

Poster Reprint

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# Machine learning statistics with chromatography and high-resolution mass spectrometry aid in combatting illegal logging and trafficking of endangered timber species

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### Targeted TQ Workflow for Metabolomics

Targeted metabolomics methods are commonly used to quantify and profile the metabolome in large scale metabolomic studies. Researchers often aim to have the most analytically-sensitive analysis with the most comprehensive list of metabolites to increase biological insights from the samples tested. To achieve this, a Poroshell 120 HILIC-Z column was selected to provide good retention of polar analytes while permitting ionization in both positive and negative mode. It was paired with a 1290 Infinity II Bio LC (Fig. 1) which is coated with MP35N metal alloy to obtain sharp peak shapes and low detection limits for metal-sensitive analytes. A database of over 400 metabolites was built on the 6495C LC/TQ (Fig. 1), which was used to characterize the samples.

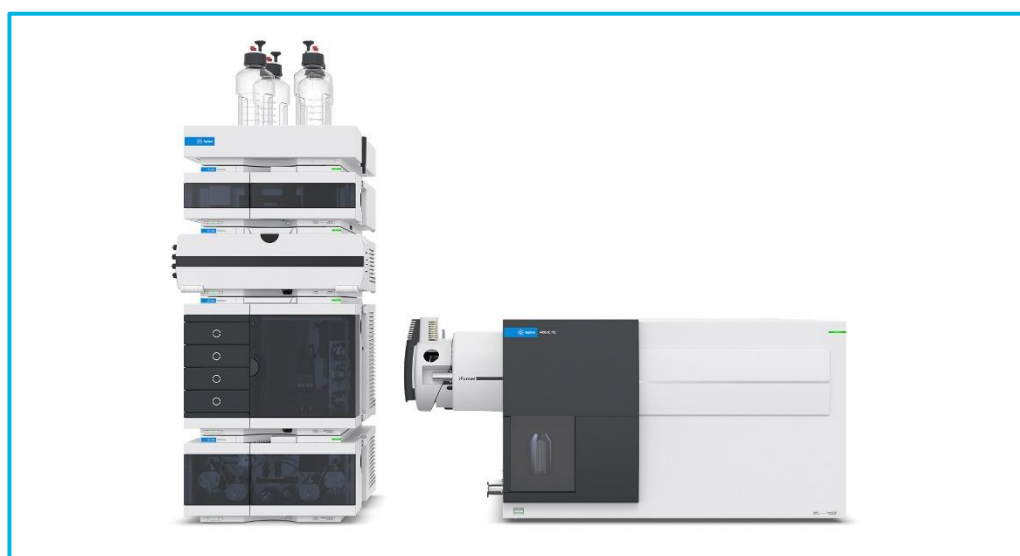


Figure 1: 1290 Infinity II Bio LC (left) shows improved out-of-the box peak shape and detection limits for metal-sensitive metabolites. When coupled with the ion funnel 6495C LC/TQ (right) low detection limits with low RSDs can be achieved even with 5 ms dwell times.

The LC/MS method described herein can be used to analyze any extract of a complex matrix. In the present work, plasma extracts were prepared with the low volume plasma Bravo Metabolomics workbench workflow<sup>1</sup> (Fig. 2) which utilizes the Captiva EMR-Lipid solid phase extraction plate technology to remove proteins and lipids but selectively elute polar metabolites of interest in a reproducible manner.

**The data collected here characterized a pooled bovine plasma sample using a transferable workflow that combined automated sample prep, with robust chromatography, and a sensitive targeted TQ analysis. This comprehensive workflow aims to support biological research in various settings.**

### Emphasis on HILIC robustness and metabolome coverage.

The previously described protocol<sup>1</sup> was used to extract metabolites from 20  $\mu\text{L}$  of a pooled bovine plasma sample (BioIVT). After drying the sample with nitrogen, it was reconstituted in 100  $\mu\text{L}$  85% acetonitrile, spun down to remove any remaining solids, and 2  $\mu\text{L}$  of the supernatant was injected.

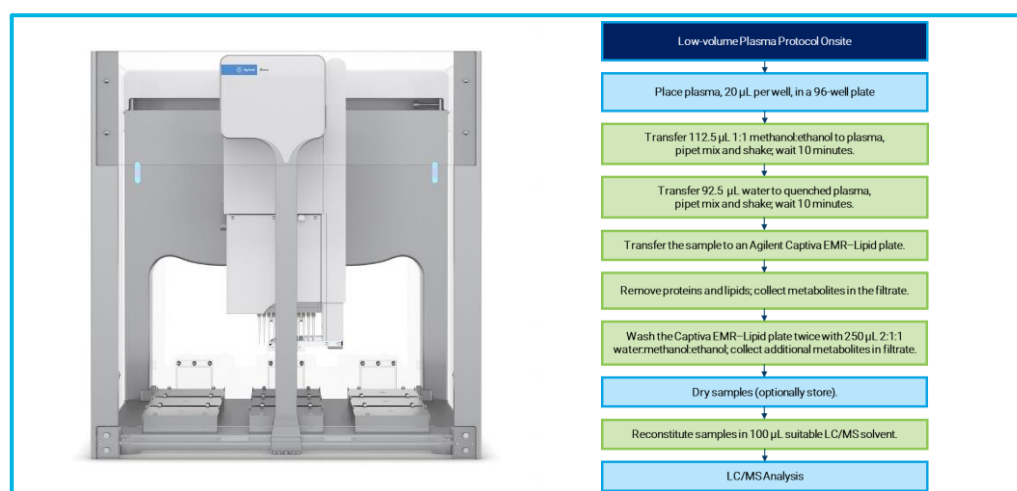


Figure 2: Bravo Metabolomics Workbench (left) used with the low plasma method (right) provide an automated reproducible, low-volume plasma sample prep workflow.

A 2.1 x 150 mm, 2.7  $\mu\text{m}$  Poroshell 120 HILIC-Z (Agilent, PN 683775-924) column was flushed with a standardized phosphorylation procedure. Subsequently, the column was equilibrated for 1 hour with analysis buffers (30:70 A:B). The buffer system was 20 mM, ammonium acetate, pH 9.3 + 5  $\mu\text{M}$  medronic acid in water (mobile phase A) and pure acetonitrile (mobile phase B). A nonlinear gradient was used for analysis- elution times span from 0.5 -16 min over the gradient. Each injection is 24 min including re-equilibration time.

**This buffer system and gradient were optimized for reproducibility of retention times and tested on multiple lots of columns, in different labs, and with different end users to ensure RT alignment and that it is feasible to transfer databases to different labs.**

Standards were used to optimize ion transitions on the 6495C. A dynamic MRM (dMRM) database containing 2 ions transitions for each analyte was built. Analytes in both positive and negative mode were detected, and the most sensitive transitions were kept in the database. With the speed of the 6495C pos/neg switching was used without detriment to the cycle time. Some experiments operated in MRM mode with a fixed dwell time of 5ms while others operated in dMRM mode where the retention time was used to collect the transition only when the analyte eluted.

## Reproducible methods are transferable to other labs and between lab members.

Unlike C18 methods, HILIC has the capability of retaining and separating the most polar analytes without the use of ion pairing reagents (Fig 3, Leucine/Isoleucine).

This is critical since most small metabolites will elute within the first minute on a traditional C18 method. Ion pairing reagents can help retain the analytes however they contaminate LC/MS systems and prevent any analysis in positive mode due to their pre-charged structure. HILIC doesn't require these so metabolites can be tested in both ion modes in the same method which increases the metabolome coverage.

However, generic HILIC chromatography has a reputation to be irreproducible and requires great skill and column treatment steps to perform successfully. This project created a robust method with standardize protocols to ensure HILIC is reproducible for any end user. The chromatography was thoroughly tested with complex samples using different column lots, different instruments, and in various labs (Figure 4). In all cases, the RTs were within expected variation and elute in the dMRM window. Thus, a dMRM method for targeted metabolomics can be routinely used and transferred to other labs.

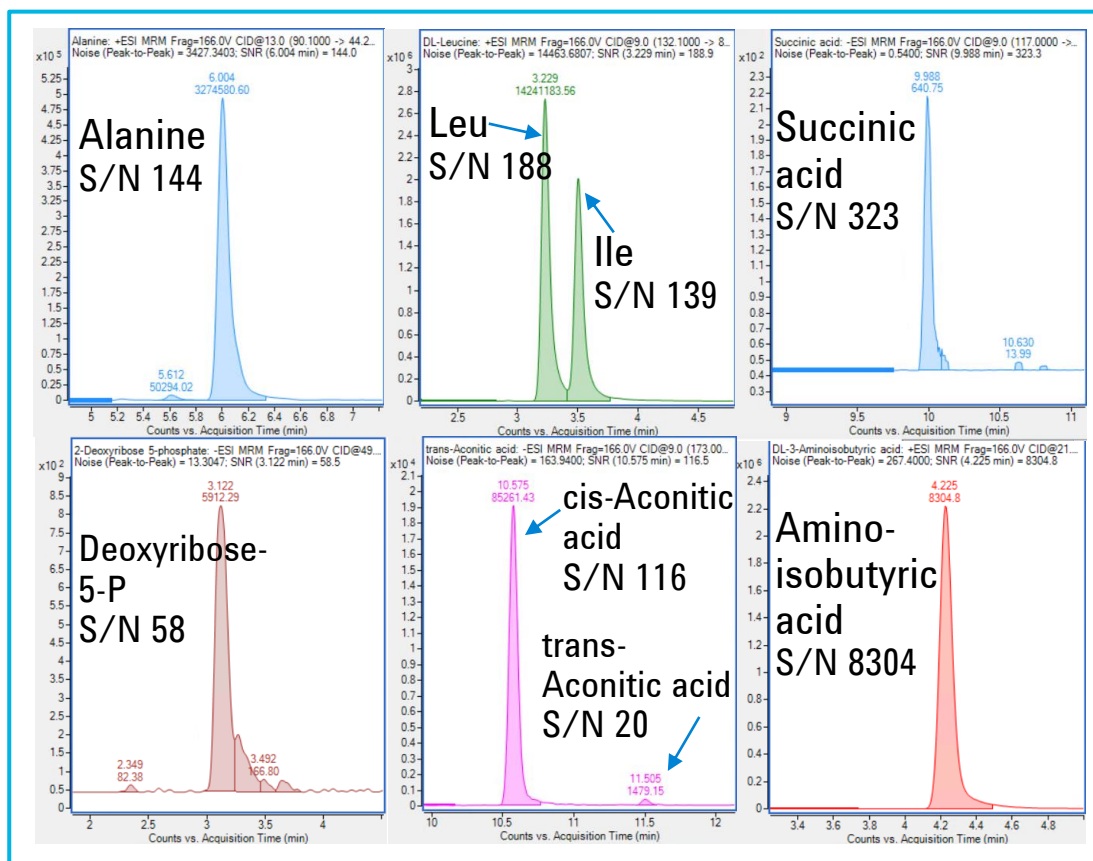


Figure 3: Example chromatograms of analytes in the plasma extract with different chemistries, some are isobaric and have good separation with HILIC. Data was collected at 5 ms dwell times and signal was reproducible with RSDs <10% (n=6). S/N ratios show that the method is fit for purpose with plenty of sensitivity to detect these analytes in complex matrix extract.

## Analytical sensitivity using the 6495C LC/TQ can detect metabolites from Bravo plasma sample with ease.

The methodology was successfully transferred to another lab offsite from where it was initially developed. A scientist with no previous HILIC experience set up and showed good RT alignment with the multicolumn study. In all studies, the RSD of the RTs was under 5% proving reproducible results even when pressure tested.

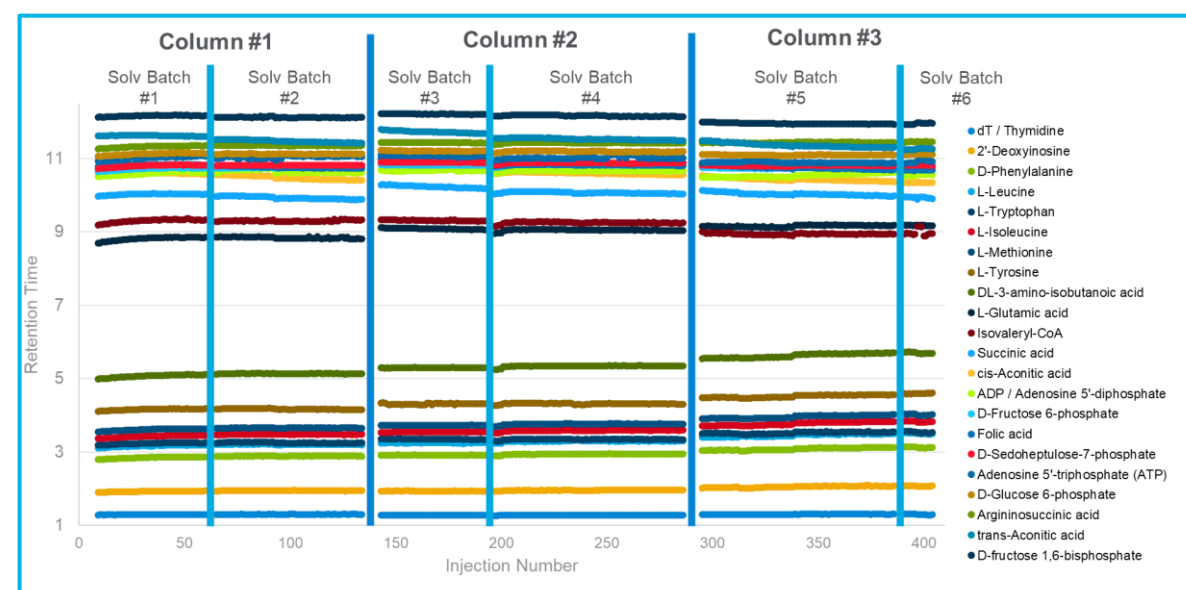


Figure 4: Retention times of different analytes in plasma extract different lots of columns on different days with different batches of solvent and different users preparing the solvents. None of the metabolites had a retention time error outside of the suggested dMRM window. In total, over 400 injections were tested.

The database contains over 400 analytes providing good coverage of amino acids, coenzymes, tricarboxylic acid (TCA) cycle, and glycolysis pathway analytes. Nearly every analyte has two ion transitions for confident identification of a peak. In bovine plasma, over 200 analytes are detectable and can be measured in one injection allowing a wide variety of analytes to be measured.

Targeted metabolomics methods can be used for profiling trends or to obtain quantitative results. When absolute quant is needed, isotopically labeled internal standards can be used for normalization. Here, these standards were used to test the absolute analytical sensitivity of the analytes in matrix. Different concentrations of  $^{15}\text{N}_5$ -Adenosine-5'-diphosphate ( $^{15}\text{N}_5$ -ADP) and  $^{13}\text{C}$ -Phenylalanine ( $^{13}\text{C}$ -Phe) were spiked into pooled bovine plasma extracts. Each sample was injected 4 times using a method with a 5 ms dwell time. 20 fmol of ADP and 1.2 fmol Phe was detected at a quantifiable level with this method. They had an RSD of 10% and 1%, respectively (Fig. 5). Additionally, calibration curves using these LOQs had  $R^2 > 0.98$  for the analytes (n=4).

Note, these results were obtained with a 5 ms dwell time demonstrating the TQ can obtain stable results even with a heavily multiplexed method and complex matrix.



## Methodology can readily be deployed for metabolomics projects in biological research.

The methodology developed here was designed with the principles of analytical sensitivity, versatility, transferability and ease of use. By providing a larger dMRM database with separation of critical biological isomers without ion pairing reagents this method offers more versatility than C18 methods. By taking additional steps to investigate and eliminate key sources of variation, RTs used in the database have proven robustness.

This enabled a successful inter-lab transferability study indicating the method is not only a robust but readily transferrable methodology to quickly enable the user to move from data acquisition to biological insights.

Further, detection limits were investigated if the method can sensitively detect analytes with ease in plasma extract. In the same injection containing isotopically labeled analytes the endogenous analytes are shown in Figure 5. these levels are 20X and 80X higher in ADP and Phe, respectively.

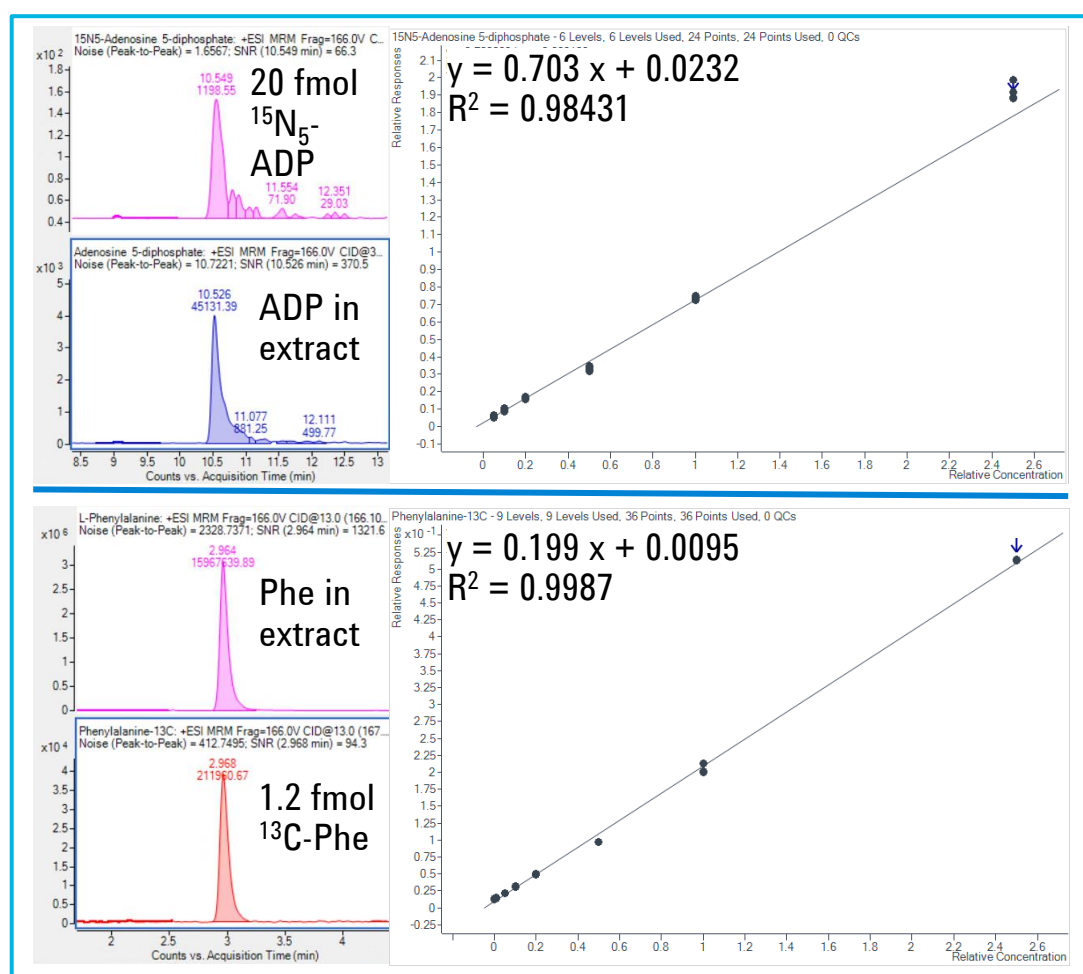


Figure 5: Phosphate containing analytes have good detection limits (top left). 20 fmol of  $^{15}\text{N}_5$ -ADP is detected in matrix with 10% RSD ( $n=4$ ) when using 5 ms dwell time. The endogenous amount detected is 20X more. The calibration curve (top right) is linear with  $R^2$  of 0.98. Amino acids had better detection limits (bottom left). 1.2 fmol of  $^{13}\text{C}$ -Phe is detectable in matrix with 1% RSD ( $n=4$ ) when using 5 ms dwell time. The endogenous amount detected is 80X more. The calibration curve (top right) is linear with a  $R^2$  of 0.99.

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## Targeted methods can cover a large number of analytes when TQ measures analytes reproducibly and sensitively at low dwell times.

The dMRM database is focused on metabolites that help the investigator maximize their biological insights by focusing on coverage of critical pathways. Specifically, the TCA, glycolytic pathways, and critical Coenzymes give insights to key cellular energetic processes. Additional coverage of amino acids and nucleotides provides a good understanding of key biological building blocks that can indicate metabolic status or health of the cells or organism. This coverage is supported by a LC/TQ that can sensitively and reproducibly measure hundreds of desired analytes of interest in complex samples.

## Conclusions

### Jump-start biological research projects with a comprehensive workflow that includes sample prep, robust chromatography and extensive dMRM database

Metabolites were effectively extracted bovine plasma using the Metabolomics Bravo protocol.

- The sample was clean enough to be injected hundreds of times on a HILIC-Z column without degradation to the chromatography.
- The 6495C LC/TQ provided needed analytical sensitivity to detect 200+ analytes in plasma reproducibly. Limits of quantitation vary but are in the fmol range in matrix for the isotopically labeled standards.
- The RTs have proven stability when pressure tested allowing for a dMRM database to be transferred to other sites for method adoption.
- Additional matrices should be explored with the 400+ metabolite dMRM database.

## References

<sup>1</sup>Sartain, M, Gomez, M, Van de Bittner, G, Shu, H. Enabling Automated, Low-Volume Plasma Metabolite Extraction with the Agilent Bravo Platform. Agilent Application Note 5994-2156EN.