**Supplementary Materials**

**Table 1.** Detailed methods for measurement of plasma and urine COT and 3HC at each Test Site.

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| **Method ID** | **Test Site Name** | **Type(s) of biological samples analyzed** | **Fluid volume (µL)** | **Extraction Procedure** | **Resuspension Fluid** | **Instrumentation and Conditions** | **Ref.** |
| 1A | University of Toronto | Plasma, urine | Plasma: 100 µL  Urine: To 50 µL urine was added 950 µL water (1:20 dilution),100 uL aliquot was used for sample preparation | (Protein precipitation and LLE)  Samples were diluted with 900 µL HPLC-grade water, 100 uL 30% perchloric acid added to precipitate protein, vortexed, centrifuged at 2200 g, supernatant combined with 2 mL tripotassium phosphate (50% w/v in water, pH ~14), 5 mL methylene chloride added, vortexed, organic layer removed, 10% HCl in methanol added, evaporation under nitrogen. Identical procedure to plasma was performed for urine samples. | 100 µL buffer (100 mM ammonium acetate in 80/20 water/methanol and 1% acetic acid) | Instrumentation: Agilent 1260 HPLC and Agilent 6430 Triple Quadrupole LC/MS system  Column: Synergi Polar RP column (150 x 4.6 mm I.D.; particle size 4 micron)  Mobile phase: 10mM ammonium acetate/0.1% acetic acid in water (solvent A) and 10mM ammonium acetate/0.1% acetic acid in methanol (solvent B)  Gradient: The initial composition was 20% solvent B, changing to 100% solvent B over 6.5 minutes, maintained at 100% solvent B until 8.0 minutes, changing to 20% solvent B at 8.1 minutes and maintained at 20% B until the end of the run at 13.0 minutes.  Flow Rate: 0.7 ml/min  Ionization: atmospheric pressure ionization (APCI), positive mode  Mass Spectrometry: collision energy of 35 eV for cotinine and cotinine-d3, 30 eV for 3HC and 3HC-d3  Ion transitions: Cot, 177🡪80; Cot-d3, 180🡪80; 3HC, 193🡪80; 3HC-d3, 196🡪80, corona current 4 uA | (1) |
| 1B | University of California, San Francisco | Plasma, urine | Plasma: 100 µL  Urine: 100 µL | (Protein precipitation and LLE)  Samples were diluted with 900 µL HPLC-grade water, 100 µL of 30% perchloric acid added to precipitate protein, vortexed, centrifuged, supernatant combined with 2 mL tripotassium phosphate (50% w/v in water, pH ~14), 6 mL methylene chloride added, vortexed, organic layer removed, 100 µL of 10% HCL in methanol added and evaporated in centrifugal vacuum evaporator.  Identical procedure to plasma was performed for urine samples. | Plasma: 150 µL 100 mM aqueous ammonium formate  Urine: 1 mL of 100 mM aqueous ammonium formate | Instrumentation: Agilent 1200 HPLC interfaced with a Thermo Electron TSQ Quantum Ultra triple-stage quadrupole mass spectrometer with an Ion Max APCI  Column: 4.0 mm x 150 mm Supelco Discovery HSF5 column (5 micron) fitted with an 4.0 x 20 mm HSF5 guard column  Mobile phase:10mM ammonium formate in water (solvent A) and 10mM ammonium formate in methanol (solvent B)  Gradient: The initial composition was 20% solvent B, changing to 100% solvent B over 6.5 minutes, maintained at 100% solvent B until 8.0 minutes, changing to 20% solvent B at 8.1 minutes and maintained at 20% B until the end of the run at 10.0 minutes. Flow Rate: 0.7 ml/min  Ionization: atmospheric pressure ionization (APCI), positive mode  Mass Spectrometry: collision energy of 33 eV for cotinine and cotinine-d9, 35 eV for 3HC and 3HC-d9  Ion transitions: Cot, 177🡪80; Cot-d9, 186🡪84; 3HC, 193🡪80; 3HC-d9, 202🡪84, corona current 5 uA | (1) |
| 1C | Centre for Addiction and Mental Health | Plasma | 200 µL | (2)  To 200 µL specimen was added 100 µL water and 50µL of deuterated internal standard working solution (400ng/mL in 10%methanol), samples were deproteinized with 50µL of 10% perchloric acid; after vortexing and centrifugation 225 µL ofsupernatant was alkalinized with 75 µL of 1N ammonium hydroxide to pH 9-10, transferred to Chem Elut cartridges and eluded with 2 portions of 1.5 mL of 9:1 dichloromethane:isopropanol, 50 µL of acidified methanol to all eluant , eluate dried | 100 µL acetonitrile | Instrumentation: Finnigan Surveyor HPLC system and Thermo Fisher TSQ Quantum mass spectrometer  Column: PolyHydroxyEthyl A 100 x 4.6 mm 3um, 300 Å at 40 0C  Mobile phase: acetonitrile (solvent A), 0.1% formic acid pH 3.5 (solvent B) and 10 mM ammonium acetate pH 5.0 (solvent C)  Gradient: The initial composition was 70% solvent A, 20% solvent B and 10% solvent maintained over 0.5 minutes, changing to 30% solvent A, 60% solvent B and 10% solvent C at 1.0 minutes, maintained at this composition until 2.0 minutes, changing to 70% solvent A, 20% solvent B, 10% solvent C and a flow rate of 1.0 mL/minute at 2.01 minutes, maintained at this composition and flow rate until 4.0 minutes, maintaining same composition but changing flow rate to 0.8 mL/minute at 4.01 minutes and kept at same composition and flow rate until the end of the run at 4.50 minutes  Flow Rate: 0.8 and 1.0 mL/min  Ionization: atmospheric pressure ionization (APCI), positive mode  Mass Spectrometry: collision energy 35 eV for Cot and Cot-d3, 31 eV for 3HC and 3HC-d3 Ion Transititions: Cot, 177🡪80; Cot-d3, 180🡪80; 3HC, 193🡪80; 3HC-d3, 196🡪80, corona current 5 uA |  |
| 2A | Yale University | Plasma | 200 µL | (Protein precipitation and LLE)  50 µL working deuterated internal standard solution added to to 200 µL of samples, standards and controls, vortexed, added 50 µL 0.1M zinc sulfate, followed by 400 µL methanol, vortex, centrifugated in micro centrifuge for 20 min, supernate collected and recentrifuged for ten minutes | 10 uL of final supernate injected | Instrumentation:Waters Acquity HPLC and Waters Quattro Micro API Micromass QAA1268 (triple quadrupole MS/MS)  Column: WATERS Acquity UPLC HSS C18 1.8 um 2.1X150mm  Mobile phase: Gradient elution: Solvent A: 0.1% formic acid and 0.2% 1M ammonium acetate in water; Solvent B: 0.1% formic acid and 0.2% 1M ammonium acetate in methanol. Initial composition 90% solvent A (aqueous) and 10% solvent B (organic), ramped up to 100% solvent B at 4 minutes.  All peaks eluted before 4 minutes. Total run time four minutes.  Flow Rate: 0.4mL/min  Ionization: Electrospray ionization (ESI), positive ion mode  Mass Spectrometry: collision energy of 20 eV for cotinine and cotinine-d3, 25 eV for 3HC and 3HC-d3  Ion transitions: Cot, 177🡪80 (Quantifier),177🡪98 (Qualifier); Cot-d3, 180🡪80; 3HC, 193🡪80 (Quantifier),193🡪134 (Qualifier),3HC-d3, 196🡪80 | (2) |
| 2B | National Institute on Drug Abuse | Plasma | 500 µL | (Protein precipitation and SPE)  10 µL working deuterated internal standard solution added to to 200 µL of samples, , standards and controls followed by 2 mL 0.1% formic acid, centrifugated at 4000xg for 5 min at 4°C, supernatant collected and submitted to solid phase extraction using Strata-XC ( 33µm, 60 mg cartridges, Phenomenex) conditioned with 2 mL methanol and 2 mL water. Cartriges were wahed withwashing with 2 mL 0.1 M acetic acid and 2 mL methanol and eluded with 3 mL 3% NH4OH in MeOH. 100 µL of 1% HCl in methanol was added prioer to evaporation under a stream of nitrogen. | Reconsititution in 100 µL 1mM ammonium formate with 0.01% formic acid pH 3.25 | Instrumentation: Shimadzu liquid chromatography system interfaced to a 3200 QTrap with a Turbo V ESI source.  Column: Synergi Polar-RP 100A, 100x2.1mm, 2.5um, with a 4x2mm identically packed guard column.  Mobile phase: 1mM ammonium formate pH 3.5 with 0.1% formic acid (Solvent A), and acetonitrile (Solvent B)  Gradient elution was with mobile phase A (1 mM ammonium formate pH 3.3 with 0.1% formic acid) and mobile phase B (acetonitrile) at a flow rate of 0.3 mL/min. The initial mixture (94A:6B) was maintained for 3 min, then mobile phase B was increased to 60% at 5 min and held for 3 min. The mixture returned to the initial conditions at 10 min, followed by 2 min equilibration. The total run time was 12 min  Flow rate: 0.3 mL/min.  Ionization: Electrospray ionization (ESI), positive mode  Mass Spectrometry: collision energy of 29 eV for cotinine, 31 eV for cotinine-d3 and 27 eV for 3HC and 3HC-d3  Ion transitions: 177.2>80.1 (quantifier) and 177.2>98.1 (qualifier) for cotinine; 193.2>80.2 (quantifier) and 193.2>134 (qualifier) for3HC; 180.2>80.2 (quantifier) and 180.2>101.2 (qualifier) for Cotinine-d3; 196.2>79.9 (quantifier) and 196.2>134.1(qualifier) for 3HC- d3- | (3) |
| 2C | University of Minnesota | Plasma | 50 µL plasma, | (2)  400 µL filtered water and 50 µLdeuterated internal standard solution were added to Oasis MCX 96well plate (2 mg sorbent material, particle diameter 30µm and 1ml cartridge volume, Waters) conditioned with 200 µL methanol and 200 µL 0.5% formic acid (by volume), the columns were washed with 200 µl 0.5% formic acid and 200 µl methanol then COT and 3HC eluted with 60:40 acetonitrile: methanol (v/v) + 2% ammonium hydroxide into tapered 96-well plates, solvent removed under gentle stream of nitrogen | 16 µL 20 mM ammonium acetate (pH 6.7), | Instrumentation: Eksigent nanoLC-Ultra interfaced to a Thermo TSQ Vantage triple quadrupole mass spectrometer.  Column: Luna C18 150x0.50 mm (3 um particle size), Phenomenex  Mobile phase: 85% 20 mM ammonium acetate, 15% acetonitrile  Gradient: isocratic  Flow Rate: 10μL/min  Ionization: Electrospray ionization (ESI), positive ion mode  Mass Spectrometry: collision energy of 25 eV for COT and 20 eV for 3HC;  Ion transitions: cotinine (m/z 177-80 and m/z 177-98), -cotinine-d3 (m/z 180-80 and m/z 180-101), 3HC (m/z 193-80 and m/z 193-134), and 3HC-d3 (m/z 196-80 and m/z 196-134) | (4) |
| 3 | National Institute for Health and Welfare (THL) / University of Helsinki | Plasma | 500 µL | (2)  Extraction was performed with BondElut Plexa PCX 1 cc 30 mg solid phase extraction cartridges conditioned with 160 µL of methanol and 600 µL of purified water, samples were loaded with 500 µL of NaH2PO4 buffer containing the ISs Cot-d3 and 3HC-d3. The cartridges were washed with 600 µL of purified water and 600 µL of methanol, dried, the analytes were eluted with 3-% NH3 in acetonitrile, and the solvent was evaporated to dryness with a vacuum evaporator. After dissolving the residue in 60 µL acetonitrile, 30 µL derivatization reagent, N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) added, and incubated at 80C for 30 min. | 2-µL aliquot of the derivatized sample was injected | Instrumentation: The analyses were performed with an Agilent Technologies 6890N GC system combined with a 5975 B inert XL mass-selective detector, and a 7683 B series autosampler (Palo Alto, CA, USA). The MSD was operated in electron impact (EI) mode using an ionization energy of 70 eV.  Column: The GC column was a DB-17 ms of length 20 m, internal diameter 0.18 mm and film thickness 0.18 µm (J&W Scientific Inc.)).  Carrier gas: Helium.  Temperature gradient: The column temperature was initially 120C with a hold time of 3 min, and was increased 45C/min to 300C.  Ionization: Electron ionization (EI), positive mode  Mass Spectrometry: ionization energy 70 eV for all analytes  Ion Transitions: MS detection of Cot (m/z 176-98 ) and 3HC (m/z 249-144) was performed in selected ion monitoring (SIM) mode. | (5) |
| 4A | Cliniques Universitaires UCL Mont-Godinne | Urine | 1 mL | (LLE)  Samples were fortified with internal standard 2-phenylimidazole, extracted with 12 mLchloroform and mixed vigorously for 15 minues. After centrifugation, the organic phase was evaporated until dryness in centrifugal vacuum evaporator | 200 µL acetonitrile: potassium phosphate buffer 10:90, v/v | Instrumentation: Waters Alliance HPLC with diode array detector  Column : Interstil ODS-3V 4.6x150 mm, 5µM, (Alltech) with matching guard column  Mobile Phase: Acetonitrile/potassium phosphate buffer (10:90, v/v, pH 4.3)  Gradient; isocratic  Flow rate : 0.8 mL/min  Detection : UV 260 nm |  |
| 4B | University of Toronto | Urine | To 50 µL urine was added 950 µL water (1:20 dilution), 500 uL aliquot was used for sample preparation | (2)  Samples were fortified with internal standard 5-methylcotinine  (70 μg), loaded onto ISOLUTE HM-N columns, extracted with 13 mL dichloromethaneand dried under nitrogen | 105 µL 0.01 M HCl | Instrumentation: Agilent 1200 series HPLC with UV detection  Column: ZORBAX Bonus-RP column 150 x 4.6 mm,5 uM (Agilent Technologies))  Mobile Phase:Acetonitrile/potassium phosphate buffer (10:90 v/v, pH 5.07) containing 3.3 mM heptane sulfonic acid and 0.5% triethylamine  Gradient: isocratic  Flow Rate: 0.9 ml/min  Detection: UV 260 nm | (6) |

a. LLE (Liquid-Liquid Extraction); SPE (Solid Phase Extraction)

**References**

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