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Analytical strategies for LC-MS-based targeted metabolomics

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Abstract

Recent advances in mass spectrometry are enabling improved analysis of endogenous metabolites. Here we discuss several issues relevant to developing liquid chromatography-electrospray ionization-mass spectrometry methods for targeted metabolomics (i.e., quantitative analysis of dozens to hundreds of specific metabolites). Sample preparation and liquid chromatography approaches are discussed, with an eye towards the challenge of dealing with a diversity of metabolite classes in parallel. Evidence is presented that heated electrospray ionization (ESI) generally gives improved signal compared to the more traditional unheated ESI. Applicability to targeted metabolomics of triple-quadrupole mass spectrometry operating in multiple reaction monitoring (MRM) mode and high mass-resolution full scan mass spectrometry (e.g., time-of-flight, Orbitrap) are described. We suggest that both are viable solutions, with MRM preferred when targeting a more limited number of analytes, and full scan preferred for its potential ability to bridge targeted and untargeted metabolomics.

Introduction

Metabolomics, in its most ambitious global form, tries to comprehensively analyze all known and unknown metabolites in a given biological sample [1]. Targeted metabolomics has the more modest goal of quantitating selected metabolites, most typically dozens to hundreds of known compounds. This requires the ability to differentiate the targeted analytes from other interfering compounds, which may be achieved based on chemical shift in a nuclear magnetic resonance (NMR) spectrum, mass-to-charge ratio on a mass spectrometer (MS), retention time in chromatography, or a combination thereof. NMR has several notable advantages relative to MS—it can deal with the biofluids without the need for sample preparation and it produces signals that correlate directly and linearly with compound abundance [2]. However, NMR has relatively low sensitivity, and accordingly only the most abundant species can generally be detected [3]. On the other hand, mass spectrometry, when combined with effective sample preparation and chromatographic separation, has high sensitivity and specificity, as well as good dynamic range [4–7].

There has been tremendous progress in mass spectrometry-based metabolomics recently, leaving researchers with a variety of choices for chromatographic separation, ionization, and mass spectrometric analysis. Separations may be achieved by gas chromatography (GC) [8], capillary electrophoresis (CE) [9], or liquid chromatography (LC) [10], with LC approaches continuously evolving (e.g., to include capillary monolithic chromatography [11–14] and ultra

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performance liquid chromatography [13,15–18]). In conjunction with liquid chromatography, ionization may be achieved using either electrospray ionization (ESI), atmosphere pressure chemical ionization (APCI), or atmospheric pressure photoionization (APPI) [19]. Mass spectrometer options include quadrupoles and ion traps which offer good sensitivity but limited resolving power [20], or higher mass-resolution instruments such as time-of-flight (TOF) [21,22], Fourier transform ion cyclotron resonance (FTICR) [23,24] or Orbitrap [25,26]. The mass spectrometer can also be arranged in a tandem configuration, such as a triple quadrupole mass spectrometer. Different types of analyzer can also be combined to form a hybrid mass spectrometer [20], such as a quadrupole-TOF (Q-TOF) instrument or an ion trap-Orbitrap (currently commercially available solely as the LTQ-Orbitrap from Thermo Fischer Scientific).

This brief review focuses on targeted metabolomics using liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). We do not intend to give a comprehensive review of all aspects of metabolomics (upon which there are already a number of excellent reviews [5,27–32]), nor do we directly address issues relating to absolute (rather than relative) metabolite quantitation (although the concepts described herein, when combined with either an external standard curve or isotope-labeled internal standards, can be used to provide absolute quantitation [33–35]). Largely drawing from our own experience, we discuss four technical issues: the choices of sample preparation procedure, chromatographic separation methods, electrospray ionization approach (heated versus unheated), and mass spectrometry technology.

Basics of sample preparation

While urine is sufficiently rich in metabolites to enable direct analysis (often after diluting in a volume of water selected to maintain a fixed salt concentration across samples), in most biological samples, metabolites of interest comprise a small minority of the starting material. Accordingly, prior to metabolomic analysis, some enrichment for metabolites is generally desirable. This processing step should ideally quantitatively retain metabolites of interest, which may span a wide range of chemical properties. It should also be fast, convenient, and avoid conditions likely to lead to metabolite degradation or interconversion. For serum or plasma, precipitation of protein using cold methanol is a simple but effective method [36].

For analysis of metabolites from cells or tissues, a concern is speed of quenching of metabolism. In live cells, the half-lives of biologically important metabolites, such as ATP or glutamine, can be on the order of a few seconds (and sometimes less), and seemingly innocuous steps such as pelleting of cells via centrifugation can lead to dramatic alterations in intracellular metabolite levels. Accordingly, rapid cooling of the cells (e.g., by mixing of cultured cells with cold organic solvent [37], or grabbing of tissue samples using liquid nitrogen-temperature tongs [38,39] is commonly employed to stop metabolism. Alternatively, rapid quenching of enzymatic activity via heat or acid denaturation can be used, if the analytes of interest are adequately heat or acid stable [40].

Once metabolism is quenched, metabolites must be released from cells. The most appropriate means of extraction depends greatly on both the sample type and the targeted metabolites of greatest interest. For example, extraction of nucleotide triphosphates from *Escherichia coli* or yeast, but not mammalian cells, is greatly enhanced in mixtures of acetonitrile:methanol:water relative to methanol:water alone [7,41]. For many other metabolites, however, methanol:water is adequate and results in cleaner samples than acetonitrile:methanol:water, which less fully precipitates protein and lipids. For tissue samples, many protocols involve freezing of the sample in liquid nitrogen, grinding to a powder, and subsequent extraction.

Once an extract is obtained, care must be taken to preserve its integrity prior to LC-MS analysis. For cell or tissue samples, which often contain redox active or multiply phosphorylated compounds, this can be a major concern. In our laboratory, we typically store extracts at 4°C

and analyze them within 24 h of their generation, and yet more rapidly if particularly labile species like folates are involved.

Chromatographic separation

Metabolomics deals with a diversity of small molecules that differ greatly in their physical and chemical properties of size, polarity/hydrophobicity, and charge. While no single chromatographic method is ideal for all classes of metabolites, we have found that two methods—one for positive ionization mode and one for negative ionization mode (described below), provide a reasonable breadth of coverage. The route by which we arrived at this approach exemplifies some of the tradeoffs in targeted metabolomics.

Our initial approach was a reversed-phase chromatography method, on a C18 Fusion-RP (Phenomenex) column with acidic mobile phase [42]. This method worked adequately for ~100 compounds in positive ion mode. However, many polar compounds did not retain on this column, eluting near the void volume, and nucleotide triphosphate compounds like ATP did not elute as well defined peaks. Subsequently, we compared the performance of nine different chromatography approaches involving seven different column chemistries [10]. This study identified hydrophilic interaction chromatography (HILIC) [43–47] on an aminopropyl column as an effective method to separate a broad range of cellular metabolites including amino acids, nucleosides, nucleotides, coenzyme A derivatives, carboxylic acids, and sugar-phosphates. By separating compounds into a 40-min positive ion run and a 50-min negative ion run, a total of ~140 compounds can be readily measured with a total running time of 90 minutes, using only one mass spectrometer. The same column chemistry can also be used to analyze additional classes of molecules, such as folates, which themselves include > 50 chemical species [48]. This method is a reasonable alternative for those occasions where limited mass spectrometry resources are available.

Recent reports have demonstrated that reversed-phase chromatography with an amine ion pairing agent is a useful method for separation of a broad range of negatively charged metabolites, including nucleotides, sugar phosphates, and carboxylic acids [49–52]. These methods utilize a volatile cationic compound, such as tributylamine [51] or hexylamine [50], to form ion pairs with negatively charged analytes, improving retention and separation on a C18 column. We performed a systematic comparison between our HILIC method and a variant of the reversed-phase, ion-pairing method of Luo *et al.* [51], using both compound standards and cellular extracts under identical mass spectrometry conditions. The ion pairing chromatography in general offered better separation and higher signal for negatively charged metabolites (Fig. 1). This improved sensitivity may in part be due to improved separation leading to reduced ion suppression by co-eluting compounds. We found that this method generally did not work well in positive ionization mode, due to poor retention of amine-containing compounds and ion suppression effects by tributylamine.

Based on this study, we arrived at the dual chromatography method approach of HILIC chromatography in conjunction with positive mode ionization, and reversed-phase chromatography with tributylamine as an ion-pairing agent in conjunction with negative mode ionization, running on two separate LC-MS systems. The LC conditions for the positive mode have been previously reported [10]: an aminopropyl column with acetonitrile and pH 9.45 aqueous buffer as the mobile phases and a running time of 40 minutes. The negative mode LC method uses a Synergi Hydro column (4 μ m particle size, 150 $\times$ 2 mm, from Phenomenex, Torrance, CA), with solvent A being 10 mM tributylamine + 15mM acetic acid in 97:3 water:methanol, and solvent B being 100% methanol. The flow rate is 200 μ l/min and running time is 50 minutes. The gradient is t = 0 min, 0% B; t = 5 min, 0% B; t = 10 min, 20% B; t = 20 min, 20% B; t = 35 min, 65% B; t = 38 min, 95% B; t = 42 min, 95% B; t = 43 min, 0% B;

t = 50 min, 0% B. This dual chromatography methodology enables quantitation of approximately 250 water-soluble metabolites of validated identity including amino acids and derivatives, sugar phosphates, nucleotides, coenzyme A and derivatives, and carboxylic acids, with the number of quantifiable compound largely limited by the availability of standards, which are used for confirmation of targeted compound identity based on retention time and (for MS/MS approaches) mass spectrometry fragmentation pattern. Due to recent acquisition of several hundred additional standards from the Human Metabolome Database [53], we anticipate generating methods covering a yet greater number of validated known compounds.

In contrast to the success in coupling ion pairing chromatography with negative ionization mode, there have been a limited number of studies exploring the possibility of coupling ion pairing with positive ionization mode. Because amino ion pairing agents work well with only with negatively charged compounds, a different kind of ion pairing agent is needed for positively charged metabolites. Volatile perfluorinated acids such as trifluoroacetic acid (TFA) have been used in the separation of peptides [54]. Recently Hsieh *et al.* reported the use of two similar ion pairing agents, heptafluorobutyric acid and nonafluoropentanoic acid in the mobile phase to improve retention and separation on a reversed-phase column, using APCI for ionization [55]. Unfortunately, these volatile perfluorinated acids may cause ion suppression in ESI [56]. More work is needed to find a proper ion pairing agent for positively charged metabolites in ESI.

A variety of additional alternative analytical techniques for metabolomics have been reported in the literature. Many researchers used multiple analytical platforms in parallel in order to detect as many compounds as possible. For examples, Sabatine *et al.* used three different chromatographic modes for the analysis of plasma samples [57]. Amino acids and amines were separated on a reversed-phase column at mobile phase pH 4, sugars and nucleotides by normal phase chromatography at mobile phase pH 11, and organic acids by reversed-phase chromatography at pH 6. A total of 477 multiple reaction monitorings were performed through 6 analytical methods for each sample. More recently, van der Werf *et al.* developed a comprehensive metabolic platform that utilizes three GC-MS methods and three LC-MS methods [58]. When applying this platform to the analysis of the metabolome of *E. coli*, more than 400 compounds were detected.

In addition to using multiple chromatographic methods in parallel, two-dimensional chromatography approaches have also been described. In these methods the two chromatographic separations occur serially, and the chromatographic modes are orthogonal—they separate based upon different characteristics, in a manner similar to a 2D-nanoLC method, now widely used in proteomics for the separation of peptides [59]. Kennedy and colleagues developed a two-dimensional liquid chromatography (2D-LC) method for metabolomics [60]. In this approach, the samples were first separated on a strong anion exchange column, with fractions released to the reversed-phase for further separation by pulses of incrementally higher ionic strength. When this method was applied to the analysis of islets of Langerhans, roughly 200 peaks were detected [61]. The main disadvantage of this approach is the long analysis time, as each fraction from the ion exchange column must be separated sequentially via reversed-phase.

Other notable developments in LC include the use of monolithic capillary columns [11–14], high temperature LC [62–64], and ultra performance liquid chromatography (UPLC, i.e., pressure > 400 bar to drive flow through columns packed with < 2 μ m diameter particles [13,15–18]). Recently, Guillarme *et al.* systematically evaluated each of these three approaches compared to conventional LC [13]. They found that each of the approaches provided at least comparable quantitative precision and accuracy to conventional LC while also expediting analysis, with the greatest gains obtained with UPLC.

On occasions in which metabolites of interests are at very low abundance or have poor ionization, it is often possible to increase the signal by derivatizing the compound(s) of interest, prior to running the sample by LC-MS. Our laboratory routinely applies several derivatization procedures for this purpose. These include the derivatization of thiol compounds such as cysteine and homocysteine which usually do not ionize very well, using S-methyl methanethiosulfonate (MMTS) [65]. The derivatized compounds give substantially improved signal using the HILIC method in positive ionization mode. Similarly, reaction of amino acids with the benzylchloroformate gives improved retention in reversed-phase mode and negative mode ionization. Many other derivatizations have been reported in the literature [29,58,66, 67] and could be used to enhance the signal for specific classes of compounds at the expense of potentially impairing quantitative reliability (e.g., due to incomplete reactions or side reactions). For enhancing class-specific signals, a particularly promising approach involves schemes that simultaneously derivatize and capture metabolites on the surface of a bead from which they can subsequently be released and analyzed [68].

Unheated electrospray versus heated electrospray ionization

Metabolites eluting from the chromatographic column enter the source region of the mass spectrometer where they are ionized by electrospray ionization (ESI). While ESI source designs can vary in many respects, one important distinction is between unheated and heated ESI. Thermo Fisher Scientific's TSQ Quantum series of triple quadrupole mass spectrometers may be equipped with either an unheated or a heated ESI source (HESI). In the unheated ESI source, the sample solution exits the tip of a metal needle to form a fine spray, with nitrogen at ambient temperature as the sheath gas and auxiliary gas to assist ionization. The HESI source is similar, but includes a heating device in the ESI probe body that heats the auxiliary gas to temperatures between 200 and 600 °C. When the coaxial flow of heated nitrogen gas intersects with the charged droplets emitted from the metal needle, these droplets evaporate more rapidly than using ambient nitrogen, thereby increasing the ionization efficiency. The Turbo V™ Source on Applied Biosystems's API 4000 instrument also includes a heating element.

Recently, we performed a small experiment to investigate the utility of HESI for targeted quantitative metabolomics. We tested both an *E. coli* cellular extract and 20 metabolite standards on two different mass spectrometers, Thermo Scientific TSQ Quantum Ultra instruments with unheated or heated ESI sources and an Applied Biosystems API 4000 instrument with Turbo V™ source. The standards included 10 compounds in positive ionization mode (glycine, serine, proline, glutamine, glutamate, methionine, arginine, AMP, IMP, and riboflavin), and 10 compounds in negative ionization mode (threonine, succinate, malate, orotic acid, phosphoenolpyruvate, glycerol-3-phosphate, 3-phosphoglycerate, citrate, glucose-6-phosphate, NAD⁺). The standards were diluted to concentrations of 5, 50, 500 and 5000 ng/mL. The LC conditions were as previously reported for the HILIC aminopropyl column, with same LC gradient applied for both positive and negative modes [10]. Compounds were detected in multiple reaction monitoring mode. Analyses of the compound standards at different concentrations enabled approximate evaluation of sensitivity (i.e., limit of detection defined as the lowest concentration at which the signal to noise is at least 5) and linearity (i.e., R²). In addition, compound standards at 500 ng/mL, as well as a cellular extract sample, were injected four times to evaluate reproducibility, defined as the relative standard deviation (RSD) for raw ion count signals observed from the four injections.

In general, HESI provided enhanced sensitivity compared with the unheated ESI source on the TSQ Quantum Ultra. Most of the 20 compound standards showed a 5-fold or greater increase in terms of absolute ion counts for HESI versus unheated ESI. While the noise also increased in most cases, the overall signal-to-noise ratios were nevertheless improved. This is illustrated in Fig. 2a, where we show a 25-fold increase in terms of absolute ion counts for HESI vs.

unheated ESI for a methionine standard at 50 ng/mL, accompanied by a less dramatic increase in noise. This increase in signal was also seen for cell extract. The sensitivity benefit of a heated electrospray source was also evident in API 4000 instrument, which had excellent overall performance.

The increased performance for a HESI source may be understood in terms of the electrospray ionization mechanism. Electrospray involves solvent molecules in the initial droplets evaporating quickly to expose the charged analyte of interest [69]. Ionization efficiency depends in part on how fast the evaporation process takes place, and how close it comes to completion. Applied heat decreases surface tension and expedites solvent evaporation, improving nebulization and desolvation, and increasing ion release from droplets [70,71].

We also want to note that the performance using a HESI source is compound dependent. Not every tested compound showed a large improvement in signal. This is illustrated in Fig. 2b, where we compare the results of HESI and unheated ESI for the pyridine nucleotide NAD⁺ from a cellular extract. For this compound, HESI does not improve signal-to-noise, perhaps due to heat-induced decomposition of some of the NAD⁺ initially present. If certain compounds decompose as a result of HESI, this may introduce quantitative artifacts. Thus, caution remains warranted in application of HESI to labile metabolites until further testing is conducted.

Multiple reaction monitoring versus high resolution full scan MS

The triple quadrupole mass spectrometer has been the standard workhorse in the quantitation of small molecules and metabolites, as it offers good sensitivity, reproducibility, and a broad dynamic range. For metabolomics, it is typically used in multiple reaction monitoring (MRM) mode, where the collision energy and product ion mass-to-charge ratio are pre-optimized for each analyte of interest to give the best signal. Instrumentally, three quadrupoles are arranged in series. The first quadrupole selects the parent ion of interest, the second quadrupole works as a collision cell to fragment the parent ion, and the third quadrupole isolates the proper product ion. When multiple metabolites are being analyzed, this process is repeated for each compound in a cyclic manner. Because each MRM scan takes a certain amount of time (the scan or dwell time), each analyte is only monitored intermittently. Chromatographic peak width divided by the time to cycle through the programmed set of MRM scan events determines the number of data points available to define a MRM chromatogram peak.

Current triple quadrupole mass spectrometers, such as Thermo Fisher Scientific's TSQ Quantum Ultra instrument and AppliedBiosystems' API 4000 instrument, typically work adequately with a scan time of ~ 50 ms. For the simultaneous measurements of ~50 compounds, this means a data point for each compound every 2.5 s, which provides (for a chromatography peak width of 30 s) ~ 12 data points across the peak, enough to give reliable peak quantitation [30]. With narrower chromatographic peaks, such as those produced by a UPLC, or a larger number of compound MRMs, the time between data points will decrease the reliability of peak quantitation. One possible solution to this problem is to reduce the scan time; however, this can compromise signal-to-noise and reproducibility. Indeed, preliminary testing on our instrument suggests that while it is possible to reduce the scan time to 10 ms, the reproducibility is significantly worse than with a 50 ms scan. Although the effect of faster scanning may be instrument and analyte-dependent, some decrement in performance is expected with faster scanning, because the number of ions in the quadrupole is roughly proportional to the scan time. Less scan time means fewer ions are detected, thus higher variation of the signal, with the signal-to-noise ratio theoretically proportional to the square root of scan time. This is a particularly serious problem for low abundance ions. When using MRM, a more promising approach is dividing the chromatography run into time segments, with MRMs for specific metabolites localized to the time window in which they elute chromatographically.

One alternative approach to using a triple-quadrupole MS in MRM mode is to perform full scan MS. Full scans on a low resolution instrument, such as a single quadrupole mass spectrometer (typical resolving power of 1000, as measured by $m/\Delta m$, where Δm is full width at half maximum), while a viable option, are generally suboptimal for low molecular weight metabolites because of potential interference from solvent ions, adducts, metabolites with the same nominal mass, and environmental contaminants. Burial of the signal from metabolite of interest in background noise impedes quantitation, especially for low abundance analytes. Full scan MS on an instrument with high mass resolution mitigates many of these concerns, as mass-based separation will generally be achieved for all compounds except isomers.

FT-ICR remains the MS method with the greatest mass-resolving power ($> 500,000$) [23,24, 72]. However, time-of-flight (TOF, resolving power 10,000) [21,22] and Thermo Fisher Scientific's Orbitrap instruments (resolving power 100,000) [25,26] offer the advantages of lower cost, greater ease of maintenance and use, and easier coupling to LC by ESI. In addition, Orbitrap offers excellent mass accuracy, in the range of 1 – 2 ppm. It is typically coupled with a linear ion trap to enable determination of the fragmentation pattern of ions of interest, which is a particularly useful feature for unknown metabolite identification. While the mass accuracy of TOF instruments has historically been in the 5 – 10 ppm range, technological advances in recent years have shown that TOF can achieve a mass accuracy of 1 – 2 ppm when internally calibrated [22], rendering them competitive with Orbitrap on this dimension.

In order to determine what utility TOF-MS has in the field of targeted metabolomics, we compared the performance of an Agilent 6220 TOF to a triple quadrupole instrument (Thermo Scientific TSQ Quantum Ultra equipped with unheated ESI source) operating in MRM mode. As before, 20 compound standards (10 in positive ion and 10 in negative ion mode) at different concentrations (5, 50, 500, 5000 ng/mL) and a cellular extract were run on both machines. We evaluated the instrument sensitivity by comparing the signal to noise ratio for the same compound at same concentration, and the limit of detection (LOD) defined as the lowest concentration at which the signal to noise ratio is at least 5. Compound standards at 500 ng/mL were run four times to evaluate reproducibility, defined as the relative standard deviation (RSD) for the detected signal from four injections. For the triple-quadrupole MS, while only 10 MRM parameters were needed to detect 10 compounds in each mode, we put additional 40 dummy MRMs to mimic a typical high throughput MRM experiment, thereby somewhat penalizing the performance of the triple quadrupole instrument. On the TOF machine, full scans with a scan time of 1 s were performed.

The results showed that the TOF has good sensitivity and reproducibility, and a reasonable dynamic range. All the standards had reproducibility comparable to the triple quadrupole ($< 10\%$ RSD over 4 injections). Additionally, the TOF sensitivity was comparable to the triple quadrupole with an unheated ESI source (the more optimized triple quadrupole conditions of fewer MRM scans and heated ESI source would presumably result in a substantial sensitivity advantage for the triple quadrupole instrument, although a direct comparison was not conducted). All compounds showed a linear response over 2 to 3 orders of magnitude on the TOF, as shown for glutamate in Fig. 3. These results indicated that TOF, while not necessarily providing fully comparable quantitative performance to MRM scanning, likely has adequate quantitative performance for many targeted metabolomics applications.

The scan time for TOF can be from 0.05 to 1 s. The Orbitrap scan time is mass resolution related, with a resolution of 7500 at a scan time of 0.1 s, 30000 at a scan time of 0.4 s, and 100000 at a scan time of 1.9 s [26]. Full scan MS approaches (TOF, Orbitrap) thus can benefit from a relatively long scan time, compared to the need for fast scanning to accommodate a large number of MRMs on a triple quadrupole instrument. Longer scan times facilitate reliable quantitation, as it allows for detection of a greater number of ions. Additionally, the high mass

accuracy can effectively separate the targeted compound from background ions having the same nominal mass but a different exact mass. Overall, these features make full scan approaches a promising alternative solution for targeted metabolomics, as full scan approaches avoid the problems associated with an excessive numbers of MRM scans and the need to optimize collision energies and product ions for each analyte of interest. Most importantly, full scan approaches provide the opportunity to marry targeted and untargeted metabolomics, where the chemical identity of the metabolites is required for the former but not for the latter. Many metabolites are currently unidentified or not available as standards to purchase. One can envision mining full scan MS data for both (1) quantitative information on targeted analytes of interest and (2) other unanticipated peaks that show large or highly statistically significant changes across biological conditions.

Summary

The biological utility of targeted metabolomic data ultimately depends on the quality of the sample being analyzed, the choice of the metabolites to target, and the selection of analytical modalities to employ. Careful quenching and extraction of metabolism is critical for cell and tissue samples. Effective chromatographic separation is essential when analyzing complex biological samples by LC-MS. A combination of HILIC chromatography in positive ionization mode and reversed-phase ion-pairing chromatography in negative ionization mode provides good coverage for a broad range of metabolites. For certain classes of metabolites, derivatization facilitates separation and ionization. In analyzing dozens of metabolites at once (up to several hundred in a properly segmented LC run), multiple reaction monitoring on a triple quadrupole mass spectrometer is a good technique. For less targeted analyses (involving yet larger number of analytes), and for marrying targeted with global metabolomics, use of high mass-resolution, high mass-accuracy full-scan MS holds promise going forward.

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Figure 1.

Chromatographic traces for selected metabolites in a cellular extract comparing the performance for the HILIC (top panel) and reversed-phase ion pairing chromatographic (bottom panel) methods in negative ionization mode, under identical mass spectrometry conditions. Selected metabolites are: glucose-6-phosphate and unresolved isomers thereof (a), glycerol-3-phosphate (b), orotate (c), NAD^+ (d), pantothenate (e), succinate (f), malate (g), UDP-D-glucose (h), fructose-1,6-biphosphate (i), phosphoenolpyruvate (j), NADH (k), ATP (l). The LC condition for the HILIC mode (aminopropyl column with basic pH mobile phase, running time 50 minutes) has been reported previously [10]. The LC conditions for the ion pairing chromatography (reversed-phase Synergi Hydro column with acidic mobile phase,

running time 50 minutes) can be found in the text. Note that metabolites a, b, f, and g are not separated in HILIC method while they are well separated in ion pairing chromatography method.

Figure 2.

Chromatographic traces for methionine standard at 50 ng/mL in positive ionization mode (a) and NAD^+ in a biological sample in negative mode (b), on identical TSQ Quantum Ultra (Thermo Fisher Scientific) triple quadrupole instruments but with different ion sources, the unheated electrospray source (solid line) and the heated electrospray source (HESI, dashed line). The insets show the corresponding noise/background region. The LC method was HILIC as reported previously [10]. The source conditions for the unheated ESI were: spray voltage +3500 V or -3000 V, sheath gas 30 psi, auxiliary gas 10 psi. The source conditions for the HESI were: spray voltage +2555 V or -2555 V, vaporizer temperature 230 °C, sheath gas 22 psi, auxiliary gas 37 psi.

Figure 3. Linearity test results for glutamate standards on an Agilent TOF instrument (a) and a triple quadrupole instrument (b). Both show a linear response from 5 to 5000 ng/mL with a $R^2 > 0.999$. The LC method was HILIC as reported previously [10].