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DROP-LCMS for wastewater surveillance of viral disease

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Wastewater surveillance programs enable large-scale analysis of viral pathogens, uncovering details such as mutation types and quantitative trends in infection prevalence. To enable sensitive and precise analysis of viral variants from wastewater in a streamlined and automated manner, we developed a multifunctional digital microfluidics-polymerase chain reaction-high performance liquid chromatography-mass spectrometry (DROP-LCMS) platform. This platform integrates a digital microfluidic system for droplet manipulation, automated pipetting, magnetic bead-based purification, and thermal control, with analysis by liquid chromatography-mass spectrometry. PCR and sample cleanup were effectively performed on the automated platform, and product droplets were directly sampled from the DMF device for LC-MS detection via a custom manifold. Using this system, we successfully detected the proportions of viral variants in wastewater, enabling effective tracking and monitoring of virus mutations and transmission, particularly during the early stages of the Omicron variant outbreak of SARS-CoV-2.

Infectious disease outbreaks are among the most substantial health challenges facing human society.¹ The defining outbreak of our time, COVID-19, caused by the SARS-CoV-2 virus, presented particular challenges due to its rapid mutation and transmission.^{2,3} Specifically, the rapid evolution of COVID-19 into ever-increasing numbers of variants of concern (VOCs) impeded the timely identification and

containment of the virus.⁴ As new variants of SARS-CoV-2 continue to emerge, and in preparation for the next pandemic, there is an urgent need for large-scale and efficient monitoring methods that enable comprehensive assessment of the region's infection status with minimal testing.⁵ Massively parallel individual-subject testing is logistically and economically impractical for entire populations. Moreover, the individual-subject tests that are most scalable for massively parallel testing, rapid antigen tests, are typically incapable of differentiating between VOCs.⁶ Consequently, wastewater-based epidemiology (WBE) has emerged as a vital, cost-effective strategy to monitor population-level disease dynamics with minimal testing burden.⁷

Next generation sequencing (NGS) and quantitative polymerase chain reaction (qPCR) are used for WBE, and have been demonstrated to provide insight into disease transmission and genomic variations.⁸⁻¹⁰ NGS provides unparalleled, comprehensive insights into whole-genome variations and *de novo* variant discovery, while qPCR offers rapid, cost-effective, and highly sensitive targeted detection with excellent quantitative accuracy for predefined sequences.⁶ However, translating these powerful tools into high-frequency, routine surveillance invokes some challenges. For instance, the comprehensive nature of NGS can require more extensive library preparation, specialized bioinformatics support, and relatively longer turnaround times in routine settings, which may increase operational demands.⁹ On the other hand, while qPCR is highly efficient and affordable for targeted detection, keeping pace with a rapidly mutating virus requires a recurring development cycle to design and rigorously validate new specific primers and probes as novel mutations emerge.¹⁰

To bridge these critical gaps in routine epidemic surveillance, we recently developed a high performance liquid chromatography-mass spectrometry (HPLC-MS)-based method to detect SARS-CoV-2 in wastewater samples.^{6,11} The method features a custom nested polymerase chain reaction (nPCR) method that features the capability to identify

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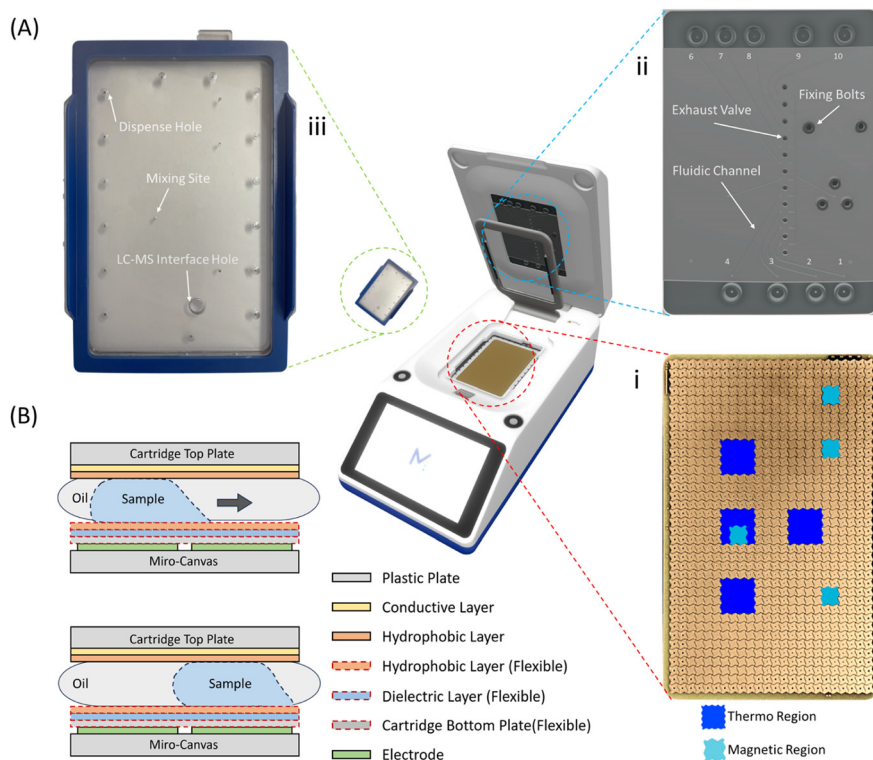


Fig. 1 Configuration of the DROP-LCMS platform for PCR amplification and DNA purification on DMF for HPLC-MS analysis. (A) Photographs of the Miro Canvas device and its DMF sample cartridge (some parts adapted with permission from <https://www.integra-biosciences.com/canada/en/ngs-automation/miro-canvas>). Key compartments are circled, each with an inset photo. i) Red inset: device electrode array overlaid with thermocycling regions (dark blue squares) and magnetic regions (light blue squares), used for PCR and purification. ii) Blue inset: syringe pump panel, which includes a pump, fluid channels, exhaust valves, and 10 ports for sample/reagent input/output. iii) Green inset: top view of the interchangeable DMF cartridge, featuring a sample dispensing port, a mixing region, and a customized interface port for HPLC-MS. (B) Side-view schematic of the DMF cartridge demonstrating the arrangement of each layer in the device. The movement of the liquid (from top panel to bottom panel) is controlled by applying electric potentials between the top and bottom plates.

single-base mutations at signature mutation sites commonly found in many strains of SARS-CoV-2 by analyzing the intrinsic mass-to-charge (m/z) of the amplified nucleic acid fragments. Critically, this method is a ratiometric approach, in which relative signals from VOCs are identified relative to total viral signatures, which makes the method robust and resistant to matrix effects, and thus appropriate for high-volume screening. In contrast to standard optical qPCR methods, the use of mass spectrometry allows for identification of many VOCs in parallel,¹² without requiring a separate probe to identify each new variant. Furthermore, compared to NGS, the HPLC-MS workflow bypasses complex library construction and sequencing steps, accelerating the sample processing pipeline and reducing the time-to-result to a matter of hours. There is growing consensus that this type of method will be a crucial tool for pandemic responses in the future, giving policy-makers the information needed to reduce and control the spread of the disease.^{13,14}

The key challenge for HPLC-MS-based wastewater analysis techniques is the requirement of complex sample preparation and processing,¹⁵ which necessitates highly trained and experienced frontline staff. Furthermore, these staff members are exposed to unknown risks while handling samples

containing new pathogens.¹⁶ There is thus high demand for technologies that can automate wastewater analysis procedures and produce accurate surveillance results, while minimizing operator exposure.

Microfluidics¹⁷⁻¹⁹ seems a perfect fit to address these limitations, to integrate with HPLC-MS-based wastewater analysis of infectious disease. While there are numerous reports of microfluidics for wastewater analysis (typically relying on amplification techniques combined with fluorimetric²⁰⁻²³ or colorimetric²⁴ detection), we are not aware of any previous microfluidic technique developed for this application combined with HPLC-MS-based analysis. Here, we report a new method relying on digital microfluidics (DMF), a technique that manipulates samples and reagents in pico- to microliter-sized droplets across an electrode array using electrodynamic forces.²⁵ This technology enables automated sample processing and reaction protocols,²⁶ which can minimize inaccuracies and contamination and enhance the efficiency of sample analysis.

The platform introduced here, DROP-LCMS, is an automated pipeline for pathogen gene amplification and purification that is coupled with analysis by LC-MS. This platform utilizes the Miro Canvas (Integra Biosciences), a



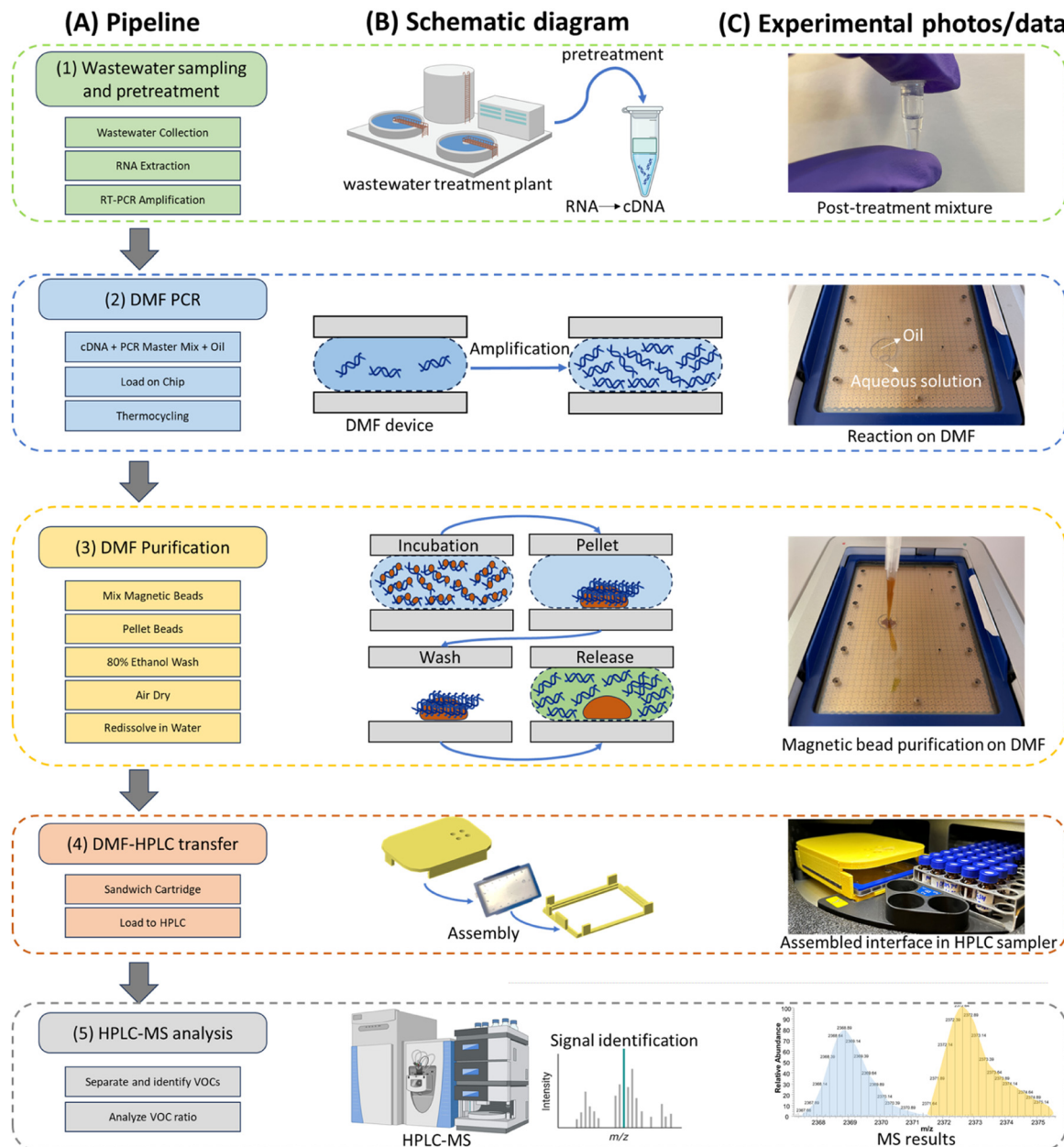


Fig. 2 DROP-LCMS pipeline. The five steps (wastewater sampling and pretreatment, DMF PCR, DMF purification, DMF-HPLC transfer, HPLC-MS analysis) are represented in (A) text, (B) schematic, and (C) photograph or experimental data. Schematics in (B) were created using BioRender (<https://www.biorender.com/>).

commercial DMF instrument that orchestrates droplet manipulation, thermal control, magnetic separation, and pump-driven liquid handling.²⁷ The Miro Canvas was designed to automate library preparation for NGS; here, we modified and repurposed the instrument to be able to automate a new workflow that integrates DMF sample preparation with HPLC-MS detection to screen wastewater for VOCs of SARS-CoV-2.

Briefly, the Miro Canvas consists of 1) an electrode control base with a touch-screen and an electrode array platform, 2) a metal fixing ring securing a DMF cartridge, and 3) a top lid equipped with a syringe pump panel (Fig. 1A). In practice,

the DMF driving electrodes are a permanent fixture in the instrument, while the cartridge is replaced between experiments (Fig. 1B). When assembled, droplets suspended in an oil shell are sandwiched between two plates in the cartridge for manipulation.

To evaluate the feasibility of applying digital microfluidics (DMF) to the automation of wastewater surveillance, a five-step detection pipeline was developed (Fig. 2): 1) manual wastewater sampling and pretreatment, 2) DMF-PCR, 3) DMF purification, 4) DMF-HPLC transfer, and 5) HPLC-MS analysis. A set of custom tubing assemblies (Fig. S1) and a custom droplet manipulation protocol comprising fifteen



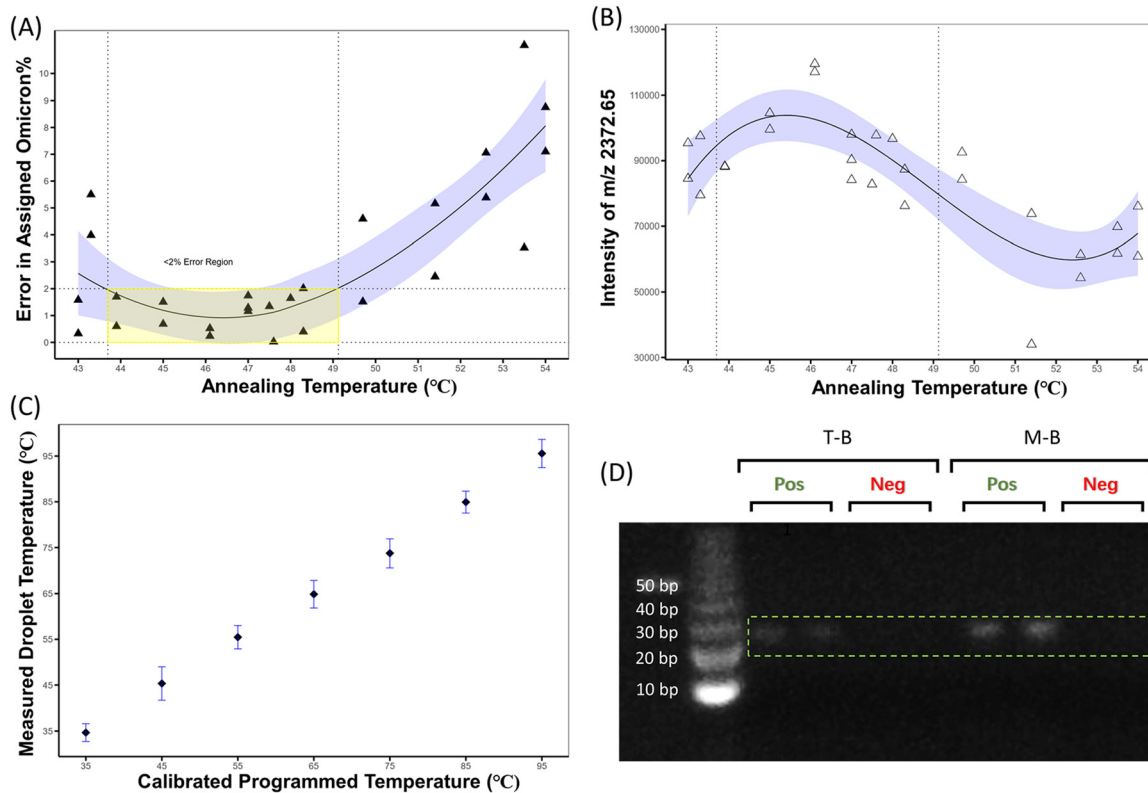


Fig. 3 Optimization of wastewater analysis pipeline on DMF. (A) Plot of error in assigned Omicron % (triangles) in a wastewater sample containing 55.75% Omicron as a function of annealing temperature. The yellow region indicates an error ranging from 0% to 2%, and the black trace represents the LOESS-fitted curve. (B) Intensity of Omicron variant peak in a wastewater sample containing 55.75% Omicron as a function of annealing temperature. The region between the dashed lines indicates Omicron variant assignment error of less than 2%, and the black trace represents the cubic spline fit. Blue shaded areas in (A) and (B) indicate standard error. (C) Plot of measured droplet temperature as a function of calibrated programmed temperature in the Miro-Canvas. Error bars represent the 95% CI from 3 replicates per temperature. (D) Gel electrophoresis results of the formation of the 31-bp amplicon (green dashed box) on-DMF and in tubes, using calibrated, programmed annealing temperature of 46 °C. Experiments for samples mixed with (Pos) and without (Neg) the DNA template showed a greater difference in band intensity when performed in a Miro-Canvas system with automated magnetic-bead-based purification (M-B, right), compared to the same procedure performed manually in tubes (T-B, left). All repeat analyses represented in this figure were technical replicates in which the same sample was processed separately through the relevant portions of the DROP-LCMS pipeline.

sub-steps was developed and optimized to automate the DMF processes in steps 2–3 in Fig. 2, which are illustrated in greater detail in the flowchart in Fig. S2.

In preliminary work, a technique derived from previous reports^{6,11} was developed to quantify the ratio of Omicron variant to total virus using HPLC–MS. Specifically, after processing wastewater samples by nested PCR, masses corresponding to the Omicron variant sequence (P13L in Table S1) can be readily distinguished from those of the wild-type sequence (Wild-type P13 in Table S1) by mass spectrometry (Fig. 2, step 5). By recording the intensities of these peaks, the relative amount of Omicron variant in wastewater can be calculated as $\text{Omicron \%} = \frac{\text{intensity of P13L}}{\text{intensity of P13L} + \text{intensity of Wild-type P13}} \times 100$. The Miro Canvas was not designed for this application; thus, the method was probed to identify sensitivities that must be addressed in the new system. For example, as shown in Fig. 3A, small variations in the annealing temperature during the thermocycling procedure were observed to result in substantial error in the relative quantitation of VOCs of SARS-

CoV-2 in wastewater. The lowest error ($\leq 2\%$) was observed for temperatures between 43.7–49.1 °C, which is the range that results in the highest intensity of MS signal for wastewater analysis (Fig. 3B). With this in mind, and knowing that variations in thermal conductivity can result in different temperatures inside and outside of a microfluidic device,²⁸ a forward looking infrared (FLIR) camera was used to measure the droplet temperature (Fig. S3A) of samples heated using the Miro heating function. The uncalibrated set-temperature on the Miro was found to be $\sim 5\%$ greater than the droplet-temperature (Fig. S3B), but this effect was reproducible, enabling the development of a calibration formula for robust prediction of droplet temperature in the Miro (Fig. 3C). Finally, when the procedure was run with a calibrated droplet temperature of 46 °C, which showed a low error in the variant ratio and high amplification efficiency, the Miro system reproducibly generated product oligonucleotides at levels greater than conventional PCR procedures carried out in tubes (Fig. 3D), giving us confidence that the DMF method was well suited for this application.



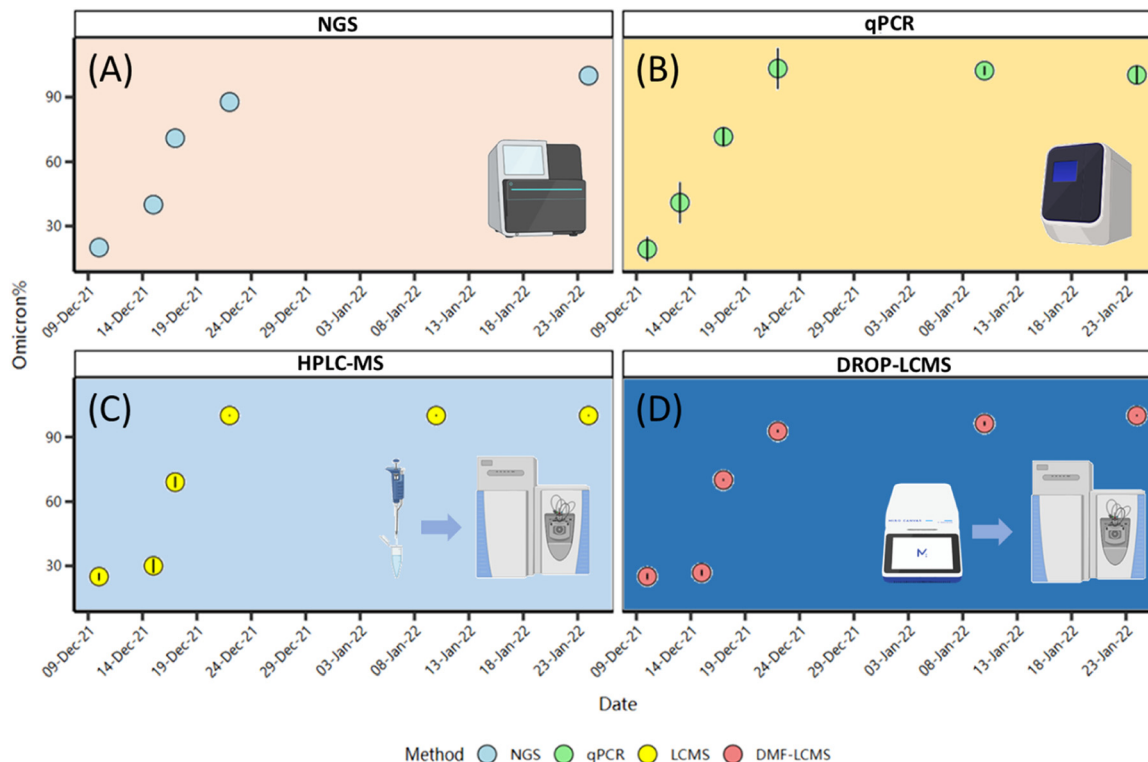


Fig. 4 Monitoring SARS-CoV-2 Omicron in wastewater. Omicron percent as a function of time measured previously⁶ by (A) NGS, (B) qPCR, and (C) manually processed HPLC-MS, are compared with (D) new data generated using the new DROP-LCMS method featured here. Error bars indicate standard deviation from 3 independent replicates (wastewater samples collected from three distinct sampling sites) for qPCR, HPLC-MS, and DROP-LCMS. Only one replicate was evaluated in the NGS results.

The value proposition for DROP-LCMS for wastewater screening requires automated integration with the HPLC autosampler, to keep the operator out of the loop and safe. A customized manifold (Fig. S4A–F) was designed and fabricated, that has the same purpose as our previous design,²⁶ enabling seamless transfer of samples from the DMF platform directly to HPLC, with the difference being that conventional liquid chromatography is used here instead of the previously used nano-LC (conventional HPLC was more than adequate for the application described here, in which analytes were amplified prior to analysis from samples that were abundant/not precious). The performance of the new method was initially evaluated by passing DNA standards through the manifold followed by analysis by HPLC-MS, which exhibited linear responses as a function of amount (Fig. S4G). These data highlight the advantage of using a ratiometric technique like this one. Recording the ratios of peak intensities (in this case for sense/anti-sense standards) allows for robust, measurements across a wide range of amounts (from 25–900 fmol). Table S2 highlights the accuracy for these measurements, which ranges from 97.0% to 103.3%, with relative standard deviations (RSDs) between 5.01% and 6.85%. The performance of the system was then evaluated for a wastewater sample with known variant ratio. The assigned omicron percentage relative to known value (Fig. S5A), assigned omicron error (Fig. S5B), and precision in assigned omicron percentage (Fig. S5C) were best for the

microfluidic method with the DMF-autosampler manifold, with results of $55.22\% \pm 0.22\%$ (relative to the known value 55.75%), 0.53%, and 0.41%, respectively. While an extensive evaluation of matrix effects was not carried out, we expect them to be minimal, given that the wild-type and variant sequences share nearly very similar chemical properties, such that matrix effects should affect both targets equally.

Finally, the new method was applied to a series of COVID-19 wastewater surveillance samples that were collected from the Oakville Southwest wastewater treatment plant (WWTP) in 2021–2022. Compared to other methods including NGS (Fig. 4A), qPCR (Fig. 4B), and LC-MS with manual sample preparation (Fig. 4C), the DROP-LCMS workflow (Fig. 4D) shows similar trends in the evolution of the Omicron variant over a critical six-week period in late 2021 to early 2022. As shown in Fig. S6, the sense strand of wild type P13 and Omicron variant P13L were verified in all mass spectra evaluated, and the chain length of the amplification product in wastewater was confirmed by gel electrophoresis. Representative mass spectra underpinning these data and method optimization data are shown in Fig. S7–S9.

In consideration of the wastewater data presented in Fig. 4, the only noticeable difference for the two HPLC-MS datasets relative to the others was the relative amount of Omicron % observed in the samples collected on Dec. 15, 2021. It is possible that this represents a general bias for the HPLC-MS techniques for underestimating the ratio at low



levels, or alternatively, this might be an artifact of how the samples on that particular day were collected and stored (that could be resolved with additional sample measurements). If the former proves true, future methods might benefit from an update in which single-base mutated sequences are separated to enable high-resolution detection of low-abundance viral mutants with differentiation in both retention time and molecular weight.²⁹

In sum, we developed a multi-functional DROP-LCMS platform for virus variant detection in wastewater. While wastewater has been analyzed by microfluidics-enabled method for this purpose previously using optical detection,^{20–24} this is the first report that we are aware of in which microfluidics was coupled with HPLC–MS, making it particularly suitable for the tracking and monitoring of virus mutations and transmission. The new DROP-LCMS platform represents a key step toward resolving the main challenge for HPLC–MS methods for WBE – the requirement of complex sample preparation and processing. We propose that this type of advance makes HPLC–MS-based WBE competitive with NGS and qPCR in terms of streamlined pipeline, reagent/consumables cost, and sensitivity. But of course, we also acknowledge the limitations of this type of approach, which is new and requires additional study and improvements, requires a larger initial instrument cost compared to standard qPCR, and lacks the capability for *de novo* discovery of entirely unknown variants, as enabled by NGS. We propose that future variations of this platform will enable the exploration of innovative methods beyond traditional detection approaches for viral pathogen analysis, opening new possibilities to combat high-risk infectious diseases.

Author contributions

J. P., H. P., and A. R. W. conceived the concept of automated DMF platform for wastewater sample analysis. J. S., V. R. and J. P. fabricated the interface between DMF and LC–MS. J. S., V. R., J. Sun and J. P. performed the experiments into the effects of sample preparation. J. P. performed the LC–MS experiments. J. P., J. S., V. R., C. C., Y. H., and H. P. carried out the data analysis. V. R. processed the R data analysis to generate the plots. J. P., C. C., J. S., V. R., and A. R. W. wrote and edited the manuscript. All authors discussed the results and commented on the manuscript.

Conflicts of interest

There are no conflicts to declare.

Data availability

The raw data associated with this manuscript is available from the corresponding author upon request.

Supplementary information is available. See DOI: <https://doi.org/10.1039/d6lc00131a>.

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