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Sharpen Your Peaks: Novel Column Hardware for Improved HILIC Polar Metabolite Chromatography

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Introduction

HILIC Metabolomics Methodology on Novel Deactivated Stainless-Steel Hardware.

Polar metabolomic separations by HILIC chromatography have been optimized and standardized in recent years to allow for new metabolomics users to collect high quality data. These methods utilized column conditioning procedures to decrease adhesion of metal sensitive analytes (e.g., ATP) to the surface of the column and LC material. Advances in Bio LC materials have provided iron free flow paths to reduce prep time, improve peak shape, and provide increased sensitivity for metabolites. In this work, a new deactivated stainless-steel (SS) column and InfinityLab Quick Change Bio Inline Filter is shown to further remove iron from the flow path, reduce column prep time by an order of magnitude and create simplified methods that produce reproducible HILIC retention times and metabolomic results allowing even novice users entry into metabolomics.

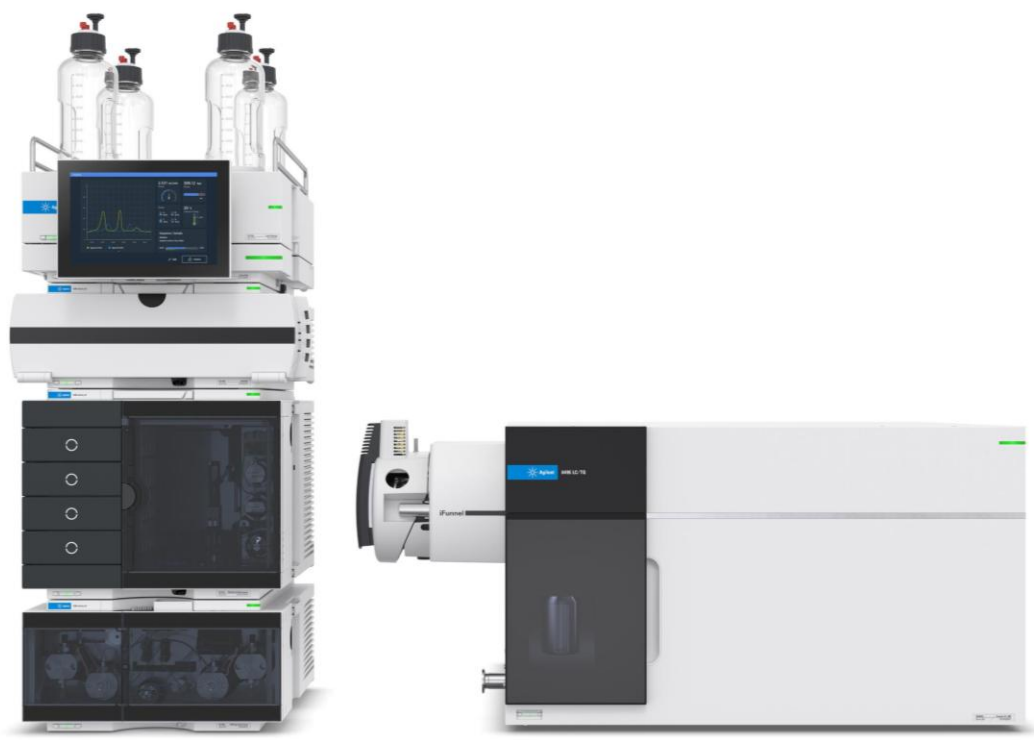


Figure 1. 6495D LC/TQ with 1290 Infinity III Bio LC.

This experiment was carried out on the 6495D LC/TQ featuring an ion funnel with improved ion optics, maximizing sensitivity for fragile analytes. Hundreds of compounds can be analyzed in both positive and negative polarities with precision in a single injection because of the speed of the 6495D LC/TQ. This makes the 6495D aptly suited for profiling a large scope of the metabolome with a targeted approach.

Experimental

Three different evaluations were carried out to compare the performance of the deactivated SS and standard SS columns, (1) direct comparison, (2) column longevity, and (3) inter-lab evaluation.

Each evaluation was carried out utilizing the same standard HILIC metabolomics methodology, previously reported.[1] This reliable method boasts predictable and stable retention times allowing for the use of a dMRM method to employ a database with 500+ metabolites (Table 1).

(1) Direct comparison: Three different column scenarios were compared, deactivated SS and SS with standard phosphonation protocols (>30 hours) and deactivated SS with abbreviated column preparation and no phosphonation (~10 hours).[2] Cell and plasma metabolomic extracts prepared with SPE were tested.[3] Each column was treated the same apart from the column preparation (Figure 3).

(2) Longevity: The deactivated SS column prepared with abbreviated column preparation protocols was tested continuously for 14 days and 700+ plasma injections, where 173 different compounds were continuously evaluated between 6 different buffer batches and 12 different sample aliquots.

(3) Inter-Lab Study: Four different labs carried out an evaluation, testing the abbreviated column preparation method and deactivated SS column and inline filter. Results were compared from 40 plasma injections. Experience of the labs ranged from first time Agilent HILIC method user to expert user. Detailed instructions and methods were provided.

Data was analyzed using MassHunter Quantitative Analysis 12.1 and MassHunter Qualitative Analysis 12.0.

Table 1. LC Conditions with 1290 Infinity III Bio LC.

LC Conditions		
Column	HILIC-Z column, 2.1 x 150 mm, 2.7 µm, 2.1 x 150 mm in SS and deactivated SS versions, guard column (821725-926), InfinityLab Quick Change Bio Inline Filter (5067-1608)	
Column temp	15 °C	
Injection	4 µL	
Autosampler	5 °C	
Mobile phase	A = 20mM ammonium acetate, pH 9.3 + 5µM medronic acid in H_2O B = pure ACN	
Flow rate	0.400 mL/min	
Gradient program	Time	%B
	0.0	90
	1.00	90
	8.00	78
	12.00	60
	15.00	10
	18.00	10
	19.00	90
	23.00	90



Results and Discussion

Maintaining Good Peak Shape and Separation with Deactivated Column.

Direct comparison of the phosphonated SS column and non-phosphonated deactivated SS column showed comparable peak shape and resolution of target analytes (Figure 2).

Longevity of HILIC Column with Ammonium Acetate Buffers and Complex Sample.

Standard protocol for the Agilent HILIC Metabolomics method recommends the use of an inline filter and guard column to further the lifetime of the analytical column. That setup was utilized for all analyses described.

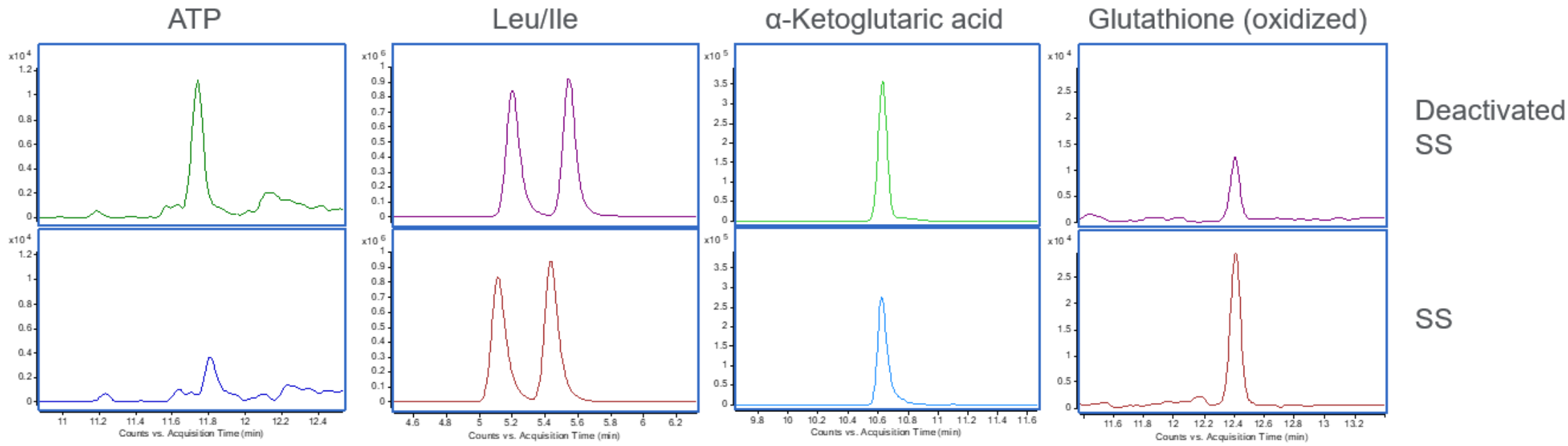


Figure 2. Comparison of metabolites in cell samples between SS and deactivated SS columns. Both display detection with good peak shape and similar retention times, independent of differences in column preparation.

Another important aspect was consistent RTs between the columns to allow usage of existing dMRM databases with newly applied column technology. Apparent in Figure 2, RTs were maintained between the columns despite deactivated SS and alternative column preparation.

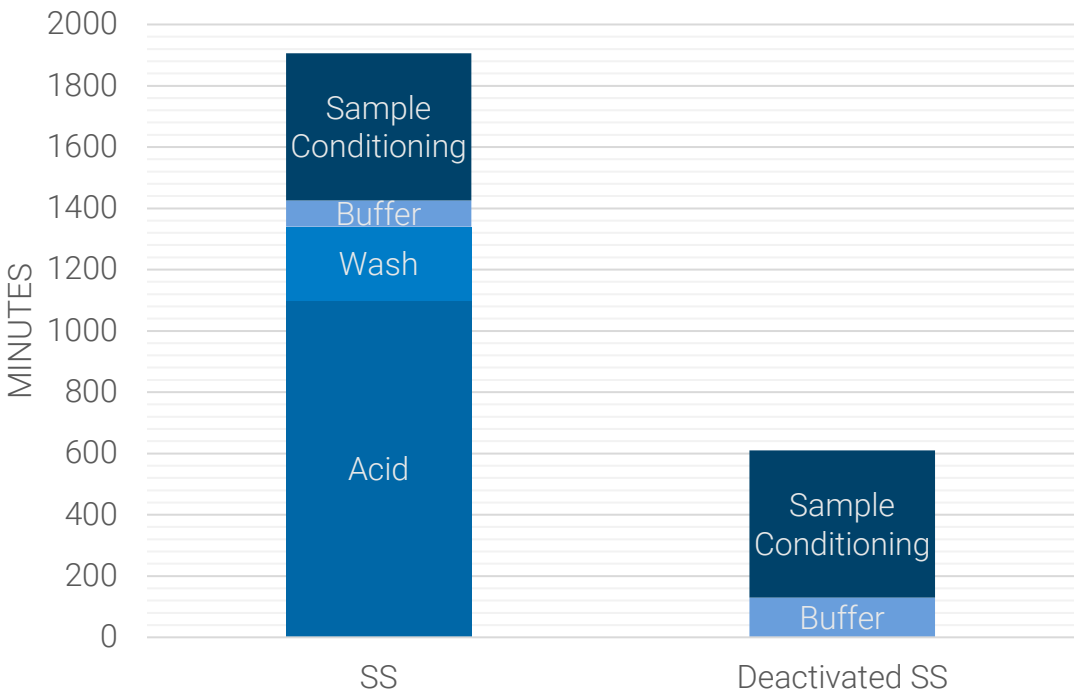


Figure 3. Difference in column preparation time from unboxing to running samples.

Standard protocol describes 1100 minutes of phosphonation with 50mM pyrophosphoric acid. This only needs to be done upon opening a new column, but without this step column preparation is reduced to just ~10 hours, significantly lowering the time between column box opening and biologically relevant samples running (Figure 3).

Along with the new deactivated SS column this study also tested a new bio inline filter. Over 700 injections were made on the same column constantly analyzing over 170 different compounds in the HILIC dMRM database within a 2-minute window.

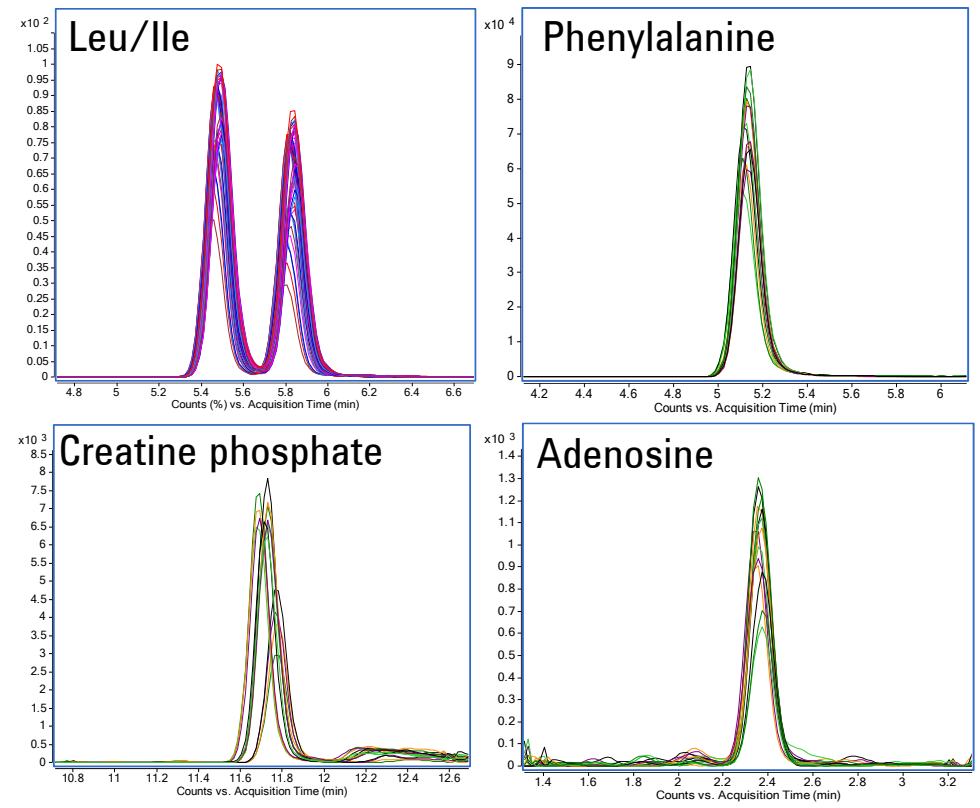


Figure 4. Overlayed compounds from longevity study, subset overlay between 1 and 700.

Across 700+ injections RTs of the compounds remained well within the set instrument acquisition window. As shown in Figure 4, the visual overlay is consistent between the first and last injection. The RSDs of all RTs remain low with over 1/2 of the compounds being below 1% RSD (Figure 5).

Results and Discussion

Inter-Lab Study: 4 Sites, 4 Systems, 4 Scientists. Transferable Retention Times and Method Stability.

Method instructions and materials were sent to four different labs across the United States to test the transferability of the methodology, as well as the newly available hardware. Scientists completed an independent analysis of sample aliquots utilizing the 6495D LC/TQ and 1290 Infinity III Bio LC.

Overall, the combination of testing the new deactivated SS analytical column hardware and Bio Inline Filter showed strong support for incorporation into current workflows with the advantage of reducing SS interaction. Further, the Agilent HILIC Metabolomics method proved to be easily transferable and reproducible in different locations and over extended injections.

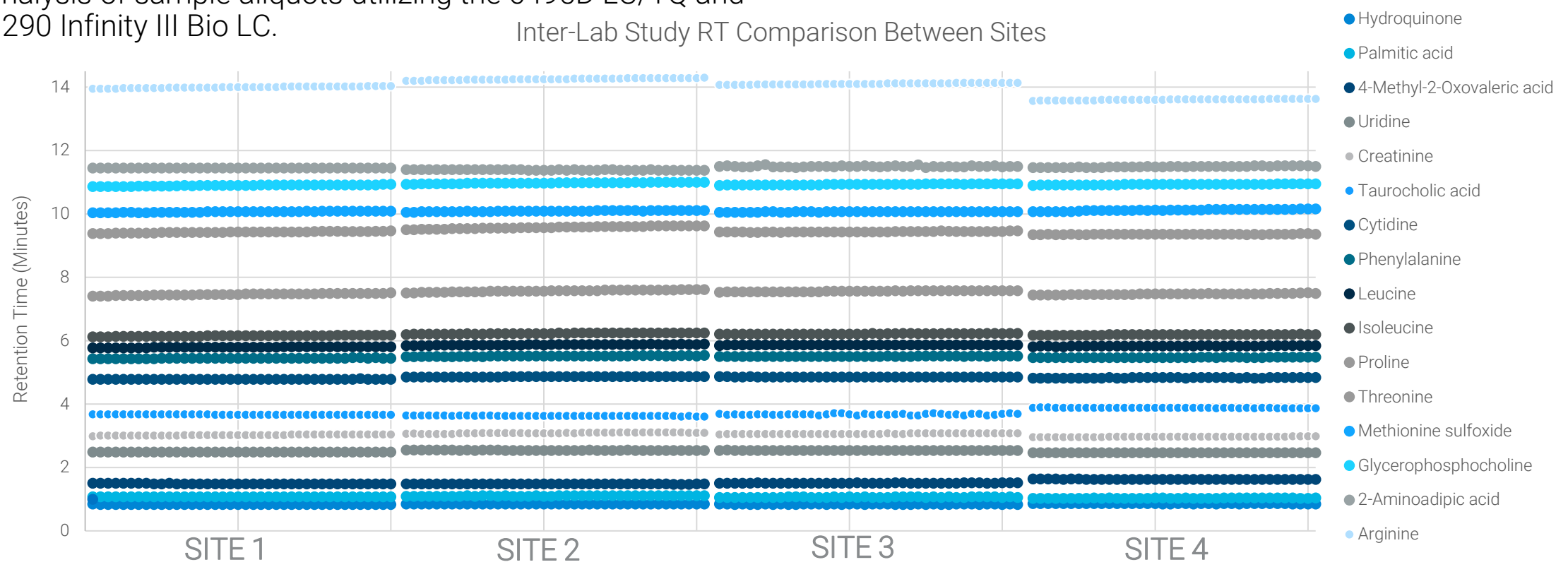


Figure 6. Retention time evaluation of 16 metabolites in plasma across the entire RT range between four sites. All four sites captured analytes within the set acquisition window.

Between lab sites most compounds were detected within the instrument acquisition windows (Figure 6). Of the over 150 compounds analyzed between the different sites the majority had a RT RSD below 5%. This consistency highlights the stability of the current Agilent HILIC Metabolomics method, but also the effective incorporation of the new hardware into applications currently using SS solutions but looking for optimization that could come from adding a more inert path.

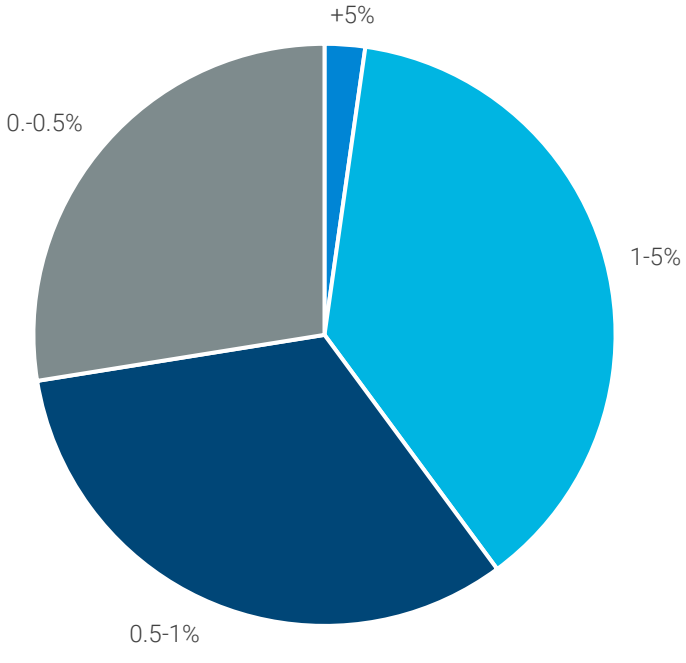


Figure 5. RT RSD profile for longevity experiment with over 700 injections included.

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Conclusions

Implementation of new deactivated SS hardware showing consistent retention times from current SS offering with improved set up and usability.

Direct comparison, longevity and inter-lab study completed to show performance of new hardware by using reliable HILIC Metabolomics method. The new hardware included:

- Deactivated and bio materials for reduction of adverse interactions with metal sensitive analytes
- 6495D LC/TQ and 1290 Infinity III Bio LC

References

[1] Yannell, KE et al. An End-to-End Targeted Metabolomics Workflow. Agilent Application Note 5994-5628EN. 2023.

[2] Yannell, KE et al. Mastering HILIC-Z Separation for Polar Analytes. Agilent Application Note 5994-5949EN. 2023.

[3] Sartain, M et al. Enabling Automated, Low-Volume Plasma Metabolite Extraction with the Agilent Bravo Platform. Agilent Application Note 5994-2156EN. 2020.

The authors declare no competing financial interest.

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