Application Note

Metabolomics

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# Absolute Quantitation of Fragile Metabolites by Isotope Dilution Mass Spectrometry on the Agilent 6495 Triple Quadrupole LC/MS


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## Introduction

The quantitative analysis of metabolites in biological samples is challenging due to the vast number of metabolites, variation in their physicochemical properties, and the wide range of concentrations in the samples. Adding to this complexity is the degradation of some metabolites in solution or in the source region of a mass spectrometer. Many metabolites are considered fragile, which can lead to poor analytical sensitivity in measurement, unreliable results that are not reproducible, and give poor analyte recovery. Derivatization of metabolites can improve chemical stability to reduce degradation, but adds another step to the analytical workflow, increasing the complexity of the method.

To address the challenge of analyzing fragile unstable analytes, Agilent has redesigned the ion funnel on the Agilent 6495 triple quadrupole LC/MS system, also known as the 6495 LC/TQ. The ion funnel can now tolerate more fragile and thermally labile analytes while drastically improving performance at fast chromatographic peak sampling times. In the updated configuration, the high-pressure ion Funnel (iFunnel) assembly has been converted to an all-metal "open" design that improves gas flow, improves ion beam sampling, and reduces RF heating. The result is improved ion collection leading to higher analytical sensitivity and greater signal robustness.

This application note presents results from a two-part study demonstrating that the iFunnel design preserves well-known fragile analytes.

- The first part of the study compared the response of fragile metabolites between a non-iFunnel LC/TQ system (Agilent 6470 LC/TQ system, product number G6470B) and the Agilent 6495 LC/TQ with redesigned iFunnel (product number G6495C). Both systems have the same ionization source and ion optics assembly, so are a useful way to demonstrate if the response of the fragile metabolites of interest is better on the iFunnel system.
- In the second part of the study, the goal was to investigate the ultimate sensitivity of the fragile metabolites in a complex matrix. A robust quantitative method was developed to determine absolute concentrations of fragile metabolites and report the level of sensitivity that can be achieved.

The data acquisition method used is a well-established targeted dynamic multiple reaction monitoring (dMRM) method for 219 central carbon metabolites in negative ion mode.[1] It is a fully tested, rugged method that provides incredibly stable retention times across large numbers of samples with excellent interday and extended retention-time reproducibility (over 4,000 biological sample injections). However, the original method workflow is intended for relative quantitation measurements.

In this application, the scope of the method was expanded for absolute quantitation using isotope dilution mass spectrometry (IDMS). The advantage of IDMS is that it compensates for ion suppression/matrix effects, as well as any bias from sample preparation and instrument conditions. Disadvantages of IDMS include the cost and availability of isotopically labeled internal standards, especially in an assay with 219 metabolites. To avoid the cost of purchasing individual internal standards, $^{13}C$ incorporated yeast from Cambridge Isotope Labs Inc. (Tewksbury, MA, USA) was used. These cells are grown in $^{13}C$ enriched media and the resulting metabolites in the extract have >99% isotopic enrichment.[2] Using this technique, an excellent response linearity for fragile metabolites over six orders of magnitude, ranging from attomole ($10^{-18}$ mol) to nanomole ($10^{-9}$ mol) concentrations on-column was obtained.

## Experimental

### Instrumentation

A 6495 LC/TQ system equipped with a third-generation iFunnel was used for the separation and detection of anionic and hydrophobic metabolites. The analytical method was developed to achieve good peak shape and for use with complex samples where matrix components may impact method performance. For comparative purposes, a 6470 LC/TQ system without the iFunnel was used.

The method used a gradient between solvent A (97/3 water/methanol with 10 mM tributylamine + 15 mM acetic acid + 3 µM InfinityLab deactivator additive) and solvent B (methanol with 10 mM tributylamine + 15 mM acetic acid + 3 µM InfinityLab deactivator additive). The Agilent ZORBAX Extend C18 column was selected for stability at the pH used with this method.

Both LC/TQ instruments were configured with a guard column and a switching valve program that enabled backflushing of both the column and guard column following each analytical run. This setup effectively cleared any potential matrix contaminants from the guard column, ensuring consistent high-quality peak shapes and superior long-term retention time stability. More detailed information about the chromatography and solvent preparation can be found in the *Metabolomics Dynamic MRM Method Setup Guide*.[2]

To ensure a robust comparison of the two instruments, care was taken not to introduce variation from the samples or sample-introduction technique into either mass spectrometer. Therefore, the same LC stack was used for both instruments, with the same mobile solvent, samples, injection volume, detector offset voltage, etc.

Table 1 shows the source conditions for the 6495 LC/TQ.

### Sample preparation

A yeast cell extract from Cambridge Isotope Labs was used as the sample. Individual $^{12}C$ metabolite standards bought from Sigma-Aldrich were spiked into the sample matrix. For the absolute quantitation study, the protocol from a paper published for the analysis of amino acids was modified to include the addition of $^{13}C$ labeled yeast from Cambridge Isotope Labs.[3] For sample and standard preparation, the volume and the concentration of the $^{13}C$ yeast extracts added to the standards and the samples were kept constant. Calibration curves were generated using the peak area ratios between the $^{13}C$ metabolites and naturally occurring $^{12}C$ metabolites (peak area ratio = $^{13}C_M/^{12}C_M$). Using peak area ratios instead of the absolute peak areas, the linear dynamic range for quantitation increased by several orders of magnitude in most cases.

The list of fragile metabolites used for this study is shown in Table 2.

**Table 1.** Agilent 6495 LC/TQ ion source conditions.

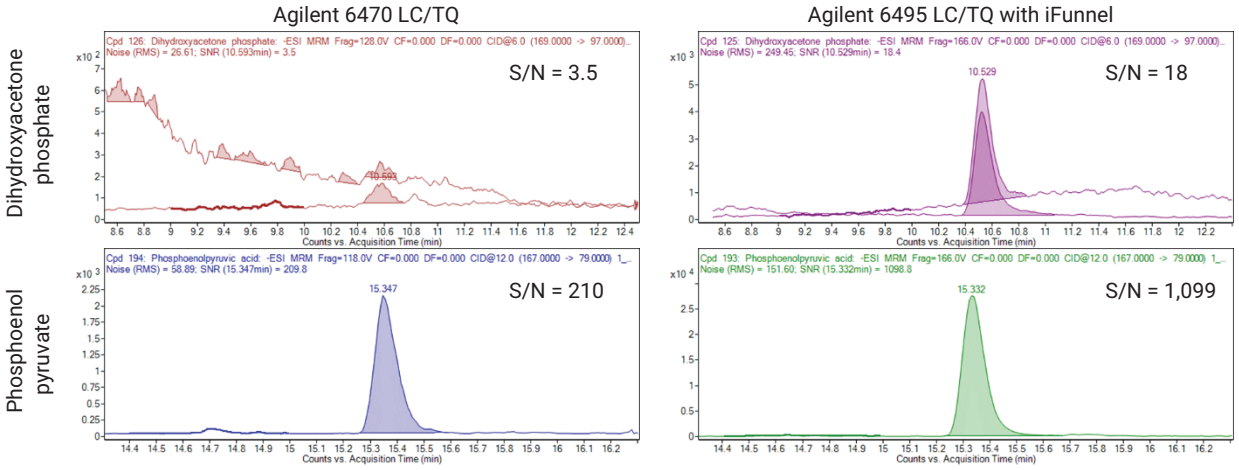
| Parameter | Setting |
|---|---|
| Product Number | G6495C |
| Agilent Jet Stream (AJS) Ion Mode | AJS Negative |
| Gas Temperature | 150 °C |
| Drying Gas Flow | 17 L/min |
| Nebulizer Gas | 45 psi |
| Sheath Gas Temperature | 325 °C |
| Sheath Gas Flow | 12 L/min |
| Capillary Voltage | 2,000 V |
| Nozzle Voltage | 500 V |
| Negative High Pressure Funnel RF | 80 V |
| Negative Low Pressure Funnel RF | 70 V |

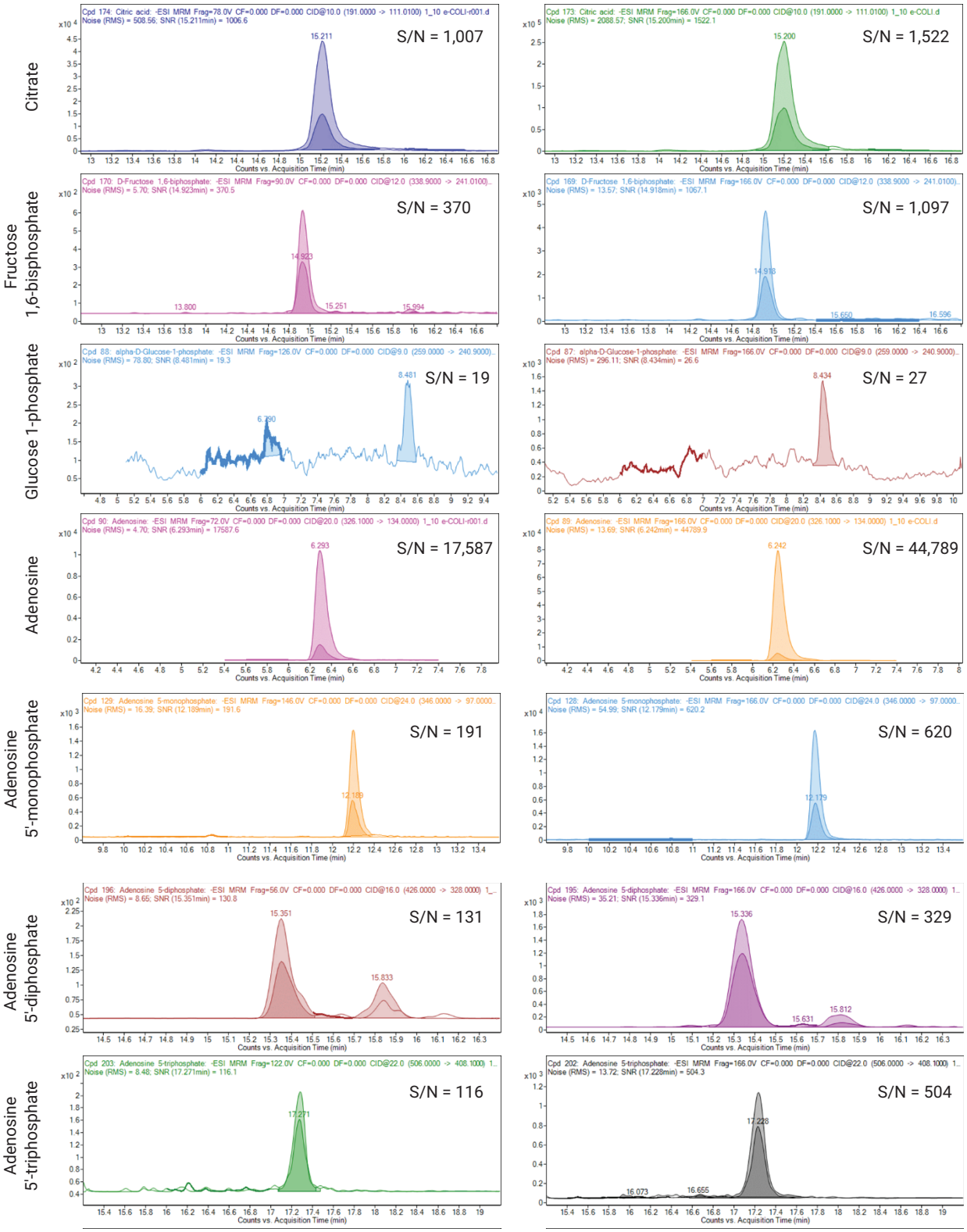
**Table 2.** List of fragile analytes used for the study.

| Central Carbon Metabolism | Nucleosides and Nucleotides |
|---|---|
| Citrate | Adenine |
| Dihydroxyacetone phosphate | Adenosine |
| Fructose 1,6-bisphosphate | Adenosine 5'-monophosphate (AMP) |
| Glucose 1-phosphate | Adenosine 5'-diphosphate (ADP) |
| Lactate | Adenosine 5'-triphosphate (ATP) |
| Phosphoenol pyruvate | Cytidine 5'-monophosphate (CMP) |
| Pyruvate | Deoxycytidine 5'-monophosphate (dCMP) |
| Leucine | Guanine |
| | Thymine |
| | Uracil |

## Results and discussion

For comparison, Figure 1 shows chromatograms for most of the compounds listed in Table 2 obtained using LC/TQ with and without iFunnel. Chromatograms for lactate and leucine are shown in Figures 2 and 3, respectively.




Citrate
Cpd 174: Citric acid: -ESI MRM Frag=78.0V CF=0.000 DF=0.000 CID@10.0 (191.0000 -> 111.0100) 1_10 e-COLI-r001.d
Noise (RMS) = 508.56; SNR (15.211min) = 1006.6
15.211
S/N = 1,007
Cpd 173: Citric acid: -ESI MRM Frag=166.0V CF=0.000 DF=0.000 CID@10.0 (191.0000 -> 111.0100) 1_10 e-COLI.d
Noise (RMS) = 2088.57; SNR (15.200min) = 1522.1
15.200
S/N = 1,522
Fructose 1,6-bisphosphate
Cpd 170: D-Fructose 1,6-biphosphate: -ESI MRM Frag=90.0V CF=0.000 DF=0.000 CID@12.0 (338.9000 -> 241.0100)...
Noise (RMS) = 5.70; SNR (14.923min) = 370.5
14.923
13.800
15.251
15.994
S/N = 370
Cpd 169: D-Fructose 1,6-biphosphate: -ESI MRM Frag=166.0V CF=0.000 DF=0.000 CID@12.0 (338.9000 -> 241.0100)...
Noise (RMS) = 13.57; SNR (14.918min) = 1067.1
14.918
15.650
16.596
S/N = 1,097
Glucose 1-phosphate
Cpd 88: alpha-D-Glucose-1-phosphate: -ESI MRM Frag=126.0V CF=0.000 DF=0.000 CID@9.0 (259.0000 -> 240.9000)...
Noise (RMS) = 78.80; SNR (8.481min) = 19.3
8.481
6.290
S/N = 19
Cpd 87: alpha-D-Glucose-1-phosphate: -ESI MRM Frag=166.0V CF=0.000 DF=0.000 CID@9.0 (259.0000 -> 240.9000)...
Noise (RMS) = 296.11; SNR (8.434min) = 26.6
8.434
S/N = 27
Adenosine
Cpd 90: Adenosine: -ESI MRM Frag=72.0V CF=0.000 DF=0.000 CID@20.0 (326.1000 -> 134.0000) 1_10 e-COLI-r001.d
Noise (RMS) = 4.70; SNR (6.293min) = 17587.6
6.293
S/N = 17,587
Cpd 89: Adenosine: -ESI MRM Frag=166.0V CF=0.000 DF=0.000 CID@20.0 (326.1000 -> 134.0000) 1_10 e-COLI.d
Noise (RMS) = 13.69; SNR (6.242min) = 44789.9
6.242
S/N = 44,789
Adenosine 5'-monophosphate
Cpd 129: Adenosine 5-monophosphate: -ESI MRM Frag=146.0V CF=0.000 DF=0.000 CID@24.0 (346.0000 -> 97.0000...
Noise (RMS) = 16.39; SNR (12.189min) = 191.6
12.189
S/N = 191
Cpd 128: Adenosine 5-monophosphate: -ESI MRM Frag=166.0V CF=0.000 DF=0.000 CID@24.0 (346.0000 -> 97.0000...
Noise (RMS) = 54.99; SNR (12.179min) = 620.2
12.179
S/N = 620
Adenosine 5'-diphosphate
Cpd 196: Adenosine 5-diphosphate: -ESI MRM Frag=56.0V CF=0.000 DF=0.000 CID@16.0 (426.0000 -> 328.0000) 1_...
Noise (RMS) = 8.65; SNR (15.351min) = 130.8
15.351
15.833
S/N = 131
Cpd 195: Adenosine 5-diphosphate: -ESI MRM Frag=166.0V CF=0.000 DF=0.000 CID@16.0 (426.0000 -> 328.0000) 1...
Noise (RMS) = 35.21; SNR (15.336min) = 329.1
15.336
15.631
15.812
S/N = 329
Adenosine 5'-triphosphate
Cpd 203: Adenosine 5-triphosphate: -ESI MRM Frag=122.0V CF=0.000 DF=0.000 CID@22.0 (506.0000 -> 408.1000) 1...
Noise (RMS) = 8.48; SNR (17.271min) = 116.1
17.271
S/N = 116
Cpd 202: Adenosine 5-triphosphate: -ESI MRM Frag=166.0V CF=0.000 DF=0.000 CID@22.0 (506.0000 -> 408.1000) 1...
Noise (RMS) = 13.72; SNR (17.228min) = 504.3
17.228
16.073
16.655
S/N = 504
Counts vs. Acquisition Time (min)

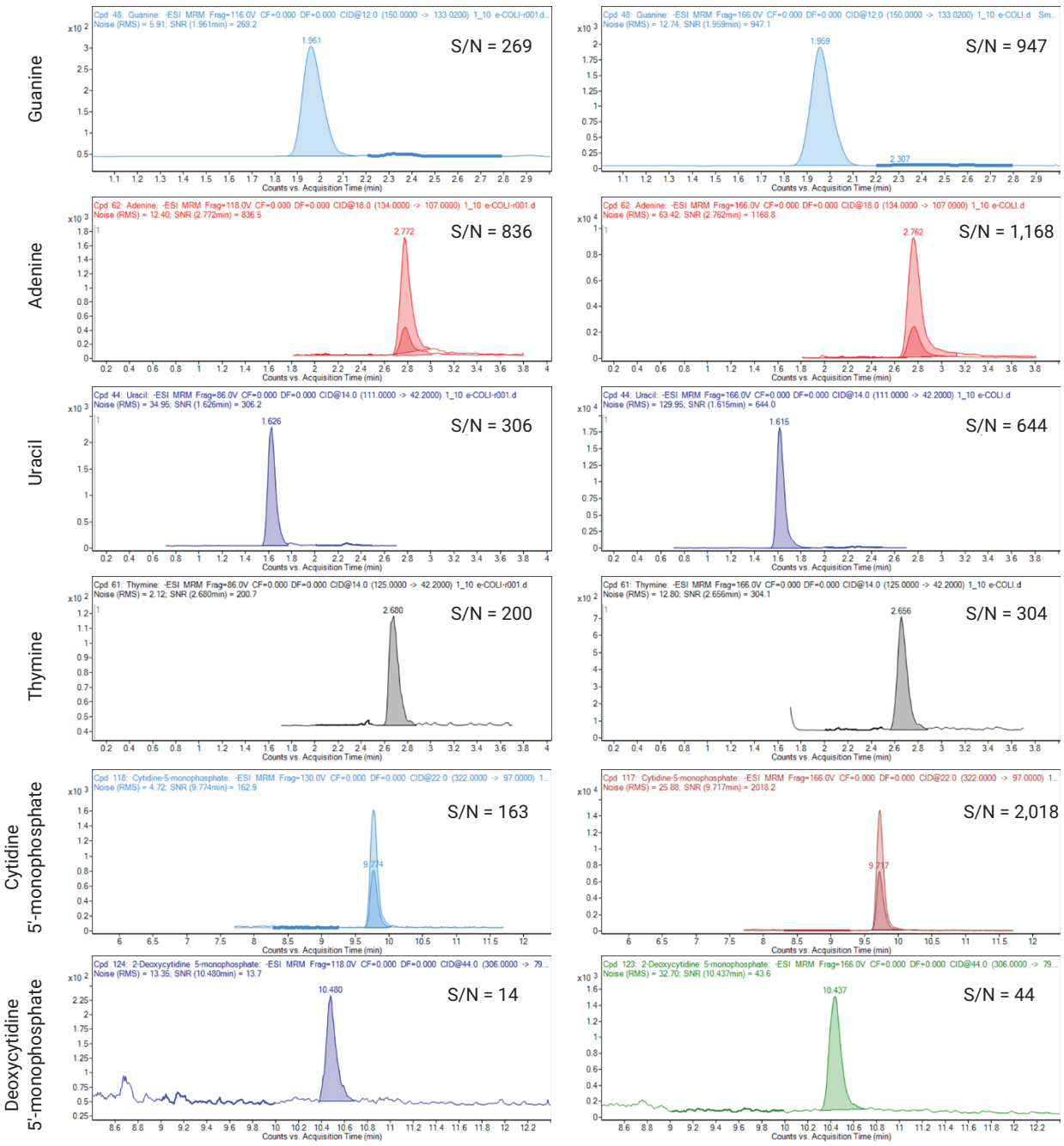



**Figure 1.** Comparison between an Agilent 6470 LC/TQ and the Agilent 6495 LC/TQ for fragile metabolites. The noise region used for S/N calculation is the same for both instruments. Values are rounded to the first decimal.

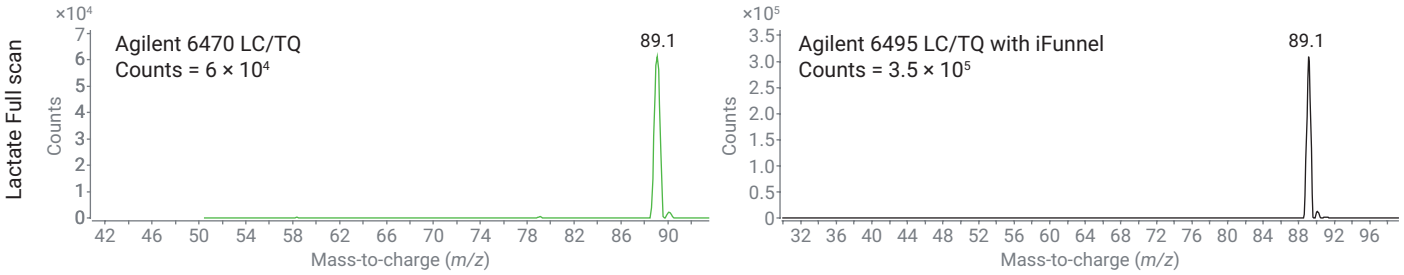



**Figure 2.** Comparison MS full scan spectrum (*m/z* 40 to 100) for lactate. No significant fragments were observed on either instrument.

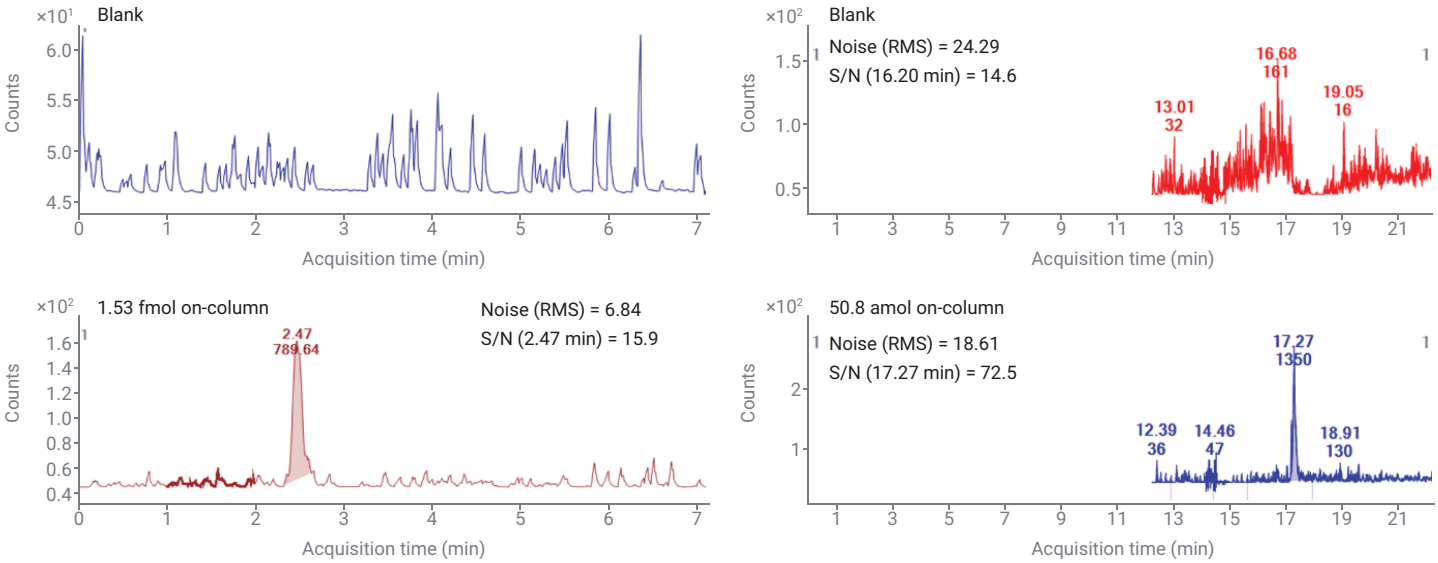



**Figure 3.** Absolute quantitation comparisons. Left: blank versus 1.53 fmol leucine on-column. Right: blank versus 50.8 amol ATP on-column.

An important aspect in the development of these methods for an iFunnel system is the proper tuning of RF amplitude for the High Pressure Funnel (HPF) and Low Pressure Funnel (LPF). RF amplitude that is set too high can improve ion beam collimation and confinement but may deposit additional energy onto the analytes by stimulating weak bonds, causing premature fragmentation in this region. If RF amplitude is set too low, there will be decreased internal energy deposition, but poor ion beam confinement and collimation, resulting in weak analytical sensitivity. For this experiment, the settings provided in Figure 1 were a good balance for ion beam transmission and reduction of premature fragmentation. In essence, the full scan spectra from both instruments were the same, as shown in Figure 2.

A visual comparison of the chromatograms shown in Figure 1 demonstrates the improvement in signal-to-noise (S/N) with the iFunnel instrument. The computed S/N value for each analyte is shown in each figure, based on the root-mean-square (RMS) noise algorithm using the same time window per comparison. For the RMS noise algorithm, the program calculated the RMS variation of abundance over a defined noise region, taking the maximum of the RMS values over all regions. Improvement in the S/N ratio is analyte-dependent and varied between a slight improvement for the nucleoside bases to a dramatic five to ten-fold improvement for some of the phosphates.

## Absolute quantitation of fragile metabolites

The advantages of using isotopically labeled standards for absolute quantitation are well known.[4] The best approach to reduce discrepancies and reduce variance between sample measurements is to determine the absolute metabolite concentrations by generating calibration curves with internal standards. However, for a panel of hundreds of metabolites, it is costly to purchase individual isotopically labeled standards. It is also tedious to make up

hundreds of solutions that may not be stable over time. Instead, $^{13}C$-labeled yeast extract was spiked into the standards and samples. The use of $^{13}C$ incorporated yeast relies on the natural metabolic processes to incorporate these labeled "standards". Using labeled standards reduces variability, improves precision and accuracy, increases the level of confidence in a reported metabolite, is easier for method development, and is cost-effective.

The procedure for making up standards and samples using $^{13}C$-labeled yeast extract is by a process of double-dilution. Standards for the calibration curve are made by serial dilution in autosampler vials. The next step is to aliquot the same volume of standard from each of these vials into separate vials for each point in the calibration curve. Next, $^{13}C$-labeled yeast extract is added to the vial. The same quantity and volume of extract is used for each of the standards and the samples, making sure that the total volume of each vial remains the same. This approach ensures that the amount of $^{13}C$-labeled yeast extract is held constant for the standards and samples. The observed peak area ratio of the $^{12}C$ and $^{13}C$-labeled analytes is used to generate calibration curves and to calculate the analyte amounts. In this study, the concentration of matrix in each standard and sample was 0.75 mg/mL or $1 \times 10^8$ Pichia Pastoris cells. Absolute quantitation results in Figure 3 show a comparison between the amount on-column versus blank, while Figure 4 shows a comparison between the $^{13}C$ internal standard from the yeast.

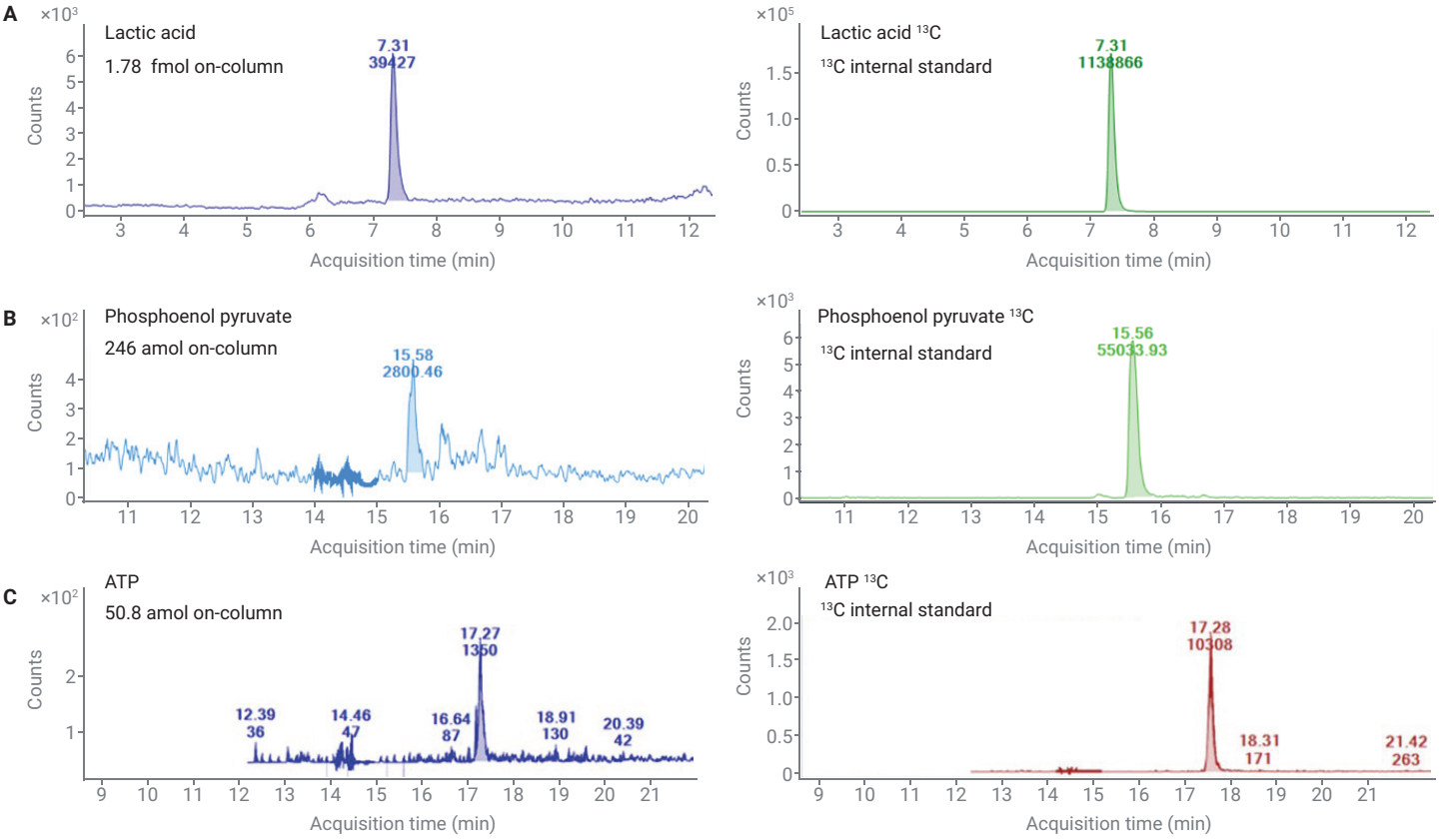
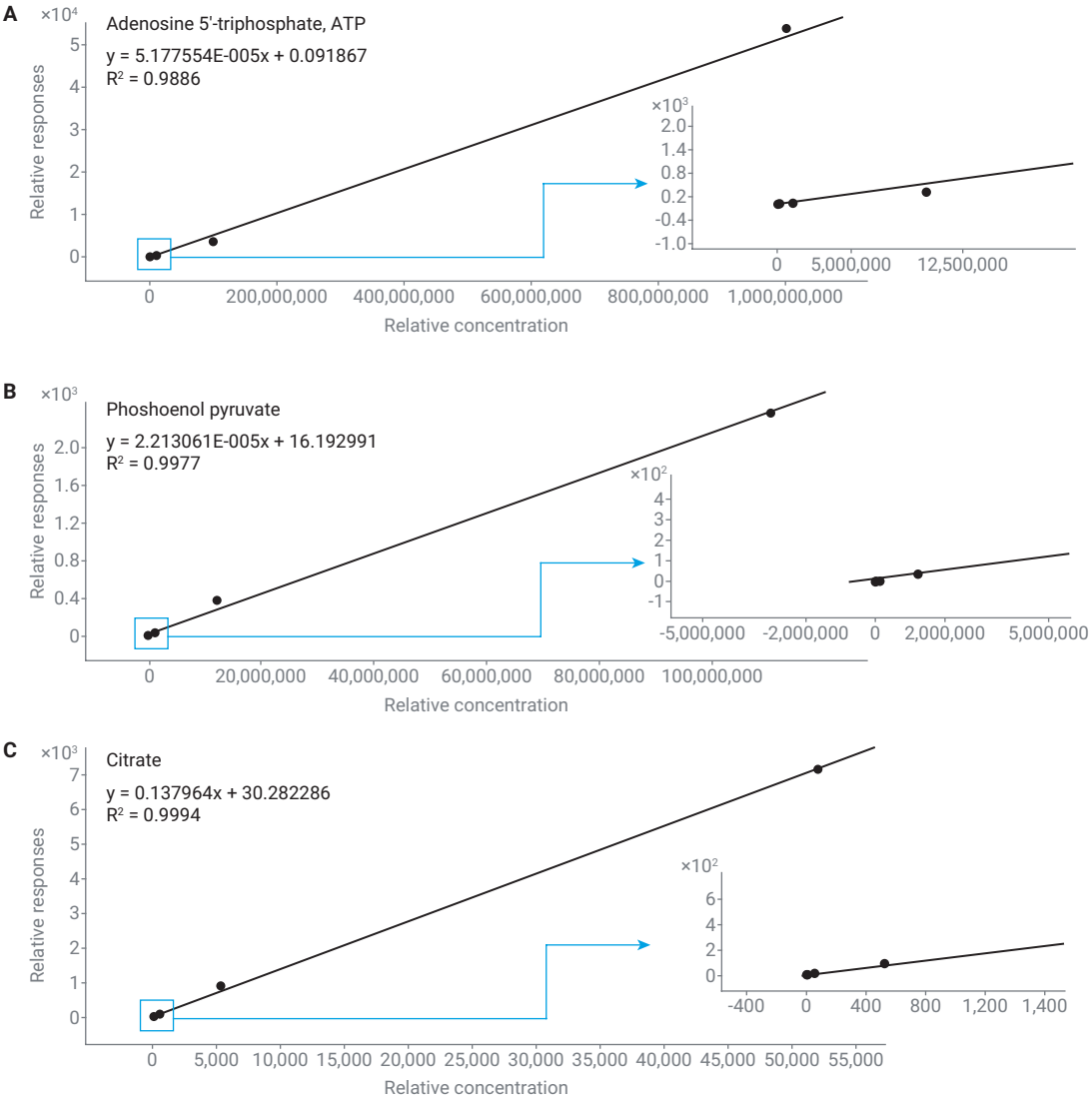



**Figure 4.** Amount on-column versus $^{13}C$-labeled internal standard. (A) 1.78 fmol lactic acid on-column. (B) 246 amol phosphoenol pyruvate on-column. (C) 50.8 amol ATP on-column.

Figure 5 shows calibration curves for some fragile analytes demonstrating the linear dynamic range spanning low-femtomole to nanomole levels, with a correlation coefficient of $R^2$ >0.99.

Metabolites in biological samples are generally characterized by a huge effective range of concentrations, which is a major challenge for analytical instruments. However, the use of ratios for the response of the $^{12}C$-labeled analyte to the ratio of the response of the $^{13}C$-labeled analyte allowed the quantitative linear dynamic range to be extended. The extension to around 6 to 8 orders of magnitude would not be possible when using absolute areas for generating calibration curves. The extended dynamic range of quantitation from attomole or low-femtomole to nanomole levels covers the effective range of analyte concentrations that can be encountered in biological samples. The wide dynamic range also means that metabolites of interest in the sample can be analyzed without the need to further concentrate or dilute the samples.



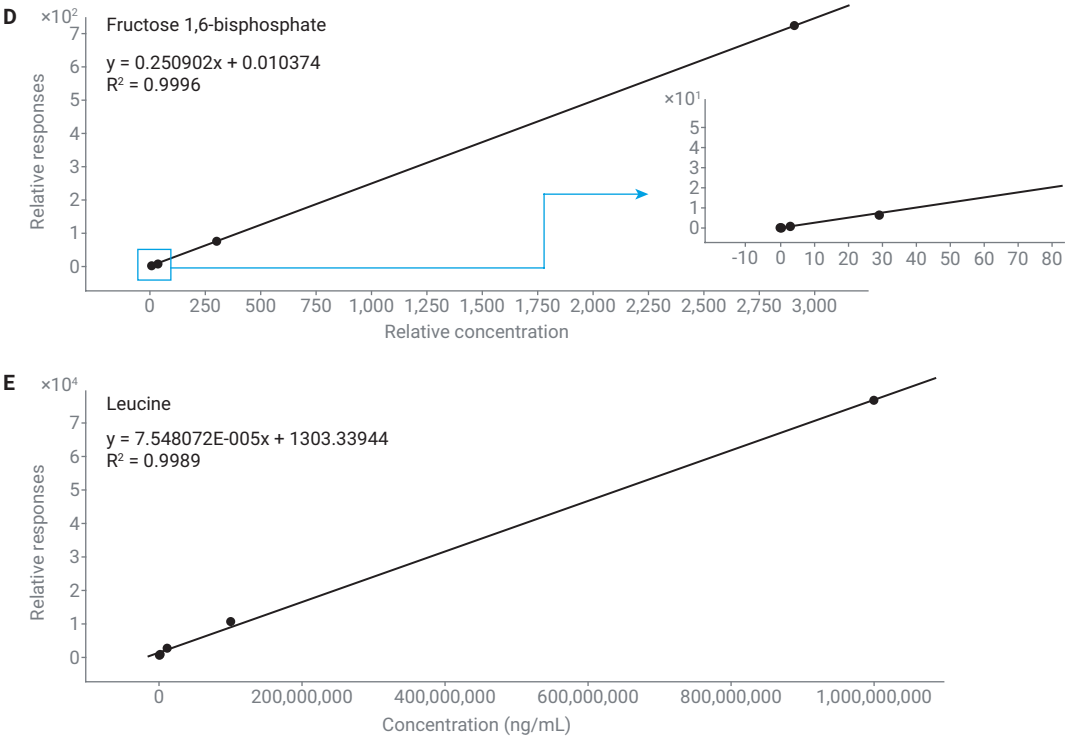



**Figure 5.** Calibration curves for select fragile analytes, spanning concentration ranges between 6 to 8 orders of magnitude.

## Conclusion

In the first part of the study, the performance of the Agilent 6495 LC/TQ with iFunnel was shown to preserve fragile analytes while also improving sensitivity compared to a non-iFunnel Agilent 6470 LC/TQ instrument. The improved response of fragile metabolites is due to the ability of the iFunnel to capture more ions over a wider linear dynamic range.

In the second part of the study, a complex matrix containing various metabolites was analyzed using a highly sensitive, robust quantitative IDMS method. The LC/TQ IDMS method provided absolute concentrations of fragile metabolites. The use of $^{13}C$ incorporated yeast avoided the cost and time-consuming labor of preparing isotopically labeled standards. Using the ratio of the response from the $^{12}C$ analyte to the ratio of the response of the $^{13}C$-labeled analyte, the linear dynamic range of quantitation was extended to between 6 to 8 orders of magnitude.

Compared to traditional workflows, the method reduces sample variability, improves precision and accuracy, increases level of confidence in a reported metabolite, simplifies method development, and is cost-effective. Also, the extended dynamic range of quantitation from attomole/low-femtomole level to nanomole levels covers the wide range of concentrations that are observed in biological samples, reducing the need for further sample concentration or dilution.

## References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