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How does your HILIC Method Stack Up? Optimization and Comparison of Common HILIC Columns, Mobile Phases, and Additives for Metabolomics

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Introduction

Reproducible HILIC Chromatography for Targeted and Untargeted Metabolomics Workflows.

Targeted and untargeted metabolomics methods facilitate measurement of metabolites across a wide dynamic range. Previously described is a HILIC polar metabolite workflow including the Bravo Sample Prep Platform with cells or plasma, a 1290 Infinity II Bio LC for improved performance of metal sensitive analytes, and either a 6495D LC/TQ mass spectrometer with a database of 500+ metabolites[1] and retention times for targeted analysis or a 6546 LC/Q-TOF mass spectrometer with a matching library of over 500 metabolites with spectra and retention times for untargeted discovery.[2] The performance and high reproducibility of the HILIC chromatography is essential for both the targeted and untargeted metabolomics workflows.

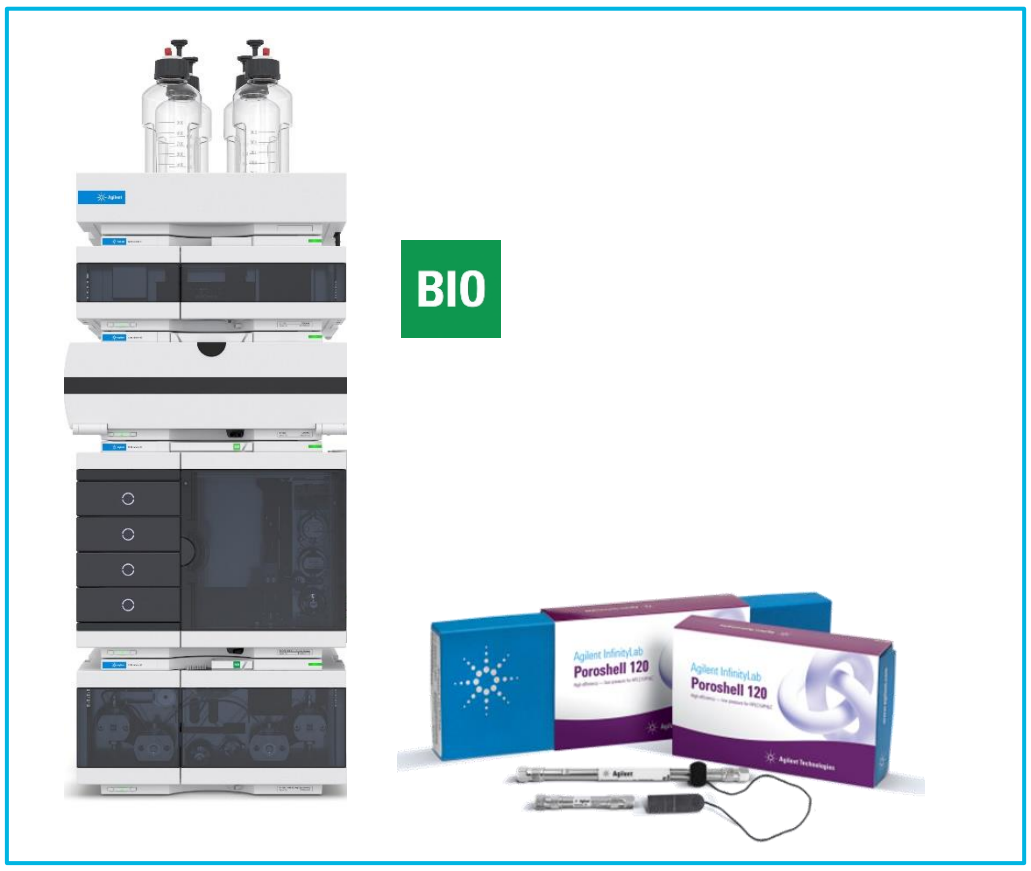

Figure 1. 1290 Infinity II Bio LC system with Poroshell 120 HILIC-Z columns.

Mobile Phase Composition and Reliable Columns are the Key to HILIC Chromatography.

Despite its reputation, reproducible retention times can be achieved for HILIC metabolomics analysis with the right column, column conditioning, and mobile phase conditions. Agilent Poroshell 120 HILIC-Z columns, when phosphonated following a previously outlined protocol,[3] and using pH-adjusted buffered aqueous mobile phase show reproducible retention times across days, users, and column production lots. Reproducible retention times allow for narrower dMRM windows for LC/TQ analyses and smaller data alignment windows for untargeted LC/Q-TOF studies.

Experimental

Prominent Metabolite Standards and Plasma Metabolite Extracts to Monitor Method Performance.

Individual analyte standards were acquired from Sigma Aldrich and prepared as equimolar solutions in 70:20:10 acetonitrile:water:methanol. Metabolite extract from plasma (20µL, bovine, BioIVT) were prepared using a Captiva EMR-Lipid SPE plate and optimized protocol for the Bravo Sample Prep Platform.[4,5]

Prepared standard mixtures and plasma extracts were analyzed under a series of chromatographic conditions using the 1290 Infinity II Bio LC, fit with a HILIC-Z column (2.7µm or 1.9µm, 2.1 x 100mm or 150m). Data for other Agilent HILIC columns not shown due to poor inherent separation for key metabolite classes.

Table 1. Optimized HILIC Metabolomics LC Conditions

LC Conditions	
Column	Agilent Poroshell 120 HILIC-Z, 2.1 x 150mm, 1.9µm PN: 683675-924
Column temperature	15°C
Autosampler temperature	4°C
Needle wash	Multiwash, 3s each: IPA, water, ACN
Mobile phase	A: 20mM ammonium acetate, pH 9.3 + 5µM medronic acid B: Pure acetonitrile
Flow rate	0.4 mL/min or 0.6 mL/min
Gradient program	Time %B 0.0 90 1.0 90 8.0 78 12.0 60 15.0 10 18.0 10 19.0 90 23.0 90
Total run time	24 min

Table 2. LC/TOF MS Source Conditions

Dual AJS ESI Source Conditions	
Gas Temperature, Flow	225°C, 9L/min
Nebulizer Pressure	30 psi
Sheath Gas Temperature, Flow	375°C, 12L/min
Nozzle Voltage	500V
Capillary Voltage	3000V

Mobile Phase Buffer Impacts Peak Shapes, Retention Times, and Background Ion Abundances.

The chromatographic performance of five buffered aqueous mobile phases commonly used for HILIC metabolomics were compared.

- 20mM Ammonium acetate, pH 5.7
- 20mM Ammonium acetate, pH 9.3
- 20mM Ammonium bicarbonate, pH 8.2
- 20mM Ammonium bicarbonate, pH 9.3
- 20mM Ammonium carbonate, pH 9.3

Lower pH aqueous buffer conditions exhibited substantial broadening of peak shape for key metabolites, including many organic acids. The use of 20mM ammonium acetate pH 9.3 buffer as mobile phase A showed 15x lower background signal from prominent background ions, compared to ammonium carbonate and ammonium bicarbonate at the same concentration and pH.

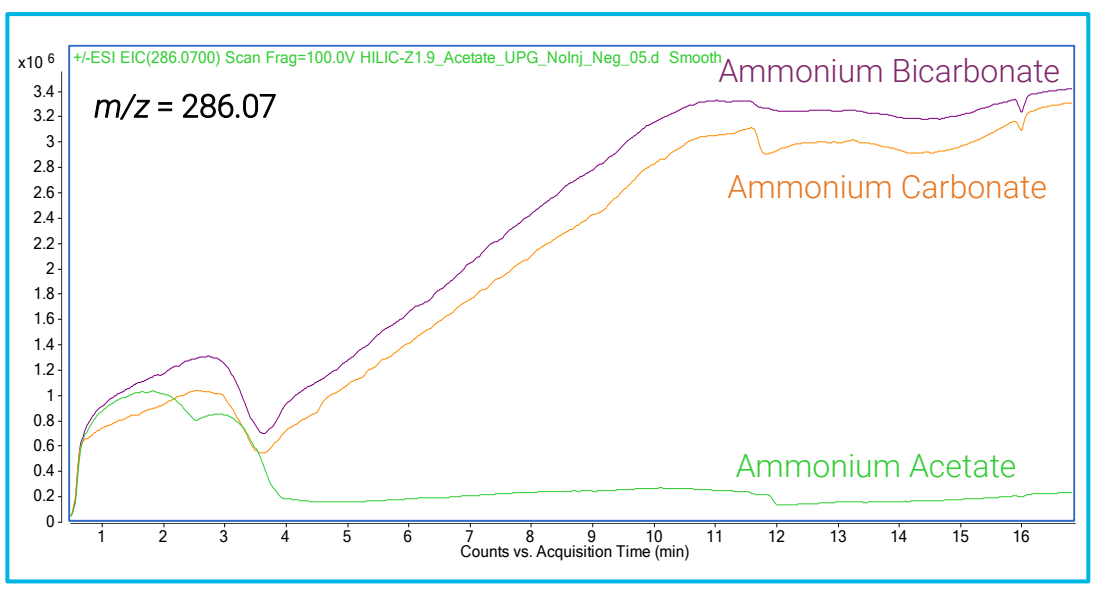

Figure 2. EICs of *m/z* 286.07 background ion with 3 HILIC mobile phase buffers, pH 9.3 over the same gradient.

Medronic Acid Deactivator Improves Sensitivity and Peak Shape for Metal-Sensitive Metabolites.

The Agilent InfinityLab deactivator additive is a medronic acid-based modifier that actively chelates metal ion contamination within the LC flow path, reducing metal-analyte interactions and resulting in sharper peak shapes and improved sensitivity for metal-sensitive analytes.[3]

The addition of up to 5µM medronic acid deactivator showed marked improvement in sensitivity and peak shape for metal-sensitive metabolites. This positive impact of medronic acid deactivator addition was observed even on Bio LC systems with primarily inert hardware as well as passivated stainless steel LC systems, indicating the importance of its inclusion for all HILIC metabolomics users.

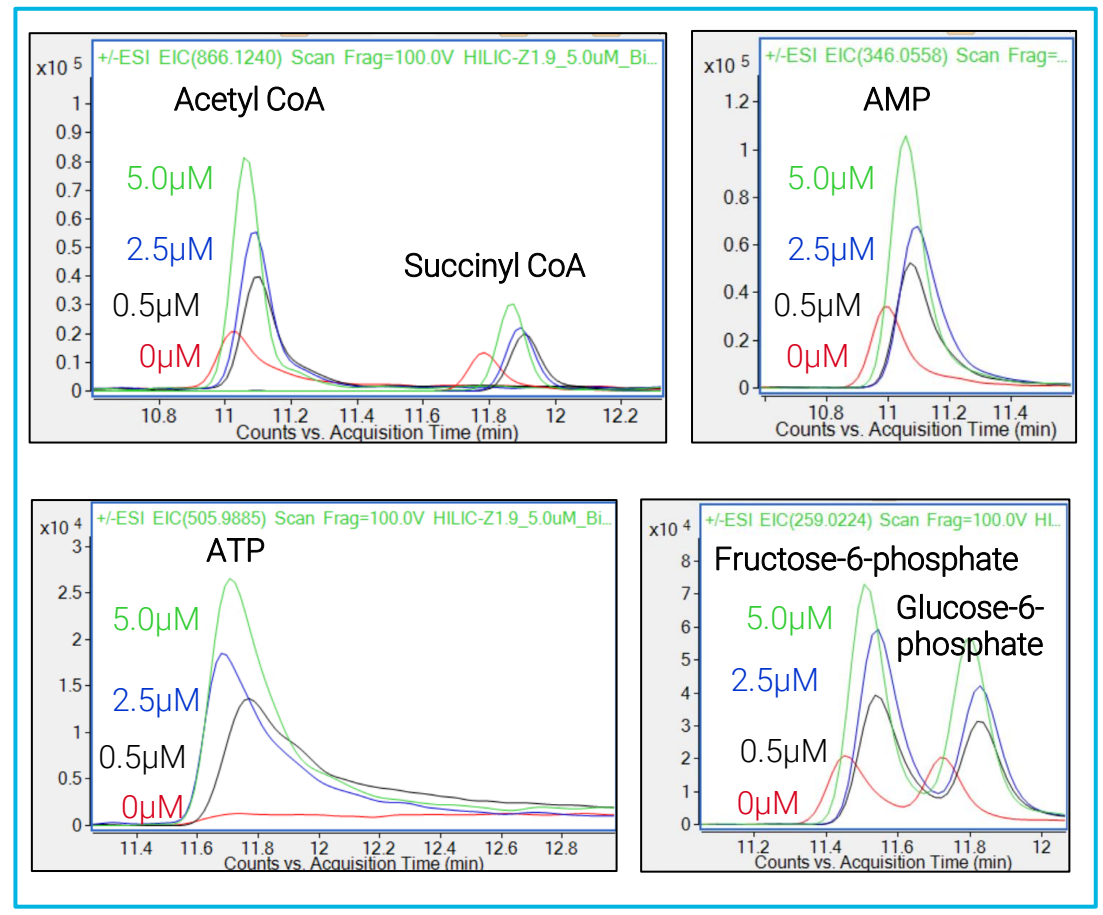

Figure 3. EICs of valuable metal-sensitive metabolites show improved sensitivity with the addition of Agilent InfinityLab deactivator to the mobile phase, with peak performance at 5µM medronic acid.

Medronic Acid Deactivator Does Not Cause Ion Suppression or Additive Carryover

Unlike EDTA or other chelators, the addition of the Agilent InfinityLab deactivator additive at medronic acid levels up to 5µM does not cause ion suppression and is easily flushed from the LC system. No adverse effects of medronic acid addition to the aqueous mobile phase were observed for non-metal-sensitive metabolites, and nearly all metabolites showed increased sensitivity with the inclusion of medronic acid. This additive should never be added into acetonitrile-based solvents, as it can promote polymerization, leading to clogging of the pump heads.

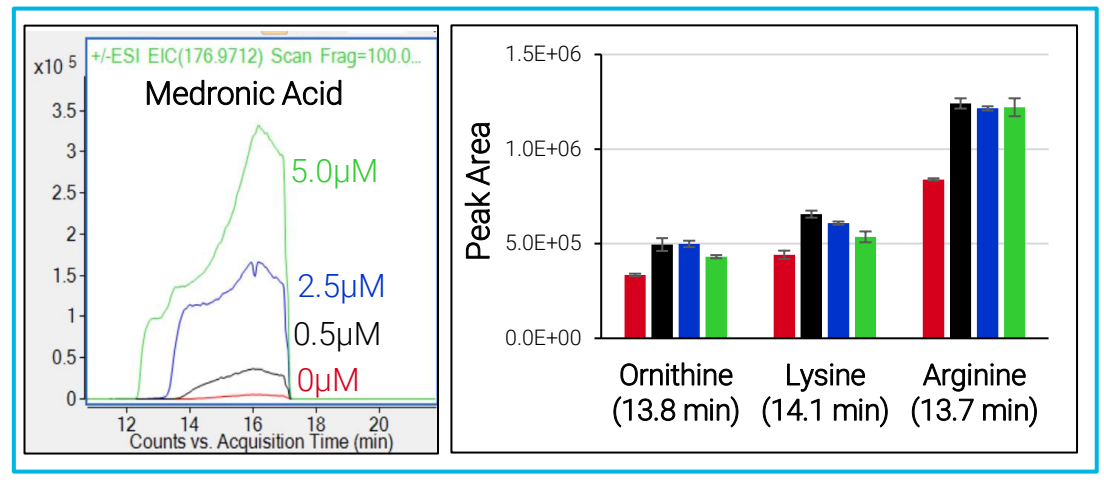

Figure 4. EICs of medronic acid at increasing concentrations (left) and peak areas of metabolites eluting in the same retention time window showing no ion suppression with medronic acid addition (right).

Results and Discussion

Flow Rate, Column Length, and Stationary Phase Particle Size Impact Separation of Near-Eluting Isobars.

The performance of Agilent InfinityLab Poroshell 120 HILIC-Z columns of different particle sizes (2.7 vs 1.9µm) and lengths (100 vs 150mm) were compared at flow rates of 0.4 and 0.6 mL/min. Metabolite peak shape and separation of near-eluting isomers were evaluated.

The combination of a 1.9µm particle size, 2.1x150mm column, and 0.4mL/min flow rate provided reproducible performance with narrow metabolite peaks, and the greatest separation between near-eluting isomers. Similar performance was also achieved with a 2.1x100mm, 1.9µm column at 0.6mL/min, yielding a more condensed metabolite elution pattern.

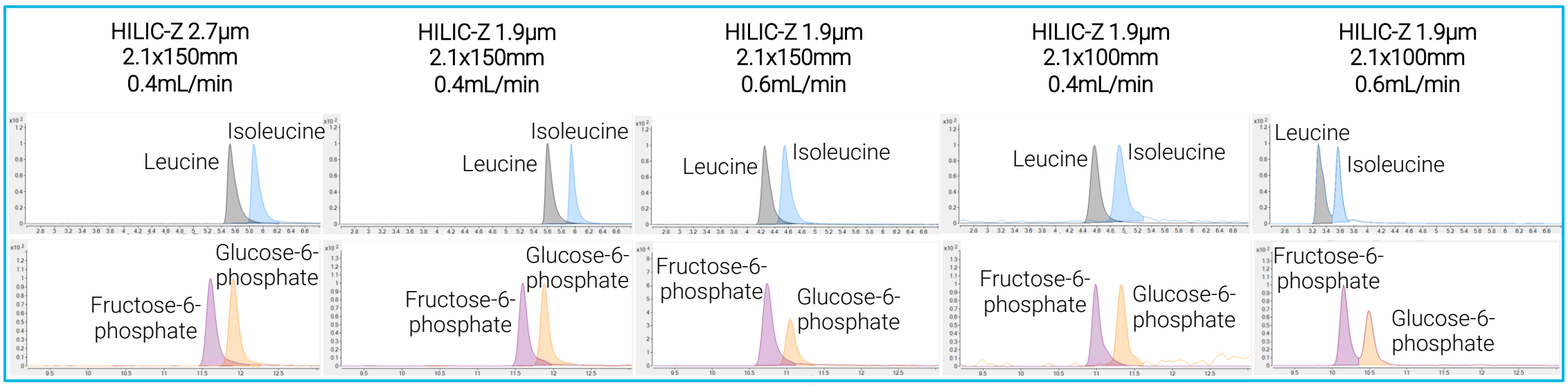

Figure 5. EICs showing the separation of near-eluting isobars (leucine & isoleucine, top; fructose-6-phosphate & glucose-6-phosphate, bottom) with varying column particle size, column length, and flow rate parameters.

Addition of a Guard Column Improves Column Life with No Negative Impact on Chromatography or Retention Times.

An Agilent Infinity Lab Poroshell 120 HILIC-Z guard column (2.1x5mm, 1.9µm, PN 821725-944) was added to the front of the analytical column to aid in extension of column life and performance. Addition of the guard column resulted in minimal change in metabolite retention times, with all peaks remaining within typical LC/TQ dMRM windows. No change in average peak area was observed with the addition of the guard column (n=6) and consistent guard column performance was observed lot-to-lot.

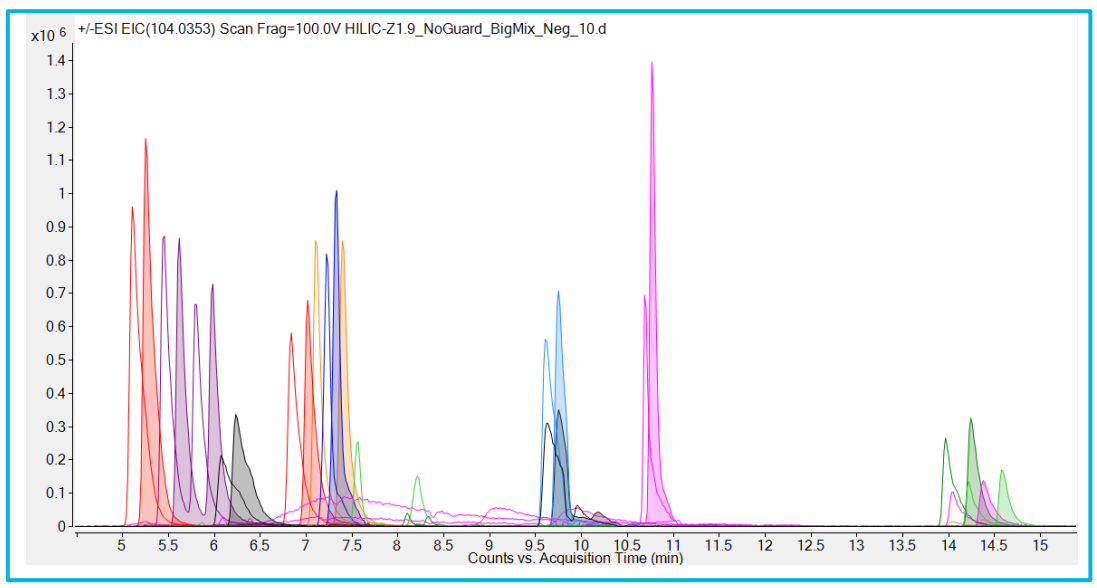

Figure 6. Amino acid chromatograms with (shaded) and without (unshaded) the inclusion of a guard column. All RT shifts with the addition of a guard column were minimal, remaining within typical LC/TQ dMRM windows.

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Conclusions

Mobile Phase Buffer & Additives and Column Dimension Choice Drive HILIC Metabolomics Method Performance.

- Aqueous mobile phase with ammonium acetate buffer, pH 9.3 and 5µm medronic acid deactivator provide reproducible chromatography with maximized sensitivity and low background.
- Smaller particle size columns yield narrower peaks and improved separation of near-eluting isomers.

References

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

[2] Yannell, KE et al. A Comprehensive Untargeted Metabolomics LC/Q-TOF Workflow with an Unknowns Identification Strategy to Identify Plasma Metabolite Shifts in a Mouse Model. ASMS, 2022.

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

[4] Van de Bittner, GC et al. An Automated Dual Metabolite + Lipid Sample Preparation Workflow for Mammalian Cell Samples. Agilent Application Note 5994-5065EN. 2022.

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

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