Supelco®
Analytical Products

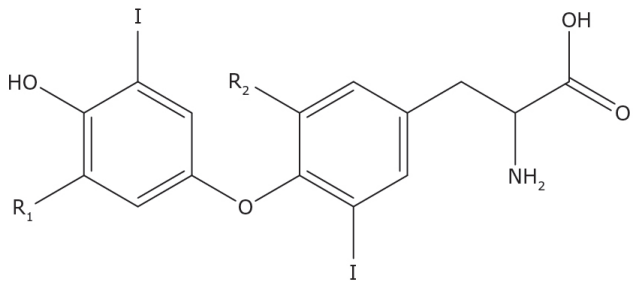
Application Note

Simultaneous Determination of Free Triiodothyronine (T3) and Free Thyroxine (T4) from Serum using the Supel™ BioSPME for Sample preparation.

Introduction

The thyroid hormones triiodothyronine, T3 or 3, 5, 3′-triiodo-l-thyronine, and thyroxine, T4 or 3, 5, 3′, 5′-tetraiodo-l-thyroxinine, are biomarkers used to monitor thyroid activity (**Figure 1**). Approximately 0.04% of the total T3 and 0.02% of the total T4 is available as the free form in circulation, with the remainder bound primarily to thyroxine-binding globulin and, to a lesser extent, albumin and transthyretin.[1] Direct analogue immunoassays, the most common tests performed at most clinical laboratories, suffer from interferences and lack of specificity leading to criticism for poor quality.[2] The most accepted sample preparation for determining the free concentration of these hormones involves the lengthy process of equilibrium dialysis prior to quantification by LC-MS/MS. This application note presents a novel approach to accurately monitor and determine the free T3 and T4 in under an hour of sample preparation using a technique called BioSPME, bioanalytical solid phase microextraction, prior to analysis by LC-MS/MS. **Figure 2** shows the Supel™ BioSPME C18 device utilized for sample preparation.

The BioSPME device is a 96-pin array with a 2 mm C18 sorbent and biocompatible coating that is on the outside of each pin. This device operates by direct immersion into the sample solution and requires no active pipetting as there is no liquid transfer in or out of pin.



R_1 = I R_2 = I L-Thyroxine, T4
R_1 = H R_2 = I 3, 5, 3`-triiodo-l-thyronine, T3
R_1 = I R_2 = H 3, 3`, 5`-triiodo-l-thyroinine, rT3

Figure 1. Structure of T4, T3, and rT3.

Figure 2. (left) Supel™ BioSPME device. (right) close-up of two pins to show the C18 coating of 2 mm on the outside of the pin.

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Experimental/Methods

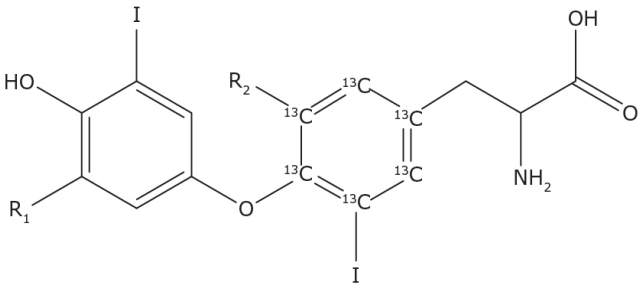
Handling and storage of serum samples

Bulk serum, 50 mL, arrived frozen and were thawed and separated into 4 mL aliquots in 5 mL polypropylene cryotubes. A 4 mL aliquot of each serum was submitted to an external clinical laboratory for independent determination of free T3 and free T4 by equilibrium dialysis, ED, prior to quantification by LC-MS/MS. The remainder 4 mL aliquots of the serums were stored in a -80 °C freezer until utilization.

Preparation of working stock solutions and internal standards

Stock solutions of T3 and T4 started with 1.0 mg/mL ampule of the corresponding Cerilliant® solution. Dilution of the stock solutions to 100 ng/mL were prepared in methanol containing 0.2% ammonia and aliquoted into 1 mL glass vials and stored in the -20 °C freezer. A new aliquot of these frozen diluted standards was used daily.

Three internal standards (**Figure 3**), $^{13}C_6$-T4,$^{13}C_6$-T3, and $^{13}C_6$-rT3 started as individual 100 µg/mL ampules of the corresponding Cerilliant® solution. A diluted mixture with a final concentration of 100 ng/mL $^{13}C_6$-T4, 50 ng/mL $^{13}C_6$-T3, and 50 ng/mL $^{13}C_6$-rT3 was prepared in methanol containing 0.2% ammonia and aliquoted into 1 mL glass vials and stored in -20 °C freezer. A new aliquot of the frozen diluted internal standard mixture was used daily.



$^{13}C_6$ - T4 when R_1 = I R_2 = I
$^{13}C_6$ - T3 when R_1 = H R_2 = I
$^{13}C_6$ - rT3 when R_1 = I R_2 = H

Figure 3. Structure of three internal standards used. All three internal standards have the $^{13}C_6$ substitution in the same location, the internal phenyl ring.

LC-MS/MS Method on AB Sciex 6500+

Samples prepared in a final solution of methanol were diluted to 50:50 with water using a needle command sequence of the Agilent 1290 Autosampler. Separation of the analytes and internal standard mixture utilized an Ascentis® Express Biphenyl Column (10 cm x 2.1 mm I.D., 2.7 µm) on an Agilent 1290, **Table 1**. The transitions monitored along with the source parameters of the Sciex Triple Quad™ 6500+ are listed in **Table 2**.

Table 1: LC Conditions and Injection Sequence

Column:	Ascentis® Express Biphenyl Column (10 cm x 2.1 mm I.D., 2.7 µm) with 0.2 µm EXP® Pre-column filter cartridge				
Mobile phase A:	Water with 0.1% (v/v) formic acid				
Mobile phase B:	Methanol with 0.1% (v/v) formic acid				
Column Temp.:	40 °C				
Injection Volume:	40 µL				
Flow Rate:	0.4 mL/min				
Needle wash:	1:1:1 isopropanol: methanol: water (v/v/v)				
Gradient:					
Time (min)	0	1.5	1.6	2.6	2.7
A%	35	26	5	5	35
B%	65	74	95	95	65

Step	Function
1	Needle wash for 2 s
2	Draw 40 µL of water into needle at 200 µL/min
3	Eject water in sample at 400 µL/min
4	Mix 60.00 µL from sample at 400 µL/min, 3x
5	Draw 40 µL from diluted sample at 200 µL/min
6	Needle wash for 2 s
7	Injection

Table 2. Mass Spectrometry Parameters and Transitions

Instrument	Sciex 6500+ Triple Quad™
Source/Gas Parameters	
Curtain Gas (CUR)	25
Collision Gas	10
IonSpray Voltage (IS)	5000
Temperature (TEM)	600
Ion Source Gas 1 (GS1)	50
Ion Source Gas 2 (GS2)	70

Analyte		Q1	Q3	Dwell (ms)	DP (V)	EP (V)	CE (V)	CXP (V)
T3	Quantifier	651.8	605.8	40	21	10	39	30
	Qualifier	651.8	478.8	40	21	10	51	26
T4	Quantifier	777.7	731.7	40	61	10	43	46
	Qualifier	777.7	604.8	40	21	10	53	30
$^{13}C_6$-T3	IS	657.7	611.8	40	21	10	39	30
	IS	657.7	484.8	40	21	10	51	26
$^{13}C_6$-T4	IS	783.6	737.5	40	61	10	43	46
	IS	783.6	610.7	40	21	10	53	30

The transitions for rT3 are the same as T3 as they are isobars of one another.

External calibration

Using a previously unused frozen aliquot of 100 ng/mL T4 and T3, a daily working solution of 4 ng/mL in methanol was prepared. Similarly, an unused frozen aliquot of the internal standard (100 ng/mL $^{13}C_6$-T4, 50 ng/mL $^{13}C_6$-T3, and 50 ng/mL $^{13}C_6$-rT3) was used to prepare a 400/200/200 pg/mL internal standard solution. Separate calibrator solutions of T3 and T4 (concentration range of 2 to 80 pg/mL) with an IS concentration of 40/20/20 pg/mL were prepared using volumetric glassware. Calibrators were loaded in duplicate and processed as described under the LC-MS/MS section.

Extracted calibration curve and preparation of sample plate

Using the 4 ng/mL of T3 and T4 in methanol as described in the previous section, calibrators in the range of 2 to 80 pg/mL were prepared in a 7.5 mM HEPES aqueous solution adjusted to pH 7.5. The calibrator solutions were loaded in duplicate at a volume of 200 µL in the sample plate.

Serum samples were pH adjusted by diluting 5% (v/v) with a 1.15 M HEPES solution. pH of serum solutions prior to extraction at room temperature were in the range of 7.3 – 7.4. The sample plate was prepared by first loading 10 µL of 1.15 M HEPES solution prior to 190 µL of the respective serum. The sample plate was agitated for 3 minutes at 300 rpm prior to starting the BioSPME sample preparation.

BioSPME Sample Preparation

An overview of the BioSPME process is shown in **Figure 4**. A Hamilton® Microlab® STARlet liquid handler was programmed to perform the method (an overview of the steps is shown in **Figure 5** and deck and consumable view shown in **Figure 6**). Briefly, the Supel™ BioSPME device was conditioned for 20 min in acetonitrile, followed by a 10 seconds wash in water. The analytes were extracted into the C18 phase of the BioSPME device over the course of a predetermined time at 1200 rpm at 37 °C using a 3 mm orbital radius Hamilton Heated Shaker. The BioSPME device then underwent a wash in the same water plate as wash one for 60 seconds before being returned to the home position. The desorption plate is then filled with 40 µL of methanol containing internal standards described earlier. The analytes are then desorbed from the BioSPME device under static conditions in 5 min. The desorption plate is covered with seal tape and loaded onto the LC-MS/MS instrument for analysis.

Method Development: Time Curve Study

A sample plate containing serum sample L-680F, pH adjusted, and a T4 calibrator prepared at 30 pg/mL were prepared and extracted as described earlier. Four different extraction time points (5, 10, 20, 30 min) were utilized. At each time point, there were n=5 serum and n=3 of the 30 pg/mL calibrator. Concentration extracted were compared to a 5-point external calibration curve as described earlier.

Condition		
20 min	static, acetonitrile	400 µL
Wash #1		
10 s	static, water	400 µL
Extraction		
20 min	1200 rpm at 37 °C	200 µL
Wash #2		
60 s	static, water	same plate as Wash #1
Desorption		
5 min	static, methanol	40 µL

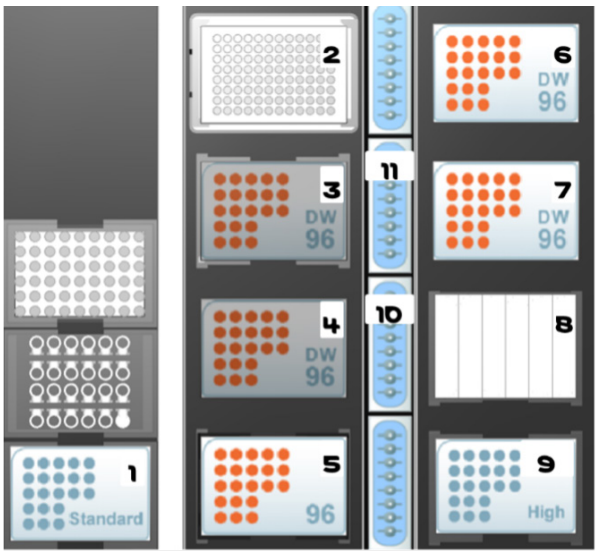
Figure 4. Overview of the BioSPME sample preparation.

Liquid Handling	
(Optional)	• Condition Plate is filled with 400 µL of acetonitrile in corresponding consecutive wells. • Wash Plate is filled with 400 µL of water in corresponding consecutive well.
Conditioning	
20 min, static	• Robot grips the BioSPME Pin Tool from the parked position and transfers to the condition plate and submerges the pins in acetonitrile for 20 min under static conditions.
Wash	
10 s, static	• Robot transfers the BioSPME from the conditioning plate to the wash plate and submerges the pins for 10 s under static conditions.
Extraction	
20 min, 37 °C at 1200 rpm	• Robot transfers the pin device to the extraction plate allowing extraction of the free testosterone to occur. The heated shaker is at 37 °C and is mixing 1200 rpm for 10 minutes. • Glass-lined well-plate to prevent non-specific binding.
Wash	
1 min, static	• Robot transfers the BioSPME device from the extraction back to the initial wash solution for 1 min under static condition.
Park	
	• Upon the second wash, the robot will transfer the BioSPME device back to the Home position. • The desorption plate is filled with 40 µL of desorption solution prepared in methanol.
Desorption	
5 min, static	• Robot transfers the BioSPME device to the desorption plate for 5 min under static condition. • BioSPME device is transfered to the Home position. • Method is complete.

Figure 5. BioSPME extraction method utilizing a Hamilton® STARlet Liquid Handler

Method Development: Reproducibility Study

Four independent calibrators were prepared in a 7.5 mM HEPES solution, pH 7.5. Six bulk sera that had externally determined free T3 and free T4 values by ED-LC-MS/MS were extracted a total of five times over multiple weeks and multiple freeze-thaw cycles. The sera pH was adjusted to 7.3 prior to extraction using a 1.15 M HEPES (5% by v/v) solution. Serum samples were loaded in quadruplets. Sample preparation was executed as described earlier.



1	Low Volume Tips for Desorption
2	Sample/extraction plate Heated Shaker *Glass-lined 96 well microplate*
3	Lid Parking
4	Desorption Plate *Thermowell® Gold PCR microplate*
5	BioSPME Home
6	Condition Plate *Axygen® Deep Well Plate*
7	Wash Plate *Axygen® Deep Well Plate*
8	Desorption Solution, Methanol w/IS
9	High Volume Tips for Conditioning and Wash
10	Solution Reservoir, Acetonitrile Conditioning solution
11	Solution Reservoir, Water Wash solution

Figure 6. Hamilton® Microlab® STARlet deck layout with consumables.

Results and Discussion

Method Development

Method development focused on two keys parameters, extraction time and reproducibility. While much work was conducted in changing solvents and organic modifiers in the BioSPME process, the factor that had the largest impact was pH. The pH adjustment was influenced by CLSI-C45-A[3], Katleen Van Uytfanghe[4], and Bingfang Yue[5]. Previously published work has also stated the impact of chloride ions and phosphate ions.[6] As a direct result, phosphate buffered saline was removed from consideration and replaced with HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); as previous reports listed that it had no impact on free thyroxine levels.

Time Curve

Using the external calibration curve, the extracted T4 values for the serum and extracted calibrator solution were determined at each time point as shown in **Figure 7** and data in **Table 3**. After 20 minutes, it is seen that the calibrator in HEPES and serum sample level off.

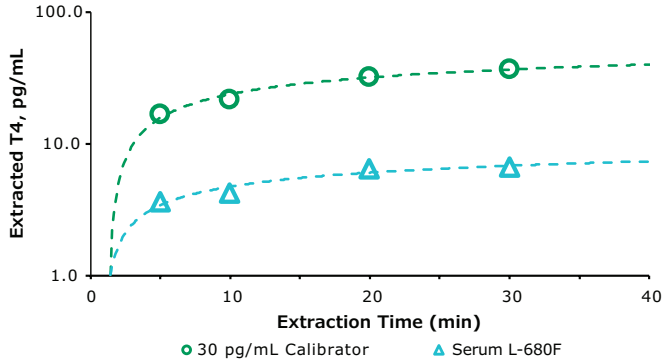


Figure 7. Time curve showing the amount of T4 extracted from serum sample L-680F (triangles) or 30 pg/mL calibrator (circles) prepared in HEPES solution.

Table 3. Concentration of T4, pg/mL, extracted against external calibration curve.

	30 pg/mL calibrator in HEPES		Serum L-680F	
Time	**Concentration (pg/mL)**	**RSD (%) n = 3**	**Concentration (pg/mL)**	**RSD (%) n = 5**
5.0	16.9	13.1	3.7	6.2
10	21.9	15.4	4.3	12.3
20	32.5	8.1	6.5	10.0
30	37.2	7.8	6.7	22.1

Reproducibility and Multiple Freeze Thaws

As highlighted earlier, a multiday freeze/thaw reproducibility study was conducted for six different sera that were previously tested externally by a validated equilibrium-dialysis LC-MS/MS method. **Figure 8** shows an example chromatogram after sample preparation by the Supel™ BioSPME device. The use of the 10 cm biphenyl column allowed separation of the isobars T3 and rT3 in addition to T4.

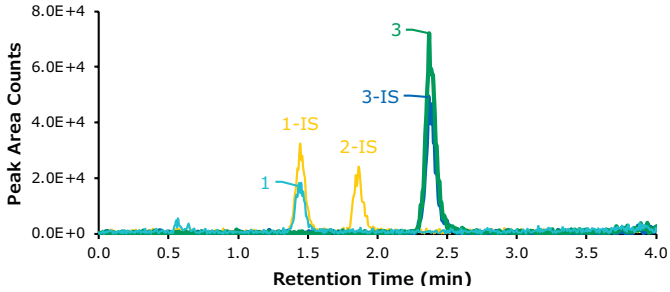


Figure 8. Representative chromatogram after extraction of **L-676M**. **1** is T3, **1-IS** is $^{13}C_6$-T3, **2-IS** is $^{13}C_6$-rT3, **3** is T4 and **3-IS** is $^{13}C_6$-T4.

In **Table 4**, the effect of dilution or no dilution with 5% 1.15 M HEPES solution can be seen. Over the three-day individual testing period, the pH of the sera was consistently between 7.3 and 7.4 prior to extraction.

Table 4. pH of bulk serum samples of unadjusted and adjusted with 5% 1.15 M HEPES solution.

Sera	L-676M	L-677M	L-678M	L-679F	L-680F	L-681F
pH undiluted	7.86	7.98	8.10	8.01	8.04	7.91
pH DAY 1 adjusted	7.30	7.32	7.31	7.33	7.35	7.33
pH DAY 2 adjusted	7.33	7.36	7.33	7.36	7.36	7.37
pH DAY 3 adjusted	7.32	7.36	7.35	7.35	7.38	7.35
pH after extraction	7.6 – 7.8					

Another important feature is the consistency in the extraction and sample preparation of the calibrators between days. As shown in **Table 5**, the reproducibility of the slopes over the course of the days for both quantitative and qualifier transitions are below 5%.

Table 5. Slopes of individual and average extracted calibration curves for monitored transitions during the reproducibility and multiple freeze thaw studies.

Slope	T3 Quantifier	T3 Qualifier	T4 Quantifier	T4 Qualifier
Day 1	3.27	3.34	4.11	0.953
Day 2	3.17	2.99	4.10	0.949
Day 3	3.14	3.08	4.29	0.919
Day 4	3.18	3.25	3.85	0.839
Day 5	3.00	3.02	4.01	0.912
AVG	3.15	3.14	4.07	0.914
RSD	3.1	4.8	3.9	5.0

With showing the consistency of the sample preparation and extraction of the calibrators and LC-MS/MS studies, the results and reproducibility for the six sera tested in quadruplets over the course of five days is shown in **Tables 6** and **7**, as well as a correlation graph in **Figure 9**. For the free T3 (range of 1.57 – 7.62 pg/mL), the interday RSD (%) was < 10%, while for free T4 (range of 4.4 – 60.3 pg/mL), the interday RSD (%) was less than 11%.

Table 6. Comparison of free T3 values, pg/mL, and reproducibility after multiple-day extraction (n=5) and freeze-thaws.

	676M	676M	678F	679F	680F	681F
ED-LC-MS/MS value (pg/mL)	3.00	3.40	7.62	3.21	1.57	2.95
AVG BioSPME-LC-MS/MS value (pg/mL)	3.75	3.13	8.01	3.00	1.48	2.84
RSD (%)	9.2	9.2	6.9	9.5	8.5	7.2

Table 7. Comparison of free T4 values, pg/mL, and reproducibility after multiple-day extraction (n=5) and freeze-thaws.

	676M	676M	678F	679F	680F	681F
ED-LC-MS/MS value (pg/mL)	13.0	13.0	60.3	10.9	4.4	11.9
AVG BioSPME-LC-MS/MS value (pg/mL)	15.6	14.1	50.8	12.1	5.8	16.5
RSD (%)	10.9	5.5	8.6	10.2	4.0	7.8

Using the data for the externally determined equilibrium dialysis prepared and internally prepared Supel™ BioSPME results, **Figure 9** highlights the correlation between the two methods for the two analytes. For the two analytes, an R^2 of >0.977 for both analytes show the strong correlation between the two methods. The slopes between the two methods (0.809 – 1.05) show the two methods further provide comparable results as well.

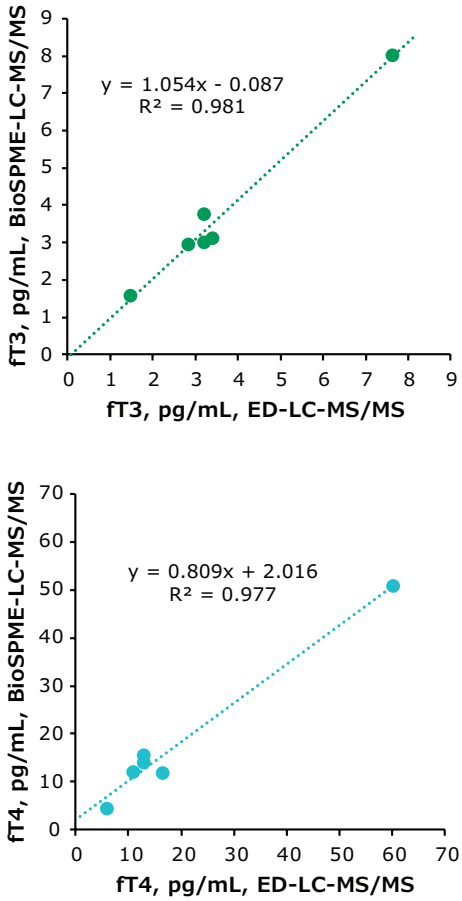


Figure 9. Correlation of free T3 (top) and free T4 (bottom) against two different sample preparation techniques. The ED-LC-MS/MS were determined externally by a validated method and laboratory.

Conclusion

In the past, free thyroid hormones, specifically free T3 and free T4, have been tested individually. However, there is now a method of sample preparation that allows for a single test to be conducted, offering a more efficient and convenient process for obtaining results. This technique, known as Supel™ BioSPME, also significantly reduces the sample preparation time from hours (as with equilibrium dialysis) to less than an hour. Consequently, this method has the potential to improve patient care by enabling quicker return of test results.

Description	Cat. No.
Supel™ BioSPME C18 96-Pin Devices, 1 pack	**59680-U**
Supel™ BioSPME C18 96-Pin Devices, 10 pack	**59683-U**
Positioning Adapter (for Automation of Supel™ BioSPME), 1 pack	**59686-U**
OmniPur® HEPES, Free Acid	**5310**
Methanol, LC-MS LiChrosolv®	**1.06035**
Water, LC-MS LiChrosolv®	**1.1533**
Formic acid 98% - 100%, LC-MS LiChropur™	**5.33002**
L-Thyroxine (T4) solution 100 µg/mL in methanol with 0.1 N NH3, ampule of 1 mL, certified reference material, Cerilliant®	**T-073**
3,3′,5-Triiodo-L-thyronine (T3) solution 100 µg/mL in methanol with 0.1 N NH3, ampule of 1 mL, certified reference material, Cerilliant®	**T-074**
L-Thyroxine-13C6 ($^{13}C_6$-T4) solution 100 µg/mL in methanol with 0.1 N NH3, ampule of 1 mL, certified reference material, Cerilliant®	**T-076**
3,3′,5-Triiodo-L-thyronine-$^{13}C_6$ ($^{13}C_6$-T3) solution 100 µg/mL in methanol with 0.1 N NH3, ampule of 1 mL, certified reference material, Cerilliant®	**T-077**
3,3′,5′-Triiodo-L-thyronine-$^{13}C_6$ ($^{13}C_6$-rT3) solution 100 µg/mL in methanol with 0.1 N NH3, ampule of 1 mL, certified reference material, Cerilliant®	**T-078**
Axygen® Deep Well Plate size 96 wells, size 600 µL, pkg of 10x5plates/cs	**P8241AXYPDW500C**
Corning® Thermowell® PCR 96 well plates 96 well plate, Thermowell® PCR plate, polypropylene, conical bottom, clear, 25/cs	**CLS6551**
Plate+ Glass Coated Microplates 96-Well Microplate, glass coated, round well; U-Shape, 7.2mm dia	**Available from NS3inc.com**
Zone-Free™ Sealing Films	**Z721646**
SealPlate Film	**Z369659**

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05/2023