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A Guide to Performing Method Development for Direct Injection Methods Using ionKey/MS in a Bioanalytical Laboratory: From Sample Preparation to LC-MS

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INTRODUCTION

In any bioanalytical laboratory, sample preparation or sample pretreatment is an extremely important aspect of assay development. There are many benefits of sample preparation, including concentration of analyte(s) of interest and removal of contaminants which can negatively impact assay performance as well as column and mass spectrometer performance. Sample preparation techniques vary greatly depending on both the biological fluid being used and the compounds being analyzed. For example, analysis of a pharmaceutical small molecule requires very different methodologies than analysis of an intact monoclonal antibody. In addition, the impact of migrating from analytical to nano- or micro-scale chromatography must also be taken into consideration at each step during the method development process, from initial stages of sample preparation, to optimization of chromatographic and mass spectrometer (MS) parameters.

This white paper describes a suggested approach to developing methods for use with the ionKey/MS™ System. The instrumentation may consist of the ACQUITY UPLC® M-Class System, and any Xevo®/SYNAPT® Mass Spectrometer that can be fitted with an ionKey® source. This currently includes the Xevo TQ-XS, Xevo TQ-S, and TQ-S micro (tandem quadrupoles) and Xevo G2-XS QTof, SYNAPT G2-S*i*, and SYNAPT G2-S (quadrupole time-of-flight/high resolution). Combined with sub-2-µm iKey® Separation Devices and MassLynx® Software, this system provides users with unparalleled sensitivity.

OVERVIEW

Many LC-MS scientists are moving from traditional analytical scale to microscale techniques for a number of reasons, including but not limited to increases in sensitivity, smaller sample volume requirements, and decreased solvent consumption. However, as with any technique, there are a number of factors that must be optimized in order to achieve high quality chromatographic and MS results.

CONSIDERATIONS

There are many sample preparation techniques available to bioanalytical scientists, including protein precipitation, liquid-liquid extraction, solid phase extraction, supported liquid extraction, dilute and shoot, protein digestion, and affinity-based methodologies. As stated above, one of the main reasons for sample preparation is to remove interferences and contaminants that may compromise the performance of the assay or equipment. In many biological matricies proteins account for a high concentration of the potential interferences and can lead to ionization related problems, such as signal suppression due to ionization competition of the proteinaceous co-eluting matrix components with the compounds of interest. High amounts of injected protein can also cause clogging, typically in the column, especially for low dispersion systems which

utilize narrow internal diameter (I.D.) tubing and columns. This effect is exacerbated when transitioning from analytical to microscale techniques where a 2.1 mm I.D. column is replaced with a 150–300 µm I.D. iKey Separation Device. This is especially a concern for direct-injection methods since the addition of a trapping device may reduce the amount of protein introduced onto the iKey Separation Device. It is generally recommended that less than 80 ng of protein be injected onto an iKey. There are 3 main factors which will affect the amount of protein introduced to the iKey: initial sample matrix, sample preparation technique, and injection volume used.

SAMPLE MATRIX

There are a number of biological matrices which are routinely encountered in a bioanalytical laboratory. Plasma, serum, whole blood, and urine are the most common, but the entire list of matrices one can encounter is quite lengthy. Plasma and whole blood both contain large amounts of protein, on the order of 60–80 g/L[1] or 60–80 µg/µL. The majority of proteins found in plasma/whole blood samples are albumin, globulins and fibrinogen,[1] which range in molecular weights from approximately 66 kDa to over 1000 kDa. Since the recommended maximum amount of protein for direct injection is limited to 80 ng, a 1 µL injection of plasma contains 1000x more protein than suggested. The plasma could be diluted at a minimum of 1000-fold prior to injection, however since applications with the ionKey/MS System are focused on achieving low detection levels, a simple dilute-and-shoot methodology is likely not realistic and instead sample preparation to remove the protein content is required. Other matrices, such as urine and bile, typically have relatively low protein content to begin with (unless the samples are originating from a diseased population which results in higher basal protein levels) and typically will not require specialized sample preparation to remove endogenous proteins but may still require some form of sample preparation to remove other interferences/contaminants.

SAMPLE PREPARATION TECHNIQUES

For high protein containing matrices, a sample preparation technique must be employed to reduce the amount of protein prior to injection. Some of the most common sample preparation techniques are listed below.

PROTEIN PRECIPITATION (PPT)

One sample preparation technique used to remove proteins is the appropriately named protein precipitation methodology. In this method, an organic solvent, typically acetonitrile (ACN) or methanol is added to an aliquot of sample matrix, commonly in a ratio of 2:1, v:v, or greater. The matrix proteins are not soluble in the organic solvent, and precipitate or crash out of solution. After centrifugation, the proteinaceous material is left as a solid pellet on the bottom of the extraction vessel. The higher the ratio of organic solvent to biological sample, the more proteins will be precipitated out of solution, resulting in a cleaner extract for injection. As depicted in Table 1, a protein precipitation methodology requires a minimum ratio of 2:1 to produce extracts amenable with injection onto an iKey Separation Device.

For sample preparation strategies that include a dry down and reconstitution step, users must be cognizant of the remaining protein material especially if the final extract is being reconstituted in a smaller volume. For example, if a 200 µL aliquot of the 2:1 PPT supernatant is transferred and subsequently dried down, there is a residual amount of protein equal to 600-3200 ng. Depending on the injection volume to be used, this extract must be reconstituted in a minimum volume of 40 µL (if using a 1 µL injection volume) or 200 µL if using a 5 µL injection volume. Often times an evaporation step is used to remove the organic extraction solvent, then the sample is reconstituted in a more aqueous solution to match the LC starting conditions. There is always a risk that the residue left after evaporation will be less soluble in an aqueous solution, leading to undissolved solid material that can clog the separation device.

Table 1. Protein amounts following common protein precipitation methodology.

	Residual Protein Concentration (ng/µL)	Amount on Column per Injection Volume (ng)	
		2 µL	5 µL
2:1 ACN:Plasma (v,v)	3–16	6–32	15–80
1:1 ACN:Plasma (v,v)	400	800	2000

LIQUID-LIQUID EXTRACTION (LLE)

Using a liquid-liquid extraction is an acceptable sample preparation strategy for removing proteins from many sample matrices. Similar to protein precipitation, LLE uses an organic solvent added to the matrix sample, but in this case, a solvent which is immiscible with water is chosen. After appropriate mixing and centrifugation, the aqueous and organic layers will partition, with the proteins remaining in the aqueous layer and ideally the compound of interest residing in the hydrophobic organic layer. The organic layer is then removed, and typically evaporated to dryness then reconstituted in a solvent mixture which is amenable to the LC method starting conditions. This is a very commonly used technique for small molecules, but may present a more significant challenge for peptides, depending on their size, polarity, etc.

SOLID PHASE EXTRACTION (SPE)

Solid phase extraction is a very versatile preparation technique due to the wide variety of possible sorbents available. In simplistic terms, SPE utilizes a stationary phase (or sorbent) that can chemically separate the different components within a sample. The sample is first loaded, typically diluted in aqueous, onto the sorbent/stationary phase. The sorbent is then washed with optimized solvent mixtures to eliminate matrix and other unwanted components while the analyte(s) of interest remains bound to the sorbent particles. After sufficient washing, the analyte(s) is then eluted from the sorbent with an optimized elution solution. Although SPE eliminates a majority of matrix components, there is often still a relatively high amount of protein being eluted in the final step that ultimately resides in the final injection solution. For this reason, it is suggested that for direct inject methods using the ionKey/MS System, a protein precipitation step be added prior to any SPE method to eliminate protein from the final solution.[2]

PROTEIN DIGESTION AND AFFINITY METHODOLOGIES

In addition to sample preparation techniques that are predominantly used for small molecules, there are other sample preparation techniques which are often used with larger molecules such as proteins, monoclonal antibodies, etc, which are suitable for direct injection with the ionKey/MS System, such as protein digestion. In this case, an enzyme is added to the biological sample, such as trypsin, lys-C, etc., which will cleave the proteins at specified sites dependent on the enzyme used. In this sample preparation, essentially all large proteins have been broken down into smaller peptides which can pass through the iKey Separation Device with less risk of clogging. While the potential for clogging has been reduced, protein digestion is not a selective method, rather it results in a multitude of smaller peptide fragments which may still provide a relatively complex sample for analysis. For this reason, affinity methods may be used in conjunction with protein digestion to improve the overall assay performance.[3,4]

LC METHOD DEVELOPMENT FOR DIRECT INJECTION IONKEY/MS

As stated in the preceding paragraphs, the sample preparation plays an important role in the method development process when using direct injection ionKey/MS because of the protein content in the injected samples. Equally important is development of the liquid chromatography method to be used in the ionKey/MS analysis. One important consideration is the comparison of column volumes when scaling from a traditional 2.1 mm I.D. column to a 150 µm I.D. column. Keeping the length of the columns at 50 mm, the column volume for the 150 µm iKey Separation Device is almost 200 times smaller than the volume of a traditional 2.1 mm column. Although the column dimensions have been scaled, the injection volumes loaded onto the iKey Separation Device are typically not scaled; rather the same injection volumes that would be used on a traditional 2.1 mm column are injected onto the much smaller iKey. Because of this, a 5 µL injection onto an iKey is equivalent to injecting almost 1 mL of sample onto a 2.1 mm column. It is therefore not surprising that chromatographic performance as well as variables likely to affect the chromatography must be seriously considered when moving from 2.1 mm to 150 µm, especially in terms of injection solvent composition and injection volume. The chromatographic effects exerted will also be dependent on the properties of the molecule(s) being analyzed, such as size, polarity, hydrophobicity, etc.

INJECTION SOLVENT COMPOSITION

The composition of the final injection solution must be considered prior to injection onto an iKey Separation Device. In the sample preparation portion of this document, the negative effects of residual protein in the injection solution, mainly in terms of clogging the iKey Separation Device, was discussed. The relative amounts of aqueous to organic solvent, or weak to strong solvent, contained within the injection solution is another concern during method development. More specifically, how the injection solution compares to the LC starting conditions in terms of solvent strength.

There are three scenarios which can occur – the injection solution solvent strength is weaker, matched, or stronger than the LC starting conditions. In any scale of chromatography, it is suggested that the injection solution match the initial LC

starting conditions to prevent any peak shape distortions. The starting LC conditions should also be weak enough that compounds are allowed time to interact with the stationary phase as opposed to being immediately eluted from the column. In the case where the injection solution is significantly weaker than the mobile phase starting conditions, it is actually possible to get concentration of the sample on the head of the column, which will result in narrower peak widths compared to the scenario where the injection solution is matched exactly in solvent strength to the mobile phase starting conditions. Again, this is only true if the LC starting conditions are weak enough to allow for ample interaction and retention on the column. The third scenario in which the injection solvent is sufficiently stronger than the LC starting conditions is problematic due to potential strong solvent effects, exemplified by peak distortions, such as fronting, smearing, or sample breakthrough (Figure 1).

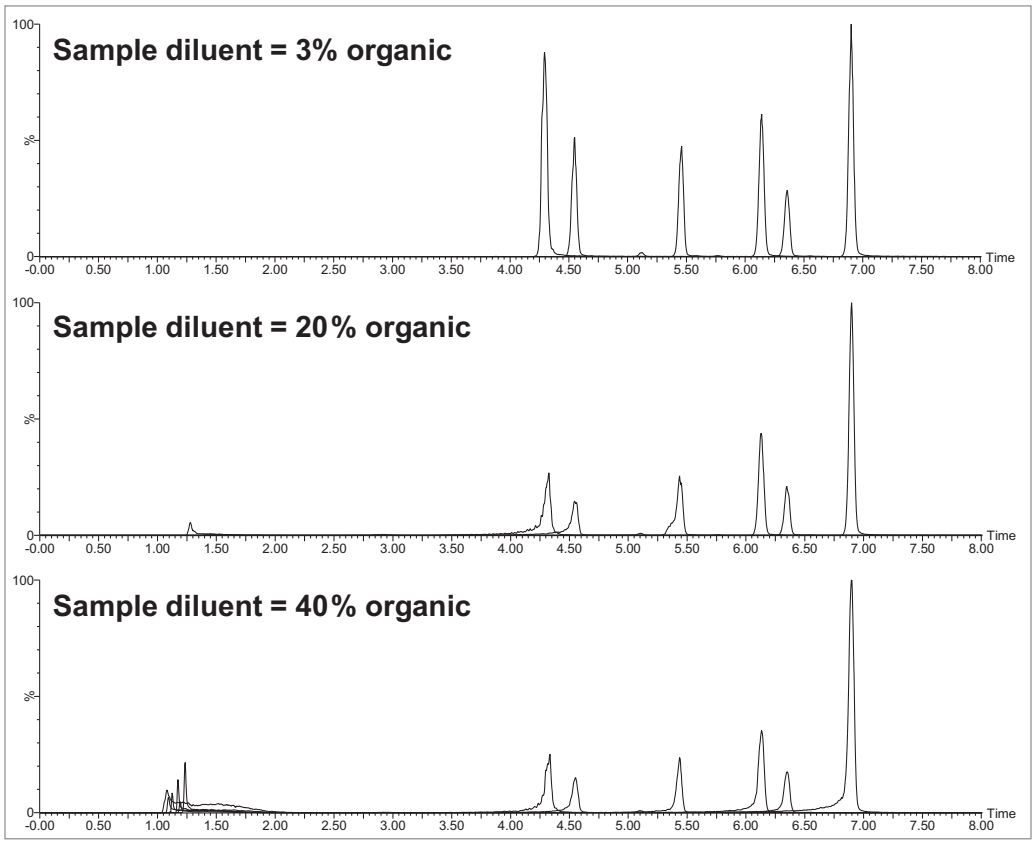

Figure 1. Example chromatograms showing the effect of increasing strength (or organic solvent composition) of sample diluents. The top chromatogram shows no peak distortions, while the middle chromatogram shows fronting for the first three peaks as well as sample break-though at ~1.2 min. The bottom chromatogram at 40 % organic shows distortion and break-through for all peaks regardless of increasing retention time.

When any sample is injected, the injection solution is diluted in the mobile phases as it travels through the sample loop, tubing and column. If the injection volume is relatively large, the plug of injection solvent travels down the column at the flow rate, becoming diluted as it travels. If the sample diluent cannot adequately mix with the mobile phase, sample molecules on the outside of the injection plug interact with only mobile phase, whereas the molecules in the middle of the plug interact only with the strong injection solvent. This essentially creates very different environments for the sample, resulting in smearing or generally poor peak shape until the injection solvent is effectively diluted within the mobile phase. For analytical scale separations, this happens relatively quickly since the injection volume is quite small in comparison to the mobile phase flow rate, i.e. a 5 µL injection with a flow rate of 0.6 mL/min. However, for ionKey/MS separations, because the injection volume is not scaled to match the iKey and LC flow rate, the injection plug is less effectively mixed in the loop and mobile phase stream, and thus strong solvent effects are more likely. In contrast to the analytical scale separation, when using the ionKey/MS System with the 300 µm ID iKey Separation Device, the same 5 µL volume is being injected, but the flow rate is now typically less than 8 µL /min. Strong solvent effects can be mitigated by either decreasing the strength of the injection solution relative to the LC starting conditions or by decreasing the injection volume (Figure 2). Additionally, because strong solvent effects are dependent on the mixing of the injection solvent with the mobile phase, later eluting peaks will have a relatively longer time to mix and will be less affected than earlier eluting peaks. Partial loop injection mode can also be used to dilute the injection solvent. When using partial loop injection mode, the

sample loop is filled with weak needle wash prior to the sample being drawn into the loop, therefore if the weak needle wash has a lower organic composition than the injection solution, it will help to dilute out or mitigate strong solvent effects. Another way to mitigate strong solvent effects is by using a trapping methodology, where the peak(s) of interest can be refocused to reduce band-broadening.[5]

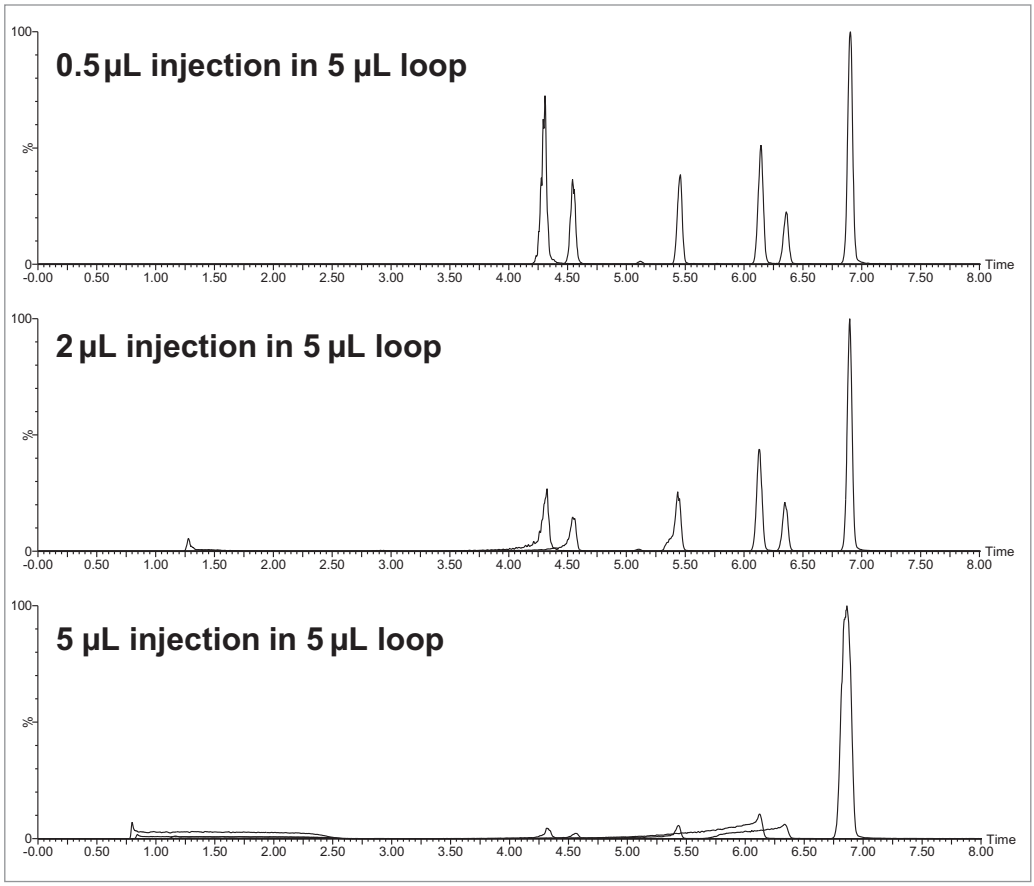

Figure 2. Chromatograms showing the effect of decreasing injection volume. The smaller injection volume is more effectively mixed within the sample loop and mobile phase stream, thus leading to less peak distortion.

Modifying the injection solvent to more closely match the LC starting conditions can be done in a number of ways. For assays which are not near their sensitivity limit, simple addition of aqueous to dilute the sample is likely the easiest option. For assays where sensitivity is critical, a change in the sample preparation may be necessary. In the case where the sample is extracted into 100% organic solvent, a step may be added to evaporate and reconstitute the sample in a more highly aqueous solution. It may also be possible to change the column chemistry and thus the selectivity of the separation. By choosing a more retentive column chemistry (in regards to the specific compounds being analyzed), the strong solvent effects can be mitigated by allowing more time for mixing between the injection solvent and mobile phase.

INJECTION VOLUME

For optimal chromatographic performance, the recommended injection volume range is largely based on the dimensions of the column being used. If we assume a near optimal flow rate and a retention factor, or k, of greater than or equal to 1, then the maximum injection volume can be determined using the equation:[6]

$$V_s < 0.14 L^{0.5} d_c^2 d_p^{0.5}$$

where Vs is the allowable sample volume, L is the column length (in mm), d_c is the diameter of the column (in mm) and d_p is the diameter of the column particle (in µm). Using this calculation a 150 µm x 50 mm 1.7 µm iKey Separation Device will have a maximum suggested injection volume of approximately 0.03 µL. However, typical injection volumes used for direct inject ionKey methods range from 1–5 µL, which is outside the optimal injection volume range. The resulting chromatographic effects, such as band-broadening, can lead to larger peak width(s) and a consequent reduction in resolution and theoretical plates.

This effect is less noticeable for later eluting peaks (larger k values), but exacerbated for early eluting peaks (lower k values). When developing direct injection ionKey methods, the injection volume should be kept as small as possible (while still maintaining the sensitivity levels required for the method) to reduce the potential for band broadening.

There is also the potential for mass overload, also sometimes referred to as column overload or concentration overload, due to the exceptionally small column volume of the iKey Separation Device. Mass overload occurs when there is an excess of sample in comparison to the stationary phase, or more specifically, sites where the sample can interact with the stationary phase. This phenomenon is characterized by a shark fin peak. General guidelines suggest injection of less than 1 mg of sample for each 1 mL of column volume to avoid potential effects of column overload. Using a porosity factor of 0.66, for a 300 µm x 50 mm, 1.7 µm iKey Separation Device the total column volume is determined to be 2.3 µL. This translates to a suggested maximum mass load of approximately 2300 ng onto the iKey Separation Device. Most ionKey applications are sensitivity driven with ultra low LODs, so mass overload is not likely to be a problem, but it is a phenomenon that users should be aware of.

From the paragraphs above regarding injection solvent composition and injection volume, it is clear that these two variables can impose a large impact on chromatography. To minimize these effects it is generally recommended, where possible, to match the injection solution solvent strength to the LC starting conditions, or have a slightly weaker injection solution. Additionally, to minimize band broadening, injection volumes should be as low as possible while maintaining the required peak height and/or peak area.

LC FLOW RATES

When developing LC methods for use with iKey Separation Devices, the optimal flow rates determined by the Van Deemter curve provide a good starting point. From the VanDeemter curve for 1.7 µm particles, the optimal linear velocity is between approximately 3–7 mm/sec.[7] For ikey Separation Device internal diameters of 150 µm and 300 µm, this translates to the flow rates listed in Table 2. It is important to note that the optimum flow rate will also be dependent on the focus of the work. For example, if throughput is the most important factor in an analysis, a higher flow rate might be used to decrease the overall run time even if it is outside of the theoretical optimal range. Conversely, if sensitivity is the ultimate goal, a lower flow rate may be used to increase the ionization efficiency,[8] thus increasing the MS signal.

Table 2. Optimal flow rates for 150 µm and 300 µm iKey separation devices.

	Optimal Flow Rate range
150 µm iKey	1 –4 µL/min
300 µm iKey	4–16 µL/min

CONCLUSIONS

The successful development of a bioanalytical method utilizing direct injection with the ionKey/MS System is dependent on a number of variables. The initial sample matrix and consequently the resulting sample preparation method used need to eliminate any potential for high protein concentrations to be injected onto an iKey Separation Device as it can induce clogging. Due to the inherently low system and column volume of the ionKey/MS System, special attention should also be paid during method development to the injection solvent composition and injection volume to prevent chromatographic distortions such as peak fronting, smearing, and break-through.

References

1. Total Protein: Analyte monograph. The Association for Clinical Biochemistry and Laboratory Medicine. 2012.
2. Doneanu, Catalin E., Rainville, Paul D., Ultrasensitive Quantification Assay for Oxytocin in Human Plasma Using the ionKey/MS System. Application Note 720005011EN. February 2016.
3. Doneanu, Catalin E., Rainville, Paul D., Robustness of the ionKey/MS System in the Analysis of Pharmaceutical Compounds in Biological Fluids. Application Note 720004953EN. February 2016.
4. 4McAvoy, T., Lassman, M.E., Spellman, D.S., Ke, Z., Howell, B.J., Wong, O., Zhu, L., Tanen, M., Struyk, A., Laterza, O.F. Quantification of Tau in Cerebrospinal Fluid by Immunoaffinity Enrichment and Tandem Mass Spectrometry. Clin Chem 2014; 60(4): 683–689.
5. Roman, Gregory T., Johnson, Jay S., Aleylyunas, Yun W., Wrona, Mark, Murphy, James P., Achieving Greater Sensitivity, Throughput, and Robustness with IonKey MS and Trapping. White Paper 720005610EN. May 2016.
6. Snyder, Llyod R., Kirkland, Jospeh J., Dolan, John W., Introduction to Modern Liquid Chromatography, 3rd Edition. Hoboken: Wiley & Sons, 2010. Print.
7. Fountain, Kenneth J., Plumb, Robert S., Diehl, Diane M., Grumbach, Eric S., Chromatographic System Requirements for Exploiting the Full Potential of Sub-2-µm Porous Particle LC. Application Note 720002619EN. April 2008.
8. Murphy, J.P., Johnson, J., Rainville, P.D., Enhancing Mass Spectrometry Sensitivity by Reducing Chromatographic Flow Rates. White Paper 720004967EN. February 2014.

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