Application Note

Clinical Research

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Dual Mass Spectrometry as a Tool to Improve Annotation and Quantification in Targeted Plasma Lipidomics

Authors

Liang Gao,
Amaury Cazenave-Gassiot,
Bo Burla, Markus R. Wenk, and
Federico Torta
Singapore Lipidomics
Incubator (SLING),
Life Sciences Institute and
Department of Biochemistry,
Yong Loo Lin School of
Medicine,
National University of
Singapore, Singapore

Carrie J. Adler
Agilent Technologies, Inc.

Abstract

High-quality data are required for future applications of human plasma lipidomic studies in the clinical research environment and are based on a clear identification of the reported lipid species. However, there can be issues in the reliable identification and quantification of these lipids, including (i) resolving peaks of polyunsaturated species, (ii) discriminating between plasmanyl-, plasmenyl-, and odd chain lipids, and (iii) resolving the isotopic overlap between coeluting lipid molecules. To improve the quality of targeted lipidomics studies, a dual MS platform was used with a reversed-phase liquid chromatography separation simultaneously connected to an Agilent 6490 triple quadrupole LC/MS and an Agilent 6550 iFunnel Q-TOF LC/MS. In one single experiment, this configuration enabled the correct identification, by high-resolution MS and MS/MS, of the peaks quantified by MRM. As proof of concept, this approach was applied to the analysis of glycerophosphocholines (GPCs) and sphingomyelins (SMs), which are highly abundant in human plasma and play crucial biological roles. Dual MS could provide a higher level of confidence in the identification and quantification of GPCs and SMs in human plasma and achieve a better specificity in the quantification of lipids in high-throughput studies.

Introduction

Plasma-based lipidomic studies are powerful clinical research tools to understand the molecular basis of pathophysiological processes and to develop new therapeutic strategies for metabolic and cardiovascular diseases.[1,2] Different LC/MS/MS-based approaches can be taken: targeted methods using triple quadrupole MS can be more sensitive and deliver better quantitative performance for a selected list of molecules. A high-resolution MS-based untargeted analysis can, however, provide a more unbiased picture of the changes in the system under study and facilitate the discovery of novel molecules, albeit with lower sensitivity. Combining untargeted experiments with targeted ones in parallel would be the most effective way to improve the discovery process. A dual MS platform was created by coupling an Agilent 1290 Infinity LC to an Agilent 6490 triple quadrupole LC/MS and an Agilent 6550 iFunnel Q-TOF LC/MS.

In one single experiment, this platform combines a confident lipid identification, by accurate mass MS and MS/MS measurement, with MRM quantification of lipid peaks.[3] As a practical example, this experiment shows how dual MS can help to correctly quantify plasmanyl-, plasmenyl-, and odd chain glycerophosphocholines (GPCs) and sphingomyelins (SMs) signals.

GPCs are composed of a glycerol backbone bearing a phosphocholine head group and two hydrophobic moieties at the *sn*-1 and *sn*-2 position. The hydrophobic substituents can consist of: (i) two acyl esters (phosphatidylcholine, PC); (ii) an alkyl ether in *sn*-1 and an acyl ester in *sn*-2 (plasmanylcholine, PC-O); or (iii) a vinyl ether in *sn*-1 and an acyl ester in *sn*-2 (plasmenylcholine, PC-P). SM consists of a phosphocholine head group, a sphingoid base and a fatty acyl chain. Although the sphingosine backbone (i.e. a sphingoid base with 18 carbon atoms and one double bond) is the most prevalent, other sphingoid bases containing 14 to 22 carbon atoms and 0 or more double bonds can also be present.

In targeted lipidomics, quantification of both GPCs and SMs is usually based on MRM transitions that include the intense product ion at *m/z* 184.1. However, a series of issues can be encountered when using these head-group-based MRM transitions when dealing with (i) resolving multiple peaks of polyunsaturated species, (ii) discriminating GPC isobars and isomers, and (iii) isotopic overlap from coeluting species with close *m/z* values.

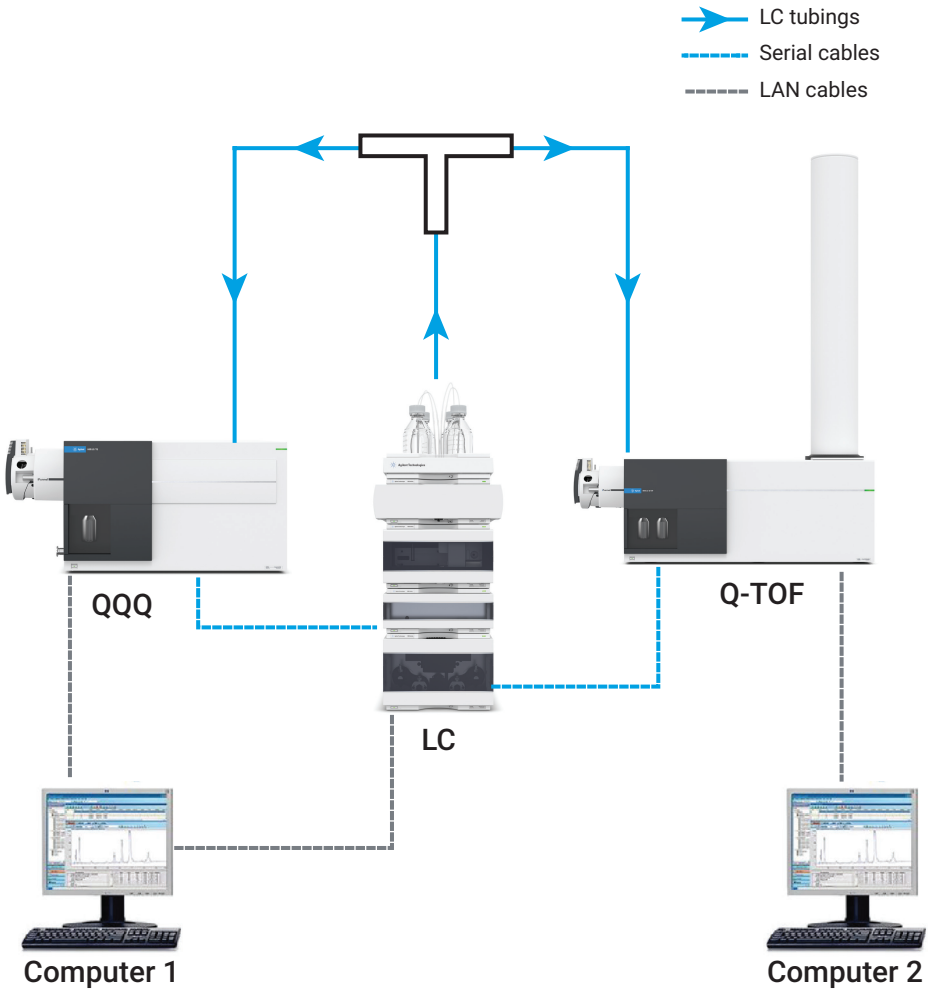

Figure 1. Schematic diagram of the dual MS platform.

Experimental

Chemicals and reagents

Ammonium formate and butanol from Sigma-Aldrich and Merck (Darmstadt, Germany); MS-grade acetonitrile, methanol, and isopropanol from Fisher Scientific (Waltham, MA); PC 14:0/14:0, PC-P 18:0/18:1, and SM d18:1/12:0 synthetic standards from Avanti Polar Lipids (Alabaster, AL). Ultrapure water (18 MΩ cm at 25 °C) was obtained from an Elga Labwater system (Lane End, U.K.). Commercial human plasma was purchased from Sera Laboratories International Ltd (Haywards Heath, U.K.).

Sample preparation

Twenty microliters of plasma were mixed with 180 µL of 1-butanol:methanol (1:1, v/v) and spiked with PC 14:0/14:0 (200 ng/mL), PC-P 18:0/18:1 (200 ng/mL), and SM d18:1/12:0 (100 ng/mL) as internal standards. The mixture was vortexed for 3 minutes, sonicated for 30 minutes, then centrifuged at 4 °C (14,000 rcf for 10 minutes). The supernatant fraction was collected for LC/MS/MS analysis.

Data analysis

Data analysis was performed with Agilent MassHunter qualitative analysis B.07.00.

LC/TQ mass spectrometer configuration and parameters

Parameter	Value
Agilent 6490 Triple Quadrupole LC/MS G6490A	
Ionization Mode	Positive
Drying Gas Temperature	250 °C
Drying Gas Flow	14 L/min
Nebulizer Pressure	35 psi
Sheath Gas Temperature	250 °C
Sheath Gas Flow	11 L/min
Nozzle Voltage	1,000 V
Capillary Voltage, Positive	3,000 V
Delta EMV, Positive	200 V

LC configuration and parameters

Parameter	Value
Agilent 1290 Infinity binary pump G4220A	
Agilent 1290 Infinity autosampler G4226A	
Agilent 1290 Infinity thermostatted column compartment G1316C	
Needle Wash	Isopropanol 3 s
Autosampler Temperature	4 °C
Injection Volume	2 µL
Analytical Column	Agilent ZORBAX Eclipse Plus C18 (2.1 × 50 mm, 1.8 µm)
Column Temperature	40 °C
Mobile Phase A	60/40 water/acetonitrile + 10 mmol/L ammonium formate
Mobile Phase B	10/90 acetonitrile/isopropanol + 10 mmol/L ammonium formate
Flow Rate	400 µL/minute
Gradient	Time (min) %B 0.00 20 2.00 60 12 100 14 100 14.01 20 15.8 20
Stop Time	15.8 minutes

LC/Q-TOF mass spectrometer configuration and parameters

Instrument and Source Conditions		
Agilent 6550 iFunnel Q-TOF LC/MS G6550BA		
Ionization Mode	AJS ESI, Positive	
Drying Gas Temperature	200 °C	
Drying Gas Flow	14 L/min	
Nebulizer Pressure	35 psi	
Sheath Gas Temperature	350 °C	
Sheath Gas Flow	11 L/min	
Capillary Voltage	3,500 V	
Nozzle Voltage	1,000 V	
MS Parameters		
Acquisition Mode	**Auto MS/MS**	**MS**
Isolation Width	Narrow (1.3 amu)	NA
MS Data Rate	2 spectra/s	2 spectra/s
MS/MS Data Rate	2 spectra/s	NA
Fixed Collision Energies	Lipid class-dependent	NA

Results and discussion

Identification of polyunsaturated GPC and SM species

Most SM and GPC species with the same total carbon and double bond numbers will have a very similar retention time on a reversed-phase LC (RPLC) system. Under RPLC-MRM experimental conditions, *m/z* values corresponding to polyunsaturated SMs and GPCs may show multiple, non-baseline-separated, chromatographic peaks (Figures 2A and 2B). However, the Q-TOF MS/MS spectra in negative ionization mode reveal that there are various possible fatty acyl/alkyl/alkenyl chain combinations for GPCs that explain the presence of these multiple peaks (Figures 2C and 2D). Similarly, different combinations of sphingoid bases and fatty acyl chains generate isobaric SMs (Figure 2B) that can be discriminated by high-resolution MS/MS in positive ionization mode (Figures 2E and 2F).

Differentiation between isobaric and isomeric GPC species

Since only unit mass resolution can be achieved on a quadrupole mass analyzer, isobaric and isomeric compounds with the same unit mass cannot be differentiated without authentic standards and chromatographic separation. The odd chain PC 33:2 has the same unit mass (*m/z* 744.6) as the plasmanylcholine PC-O 34:2 and the plasmenylcholine PC-P 34:1. Such isobaric compounds, when measured by the same transition 744.6 → 184.1 in MRM mode, show multiple peaks (Figure 3A). The lipid accurate mass can be obtained from the Q-TOF analysis for the previously mentioned isobaric and isomeric compounds. First, odd chain PC 33:2 is annotated to peak 1 by its accurate mass *m/z* 788.5447 for $[M+HCOO]^-$ (Figure 3B) and 744.5538 for $[M+H]^+$ (Figure 3E) and further confirmed by the fatty acyl chain fragmentation from $[M+HCOO]^-$ in negative ionization (Figure 3H). The isomeric PC-O 34:2 and PC-P 34:1 have the same accurate mass at *m/z* 788.5811 for $[M+HCOO]^-$ (Figures 3C and 3D) and 744.5902 for $[M+H]^+$ (Figures 3F and 3G). Therefore, the fatty acyl chain fragments from *sn-2* position in peak 2 (Figure 3I) and peak 3 (Figure 3J) in negative ionization are not specific enough to distinguish a vinyl ether bond (PC-P) and characteristic fragments from *sn-1* position are still needed. A specific fragment at *m/z* 504.3437 in peak 3 (Figure 3M), arising from neutral loss of $C_{14}H_{29}CH{=}CHOH$ as an alcohol at *sn-1* position, identified the compound as a plasmenylcholine. In contrast, the fragment at *m/z* 502.3292 was absent in peak 2 (Figure 3L), suggesting that the compound is a plasmanylcholine.[4]

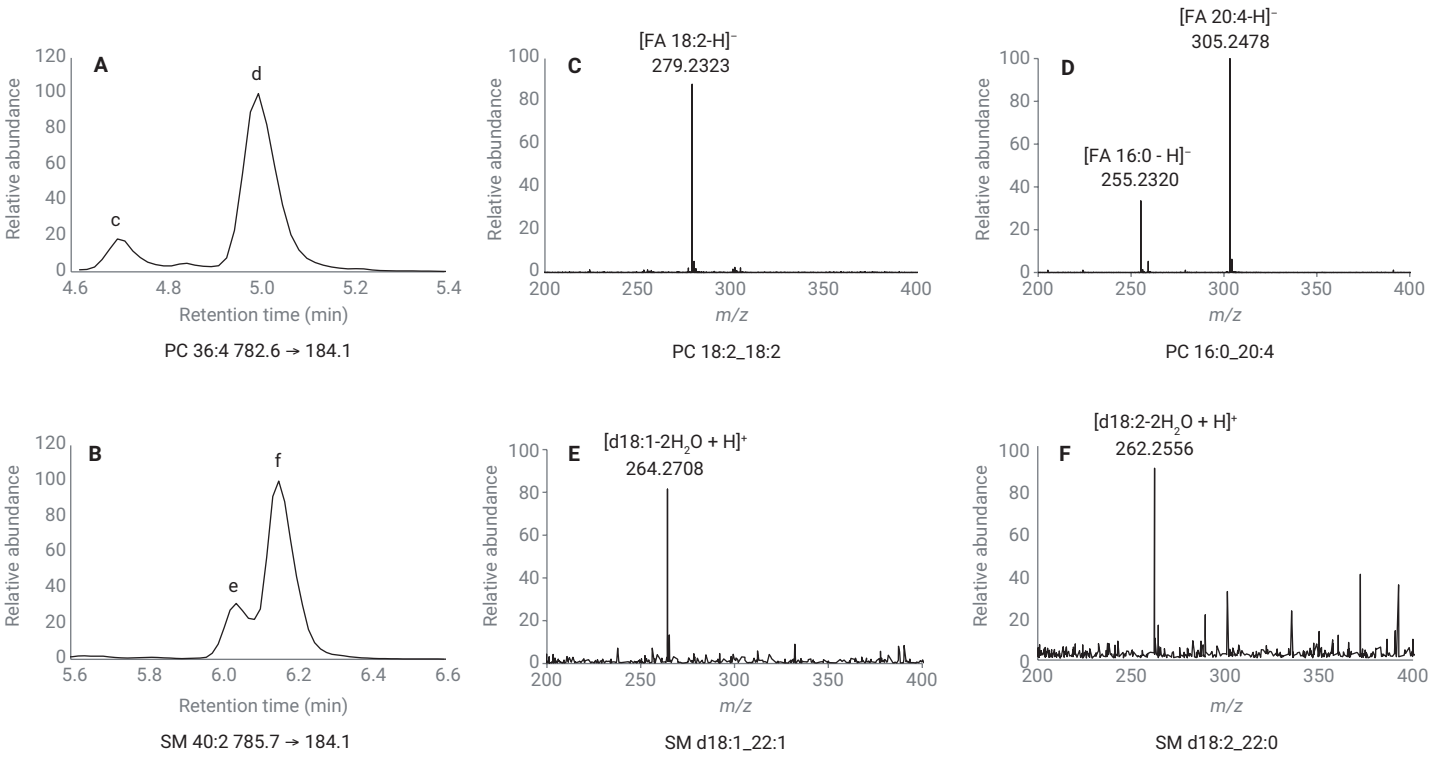

Figure 2. Reversed-phase separated chromatographic peaks measured in MRM mode of (A) PC 36:4 and (B) SM 40:2. (C and D) MS/MS spectra of peaks c and d in (A) from Q-ToF in negative ionization. (E and F) MS/MS spectra of peaks e and f in (B) from Q-TOF in positive ionization.

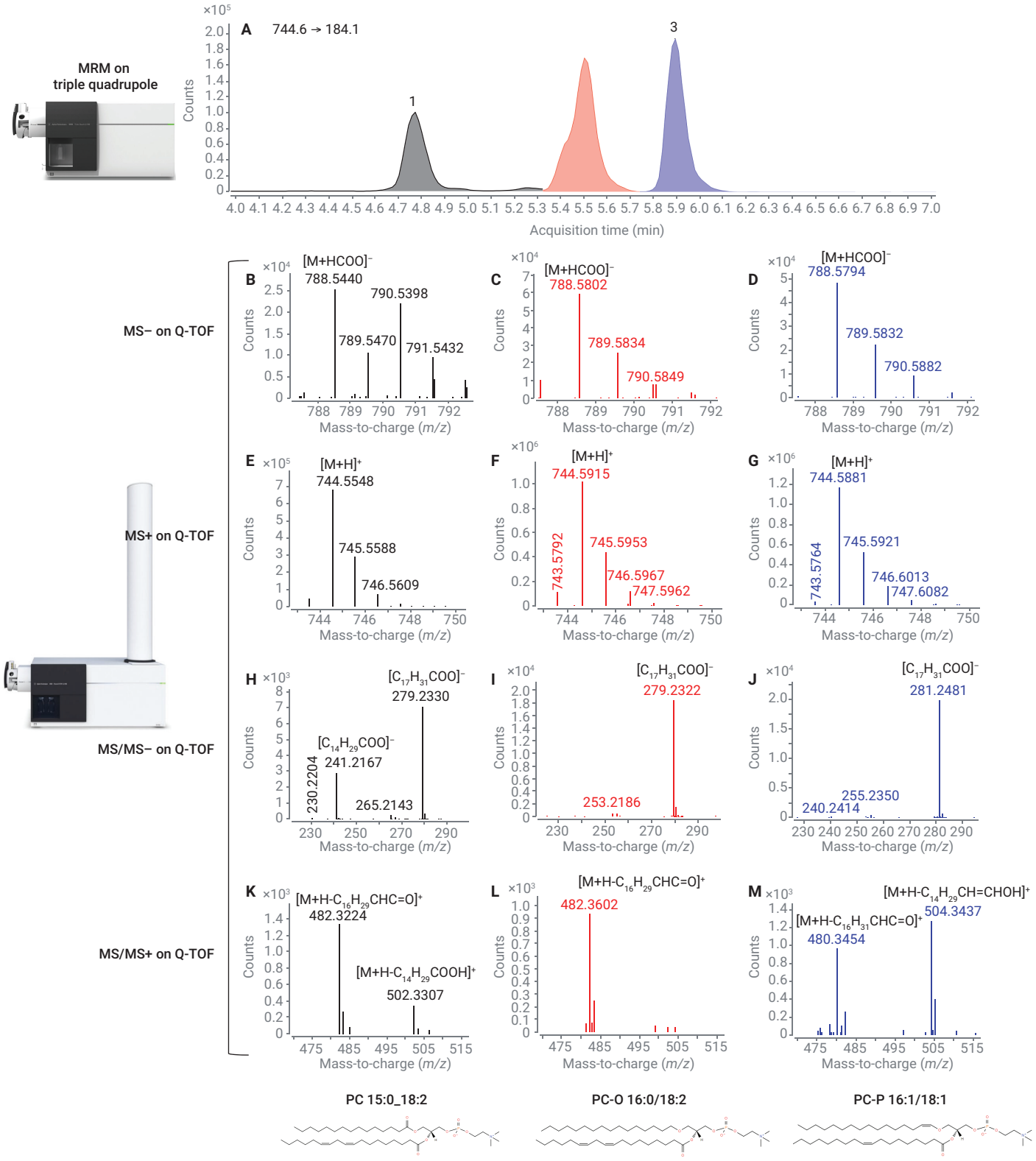

Figure 3. (A) Chromatographic peaks 1, 2, and 3 for MRM transition 744.6 → 184.1 on an RPLC-QQQ system. (B to D) MS spectra of peaks 1 to 3 in (A) from Q-TOF in negative ionization. (E to G) MS spectra of peaks 1 to 3 in (A) from Q-TOF in positive ionization. (H to J) MS/MS spectra of peaks 1 to 3 in (A) from Q-TOF in negative ionization. (K to M) MS/MS spectra of peaks 1 to 3 in (A) from Q-TOF in positive ionization.

Conclusion

The dual MS approach presented here aims at combining the advantages of targeted and untargeted MS approaches, to improve data quality in lipidomics. As low-resolution MRM-based measurements are prone to misannotation when applied to molecules with similar *m/z* and elution times, high-resolution MS and MS/MS can be used to confirm molecular compositions. At the same time, the higher sensitivity of MRM measurements is fundamental for the quantification of low abundance lipids. Shown here is the application of dual MS to resolve ambiguities when measuring GPCs and SMs in human plasma.

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