TN-0150

Quantitation of Pain Panel Analytes from Oral Fluid Utilizing Microelution Solid Phase Extraction Coupled with LC-MS/MS

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Introduction

Oral fluid has emerged as a popular biological matrix for analysis due to its non-invasive nature and ease of sample collection. It has wide applicability for drug testing and screening in clinical research. However, the analysis of these compounds in oral fluid becomes challenging due to the presence of the excipients, surfactants, and preservatives in the collection buffer of commercially available oral fluid collection (OFC) devices. These additives are necessary to ensure the stability and authenticity of the sample during collection and transport. However, the presence of these additives can foul the optics of the mass spectrometer and diminish the signal response if the samples are not cleaned up adequately before injection. In this technical note, we present an effective sample cleanup method for oral fluid analysis that targets 32 pain panel analytes, utilizing a mixed mode strong cation exchange Strata™-X-C microelution 96-well plate. A Kinetex™ core-shell 2.6 µm Biphenyl, 50 x 4.6 mm column was employed for fast chromatographic separation.

Sample Preparation

Pretreatment:	Drug-free human saliva was spiked (concentration used as per **Table 1** and **2**) with standards. 1 mL of oral fluid was pipetted onto the cellulose pad and allowed to absorb until the indicator window turned blue. The saturated pad was placed into a transport tube containing buffer solution and allowed to sit overnight. The plastic nipple at the end of transport tube was removed, and the tube was placed in a centrifuge at 6000 rpm for 10 minutes. The supernatant was collected.
Condition:	Strata-X-C, 2 mg/well 96-well microelution plate (8M-S029-4GA) with 200 µL Methanol
Equilibrate:	Plate with 200 µL Water
Load:	150 µL supernatant diluted with 150 µL 1 % Formic Acid in Water
Wash 1:	With 200 µL Water
Wash 2:	With 200 µL 50 % Acetone in 1 % Formic Acid
Dry down 1:	For 30 sec at high vacuum (15-20 in. Hg)
Elute:	With 2 washes of 50 µL Methanol / Acetonitrile / Ammonium Hydroxide (5:5:2, v/v/v)
Dry down 2:	Evaporate to dryness under a gentle stream of Nitrogen at 40-45 °C
Reconstitute:	With 100 µL initial mobile phase

LC Conditions – Quantitative Analysis for Pain Panel Analytes

Column: Kinetex 2.6 µm Biphenyl
Dimension: 50 x 4.6 mm
Part No.: 00B-4622-E0
Mobile Phase: A: 10 mM Ammonium Formate
B: Methanol

Gradient:

Time (min)	%B
0	15
1	70
3	95
5.5	85
5.51	15
7	15

Flow Rate: 0.6 mL/min
Injection Volume: 5 µL
Temperature: Ambient
LC System: Agilent® 1260 Infinity
Detection: MS/MS
Detector: SCIEX® 4500 Triple Quad™

LC Conditions – Qualitative Q1 Scan (200-2000m/z)

Column: Kinetex 2.6 µm C18
Dimension: 50 x 2.1 mm
Part No.: 00B-4462-AN
Mobile Phase: A: 0.1 % Formic acid in water
B: 0.1 % Formic acid in Methanol

Gradient:

Time (min)	%B
0	10
5	95
6.5	95
6.51	10
8	10

Flow Rate: 0.5 mL/min
Injection Volume: 1 µL
Temperature: Ambient
LC System: Agilent 1260 Infinity
Detection: MS/MS
Detector: SCIEX 4500 Triple Quad

MS/MS Conditions

Ion Source: ESI
Polarity: Positive
Source Temperature: 650 °C
GS1: 70
GS2: 70
CUR: 25
IS: 5000

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Revision: 0
PHEN-RUO-00165

Results and Discussion

The Kinetex™ 2.6 µm Biphenyl column provides fast chromatographic separation and good selectivity for critical isomeric (codeine/hydrocodo ne, morphine/hydromorrphone, 6-Monoacetylmorphine/Naloxone) pairs (**Table 1**). To remove the harmful effect of the components of the OFC device on the LC-MS/MS, an aggressive organic wash was necessary. The prescribed SPE method resulted in a clean oral fluid extract with minimal interference as observed in the Q1 scan monitored from 200 to 2000 Da (**Figure 2**). A qualitative matrix effect experiment by post-column infusion was conducted. Upon continuous infusion of codeine, multiple suppression zones were revealed for the injection of unextracte d preservative buffer. The microelution SPE successfully removed most of those interferences that were responsible for ion suppression (**Figure 3**).

The QC samples for replicate extraction at 3 different concentration levels showed precision and accuracy data between 1.4 to 20.3 % and 80 to 118 %, respectively, which are within acceptable industry standards (**Table 2**). The dynamic range of this method was tested with seven calibrators over a 300-fold concentration range with linearity values of $R^2 \geq 0.995$ (**Figure 4**, **Table 1**). The simplified microelution sample extraction method provides the ideal combination of automatability and high throughput with minimum solvent usage.

Table 1. MRM Transitions and Linearity Data for 32 Pain Panel Analytes Extracted from Oral Fluid Using the Strata™-X-C 96-well Microelution Plate

Analyte Name	RT (min)	Reference conc. (ng/mL)	Q1 (m/z)	Q3 (m/z)	Linearity Range (ng/mL)	Linear regression (R^2)
Hydroxyalprazolam	4.1	100	325.1	297	1-300	0.998
Amphetamine	2.8	500	136.1	91.1	5-1500	0.999
Benzoylecgonine	2.8	150	290.1	168.1	1.5-450	0.999
Codeine	3.8	100	300.2	152.1	1-300	0.999
Diazepam	4.6	100	285	193.2	1-300	0.998
Methylenedioxy-methamphetamine (MDMA)	3.2	250	194.1	105.1	2.5-750	0.999
Methamphetamine	2.99	500	150.1	91	5-1500	0.998
Oxymorphone	3.3	100	302.1	227	1-300	0.997
Phencyclidine (PCP)	4.9	25	244.3	91	0.25-75	0.999
Sufentanil	4.9	3	387.2	238.1	0.03-9	0.995
6-Monoacetylmorphine	3.5	10	328.1	165.1	0.1-30	0.998
Clonazepam	3.8	100	316.1	270.1	1-300	0.995
2-Ethylidene-1,5-dimethyl-3,3-dipehnylpyrrolidine (EDDP)	5.2	100	278.2	234.2	1-300	0.997
Fentanyl	4.6	3	337.3	105.1	0.03-9	0.998
Flunitrazepam	4.3	100	314.1	268.2	1-300	0.998
Flurazepam	4.7	100	388.2	315.2	1-300	0.996
Hydrocodone	4.2	100	300.2	199	1-300	0.999
Hydromorphone	3.4	100	286.1	185.1	1-300	0.999
3,4-Methylenedioxymethamphetaminen (MDA)	2.9	250	180.1	133	2.5-750	0.999
Methyl diethanolamine (MDEA)	3.2	250	208.2	163	2.5-750	0.998
Meperidine	3.9	250	248.2	220.2	2.5-750	0.999
Methadone	5.4	100	310	265	1-300	0.999
Midazolam	4.5	100	326.1	291.1	1-300	0.999
Morphine-	3.1	100	286.1	152.1	1-300	0.997
Naloxone	4.17	100	328.2	212	1-300	0.995
Naltrexone	4.19	100	342.2	267.1	1-300	0.996
Nordiazepam	4.15	100	271	140	1-300	0.997
Normeperidine	3.5	100	234.1	160.1	1-300	0.995
Oxycodone	4.2	100	316.1	241.2	1-300	0.999
Temazepam	4.3	100	301.1	255.1	1-300	0.996
Tramadol	3.5	100	264.1	58.1	1-300	0.999
Cocaine	4.2	100	304.2	150	1-300	0.998

Figure 1. Representative Chromatogram of 32 Pain Panel Analytes Extracted from Oral Fluid Matrix Utilizing a Kinetex™ 2.6 µm Biphenyl Column

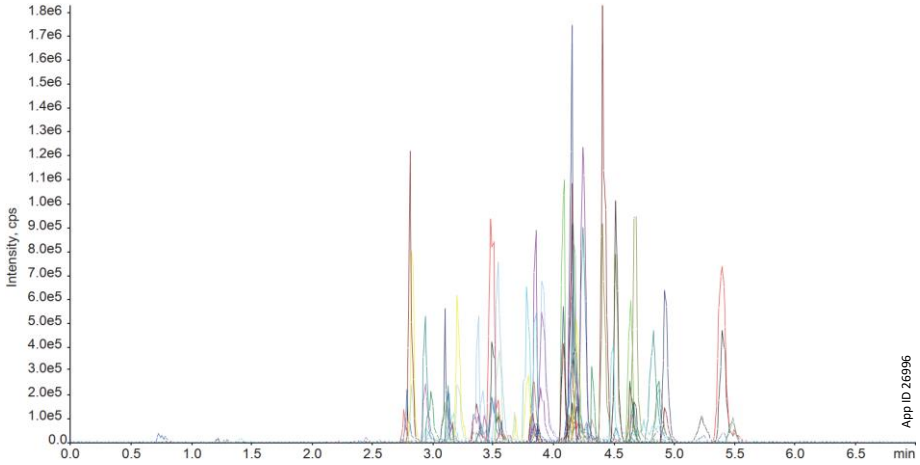


Figure 2. Representative Q1 Scan Comparison for Extracted Oral Fluid Sample Before and After SPE Cleanup

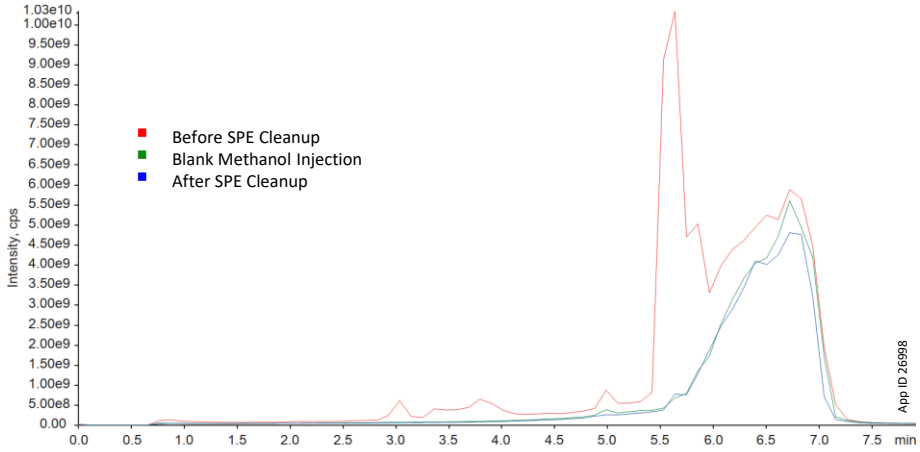


Figure 3. Representative Chromatogram Obtained from Post Column Infusion of Codeine, for an Extracted Oral Fluid Sample Before and After SPE Cleanup

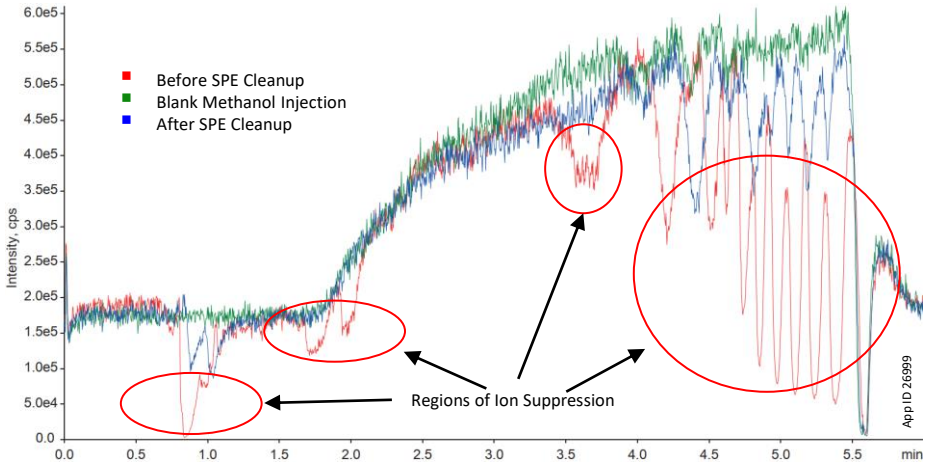
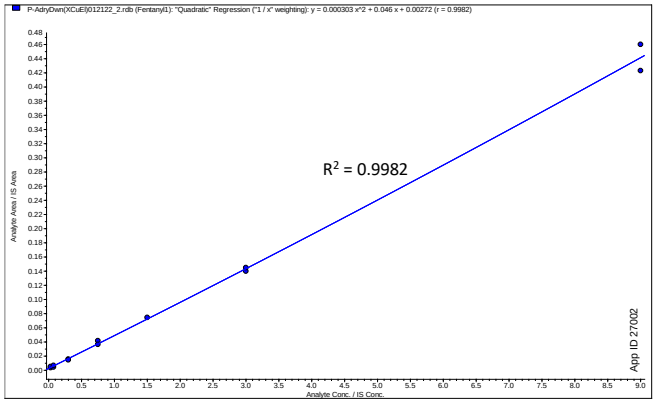


Table 2. Precision and Accuracy Data for 32 Pain Panel Analytes Extracted from Oral Fluid Using the Strata™-X-C 96-well Microelution Plate

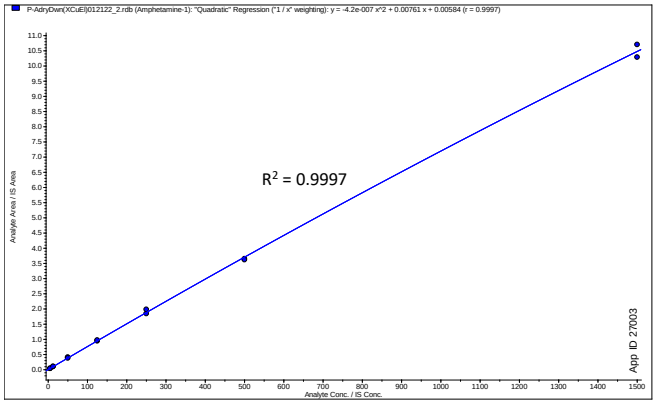
Analyte Name	QC-1 (5% of Reference) (ng/mL)		QC-2 (40% of Reference) (ng/mL)		QC-3 (2x Reference) (ng/mL)	
	% Accuracy	% CV (N=4)	% Accuracy	% CV (N=4)	% Accuracy	% CV (N=4)
Hydroxyalprazolam	104.2	6.4	105.9	7.4	108.4	13.1
Amphetamine	103.7	13.5	99.3	5.2	94	6.2
Benzoylecgonine	111.3	11.3	114.3	13.4	105.5	2.9
Codeine	108.9	12.3	108.6	6.2	103.2	3.3
Diazepam	101.3	6.2	105.3	9.9	104.9	9.9
Methylenedioxy-methamphetamine (MDMA)	109.4	10.1	99.8	4.7	99.9	2.2
Methamphetamine	99.1	6.4	95.6	3.7	93.4	3.6
Oxymorphone	106.8	5.3	109	7	100.6	4.8
Phencyclidine (PCP)	95	4.8	100.3	7.6	97	1.5
Sufentanil	103.9	18.1	118.1	10.9	89.6	11.3
6-Monoacetylmorphine	99.3	16.7	104.9	4.3	103.8	4.2
Clonazepam	108.3	16.4	99.6	11	95.2	7
2-Ethylidene-1,5-dimethyl-3,3-dipehnylpyrrolidine (EDDP)	114.3	15.6	99.8	12.8	105.4	10.9
Fentanyl	107.4	14.9	117	8.8	98.8	1.9
Flunitrazepam	107.3	18.1	113	15.6	115	10.2
Flurazepam	98.5	20.3	114.4	14.9	117.8	1.9
Hydrocodone	112.4	8.8	107.4	7.4	95.7	1.8
Hydromorphone	116.3	13.5	112.9	3.4	108.7	1.6
3,4-Methylenedioxymethamphetaminen (MDA)	100	6.7	109.4	4.9	94.9	5.3
Methyl diethanolamine (MDEA)	110.8	10.6	102.3	4.5	101.1	5.4
Meperidine	91.6	15.3	98.6	3.4	91.3	3.2
Methadone	86.5	9.3	89.5	9	92.5	5.9
Midazolam	12.2	13	111.1	7.6	105.7	8.5
Morphine	102.6	7.1	102.5	4.3	93.9	5.3
Naloxone	113.5	19.3	102.7	14.9	98.7	6
Naltrexone	105.2	8.5	109	10.2	97.6	6.5
Nordiazepam	93.6	14.5	118	15.5	97.1	14.4
Normeperidine	80.8	18.3	90.2	13.2	95.2	11.2
Oxycodone	115.5	5.1	112.1	8.1	97.9	1.4
Temazepam	96.1	10.7	116.9	11.2	109.3	12.5
Tramadol	92.3	9.6	109.1	10.3	85	3
Cocaine	102.7	5.3	114.8	7.7	91	15.1

Figure 4. Linearity Curves for Selected Analytes in an Oral Fluid Sample Extracted Using a Strata™-X-C, 96-well Microelution Plate Over a 300-fold Dynamic Concentration Range

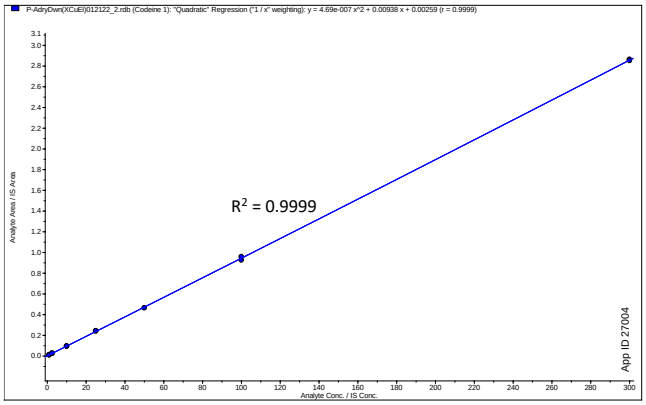
Fentanyl (0.03-9 ng/mL)



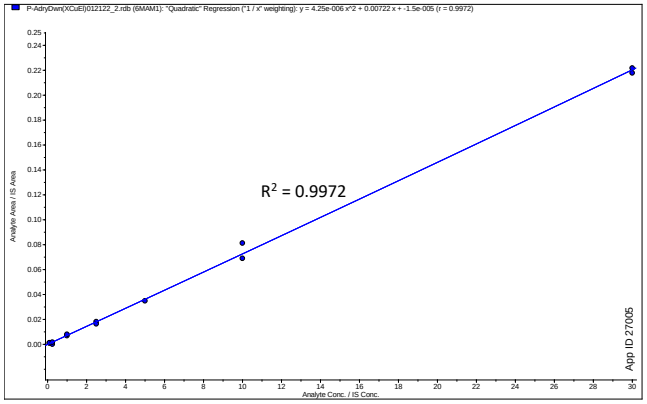
Amphetamine (5-1500 ng/mL)



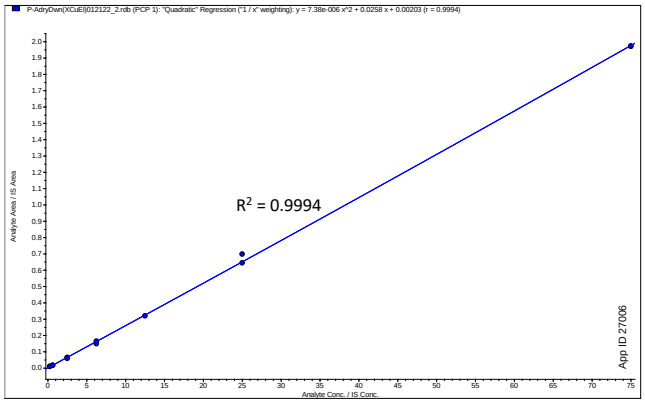
Codeine (1-300 ng/mL)



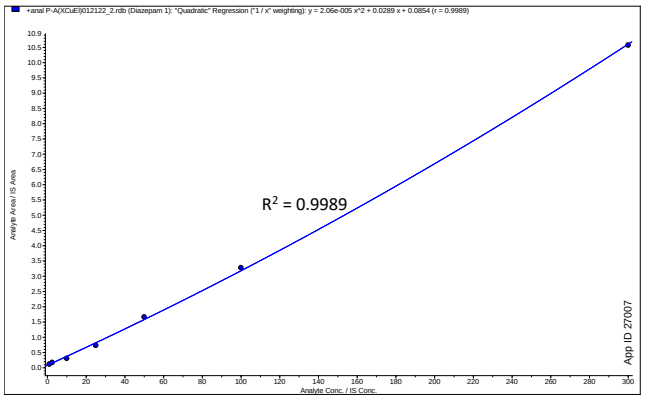
6-Monoacetylmorphine (0.1-30 ng/mL)



Phencyclidine (0.25-75 ng/mL)



Diazepam (1-300 ng/mL)



Conclusion

The prescribed sample prep method utilizing microelution SPE resulted in a simple, rapid extraction for identification and quantitation of 32 pain panel analytes from oral fluid which is cost effective and can efficiently be incorporated in clinical workflow analysis.

Kinetex™ Ordering Information

2.6 µm Analytical Columns (mm)						SecurityGuard™ ULTRA Cartridges (mm)‡	
Phases	**30 x 4.6**	**50 x 4.6**	**75 x 4.6**	**100 x 4.6**	**150 x 4.6**	**250 x 4.6**	**3/pk**
EVO C18	00A-4725-E0	00B-4725-E0	—	00D-4725-E0	00F-4725-E0	00G-4725-E0	AJ0-9296
PS C18	00A-4780-E0	00B-4780-E0	—	00D-4780-E0	00F-4780-E0	00G-4780-E0	AJ0-8949
Polar C18	00A-4759-E0	00B-4759-E0	—	00D-4759-E0	00F-4759-E0	—	AJ0-9530
Biphenyl	—	00B-4622-E0	—	00D-4622-E0	00F-4622-E0	—	AJ0-9207
XB-C18	—	00B-4496-E0	00C-4496-E0	00D-4496-E0	00F-4496-E0	—	AJ0-8768
C18	00A-4462-E0	00B-4462-E0	00C-4462-E0	00D-4462-E0	00F-4462-E0	—	AJ0-8768
C8	—	00B-4497-E0	00C-4497-E0	00D-4497-E0	00F-4497-E0	—	AJ0-8770
HILIC	—	00B-4461-E0	00C-4461-E0	00D-4461-E0	00F-4461-E0	—	AJ0-8772
Phenyl-Hexyl	—	00B-4495-E0	00C-4495-E0	00D-4495-E0	00F-4495-E0	—	AJ0-8774
F5	00A-4723-E0	00B-4723-E0	—	00D-4723-E0	00F-4723-E0	—	AJ0-9230

for 4.6 mm ID

2.6 µm Minibore Columns (mm)					SecurityGuard ULTRA Cartridges (mm)‡	
Phases	**30 x 2.1**	**50 x 2.1**	**75 x 2.1**	**100 x 2.1**	**150 x 2.1**	**3/pk**
EVO C18	00A-4725-AN	00B-4725-AN	—	00D-4725-AN	00F-4725-AN	AJ0-9298
PS C18	00A-4780-AN	00B-4780-AN	—	00D-4780-AN	00F-4780-AN	AJ0-8951
Polar C18	00A-4759-AN	00B-4759-AN	—	00D-4759-AN	00F-4759-AN	AJ0-9532
Biphenyl	00A-4622-AN	00B-4622-AN	—	00D-4622-AN	00F-4622-AN	AJ0-9209
XB-C18	00A-4496-AN	00B-4496-AN	00C-4496-AN	00D-4496-AN	00F-4496-AN	AJ0-8782
C18	00A-4462-AN	00B-4462-AN	00C-4462-AN	00D-4462-AN	00F-4462-AN	AJ0-8782
C8	00A-4497-AN	00B-4497-AN	00C-4497-AN	00D-4497-AN	00F-4497-AN	AJ0-8784
HILIC	00A-4461-AN	00B-4461-AN	00C-4461-AN	00D-4461-AN	00F-4461-AN	AJ0-8786
Phenyl-Hexyl	00A-4495-AN	00B-4495-AN	00C-4495-AN	00D-4495-AN	00F-4495-AN	AJ0-8788
F5	00A-4723-AN	00B-4723-AN	—	00D-4723-AN	00F-4723-AN	AJ0-9322

for 2.1 mm ID

‡ SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-9000

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