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Proof-of-Concept: Interfacing the Liquid Sampling-Atmospheric Pressure Glow Discharge Ion Source with a Miniature Quadrupole Mass Spectrometer Towards Trace Metal Analysis in Cell Culture Media

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Abstract

In an effort to provide a mass spectrometry system capable of at-reactor trace metal analysis of bioprocess media and clarified cell culture fluid, the liquid sampling-atmospheric pressure glow discharge (LS-APGD) ion source was interfaced with a Waters QDa mass spectrometer. The LS-APGD is capable of elemental analysis, as well as organic compound determinations. The Waters QDa is a compact, rack-mounted single quadrupole mass analyzer commonly employed in an integrated liquid chromatography system. By replacing the QDa's standard electrospray ionization (ESI) source with the LS-APGD, trace metal analysis of bioprocess stock media samples can be performed, alleviating use of high-end, inductively coupled plasma (ICP-MS) instruments. Presented here is a proof-of-concept effort, interfacing the microplasma to this platform for the first time. Preliminary optimization of the LS-APGD operating parameters and the QDa's in-source collision induced dissociation (CID) conditions was performed, yielding signal intensities of >6 $X10^7$ AU for a multi-element test solution containing 25 µg mL^{-1} of Cu, Ag, and Tl. As further proof-of-concept, Chinese hamster ovary (CHO) cell culture media was spiked with the same elemental concentrations and analyzed on the LS-APGD/QDa system. Stable plasma response allows spectral background subtraction of the media components, yielding analytically-relevant elemental signals. These results suggest that the LS-APGD/QDa coupling may be a viable approach for at-bioreactor, elemental analysis.

Key Words

Liquid Sampling-Atmospheric Pressure Glow Discharge, Chinese Hamster Ovary Cells, Cell Culture Media, QDa

Introduction

For decades the pharmaceutical industry has used bioreactors for the recombinant production of therapeutic proteins, such as monoclonal antibodies (mAb).[1, 2] While this mature field continues to grow, there remains a large amount of research and development needed in order to better understand and control the processes taking place inside the reactor and to prepare for future manufacturing platforms including semi continuous and continuous production.[3, 4] One opportunity to improve biopharmaceutical process is to understand the level of metals (which could be in various ionic/chemical forms) present in the cell culture media. In this study, the Chinese hamster ovary (CHO) cells used in mAb production are particularly sensitive to the levels of trace metals because they impact the cell culture process performance and modify the protein of interest. Metal ions present in cell culture media can be manipulated to generate desirable culture conditions (e.g., productivity enhancement and metabolic manipulation[5]) or deliver an optimized product quality profile (e.g., control of C-terminal lysine heterogeneity[6] or glycosylation profile[7]) for both biosimilar and novel molecules. Additionally, variations in metal content can effect process consistency, or in the extreme result in concentrations outside of regulatory limits.[8] For example, in the case of mined minerals, especially iron, manganese contamination has been observed. If unintended manganese is present at levels on the order of 5-50 ppb, galactosylation can be increased unexpectedly, which has the potential to affect the product quality profile of the therapeutic protein. There exists a critical need for sensitive, rapid analysis of metals within a cell culture process in both process development and commercial production; therefore the development of an at-line mass spectrometer system capable

of elemental analysis is desirable.[9-11] While inductively coupled plasma mass spectrometry (ICP-MS) instruments remain the work horse in the field of elemental analysis in the pharmaceutical industry, their large size and intensive operational overhead requirements, make them unsuitable for production floor, at-reactor, analysis.

To this end, the liquid sampling-atmospheric pressure glow discharge (LS-APGD) microplasma was interfaced with a compact-footprint, Waters QDa mass spectrometer. The LS-APGD is an alternative “atomic” ion source that is compatible with diverse atmospheric pressure MS sampling interfaces.[12-14] Prior research has shown the versatility of the microplasma for elemental analysis, as well as its ability to ionize organic compounds while retaining relevant structural information.[15-19] The Waters QDa mass spectrometer is a rack-mounted, single quadrupole system that is typically used as a detector within a Waters UHPLC (ultrahigh performance liquid chromatography) component systems. By replacing the ESI source equipped with the QDa with the LS-APGD, it was hoped that at-reactor, quantitative trace metals analysis could be achieved without the use of a standard format ICP-MS instrument.

The work presented here reflects a proof-of-concept demonstration project occurring over the period of approximately 1 week at the Merck facilities, involving the initial coupling and elementary-level optimization of the LS-APGD and QDa operating parameters. A synthetic multi-element solution containing Cu, Ag, and Tl (representing elements of diverse mass and chemistries) at concentrations of 25 μg mL^{-1} (each) was used to evaluate the operating parameters of the LS-APGD (gas flow, liquid flow, and operating current) with respect to signal intensity and signal-to-background ratio. Variation of the QDa sampling cone voltage to affect the collision induced dissociation

(CID) efficiency towards removing concomitant ions, primarily water clusters, with respect to metal analytes. This effort culminated in the analysis of commercial cell culture media containing the 25 μg mL^{-1} multi-element spike. Based on these preliminary results, it is concluded that the LS-APGD ion source, interfaced with the Water's QDa mass spectrometer, is capable of measuring metals in complex media samples, suggesting a pathway towards developing a reduced-format system for the trace metal analysis of bioprocess media.

Experimental

The LS-APGD ionization source, as described in detail previously,[13] was interfaced with a Waters QDa mass spectrometer, shown diagrammatically and in photographs in Fig. 1. The basic components of the LS-APGD ionization source are presented in Supplementary Information Table 1. As seen in Fig. 1, the electrodes were perpendicular to one another, with the solution electrode in-line with the QDa sampling cone. An abbreviated evaluation of the LS-APGD operating parameters was completed during which continuous sample flow was sustained using a syringe pump. A test solution containing Cu, Ag, and Tl at 25 μg mL^{-1} each, was prepared in 2% HNO_3. In order to access the ability of the LS-APGD/QDa to analyze metals in a cell media, 2 samples were prepared using Chinese hamster ovary (CHO) cell culture media (CD CHO Medium, Thermo Scientific, Waltham, Ma, USA). The media samples were diluted 25X using 2% HNO_3. While acidic environments may have deleterious effects on the biological/organic components of the media, such perturbations should not adversely affect the metal determinations here. The first sample contained fresh CHO cell media. The second was diluted and then spiked with the multielement mixture to achieve a final

concentration of 25 μg mL^{-1}, each. (Of this suite of elements, only Cu would be expected to have significant biological consequences in antibody production.[20]) A six-port Rheodyne 7125 injection valve and a Rheodyne 50 μL injection loop (Rheodyne, LLC, Rohnert Park, CA, USA) were put in-line with the syringe pump to introduce the samples.

The Waters (Milford, MA) QDa system installed at the Merck & Co., Inc., Kenilworth, NJ, USA, facility was used with minimal modification: removal of the ESI source and the cone clamp equipped on the mass spectrometer inlet. The QDa used in these studies was the "performance" version, controlled using the Empower 3 Software. An optimization of the in-source CID was performed with the goal to reduce the number of concomitant ions (principally H_2O-related clusters). For these experiments, a mass scan rate of 10,000 amu sec^{-1} was used and the scan range was set from *m/z* = 50 to *m/z* = 250 for the source evaluation experiments and *m/z* = 50 to *m/z* = 1000 for the CHO cell media analysis. Scans were accumulated for a 1-minute time period and then averaged to constitute one data point for a given set of experimental conditions. For the analysis of the media samples, scans were collected in the system Profile mode for a 5-minute time period and the total ion chromatograms (TIC) collected.

Results and Discussion

Parametric Evaluation

The coupling of the LS-APGD ion source with the QDa represents the first instance where the LS-APGD was not interfaced with a trap-type mass analyzer. Previous work has relied on either Thermo Scientific LCQ Advantage Max (Paul trap) mass analyzers or Thermo Scientific Exactive-family Orbitrap mass analyzers.[16, 17, 21]

The atmospheric pressure interfaces of the Thermo instruments employ an ion transfer capillary. Alternatively, the "Performance" QDa employed here uses an ~0.2 mm aperture followed by a second orifice for the interface. Given these differences in ion sampling, but the very limited amount of available instrument time, a cursory evaluation of operating parameters was necessary. Previous studies have demonstrated that each of the individual operating parameters, i.e. current, liquid flow and gas flow rates, has an effect on the observed ion signal intensities,[16, 21] For this evaluation, an aqueous 2% HNO_3 solution containing Cu, Ag, and Tl at 25 μg mL^{-1} each was used, with the range of tested parameters presented in Table 1. To minimize the number of variables, the LS-APGD electrodes were kept ~1.5 mm from the orifice of the QDa and the inter-electrode spacing was set at 1.5 mm. The potential of the in-source CID was initially set at 25 V, though the impact of the CID energy on the performance of the system was investigated following the source parameter evaluation.

The optimization results for the ^{205}Tl ion response are presented in Supplementary Information Fig. 1, however the same trends hold true for the other isotopes analyzed (^{63}Cu, ^{65}Cu, ^{107}Ag, ^{109}Ag, and ^{203}Tl). At each of the gas flow rates, the responses across the solution flow/current grid are qualitatively very similar to previous works, in that there exists a discrete combination of the two which yields the highest analyte signal levels for both the orbitrap and ion trap instruments.[16, 21] As Zhang et al. suggested, the sheath gas should aid in the nebulization and desolvation of analyte species.[21] Increased responses at higher gas flow rates would suggest more efficient transport of ions towards the entrance aperture as well.

As seen in all LS-APGD-MS works, the most common spectral background ions are related to the electrolytic solution,[13] most commonly of the general form $(H_2O)_nH^+$. Such species add appreciable spectral complexity in the case of a unit resolving power mass analyzer. Previous work by Hoegg et al.[17] showed that in-source CID and collisions in the Orbitrap's higher energy collision induced dissociation (HCD) cell were effective at removing unwanted concomitant (background, analyte oxides, etc.) ions. In order to mitigate the presence of these species when using the QDa, in-source CID was employed. Supplementary Information Fig. 2 shows the effect of increasing the cone voltage on the signal-to-background ratio (S/B) of the ^{205}Tl signal as well as its raw intensity (again the other elements responded similarly). The calculated S/B increases from ~30 to over 200 as the cone voltage is increased from 10 V to 30 V. The initial increase is predominately due to improved response of $^{205}Tl^+$ (by virtue of better ion transmission or dissociation of metal-containing moieties) and to a lesser extent background reductions through removal of cluster species (this was generally true for the other corresponding background species). At higher cone voltages decreases in S/B corresponds to an overall signal suppression. Based on these results a cone potential of 30 V was used for the analysis of the CHO cell media.

Based on the parametric responses, the preferred LS-APGD operating parameters were: current = 30 mA, gas flow rate = 0.5 L min^{-1}, and liquid flow rate of 10 µL min^{-1}, with a CID energy of 30 V. Using these operating parameters, preliminary limits of detection for the three elements were determined and are shown in Table 2. Due to the limited nature of these efforts, the LODs were calculated based on the method described by Boumans et al.,[22] which is given by equation 1:

$$LOD = \frac{(0.01)k(RSDB)m}{S/B} \quad (1)$$

where RSDB is the relative standard deviation in the spectral background (thus the 0.01 multiplier), S/B is the signal to background ratio, and m is the concentration of the analyte used in the determination, with a value of k = 3 being used to give a >99% confidence level. Looking at the concentration-based LODs calculated, the values are significantly impacted by the signal-to-background values. The lower S/B values for Cu and Ag in comparison to Tl correspond to higher levels of background ions at the lower masses. The calculated LOD for Ag compares well with previous ion trap work presented by Zhang et al.;[21] the Cu and Tl values had not been determined. To be sure, this method of calculating LODs is not entirely satisfying in terms of a comprehensive analytical evaluation where calibration functions would be incorporated. The RSDB method simply provides a simple reference point to previous efforts on other analyzers as well as the following observations with CHO cell media. Future efforts will clearly use more rigorous analytics.

CHO Cell Media Analysis

To conclude this preliminary assessment of the LS-APGD/QDa coupling for analysis of metals in cell culture media, commercial CHO media was used as a test matrix. Media tend to be chemically heterogeneous and salt laden, requiring dilution for many forms of analysis. The complete assay of commercial CHO cell media is typically proprietary knowledge, with few specifics provided on certificates of analysis. As a general rule, sodium content (as bicarbonate and pyruvate) is typically ~120 mM with the total electrolytes, given as osmolality, being 300 – 350 mOsm kg^{-1}. This would

suggest a total "salt" content of approximately 350 mM. In addition, various nutrients (amino acids and sugars) are added, as are some emulsifiers. As such, the media was diluted 25X using 2% HNO_3 to minimize clogging within the microplasma solution capillary. Clogging of the MS entrance aperture is also problematic with the direct introduction of culture media. Even with the 25X dilution, it was necessary to increase the liquid flow rate to 20 μL min^{-1} to prevent the sample from desolvating inside of the solution capillary, which leads to clogging. The other parameters were kept at the optimized settings, current = 30 mA, gas flow = 0.5 L min^{-1}, and in-source CID potential = 30 V.

Figure 2a shows a representative mass spectrum of the CHO cell media. The spectrum is predominately composed of ion signals at masses of less than m/z = 500 Da, however there are a large number of species at higher masses, likely related to highly-hydrated media components (including peptides and other organics) or protonated water clusters. Such species would need to be subjected to MS/MS analysis to determine their identity. During these experiments, the plasma appeared orange in color rather than the violet hue seen in Fig. 1c, likely due to the Na content in the media. The Na ion signals could not be detected because the low mass cut-off of the instrument is fixed at m/z = 30 by the system electronics (a limitation that must be addressed in future endeavors).

In order to show the efficacy of the system to measure metals in this complex medium, a second sample was prepared that included a spike of Cu, Ag, and Tl to in-media concentrations of 25 μg mL^{-1}, each. Given the low concentrations of the spike species, and the spectral complexity of the medium (Fig. 2a), the only way to easily

distinguish the added elements was background subtraction. In this method, native media was introduced continuously, while spiked media (50 μL) was injected into the flow. Figure 2b shows the resulting mass spectrum after background subtraction (spike – continuous at each mass point), clearly revealing the presence of the spiked metal species (in red). The differences in the recoveries of the three elements reflects the fact that Tl is extremely well ionized in this source, while the suppression of Cu relative to Ag is a function of the lower transmission efficiency of the quadrupole mass filter near its low-mass cut-off. The net values of Fig. 2b, being relatively close to zero, for the intense signals of the media components is testament to the high temporal stability of the LS-APGD ionization source.

It is noted that the signal intensities of the analyte signals are significantly (10-100X) lower in the media sample compared to the optimization studies above, for the same metal concentrations in neat nitric acid media. The reasons for this require further thought and investigation. That said, one contributing factor is the different scan ranges used in the optimization as compared to the commercial CHO media analysis. The m/z range for the CHO media analysis was 4.75 times greater than the aqueous sample, and so the number of spectral acquisitions per unit time was ~5X less. Therefore, lower ion counts would be expected. Likewise, it would certainly not be surprising if the total ionization capacity of the microplasma was exceeded in the case of media introduction, i.e., a matrix effect. Cleary, much work remains in quantifying any such effects. Matrix effects have not been noted in the analysis of aqueous samples using the LS-APGD, but clearly this is a more complex matrix than has been introduced previously. As a point of simple reference, the LODs in the CHO cell matrix samples were calculated for

each metal in the spike, Table 2. The LODs were calculated, after background subtraction, using Eq. 1. There is a slight increase in the LODs for the Cu and Ag additives, with a larger relative increase in LOD seen for Tl. The greater degradation in performance for Tl is likely due to the fact that there is great background spectral complexity in that mass region that is not fully compensated in the background subtraction step. Likewise, Fig. 2a shows little content in the mass regions of Cu and Ag, resulting in lower residual net background ion signals in Fig. 2b.

Evidence of Secondary Discharge

While the initial pairing of the LS-APGD to the QDa shows promise, a significant limitation regarding the robustness of the ion sampling interface must be addressed. After approximately 8 hours of continuous sample introduction (predominately the acid-matrix materials), the system performance degraded rapidly, with vacuum system warnings suggesting a leak. Upon visual inspection of the 0.2 mm diameter aperture, there was clearly a widening of the aperture and indication of a discharge being struck within the orifice. The presence of a secondary discharge suggests that the nickel plate containing the ion inlet aperture is not grounded or at least at an offset potential versus the sampled plasma. The aperture holder includes an electrically conductive o-ring so that the sampling cone, ion aperture, and ion transfer tube are clamped together to assure electrical contact. In the removal of the clamp in mounting the microplasma it is possible that the aperture holder is not connecting correctly and may cause the cone to float intermittently. Scanning electron microscopy (SEM) with energy dispersive x-ray (EDS) analysis showed that the aperture had nearly doubled in size with significant oxidation (Ni and O presence in the EDS spectra) around the aperture. These sorts of

effects suggest the occurrence of a secondary discharge within the aperture; not an unknown phenomenon in plasma source mass spectrometry.[23, 24] The alleviation of the secondary discharge is a point that must be addressed in future efforts, but it is not seen as a major challenge moving forward.

Conclusions

The primary goal of this effort, affecting a means of performing elemental analysis of bioprocess media on a platform suitable for at-reactor monitoring in the biopharma cell culture environment, was successfully realized. Interfacing of the LS-APGD ionization source with the Waters QDa miniature mass spectrometer was demonstrated in this ~1-week effort. While these preliminary efforts only tested a limited number of analytes, the LS-APGD/QDa system is capable of measuring metals in a CHO media matrix at sub-μg mL^{-1} levels. Greater sensitivity will certainly be needed, though precisely how much will be element- and culture-specific. In future cases of spent media analyses, methods of cell and debris removal (filtration/centrifugation) will likely be required, as are routine in this application area. Indeed, an integrated approach including aseptic sampling and clarification could be implemented.

Clearly much work remains beyond this demonstration project. Challenges exist in terms of the alleviation of the secondary discharge. This challenge was overcome in the early days of ICP-MS, providing a route to do so in this case. Going forward, it will be necessary to rigorously determine the analytical figures of merit of the LS-APGD/QDa system, on neat aqueous solutions as well as spent media. There is a need for refinement of the source hardware and optimization of the actual analytical

protocols. Beyond bulk elemental analysis, one can easily imagine implementation of ion chromatography or some other form of solid phase extraction (SPE) as a means of isolating analyte metals from the complex matrix, and perhaps even performing speciation analysis of the metals in the media. Ultimately, a path towards a miniature/portable mass spectrometer capable of trace metal analysis of bioprocess samples, on a platform that can be implemented at-reactor, is foreseeable.

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Figure Captions

Figure 1. a) Drawing of the components of the LS-APGD interfaced with a Waters QDa mass spectrometer. b) Photograph of ion source mount within a plexiglass protective cover including sample injection valve. c) Photograph of operating plasma in proximity to ion sampling orifice with cover removed.

Figure 2. a) Representative mass spectrum of CD CHO media diluted with 2% HNO_3. Spectrum was generated by averaging 400 spectra taken during a 50 µL injection period. b) Representative mass spectrum of CD CHO media diluted with 2% HNO_3. A spike containing Cu, Ag, and Tl was added to the medium to the final concentration of 25 µg mL^{-1} for each metal. Spectrum generated using a 50 µL injection with the resulting mass spectrum representing background subtraction (spike – neat CHO cell media) at each mass point. LS-APGD operating parameters: current = 30 mA, gas flow rate = 0.5 L min^{-1}, and liquid flow rate of 20 µL min^{-1} and CID potential = 30 V.

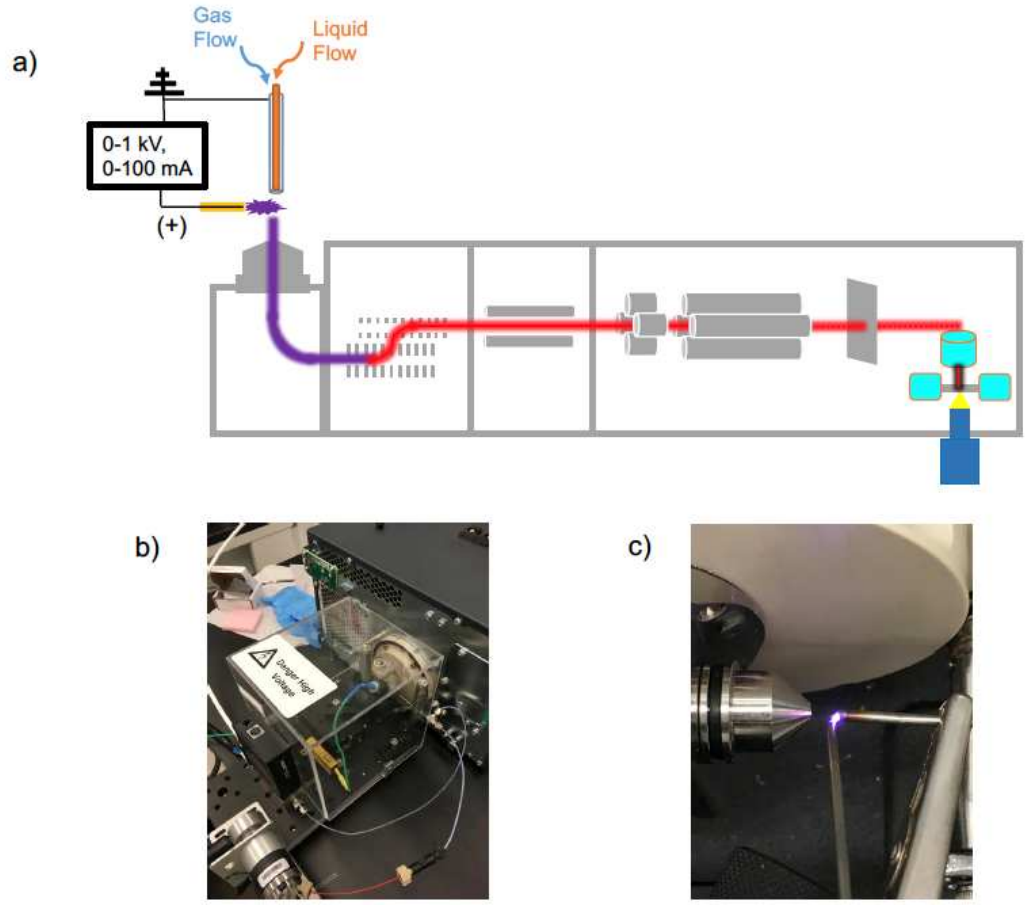
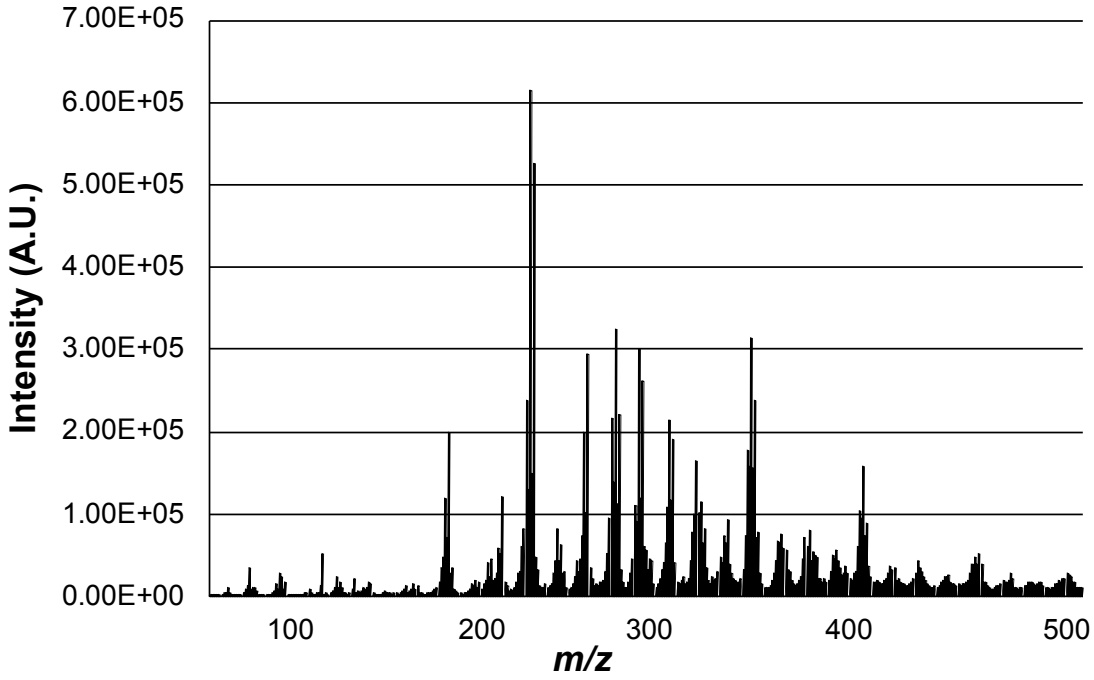


Fig. 1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6.00E+05
5.00E+05
4.00E+05
3.00E+05
2.00E+05
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Intensity (A.U.)
100
200
300
400
500
m/z

Fig. 2a

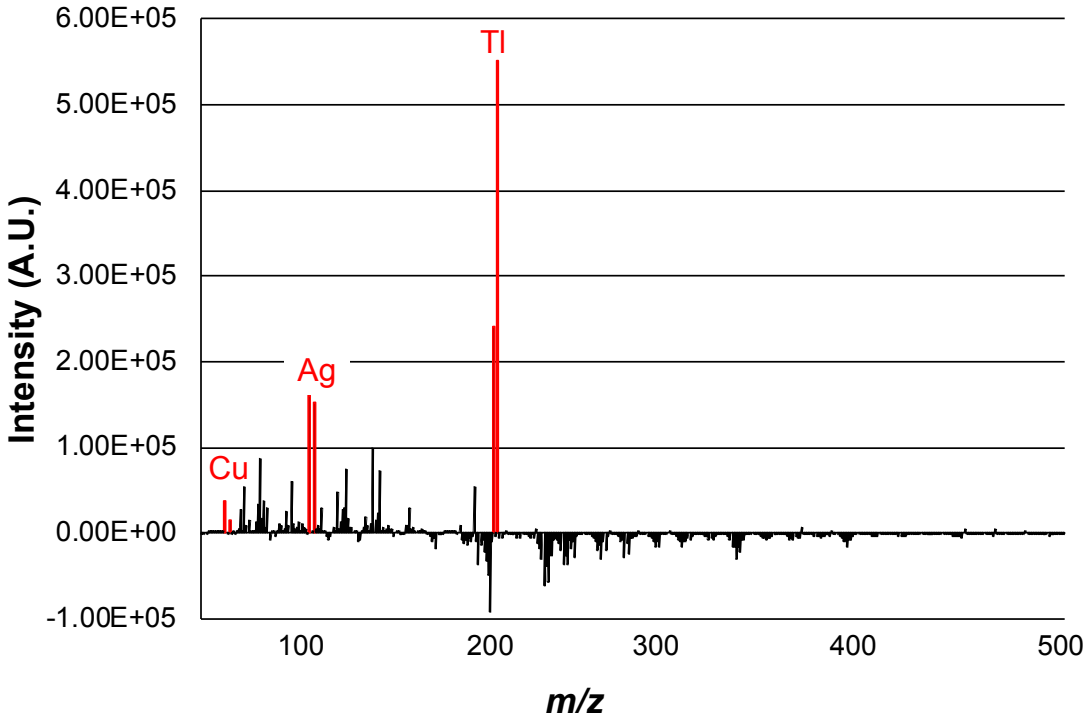
6.00E+05
5.00E+05
4.00E+05
3.00E+05
2.00E+05
1.00E+05
0.00E+00
-1.00E+05
Intensity (A.U.)
Tl
Ag
Cu
100
200
300
400
500
m/z

Fig. 2b

Table 1. Ranges of operational parameters probed in preliminary evaluation.

Parameter (unit)	**Range Tested**
Discharge current (mA)	15-40
He gas flow rate (L min^{-1})	0.250-1.00
Liquid flow rate (µL min^{-1})	5-30
Cone voltage (V)	10-100

Table 2. Limits of detection determined using the Boumans method presented in Eq. 1.

Matrix	Element	m (µg mL^{-1})	RSDB (%)	S/B	LOD (ng mL^{-1})
2% HNO_3	Cu	25	1.3	77	13
	Ag	25	0.8	20	28
	Tl	25	1.0	3200	0.2
Cell Culture Media	Cu	25	2.1	70	23
	Ag	25	1.1	22	38
	Tl	25	1.2	19	48