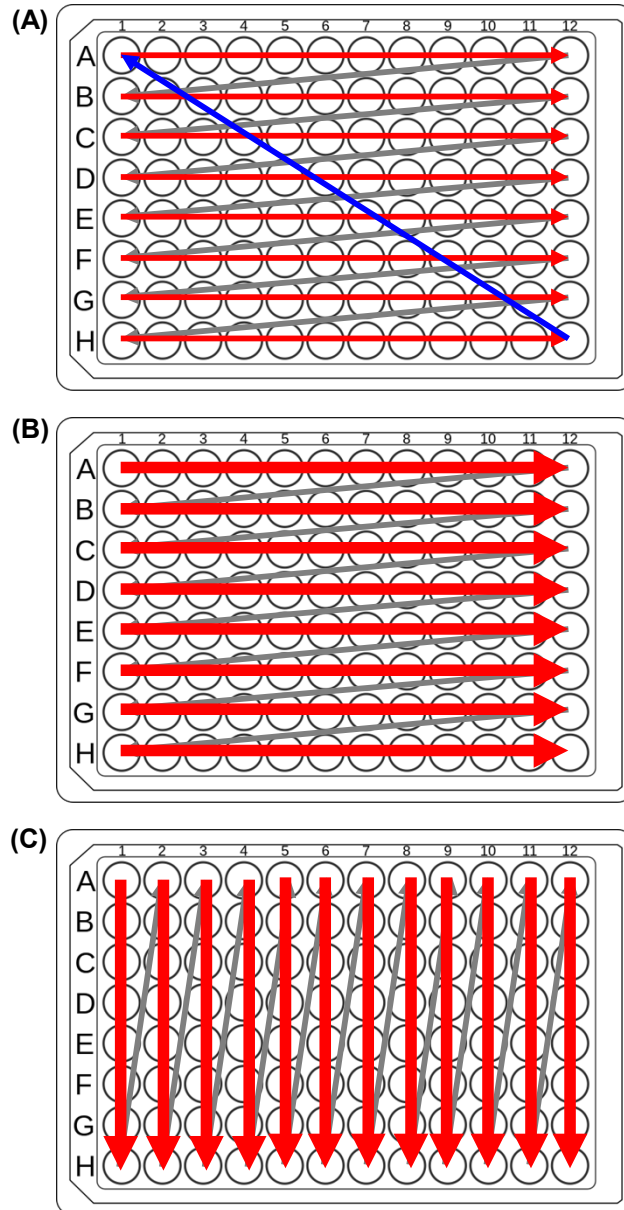


Figure 1. Sampling tactics for 96-well plate analysis. (A) WbW, (B) RbR- and (C) CbC sampling. Red and gray arrows indicate sampling from the wells along the arrow or only moving from one well to another, respectively. Thin and thick red arrows represent single or triplicate analysis from the given well, respectively. Using WbW sampling the whole plate was analyzed three times.

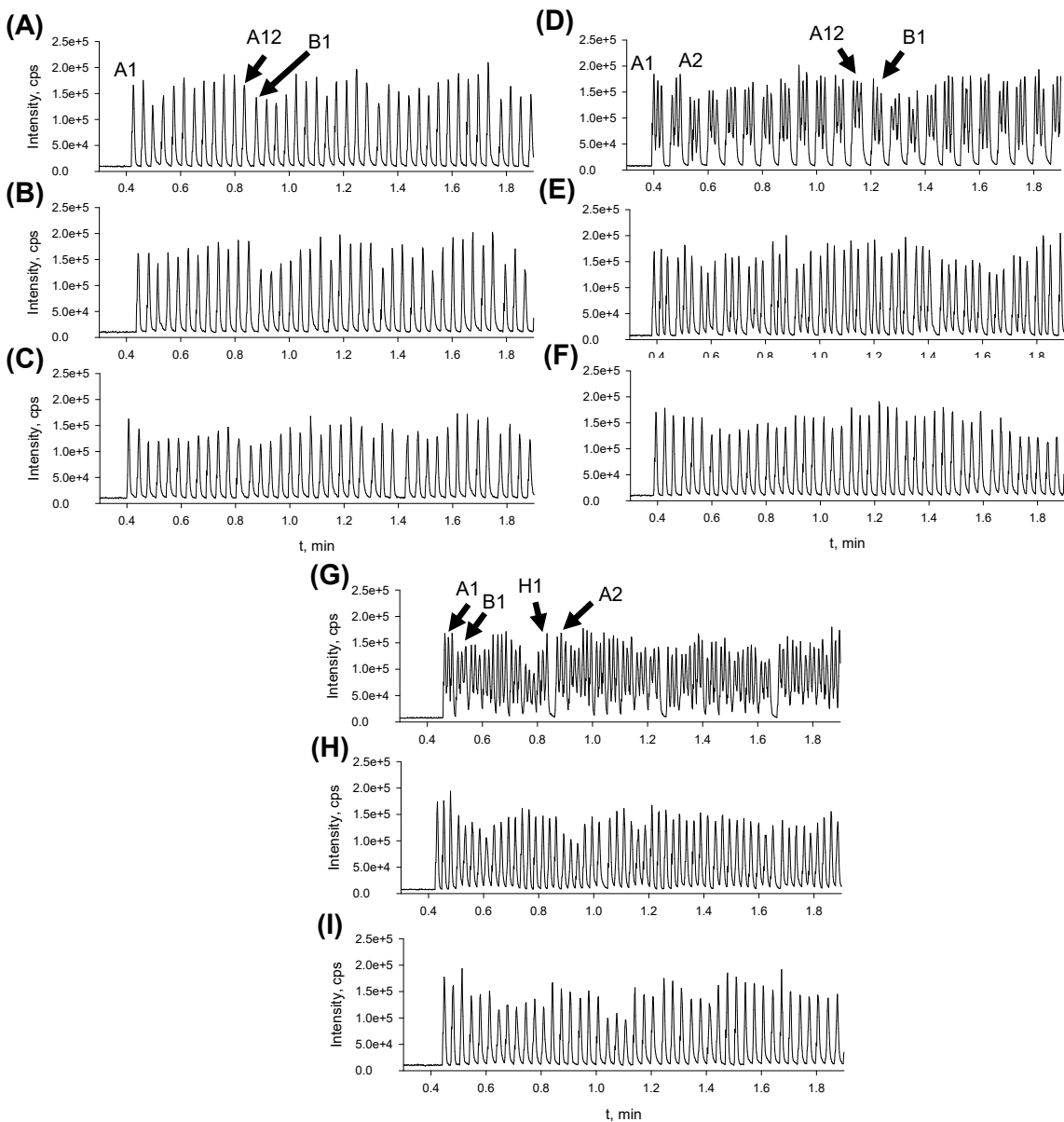


Figure 2: MS signals of ejecting 25 nL of 500 nM propranolol when using WbW with (A) no minimum time between sampling, and minimum times of (B) 1.5 s and (C) 2 s; using RbR with (D) no minimum time between sampling, and minimum times of (E) 1.5 s and (F) 2 s; and using CbC with (G) no minimum time between sampling, and minimum times of (H) 1.5 s and (I) 2 s. Some wells are indicated by arrows to highlight sampling order and strategy.

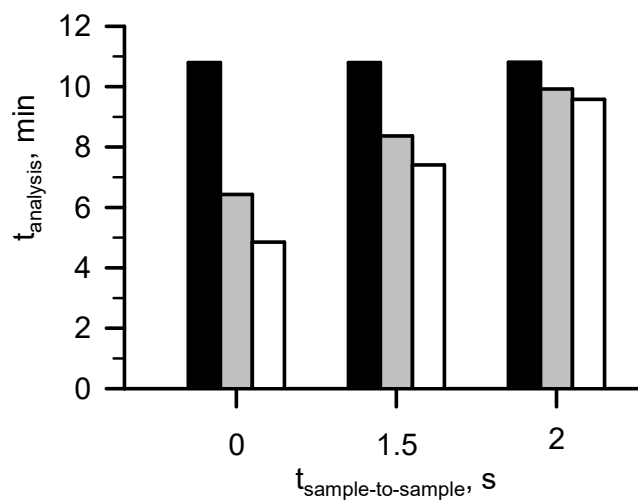


Figure 3. Total analysis time of a 96-well plate analyzed in three technical replicates using (black bars) well-by-well, (gray bars) row-by-row and (white bars) column-by-column sampling. Minimum sample-to-sample time was unrestricted (“0”) and restricted to 1.5 s or 2 s.

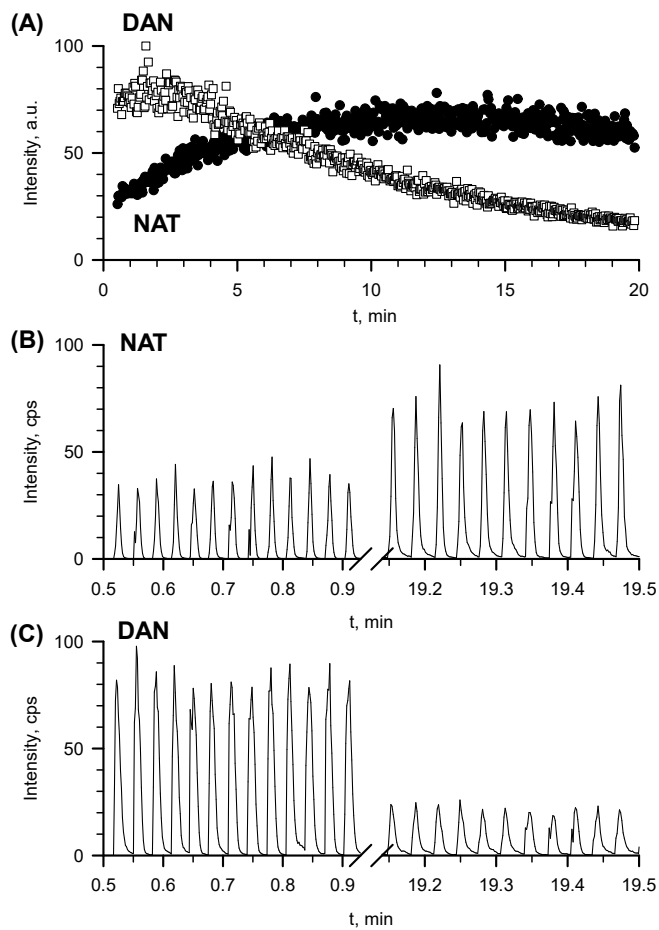


Figure 4. (A) Calculated peak areas of (filled circle) NAT and (empty squares) DAN signals using a 10/1 (c/c) DAN/NaNO₂ mixture in a well of 96-well source plate and analyzed at a 2 s/sample throughput. Observed MS signal traces of (B) NAT and (C) DAN at the start and end of the analysis.

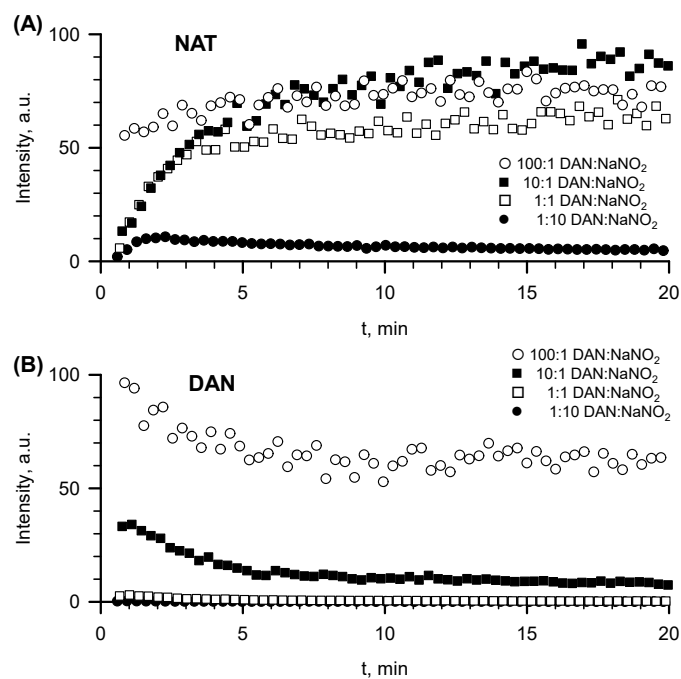


Figure 5. Calculated peak areas of (A) NAT and (B) DAN signals using DAN:NaNO₂ mixtures of (empty circle) 100:1, (filled square) 10:1, (empty square) 1:1, and (filled circle) 1:10 concentration ratios and analyzed at a 5 s/sample throughput.

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