Acoustic Ejection/Full-Scan Mass Spectrometry Analysis for High-Throughput Compound Quality Control

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Abstract
High-throughput analysis of compound dissolved in DMSO and arrayed in multiwell plates for quality control (QC) purposes has widespread utility in drug discovery, ranging from the QC of assay-ready plates dispatched by compound management, to compound integrity check in the screening collection, to reaction monitoring of chemical syntheses in microtiter plates. Due to the large number of samples (thousands per batch) involved, these workflows can put a significant burden on the liquid chromatography–mass spectrometry (LC-MS) platform typically used. To achieve the required speed of seconds per sample, several chromatography-free MS approaches have previously been used with mixed results. In this study, we demonstrated the feasibility of acoustic ejection–mass spectrometry (AE-MS) in full-scan mode for high-throughput compound QC in miniaturized formats, featuring direct, contactless liquid sampling, minimal sample consumption, and ultrafast analytical speed. The sample consumption and analysis time by AE-MS represent, respectively, a 1000-fold and 30-fold reduction compared with LC-MS. In qualitative QC, AE-MS generated comparable results to conventional LC-MS in identifying the presence and absence of expected compounds. AE-MS also demonstrated its utility in relative quantifications of the same compound in serial dilution plates, or substrate in chemical synthesis. To facilitate the processing of a large amount of data generated by AE-MS, we have developed a data processing platform using commercially available tools. The platform demonstrated fast and straightforward data extraction, reviewing, and reporting, thus eliminating the need for the development of custom data processing tools. The overall AE-MS workflow has effectively eliminated the analytical bottleneck in the high-throughput compound QC work stream.

Keywords
acoustic ejection, mass spectrometry, compound QC, high-throughput, DMSO sample

Introduction
In modern drug discovery, the advancement of automation and low-volume dispensing has propelled the miniaturization of many areas, including compound management and storage,¹,² high-throughput chemistry (HTC),³,⁴ and high-throughput screening (HTS).⁵-⁷ The operation of these processes in 384- or 1536-well or even higher-density plate formats has enhanced assay and process capacity while at the same time reducing the consumption of reagents, compounds, and the associated cost. However, they also create a need for quality control (QC) of the compound plates generated from areas such as reaction monitoring of parallel chemical synthesis, profiling of compound integrity in the screening collection, and quality checking of assay-ready plates dispatched by compound management for various assays. This QC workflow has been typically supported by high-throughput analysis using liquid chromatography–mass spectrometry (LC-MS). However, the huge number of samples involved in these workflows can place a significant burden on the LC-MS platform, making the analytical measurement the bottleneck of the workflow. LC-MS is a serial process, with each sample undergoing injection, chromatographic separation, and mass analysis. The fastest LC-MS process typically takes at least 1–2 min per sample; however, that is still too slow for most high-throughput compound QC evaluations from high-density...
spray ionization. The first attempt to couple acoustic transfer with MS was AMI, which uses a heated transfer tube for gas-phase transportation and desolvation of charged droplets generated by the application of high voltage to the liquid sample, prior to MS analysis. Due to the need for adequate desolvation, picoliter-sized droplets have to be generated (hence the name “mist”). AMI has been shown to exhibit somewhat high variability when analyzing biological samples, owing to the difficulties associated with both reproducible mist generation and ion suppression.

The more recently emerging AE-MS features the utilization of acoustic droplet ejection, a sample dispensing and transferring tool as previously used. In this acoustic technology, the acoustic ejection applied focuses acoustic energy to liquid samples in the source plate, ejecting droplets of nanoliter size from the meniscus of the liquid with rapidity, as well as excellent precision and accuracy (<2% coefficient of variation at 2.5 nL). The acoustic droplet ejection has also demonstrated suitability for high-density, 1536-well microtiter plates, which relies upon the precise location of the targeted wells in the plate. As an inlet to MS analysis, this compatibility with 1536-well plates is not yet easily achievable by AMI, or liquid analysis approaches using a contact liquid handler for injections such as LC-MS. The AE-MS also features the incorporation of an open port interface (OPI) with a continuous carrier flow (e.g., methanol at 250 µL/min) for the liquid-phase transfer of acoustic ejected sample droplets for ESI ionization and detection. Originally developed for direct MS analysis of unprocessed samples as an easy “open-access” operation, the OPI was modified in AE-MS to be coupled with an acoustic dispensing unit to transfer sample droplets generated by acoustics. The continuous carrier flow not only plays a key role in transporting the sample droplets to the MS, but also enables self-cleaning of the open port sampling interface to facilitate transportation of complex and concentrated samples without carryover. In addition, the carrier flow dilutes the samples by 1000x, effectively reducing matrix interference in unprocessed samples or dirty matrices. This online dilution also enables the compatibility of samples in any acoustic-compatible solvent with direct ESI analysis on AE-MS. This is especially beneficial for plates dispatched by compound sample management and parallel chemical synthesis, which are typically made in DMSO and known to otherwise cause ion suppression in ESI. The advantageous features enabled by the OPI and carrier flow in AE-MS make it an appealing tool for high-throughput compound QC from various assays, and in different concentrations and matrices.

In the workflow of high-throughput compound QC in high-density plate formats, especially when ultrafast chromatography-free MS approaches are used, a huge amount of data can be generated, demanding a rapid data analysis, reviewing, and reporting mechanism. Generating an extracted ion chronogram (XIC) from a total ion chronogram (TIC) to identify the presence or absence of the expected compounds of interest in each well of the microtiter plate, and establishing comparisons across wells or plates when needed, has been a manual and time-consuming process. Even though the MS inlet varies in the chromatography-free platforms (acoustic, DESI, or MALDI), the data
output is always mass spectrometric based. The uniformity of the MS data so far has not yielded a universal or commercially available data analysis tool for the miniaturized campaign. Instead, tremendous efforts and resources have been spent on the development and maintenance of custom-built analytical tools, or combinations of commercial software supported by home-built scripts.

In this study, we evaluated the feasibility of using AE-MS for high-throughput compound QC in a miniaturized format for various drug discovery applications, including integrity analysis of HTS screening hits, QC of DMSO serial dilution plates, and reaction monitoring for parallel chemical synthesis. Although LC-MS has been conventionally used in these applications, AE-MS offers an unparalleled, ultrafast analytical speed, as well as negligible (nL) sample consumption, which is especially essential for assay-ready plates to maintain the sample integrity for the subsequent assays. Furthermore, AE-MS enables direct ESI-MS analysis of DMSO plates in these QC applications, which have not been compatible with conventional LC-MS analyses. We compared the AE-MS results to those obtained by the LC-MS platform typically used for QC purposes in the areas of both qualification and relative quantification for the same compounds. The development of a data processing platform using commercially available software tools was also described as an approach to streamlining the overall process of high-throughput compound QC by AE-MS, and eliminating the bottleneck in the QC applications.

**Materials and Methods**

A prototype AE-MS was used for all sample analysis. It consisted of an ATS Gen 4 acoustic dispenser (EDC Biosystems, Fremont, CA) connected to a SCIEX triple quadrupole mass spectrometer 6500+ equipped with an OptiFlow ion source, via an OPI (SCIEX, Concord, ON, Canada). Methanol (MilliporeSigma, Burlington, MA) was used as the OPI carrier solvent and delivered by a Shimadzu 20AD VP pump (Shimadzu, Columbia, MD). The mass spectrometer was operated by Analyst software (version 1.7.2) at a fixed nebulizing gas setting of 90 (in-house nitrogen, arbitrary units). The carrier flow was fine-tuned (typically around 0.25 mL/min) in order to achieve the proper formation of a “stable vortex” at the inlet of OPI, dictated by a balanced flow of carrier solvent and nebulizing gas. The MS was operated under positive electrospray ionization, and full-scan data (m/z 150–800) were acquired in Analyst tune mode, which was started before the onset of acoustic ejection. The MS scan time was set to 0.325 s, resulting in four data points across a typical AE-MS peak. The ATS was controlled by ATS-100 software, and an ejection volume of 5–10 nL was employed, with a pause time of 2000–5000 ms between each sample ejection.

For QC of assay-ready dose–response curve plates and identity confirmation of compound plates dispatched in DMSO (MilliporeSigma) by compound management, samples were subjected directly for AE-MS analysis in Echo Qualified 384-well, flat-bottom plates (Beckman Coulter, Indianapolis, IN). For chemical synthesis in microtiter plates, reactions were carried out in 3 µL of DMSO, yielding a theoretical product concentration of 10 mM. The reaction mixture was diluted by 20× to 500 µM into 20 µL 50:50 acetonitrile/water (J.T.Baker, Phillipsburg, NJ) in 384-well plates before being subjected to AE-MS analysis, to avoid the detector saturation and hence possible mass shift of detected m/z. For the comparison study of AE-MS with LC-MS analysis, DMSO samples from a compound management dispatch were diluted by twofold post-AE-MS analysis, before being subjected to ultra-performance liquid chromatography (UPLC)-UV/MS analysis. The system used was an Acquity UPLC coupled with an SQD mass spectrometer (Waters, Milford, MA). A generic gradient of 2.5 min was employed at a flow rate of 1.0 mL/min, with a mobile phase A of 95% water, 5% acetonitrile with 10 mM ammonium acetate (MilliporeSigma), and a mobile phase B of 5% water, 95% acetonitrile with 10 mM ammonium acetate. The column used was Waters Acquity UPLC BEH C18, with a dimension of 1.7 µm, 2.1 × 30 mm. Full-scan MS data as well as UV absorbance data at 220 nm were acquired. To test the ion suppression in AE-MS analysis, a series of fluoxetine (MilliporeSigma) calibration standards with a concentration range of 1–1000 µM in 50:50 acetonitrile/water was spiked with propranolol (J.T.Baker) at a fixed concentration of 10 µM, before subjecting to AE-MS analysis.

To process the large amount of data generated by AE-MS in the high-throughput compound QC workflow, we developed a semiautomated platform using SCIEX’s Peak Splitting Tool (a research version tool) and PeakView software, MasterView module. In the Peak Splitting Tool, the user first loaded two files generated during data acquisition, the TIC file acquired by MS and the acoustic ejection timing file generated by ATS. The timing or retention time (RT) corresponding to the first ejection needed to be manually identified in TIC, based on the desired m/z in the first well ejected, since the onset of MS data acquisition and acoustic ejection were not concurrent, with the acquisition always started prior to the sample ejection. To be more confirmatory, a marker compound with known m/z could be spiked in the first well to be ejected, and the RT of the well was then identified based on the extracted m/z of the marker compound. Once the timing for the first peak was confirmed, the ejection timing for all other peaks in the TIC was automatically assigned based on the pause time set between ejections. The RT for each well was then exported to a csv file, which was subsequently imported into MasterView along with the molecular formula of the interested compounds in each well.
The MasterView then extracted XIC data for each well and ejection at the specified time defined by the csv file imported from the Peak Splitting Tool (Fig. 1). If desired, the full-scan mass spectrum of the well could also be displayed. A set of user-defined criteria, including XIC intensity threshold, signal-to-noise ratio, found m/z window width, and RT window width, was applied to flag whether an expected compound was “found” or “missing,” along with the corresponding XIC abundance in a tabulated, ready-to-export format.

Results and Discussion

Data Processing Workflow

Although MasterView was originally designed for high-resolution mass spectrometric data processing, we were able to implement this software to process unit-resolution quadrupole data, by lowering the resolution threshold during processing. This semiautomated data processing workflow significantly improved the data review speed compared with manual processing, and enabled a same-day turnaround time for both sample analysis and data review after receiving the samples. The semiautomated data review was accomplished without any investment in the development of custom software. All the tools used in the data analysis are supported by a single MS instrument vendor (SCIEX), which facilitates the technical support and maintenance of the data processing platform. Since the MasterView software was originally developed for high-resolution mass data, it can be easily adapted for occasions where high-resolution MS is used for data acquisition. The limitation of the current data processing platform is that XIC extraction can only be performed for a single-charge proton adduct of compounds of interest. The platform can be further improved by implementing the identification and extraction of multiply charged ions and other adducts of the compounds of interest. Also, it would be advantageous to process both positive and negative ionization modes simultaneously (if data are acquired in positive/negative switched mode) to reduce possible false negatives in the data review. The incorporation of these features would provide a more comprehensive data interpretation and troubleshooting capability, and further minimize the need for manual intervention in data review.

Comparison of AE-MS and LC-MS

High-throughput QC for compounds in high-density plate formats from various screening assays requires an analytical
tool with matching speed and capacity to monitor the presence or absence of the expected compounds. With an acquisition speed of 2.5 s per sample, the AE-MS instrument was successfully used to complete full-scan data acquisition for a 384-well plate in 17 min. Without any chromatographic separation, this fast turnaround of AE-MS was perfectly suitable for compound plate QC workflows that required a simple yes or no binary analysis. In these cases, the comparability of results between AE-MS and conventional LC-MS analysis would be a prerequisite for the utilization of AE-MS for these workflows. As a validation of the AE-MS approach for high-throughput qualitative compound plate QC, the same set of samples from a 384-well integrity check plate was analyzed by both conventional UPLC-MS and AE-MS. The results are summarized in Figure 2, with white indicating an XIC intensity above and gray an XIC intensity below the threshold set manually for the background noise observed across all samples. Peaks with XIC intensity higher than the threshold (cells in white) have expected compound present, and those with an intensity lower than the threshold (cells in gray) have expected compound absent. The results from both approaches showed a good correlation of 92% for all tested samples (percentage of combined number of hits and misses conformed by AE-MS and LC-UV/MS to the total number of wells). The slightly higher number of hits detected by AE-MS may be attributed to the higher concentration of samples used, as well as the better sensitivity associated with the higher-end mass spectrometer used. The good correlation between AE-MS and LC-MS analysis demonstrated the validity of the AE-MS approach for qualitative compound QC.34

In conventional LC-UV/MS analytical platforms for QC applications, UV% of the expected compound is typically used as a representation of compound purity in the test sample. Although UV offers a more universal detection than ESI-MS, which can be affected by the differential ionization across different compounds, it is a less sensitive detector, especially for highly MS ionizable compounds or compounds without UV chromophores. An example of this circumstance is demonstrated in Figure 3, whereby LC-UV/MS analysis detected five peaks (marked by ticks on the x axis) at UV 220 nm for an integrity check sample, while full-scan MS attributed none of them to the expected compound. The direct AE-MS analysis of the DMSO solution, however, successfully detected the presence of the expected compound \(m/z 354\) in addition to the impurities detected by LC-UV, as illustrated in its full-scan mass spectrum. The failure of LC-UV/MS to find the expected compound in this case was attributed to the lower sensitivity of MS, in part resulting from sample dilution in acetonitrile/water from DMSO to accommodate platform requirements, and the lack of a UV chromophore in the expected compound. However, the opposite circumstance could also occur in which compounds detected by UV may lack any MS ionization that would confirm the expected compound identity. In consideration of the potential drawbacks of any individual detection method and the low analytical speed of LC-UV/MS, we propose that it serves as a rational approach to use AE-MS analysis for the rapid survey of compound plate QC (applicable to chemical reaction monitoring as well, as discussed below), to take full advantage of its ultrafast turnaround time, and deploy UV for a purity check on an
as-needed basis. This way, the most essential data are made available to end users at a much higher speed to guide the next-step actions needed in a timely fashion.

**Ion Suppression in AE-MS**

Without any chromatographic separation, nonchromatographic MS approaches are more prone to ion suppression in ESI-MS, especially for samples in heavy matrices. However, the incorporation of OPI with carrier flow in AE-MS sets itself apart from other chromatography-free MS approaches by diluting the samples 1000× in the carrier flow, effectively reducing the ion suppression effect in MS. In most high-throughput qualitative compound QC workflows such as those used in chemical synthesis in high-density microtiter plates, and in identity confirmation from compound management plates, samples are typically present at high concentration and ion suppression usually inflicts no negative impact on the qualitative analysis. However, when relative quantification for the same compound across different samples is to be obtained with AE-MS analysis, ion suppression by components coexisting in the same sample mixture needs to be evaluated to ensure a valid relative quantification. We evaluated this ion suppression effect by making a calibration curve of fluoxetine (m/z 308) ranging from 1 to 1000 µM, spiked with a constant concentration of propranolol (m/z 260) at 10 µM in DMSO. As shown in Figure 4, from a fluoxetine concentration of 100 µM and above, the XIC signal of propranolol dropped due to suppression by fluoxetine, and the higher the fluoxetine concentration, the more severe the suppression on propranolol intensity. Based on these results, an upper limit of 100 µM was instituted for all components in each sample well in all relative quantitative AE-MS experiments to ensure that the ionization suppression effect was minimized. Owing to different assay conditions, this evaluation process of ion suppression should be based on the comprehensive consideration of the relative amount of analyte and coexisting interferences, the possible presence of ion-pairing agents, different classes of compounds, and differential MS ionization.

**QC of Dose–Response Curve Plates**

The high accuracy and precision in the preparation of dose–response curves from the DMSO solution of test compound plates are prerequisites for the accurate determination of IC$_{50}$ or EC$_{50}$ values in screening assays. These plates commonly contain varying concentrations of the test compounds in acoustic-compatible plates, ready to be transferred acoustically into assay plates containing substrates and assay buffers. The rationale for QC of these assay-ready DMSO plates lies in the fact that in typical screening assays, only the probe substrate or reporter molecule is monitored, while the identity or concentration of the test compound itself is not checked. As a result, the IC$_{50}$ or EC$_{50}$ value generated out of an erroneous dose–response curve can easily go unnoticed; such errors may include the wrong test

Figure 3. Single-sample analysis by LC-UV/MS and AE-MS. From top to bottom: 1, LC-UV of sample in well A5 (none of the found peaks are attributed to the expected compound of m/z 354); 2, XIC of m/z 354 in well A5 by AE-MS analysis; 3, full-scan spectrum of well A5.
compound being plated, erroneous concentrations due to dilution errors, and insolubility of the test compound at higher concentration points. However, the large number of samples from typical dose–response curve DMSO plates makes checking every well for QC impractical using conventional LC-MS. On the other hand, since these DMSO serial dilution plates are already in acoustic-ready plates, AE-MS is the perfect just-in-time, nondestructive QC solution due to its high-speed, low sample consumption, and seamless fit into the workflow, ensuring that all plates can still be used after the AE-MS QC step.

To demonstrate this, we performed a QC check of DMSO dose–response curve solutions for 32 compounds to confirm the identity of test compounds plated, with the results shown in Figure 5. Most compounds displayed an appropriate serial dilution profile (Fig. 5, bottom) and also had an appropriate concordant IC$_{50}$ curve in the screening assay. However, for one compound (RT at 4.31 min) that generated an atypical IC$_{50}$ curve, the AE-MS analysis revealed the absence of the desired test compound. This key information led to the timely invalidation of the IC$_{50}$ value generated for the compound. Considering that IC$_{50}$ could also be driven by the impurities present in the test compound plates, the invalidation could also be triggered by the identification of significant degradation products, which is by far the biggest cause of poor quality in a screening collection. As demonstrated with the fast turnaround and high capacity of AE-MS, the enabled high-throughput QC workflow for dose–response curve DMSO plates not only ensured the quality of the IC$_{50}$ data generated, but also provided valuable information for troubleshooting applicable screening assays. The same workflow could easily be applied to many areas of compound management and curation, examples of which include the periodic check of a screening collection, integrity determination of screening hits, and QC of externally acquired compounds for deck enhancement, among many others.

**Reaction Monitoring of Chemical Synthesis**

The advancement of HTC has allowed the synthesis of large numbers of chemically diverse compounds in a single experiment in a matter of minutes or hours, facilitating the

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**Figure 4.** Ion suppression in AE-MS. Samples 1–9 contain a standard dilution of fluoxetine (m/z 308), concentration range 1 µM to 1 mM. Each sample is also spiked with the same concentration of propranolol (m/z 260). Top: XIC of m/z 308. Bottom: XIC of m/z 260.
rapid exploration of chemical space for biological activity screening, with the requirement for milligram amounts of material.\textsuperscript{36,37} Continuous-flow chemistry has been used as a validation tool for HTC to build confidence in the screening hits due to its rapid mixing and precise control of reaction conditions.\textsuperscript{38,39} Although LC-UV/MS provided the needed data for reaction monitoring, the slow turnaround, typically more than 10 h for a single 384-well plate, easily makes it the bottleneck for monitoring reaction progress.\textsuperscript{3} It is also preferable to eliminate the extra step of reaction mixture transfer for DESI or MALDI analysis. AE-MS, operated in the full-scan mode, provides ultrafast reaction monitoring directly in plates where the reactions are carried out. It maintains the ability not only of confirming the hits or misses of the desired reaction products (as shown in Fig. 1), but also of identifying side products, as well as evaluating the extent of conversion of starting materials.

To illustrate the utility of AE-MS in reaction monitoring, we carried out an HTC experiment of amide coupling (Scheme 1) in a 384-well plate and monitored the remaining abundance of the common starting material, 2-((5-(4-chlorophenyl)-4-methyl-4\textsubscript{H}-1,2,4-triazol-3-yl)thio)acetic acid (m/z 284), in each reaction to help evaluate the relative substrate conversion in the presence of different amine coupling partners.

In Figure 6, the TIC for part of the 384-well plate was displayed, and an XIC of m/z 284 across all reactions in the plate displayed the relative abundance of the remaining acid, thus presenting a visual illustration of its conversion across reactions (namely, the higher the remaining abundance, the lower the conversion). This relative quantification by AE-MS provided the conversion efficiency of the acid among various coupling amine monomers. Due to the differential ionization in ESI-MS, the relative quantification of different compounds is usually invalid unless the compounds are known to share a similar ionization efficiency. However, in this case, the assessment of the same entity or a common starting material makes the abundance comparison across wells relevant without the concern of differential ionization. In Figure 6, besides the remaining starting material (m/z 284 and 295) for the selected well, and the desired product (m/z 354), the full-scan MS also revealed an unexpected peak at m/z 331, which was attributed to a side reaction product. The revelation of side products in the full-scan AE-MS analysis helps to identify the reaction routes that deviate from the formation of the desired products, providing helpful information toward optimizing the reaction conditions if needed. Although these can all be achieved by LC-MS analysis, the speed advantage of the AE-MS analysis makes it much more compelling in being able to provide a rapid scouting of the progress and success of HTC. We are currently trying to couple the acoustic ejection front end to a high-resolution mass spectrometer to reduce isobaric interferences and provide an even more unambiguous compound identification protocol.

To help identify the possible reasons for failed reactions, or a likely connection between conversion of starting materials.
material and the absence of the desired products in the reaction mixtures, we tabulated the XIC intensity of the remaining starting material and desired products, respectively. Among all seven wells highlighted where none of the desired product was detected (Fig. 7, left), only one of them (well M2) was also highlighted for high amounts of remaining starting material (Fig. 7, right). This observation revealed that only one of seven (14%) of the failed reactions was attributable to the lack of conversion of the starting material to product. For the remainder of the failed reactions, the starting material was converted to undesired side products rather than the expected material. This information provided a rapid survey of HTC processes that failed, offering useful guidance for further optimization of reaction conditions as needed, and, more importantly, at an unparalleled speed compared with LC-MS.

**Conclusions**

We have demonstrated the feasibility of acoustic ejection–full-scan MS for high-throughput compound QC in high-density plate formats, featuring contactless direct liquid sampling, nanoliter sample consumption, and ultrafast analytical speed with 1000-fold and 30-fold reduction, respectively, compared with LC-MS analysis. The data processing platform developed using Peak Splitting Tool and PeakView provided a quick and straightforward extraction of data, followed by rapid reviewing and reporting, eliminating the need for the development of custom data processing tools. The overall high-throughput compound QC workflow has positively impacted the method development, optimization, troubleshooting, and quality of the end result of multiple screening assays conducted in high-density plate formats.
in addition to compound management and curation, as well as HTC.

Declaration of Conflicting Interests
The authors declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Jun Zhang, Yong Zhang, Julia Nielsen, Shu Li, Harold Weller, and Wilson Shou are employed by Bristol-Myers Squibb Co., and their research and authorship of this article were completed within the scope of their employment with Bristol-Myers Squibb Co. Chang Liu and Tom Covey are employed by SCIEX, and their research and authorship of this article were completed within the scope of their employment with SCIEX.

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References