Investigating Antibiotic Mode of Action Using Targeted Metabolomics with Ion-pairing Reversed-phase LC/Q-TOF

Application Note

Metabolomics

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Abstract

Metabolomics is a growing analytical field characterizing the biochemical contents and reactions of the cell. Determining metabolic state requires separation and measurement of a diverse set of small molecules. We have developed an ion-paired reversed-phase chromatographic method that enables the robust separation of anionic and hydrophobic metabolites. This method enables simultaneous analysis of hundreds of compounds including amino acids, citric acid cycle intermediates, and other carboxylic acids, nucleobases, nucleosides, phosphosugars, and fatty acids. When used in conjunction with accurate mass TOF/Q-TOF or triple-quadrupole mass spectrometers, this separation enables rapid, robust, and reproducible quantitation of a large array of compounds in central metabolism across a wide range of concentrations. Using this method, we measured metabolite levels following treatment of the gram negative bacteria *E. coli* with the antibiotic glyphosate. Analyzing changes in metabolite levels revealed the mechanism of action of glyphosate, demonstrating the power of metabolomics for direct biochemical phenotyping, and illustrating its potential use in uncovering novel mechanisms of action.

Agilent Technologies

Introduction

A major goal of metabolomic analysis is the simultaneous quantitation of molecules possessing a variety of functional groups and ranging widely in mass and hydrophobicity. Many chromatographic modalities including normal phase, HILIC, and reversed-phase have been applied to metabolomic analysis, but are generally suboptimal due to poor selectivity, poor run-to-run reproducibility, or nonretention of polar metabolites. Ion-paired reversed-phase analysis has the advantage of providing retention of polar compounds on a conventional reversed-phase column by functionalization of the stationary phase with a bifunctional ion-pairing agent. These agents possess an ionizable moiety, and one or more hydrophobic elements. The ionizable group of the ion-pairing agent interacts with polar compounds, while the hydrophobic group interacts with a reversed-phase column stationary phase such as C18 (Figure 1).

The tertiary amine tributylamine provides a cationic functionalization of the reversed-phase packing with which many acidic metabolites can interact. Selectivity of conventional reversed-phase analytes, including hydrophobic metabolites, is maintained in the presence of tributylamine.

This application note describes an ion-paired reversed-phase LC/MS metabolomics analytical method specifically developed for long-term reliability and reproducibility. This approach can be used to perform relative quantitation of endogenous cellular metabolites, and extended to semiquantitative or quantitative analysis through the use of spike-in and isotopically labeled internal controls.
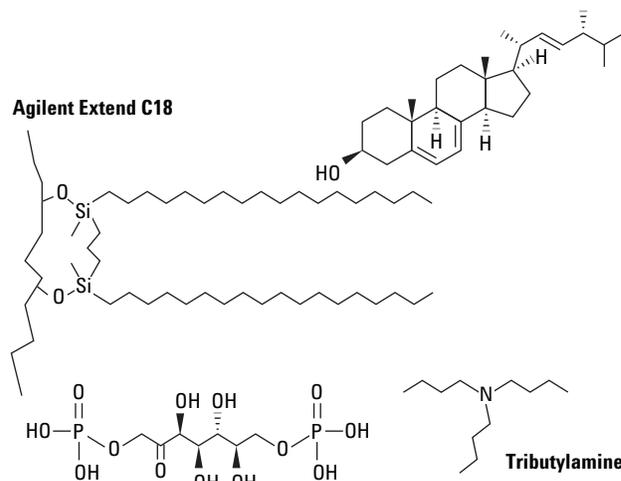

Figure 1. Ion pairing with tributylamine functionalizes C18 side chains, permitting retention of polar compounds like phosphosugars in addition to nonpolar compounds that are typically retained in reversed phase analysis.

Previous ion-paired reversed-phase methods have been limited in their application to large or extended sample sets by progressive chromatographic drift over multiple sample injections. Our approach uses a physically robust Agilent Extend C18 column packing in a UPLC configuration that permits backflushing during column regeneration. We find that this off-line backflushing and regeneration provides superior system performance and excellent run-to-run reproducibility (Figure 2). While this regenerative backflushing is possible on a single pump system, we present a method that makes efficient use of the connected mass spectrometer by using a pair of identical columns and alternating column regeneration.
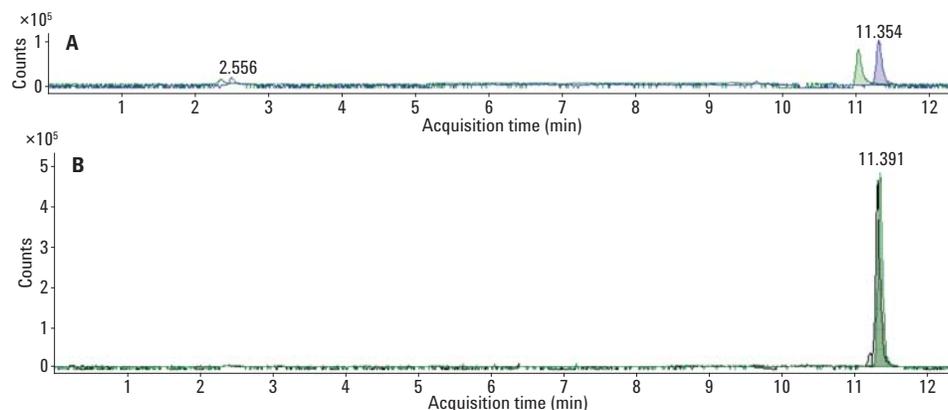

Figure 2. Backflushing during column regeneration increases retention time stability. Repeat injections of the same sample without backflushing (A) and with backflushing (B).

2

The LC system includes two binary pumps connected to two columns through a two-position, 10-port valve to allow one column to be run on an analytical gradient to the mass spectrometer, while the other column is backflushed and regenerated off-line (Figure 3). The backflush provides superior long-term stability of retention time compared to conventional forward-flow column regeneration (Figure 4). The method used the Agilent RRHD Extend-C18 column, which features high pH stability, potentially improving column life in the presence of the strongly basic ion-pairing agent.
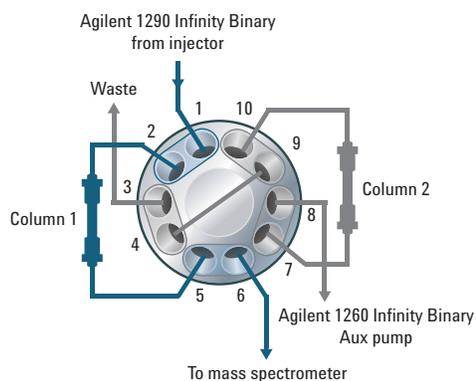

Figure 3. Alternate column regeneration permits higher throughput analysis. Using a 2-position, 10-port valve, one column is connected to the sample injector while the second is connected to a binary pump for column regeneration. In the second position, the first column is regenerated, while the second is used for sample analysis. The flow from the regeneration pump is backflushed through the column to provide superior column cleaning.

We demonstrate this method in the metabolic profiling of the gram negative bacterium *E. coli* exposed to glyphosate, an aminophosphonate analogue of glycine. Better known as the widely used herbicide Round-Up, glyphosate inhibits 5-enol-pyruvylshikimate-3-phosphate (EPSP) synthase, a critical step in the synthesis of chorismate and *de novo* biosynthesis of all aromatic amino acids[1]. Many higher animals require dietary intake of aromatic amino acids due to the absence of the *de novo* biosynthetic pathways. Glyphosate has low toxicity in mammals, and has been considered as a candidate antibiotic. We examined the metabolic consequences of glyphosate treatment of *E. coli* to demonstrate the potential of LC/MS metabolomics to measure metabolic changes induced by small molecule treatment, identify mode of action, and serve as a platform to assist in drug discovery and preclinical studies.
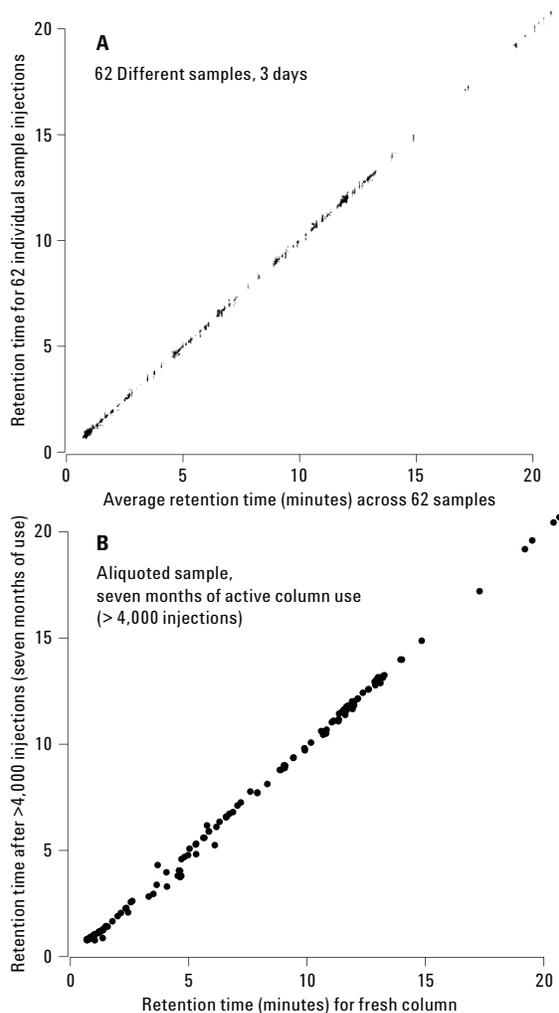

Figure 4. Chromatographic retention time is stable over many injections. Shown are chromatographic retention times for a set of known compounds. A) Retention time deviation versus retention time for 62 different biological samples (extracts of yeast cells) analyzed over the course of three days. B) Retention times for analytes in the same sample, analyzed before and after an intervening 4,000 injections of biological samples.

Experimental

Sample preparation

For preparation of metabolite extracts, *E. coli* MG1655 cultures were grown at room temperature in 24-well blocks containing 5.5 mL M5 minimal medium per well. At the mid-log phase, glyphosate or an equal volume of vehicle was added to the culture. A 5.0 mL volume of culture was transferred to a 25 mm diameter Pall Nylaflo 0.2-μm nylon membrane filter on a Hoefer FHV-225 manifold and cells were collected by vacuum filtration. Filters containing cells were immediately transferred to a 15-mL Falcon tube containing 1.2 mL of extraction solvent prechilled to –20 °C. The tube was vortexed to resuspend the cells in solvent, and immediately returned to –20 °C. Preparation of metabolite extracts from filtration to storage took ~12 seconds per sample. The extraction solvent consisted of a 40:40:20 ratio of acetonitrile, methanol, and water cooled to –20 °C. Optical density was measured from a small sample of culture at the time of metabolite extraction as a proxy of culture concentration. Cells in the extraction solvent were lysed by three freeze-thaw cycles alternating between –80 °C and –20 °C. Following the final thaw at –20 °C, filters were removed from the tube and insoluble material was collected by centrifugation at 4,000xg for 15 minutes at 4 °C. Supernatant containing extracted metabolites was transferred to a fresh polypropylene tube, and dried in a TurboVap LV evaporator under dry N_2. Dried metabolite extracts were resuspended by vortexing in a volume of HPLC-grade H_2O adjusted to the cell density determined by optical density at the time of cell harvest. Following resuspension, samples were briefly centrifuged to collect material, transferred to a Pall Acroprep 0.2-μm multiwell hydrophilic polypropylene filter plate, and vacuum-filtered into a polypropylene receiver plate. Filtered extracts were loaded into conical polypropylene HPLC vials, and placed in a 4 °C autosampler for LC/MS analysis.

Instrumentation

An Agilent 6550 Quadrupole Time-of-Flight LC/MS consisting of:

- Agilent 1290 Infinity Binary UHPLC pump
- Agilent 1260 Infinity Binary pump
- Agilent 1290 Infinity Thermostatted Column Compartment
- Agilent 1290 Infinity HTS Autosampler.

An Agilent 6550 accurate-mass Q-TOF mass spectrometer with an Agilent Jet Stream dual ionization source were used to acquire data in high resolution mode at 4 GHz at two spectra per second.

LC Conditions

Column	Agilent RRHD Extend-C18, 2.1 × 100 mm, 1.8 μm
Mobile phase	A) Water:methanol 97:3 with 10 mM tributylamine and 15 mM acetic acid
	B) Methanol for analytical separation, acetonitrile for regeneration
Gradient	**Analytical phase:** Methanol in channel B, flow rate 0.25 mL/min
	1% B at 0 minutes
	1% B at 2.5 minutes
	20% B at 7.5 minutes
	55% B at 13 minutes
	99% B at 20 minutes
	99% B at 21 minutes
	1% B at 22.9 minutes
	1% B at 23 minutes
	Regeneration phase: Switch 2-position, 10-port valve to second pump with acetonitrile in channel B. This program runs while an analytical gradient is running on the other column.
	1% B at 23 minutes, 0.2 mL/min
	99% B at 26 minutes, 0.2 mL/min
	99% B at 27 minutes, 0.4 mL/min
	99% B at 33.9 minutes, 0.4 mL/min
	99% B at 34 minutes, 0.25 mL/min
	1% B at 36 minutes, 0.25 mL/min
	1% B at 46 minutes, 0.25 mL/min
Column temperature	37 °C
Autosampler temperature	4 °C
Injection volume	2 μL

MS Conditions

Ionization mode	Jet Stream
Ionization polarity	Negative
Gas temperature	150 °C
Drying gas	14 L/min
Nebulizer pressure	45 psi
Sheath gas temperature	325 °C
Sheath gas flow	12 L/min
ESI Capillary voltage	2,000 V
Nozzle voltage	300 V
Fragmentor voltage	340 V
Octopole 1 RF voltage	600 V
Scan range	m/z 25–1,100
Reference masses	m/z 112.9855 (trifluoroacetic acid), 1033.9981 (HP-0921)

Data analysis

The chromatographic retention times of known metabolites were determined by LC/MS analysis of neat standards to generate an accurate mass/retention time database. We used Agilent MassHunter Profinder in Batch Molecular Feature Extraction mode to extract features in this database from biological sample d files. .CEF files from Profinder were imported into Agilent MassHunter Mass Profiler Professional for preprocessing, hierarchical clustering, and statistical analysis. Agilent MassHunter PathwayArchitect was used to display changes in metabolite levels on BioCyc pathways.

Results and Discussion

E. coli cells were grown to log phase, treated with glyphosate, and harvested for metabolite extraction. The quantity of each metabolite was determined by integrating an extracted ion chromatogram (EIC) centered at the known retention time of a metabolite standard, and the area under the curve was calculated and the blank subtracted. These values of abundance for each metabolite were log$_2$ transformed and centered to a median change of zero to highlight differences among the samples for each metabolite. The metabolite abundances were clustered, and the log$_2$ changes in metabolite abundances were displayed as a heatmap, in which red represents an increase in metabolite compared to the median value of that metabolite for all samples, and blue represents a decrease in the value of that metabolite for the samples.
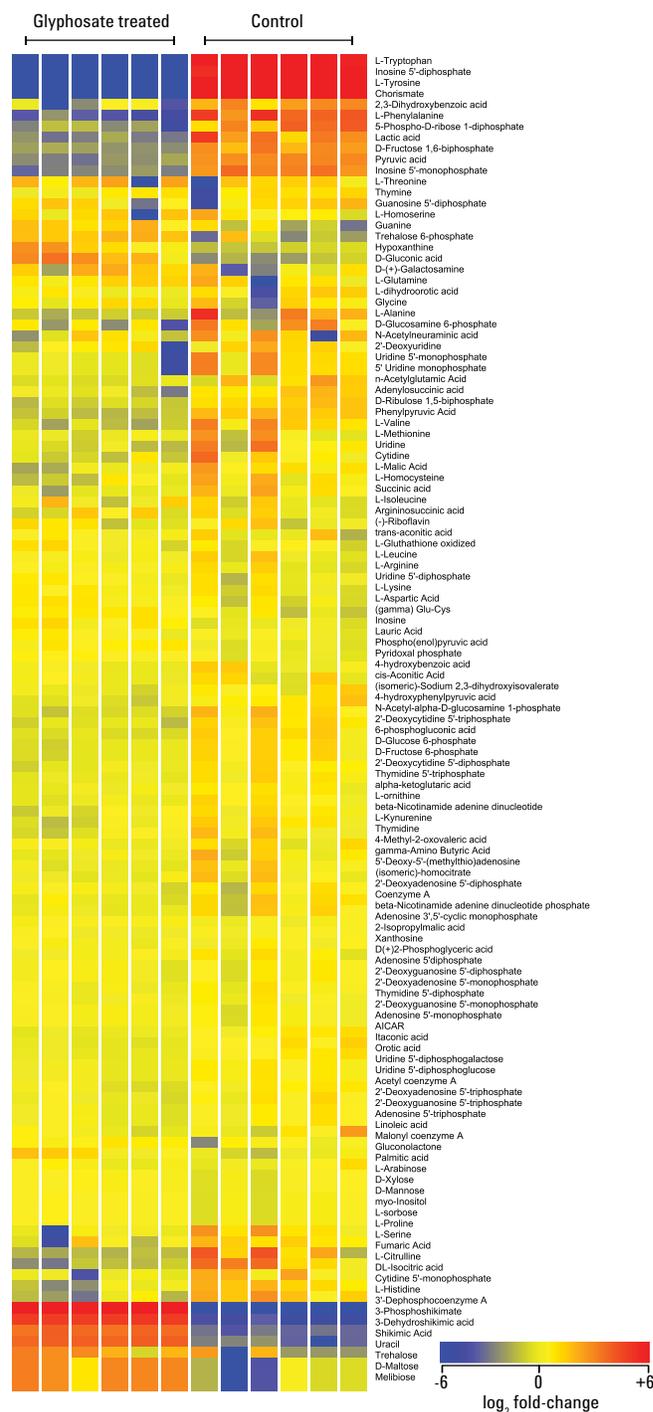

Figure 5. Levels of known metabolites following treatment with glyphosate or vehicle control. Data were extracted using Agilent MassHunter Profinder in Batch Molecular Feature Extraction mode and analyzed using Agilent MassHunter Mass Profiler Professional. Levels for each metabolite are presented as log$_2$ transformed, mean-subtracted values to identify changes between conditions.

Targeted metabolomic analysis reveals a number of significantly changing metabolites between treated and control cells (Figure 5).

Large increases in shikimate and 3-phosphoshikimate were observed following glyphospate treatment, while chorismate and all three aromatic amino acids were significantly decreased in concentration following treatment (Figure 6).

Although EPSP levels are not directly measured and two enzymatic steps exist between 3-phosphoshikimate and chorismate, the resulting changes in up- and down-stream metabolite pools point to disruption in the pathway known to be the target of glyphosate, centered around the established target of action, EPSP-synthase. Even without targeted methods for analysis for each compound in the pathway, we were able to identify the correct target of glyphosate treatment.
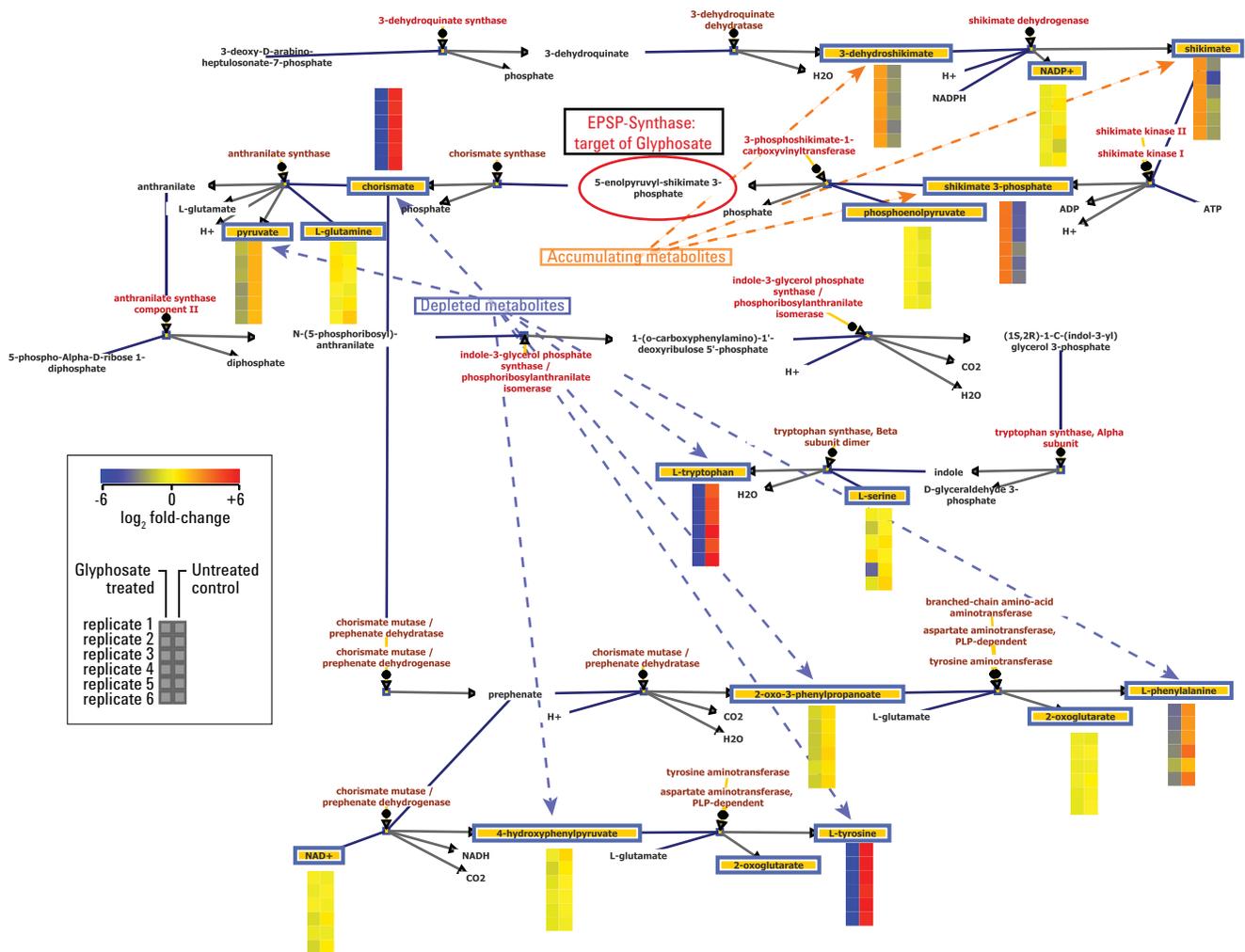

Figure 6.   Effects of glyphosate treatment are visible as a concerted change in many intermediate and end-product metabolites. Using Pathway Architect and Agilent MassHunter Mass Profiler Professional, aromatic amino acid biosynthesis was identified as the pathway most significantly affected in this experiment. Individual metabolite measurements for glyphosate-treated and control-treated cells are shown. Intermediates upstream of EPSP-synthase accumulate upon treatment, while downstream intermediates and end products are highly depleted (with several more than 4,096x decreased).

Conclusions

We have developed a reversed-phase ion-pairing method that provides a platform for the robust analysis of a wide range of endogenous anionic metabolites. Regenerative backflushing and the use of a ternary solvent for regeneration of the Agilent Extend C18 RRHD columns provides superior long-term performance compared to other ion-paired reversed-phase analytical methods, including stable retention times and consistently high quality peak shape, and enables long column life with minimal sample preparation.

The highly reproducible retention times achieved through this analytical method enable targeted metabolomic analysis by nature of minimal chromatographic drift. In addition to use on an accurate mass instrument, we have deployed this chromatography on the triple quadrupole platform, where retention time consistency enables tight windows for scheduling of a large number of MRM transitions.

Although targeted analyses are presented here, this method is readily applicable for full-scan, untargeted analysis and identification of novel metabolites.

Reference

1. H. C. Steinrücken, N. Amrhein. "The herbicide glyphosate is a potent inhibitor of 5-enolpyruvylshikimic acid-3-phosphate synthase" *Biochem. Biophys. Res. Commun.* **94**, 1207-1212, (1980).

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