Ultra-Sensitive SLE-LC-MS/MS Method Optimization for Nicotine Metabolites Measurement in Human Serum

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ABSTRACT

Tobacco smoking remains a leading cause of preventable disease and premature death in the United States and other countries. Its active ingredient, nicotine, has long been recognized to be an addictive substance. Cotinine (COT) and trans-3'-hydroxycotinine (HC) are two primary metabolites of nicotine after exposure to tobacco smoke. Because their concentrations in body fluids are greater and their half-lives are longer than nicotine, these two metabolites are generally preferred over nicotine as biomarkers of tobacco smoke exposure and are often used for biomonitoring in general population study.

A bioanalytical method for the measurement of COT and HC in human serum has been developed by CDC (Method 2017). The CDC method has been used for large-scale biomonitoring study such as the National Health and Nutrition Examination Survey (NHANES) that is ongoing. Due to a wide range of tobacco exposure in general population (e.g., smokers and non-smokers simultaneously), a higher sensitivity/selectivity and a wider range of linearity are required for a population study. The Environmental and Chemical Laboratory Services (ECLS) within the New Jersey Department of Health (NJDOH) conducted experiments to optimize the method based on the CDC method #2017, including Liquid Chromatography (LC) conditions and Mass Spectrometry (MS) parameters as well as Supported Liquid Extraction (SLE) procedure to enhance overall method sensitivity/selectivity.

By using new mobile phase compositions and switching to different ionization source, we achieved much higher signal to noise ratio (S/N), which resulted in improved sensitivity and selectivity of the method. In addition, we further refined the sample preparation step that resulted in recoveries greater than 95% for both analytes in complex human serum matrix. This optimized method was validated and will be used to support the NJ Health and Nutrition Examination Survey (NJHANES), a surveillance project of the NJ State Biomonitoring Program. The method optimization and validation results will be discussed in this poster
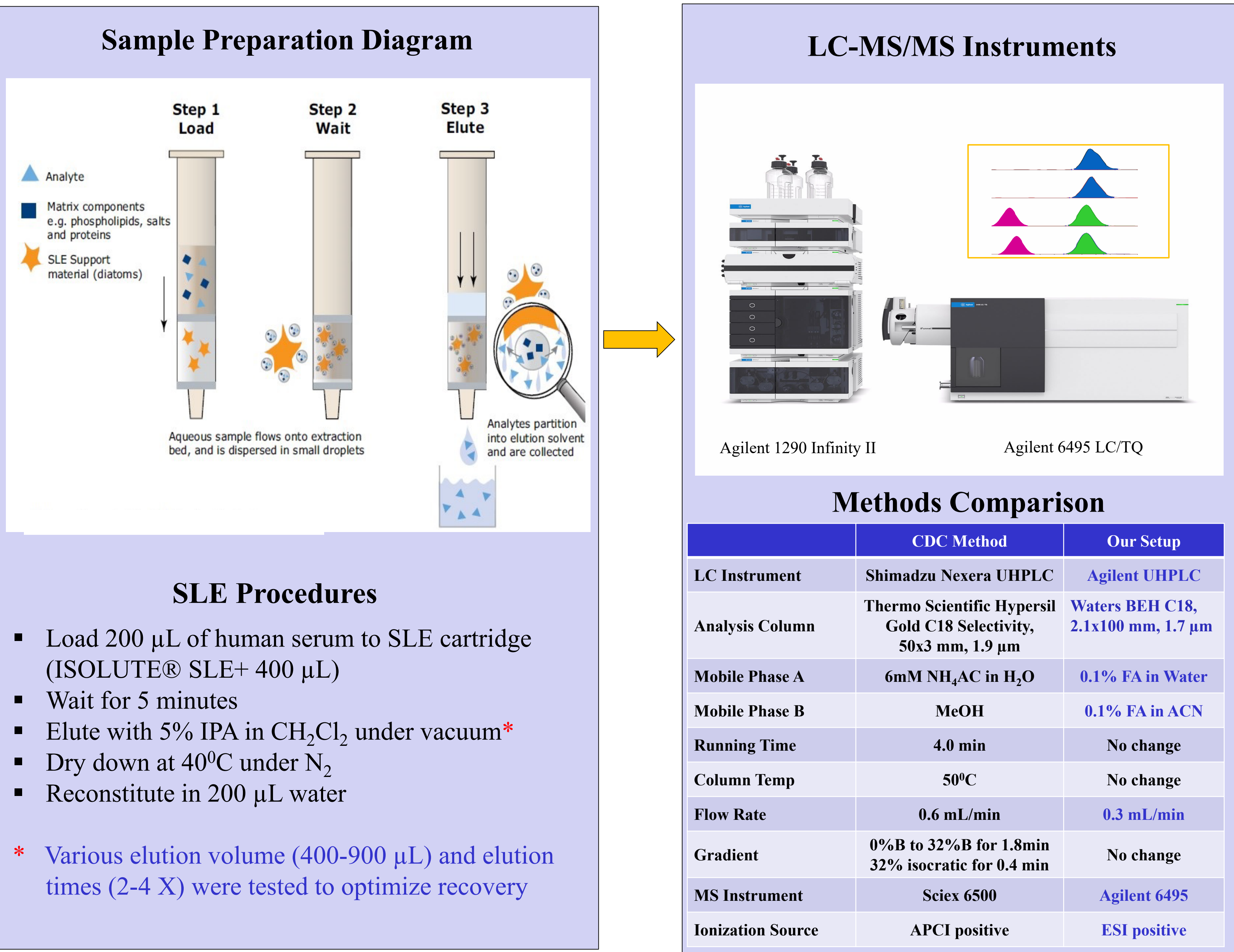
OBJECTIVES

➢ Adopt SLE-LC-MS/MS methods to measure COT and HC in human serum based on CDC's existing method (# 2017)

➢ Optimize LC-MS/MS conditions to increase sensitivity and selectivity

➢ Optimize sample preparation procedures to improve recovery

➢ Validate the method that will be used for both non-smoking and smoking population in NJHANES study
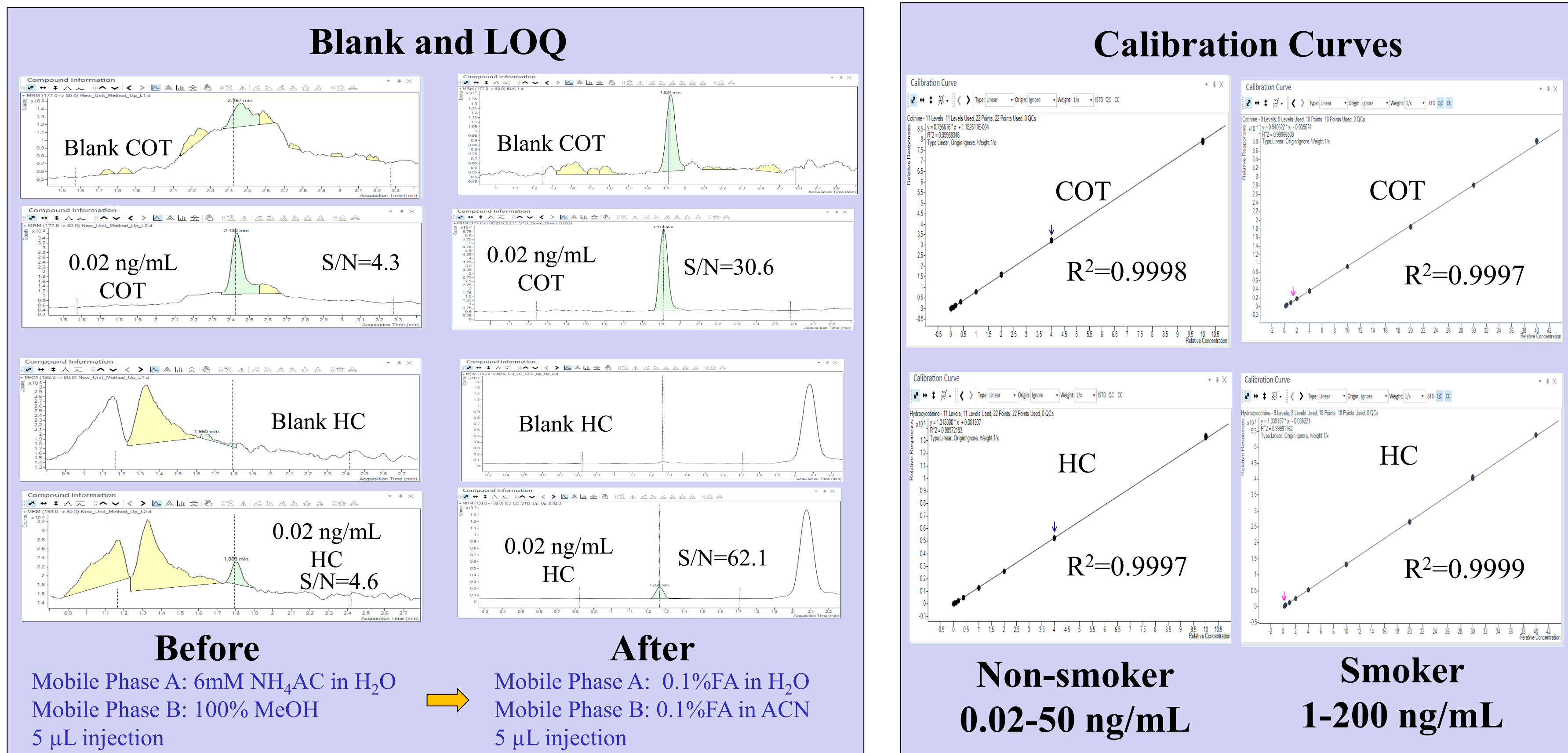
TEST PLANS

➢ Optimize MS/MS parameters using positive ESI mode.

➢ Optimize LC conditions to decrease interfering background.

➢ Test different SLE sample preparation conditions to improve recovery.
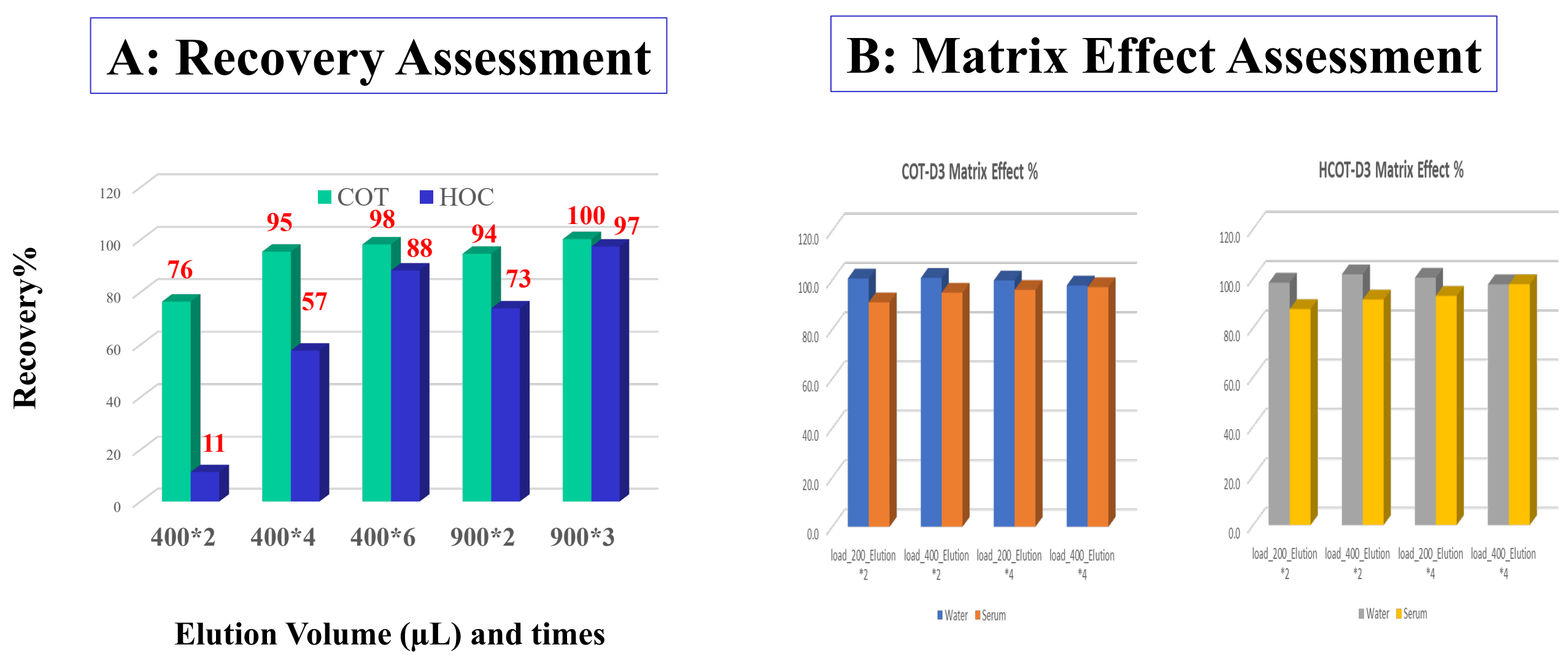
MATERIALS AND METHODS

Sample Preparation Diagram

Step 1 Load
Step 2 Wait
Step 3 Elute

▲ Analyte
◆ Matrix components e.g. phospholipids, salts and proteins
★ SLE Support material (diatoms)

Aqueous sample flows onto extraction bed, and is dispersed in small droplets

Analytes partition into elution solvent and are collected

SLE Procedures

- Load 200 µL of human serum to SLE cartridge (ISOLUTE® SLE+ 400 µL)
- Wait for 5 minutes
- Elute with 5% IPA in CH_2Cl_2 under vacuum*
- Dry down at 40^0C under N_2
- Reconstitute in 200 µL water

* Various elution volume (400-900 µL) and elution times (2-4 X) were tested to optimize recovery

LC-MS/MS Instruments

Agilent 1290 Infinity II   Agilent 6495 LC/TQ

Methods Comparison

	CDC Method	Our Setup
LC Instrument	Shimadzu Nexera UHPLC	Agilent UHPLC
Analysis Column	Thermo Scientific Hypersil Gold C18 Selectivity, 50x3 mm, 1.9 µm	Waters BEH C18, 2.1x100 mm, 1.7 µm
Mobile Phase A	6mM NH_4AC in H_2O	0.1% FA in Water
Mobile Phase B	MeOH	0.1% FA in ACN
Running Time	4.0 min	No change
Column Temp	50^0C	No change
Flow Rate	0.6 mL/min	0.3 mL/min
Gradient	0%B to 32%B for 1.8min 32% isocratic for 0.4 min	No change
MS Instrument	Sciex 6500	Agilent 6495
Ionization Source	APCI positive	ESI positive

RESULTS OF LC/MS/MS OPTIMIZATION

Blank and LOQ

Blank COT

0.02 ng/mL COT   S/N=4.3

Blank HC

0.02 ng/mL HC   S/N=4.6

Blank COT

0.02 ng/mL COT   S/N=30.6

Blank HC

0.02 ng/mL HC   S/N=62.1

Before
Mobile Phase A: 6mM NH_4AC in H_2O
Mobile Phase B: 100% MeOH
5 µL injection

After
Mobile Phase A: 0.1%FA in H_2O
Mobile Phase B: 0.1%FA in ACN
5 µL injection

Calibration Curves

COT R^2=0.9998

COT R^2=0.9997

HC R^2=0.9997

HC R^2=0.9999

Non-smoker
0.02-50 ng/mL

Smoker
1-200 ng/mL

❖Background noise significantly decreased after switching to different mobile phases for both analytes

❖Sensitivity improved significantly with improved S/N ratio from 4.3 to 30.6 for COT and 4.6 to 62.1 for HC

❖Selectivity improved for confirmation ions of both analytes by changing ionization source to ESI positive

❖Excellent linearity achieved for both analytes (R^2>0.999) in wide linear range

RESULTS OF SLE OPTIMIZATION

A: Recovery Assessment

B: Matrix Effect Assessment

COT-D3 Matrix Effect %   HCOT-D3 Matrix Effect %

❖Recovery of COT increased from 76% to 100% by increasing elution volume up to 900 µL with three times of elution.

❖Recovery of HC increased from 11% to 97% under same optimized condition.

❖No adverse matrix effect observed

Accuracy and Precision Results

Accuracy: Non-smoking

Analyte	0.1 ng/mL (n=3)	1.0 ng/mL (n=3)	10 ng/mL (n=3)
COT	90.4%	96.8%	97.9%
HC	92.9%	100.1%	97.9%

Precision: Non-smoking

Day	Analyte	Spiking Conc. (ng/mL)	Mean (n=4)	Intra-day %RSD (n=4)	Inter-day %RSD (n=12)
Day1	COT	1.00	0.95	0.8	2.3
Day2	COT	1.00	0.99	3.2	
Day3	COT	1.00	0.98	0.8	
Day1	HC	1.00	1.00	3.4	3.4
Day2	HC	1.00	0.96	2.3	
Day3	HC	1.00	0.99	3.2	

Accuracy: Smoking

Analyte	2.0 ng/mL (n=3)	20 ng/mL (n=3)	150 ng/mL (n=3)
COT	106.2%	102.2%	97.8%
HC	101.9%	101.3%	104.5%

Precision: Smoking

Day	Analyte	Spiking Conc. (ng/mL)	Mean (N=4)	Intra-day %RSD (n=4)	Inter-day %RSD (n=12)
Day1	COT	20.00	19.89	4.5	2.7
Day2	COT	20.00	19.72	1.5	
Day3	COT	20.00	20.01	1.7	
Day1	HC	20.00	19.65	5.4	3.5
Day2	HC	20.00	19.47	2.4	
Day3	HC	20.00	19.80	2.7	

CONCLUSIONS AND FUTURE PLANS

❖An ultra sensitive LC-MS/MS method (LOQ=0.02ng/mL) was established at ECLS-NJDOH to measure nicotine metabolites in human serum matrix

❖Higher sensitivity/selectivity was obtained after LC condition and MS parameter optimization. 0.005 ng/mL detection limit was achieved

❖Recovery and matrix effect were evaluated under different sample preparation conditions. The optimized procedure achieved >95% recovery for both analytes

❖Wider calibration ranges were achieved to cover both non-smoking and smoking specimens with excellent linearity (R^2>0.9990)

❖This method demonstrated acceptable accuracy (95-105% recovery) and precision (<5%)

❖This method will be used to support NJHANES and other NJ Biomonitoring programs

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