

# FDA Compliant Analysis of Nicotine Plus Nine Metabolites in Human Urine by LC-MS/MS

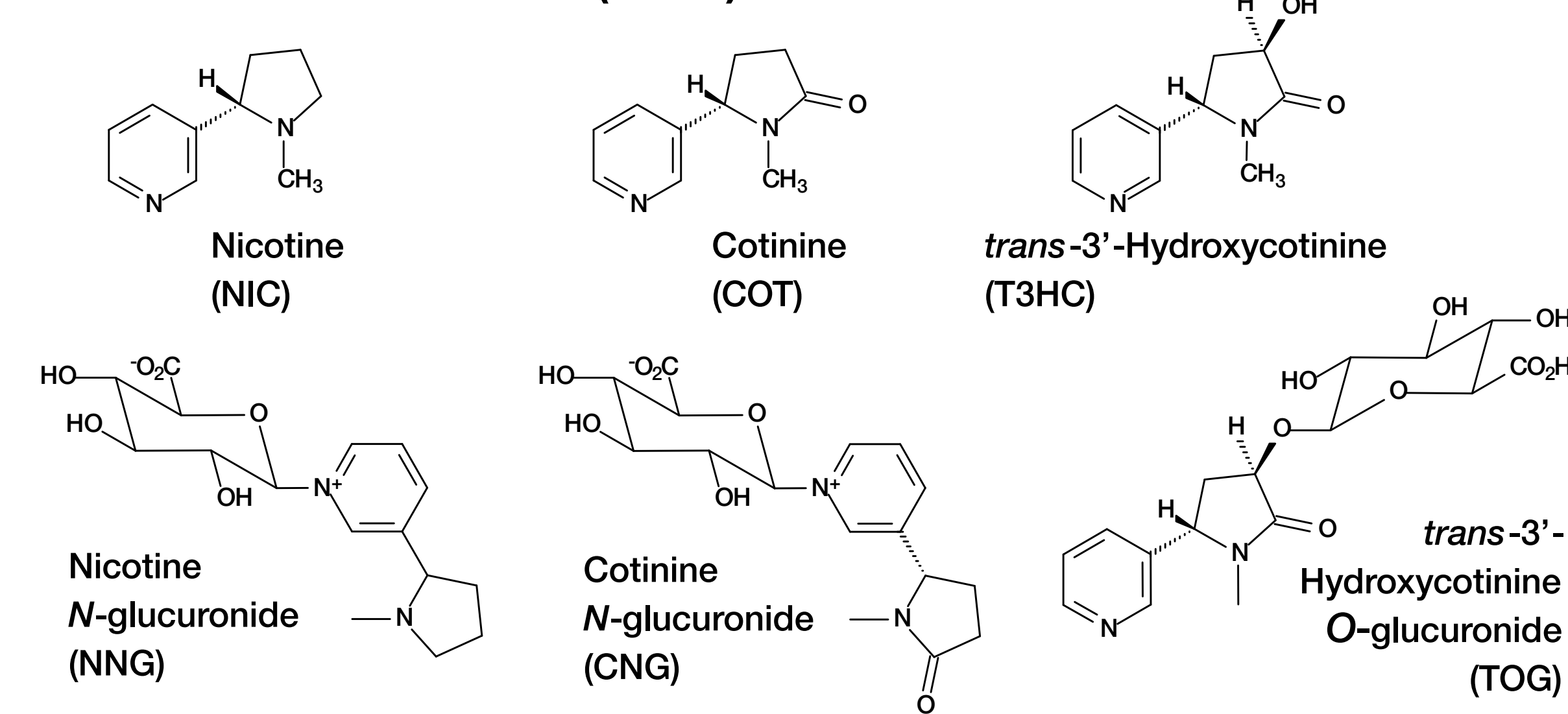
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## ABSTRACT

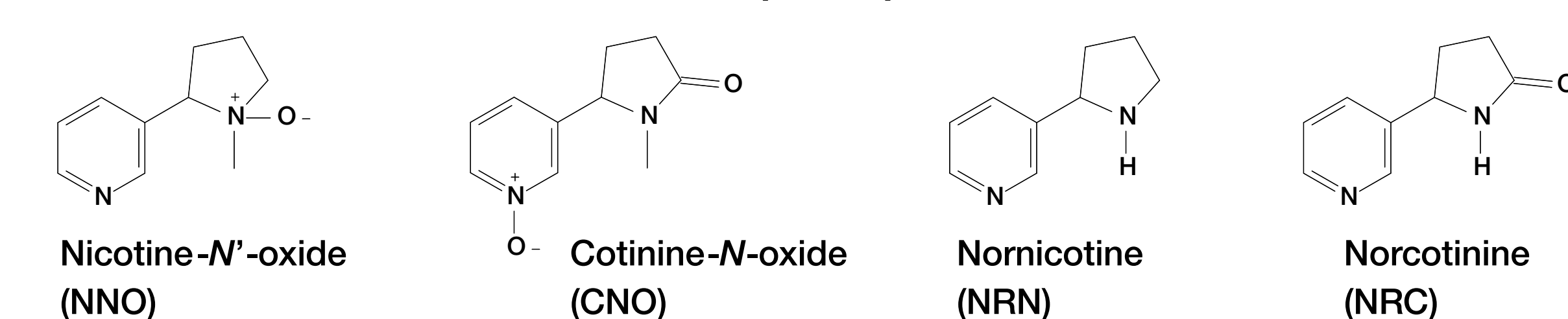
With the U.S. Food and Drug Administration (FDA) having oversight for legal distribution and marketing of tobacco products, a rigorous approach to the analysis of tobacco-related compounds in biological samples for product assessment studies is prudent to ensure compliance to regulatory guidelines. The measurement of total nicotine equivalents (i.e. nicotine plus its major metabolites) in urine is a direct and noninvasive method for assessment of human exposure to tobacco products. Selectivity for all of the analytical compounds in a method is paramount for accuracy and reproducibility. Although some methods may quantify nicotine and its metabolites from a single injection, the selectivity of such methods may not be sufficient for regulated bioanalysis (i.e. GLP-compliant).

A method for the determination of nicotine, cotinine, *trans*-3'-hydroxycotinine, and their *N*-, *N'*-, and *O*-glucuronides (respectively) from 0.250 mL of urine has been validated with lower limits of quantification (LLOQs) of 10/10/10/20/50 ng/mL, respectively. Liquid-liquid and solid-phase extraction were combined with reversed-phase gradient chromatography and detection by multiple reaction monitoring (MRM) of ions on a SCIEX API 4000™ triple quadrupole mass spectrometer. This enabled accurate and precise analysis with complete, FDA-compliant selectivity against trace urinary interferences. Development of a complementary method for analysis of norcotine, norcotinine, nicotine oxide, and cotinine oxide, from 0.100 mL of urine with target LLOQs of 2/1/5/2 ng/mL, respectively, is in progress and nearing completion.

### Nicotine Plus 5 Metabolites (Nic+5):



### Add for Nicotine Plus 9 Metabolites (Nic+9):



## CELERION ASSAYS FOR NIC+9

Concentrations of Nic+5 aglycones (NIC, COT, T3HC) and their glucuronides may be determined concomitantly using a lower range or a higher range validated assay. These analytes are extracted together using a combined liquid-liquid and solid-phase procedure. The glucuronides are analyzed using chromatography that is separate from the aglycones. Concentrations of NNO, CNO, NRN, and NRC are determined using a second solid-phase extraction and chromatography procedure (in development).

Analyte	MW (g/mol)	Lower Range (ng/mL)	Higher Range (ng/mL)
Nicotine	162.23	10 - 1000	50 - 5000
Cotinine	176.22	10 - 1000	50 - 5000
<i>trans</i> -3'-Hydroxycotinine	192.21	10 - 1000	50 - 5000
Nicotine <i>N</i> -Glucuronide	338.36	10 - 1000	50 - 5000
Cotinine <i>N</i> -Glucuronide	352.34	20 - 2000	200 - 20,000
<i>trans</i> -3'-Hydroxycotinine <i>O</i> -Glucuronide	368.34	50 - 5000	200 - 20,000
Nicotine- <i>N'</i> -Oxide	178.23	5 - 1000	--
Cotinine- <i>N'</i> -Oxide	192.22	2 - 400	--
Norcotine	148.21	2 - 400	--
Norcotinine	162.19	1 - 200	--

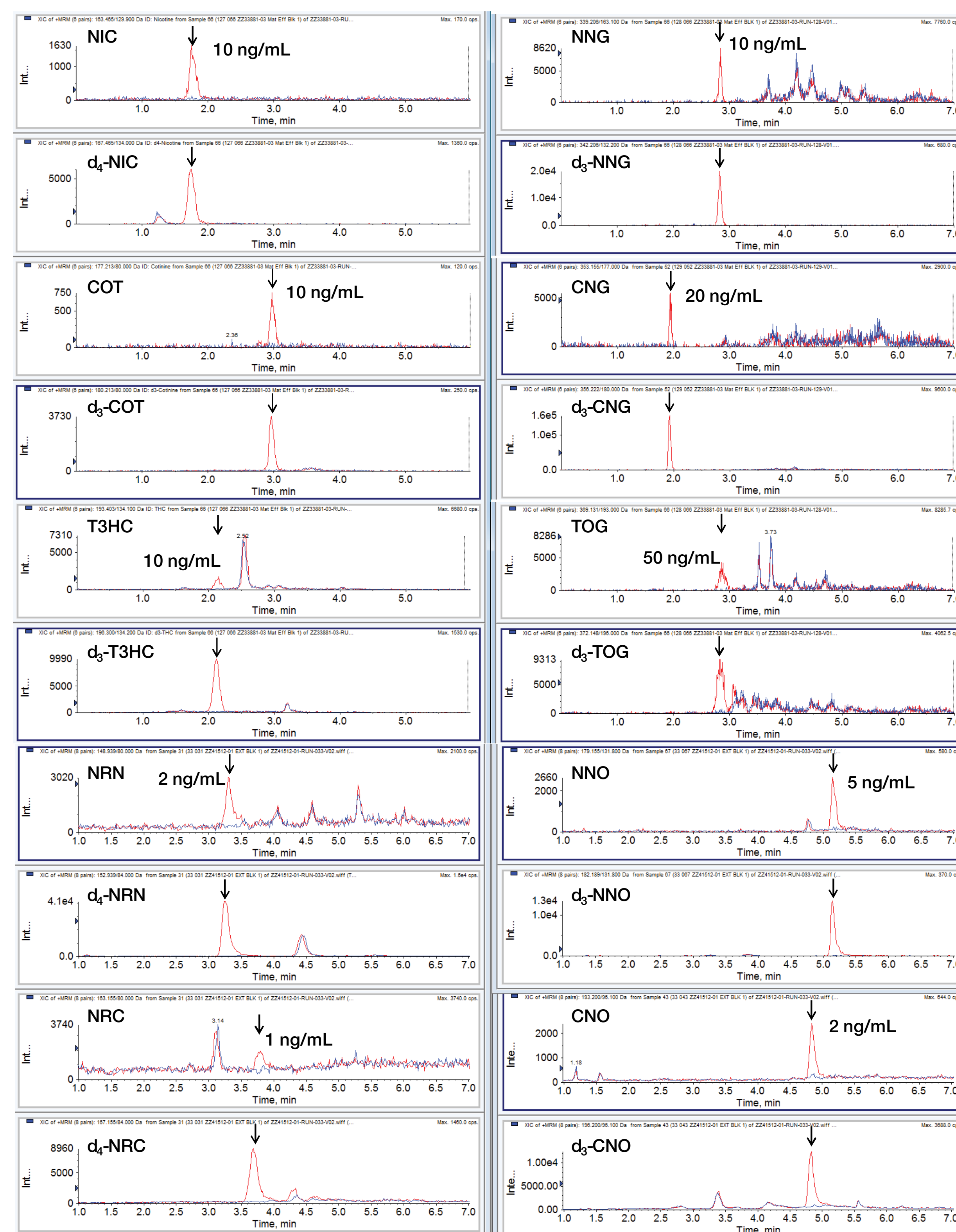
### Method for Nicotine Plus 9 Metabolites

Parameter	NIC/COT/T3HC/NNG/CNG/TOG	NNO/CNO/NRN/NRC
Analytes in Sample Injection	1) NIC, COT, T3HC 2) NNG, CNG, TOG	NNO, CNO, NRN, NRC
Extraction	1) Liquid-Liquid Extraction 2) Solid-Phase Extraction (Oasis® MCX)	Solid-Phase Extraction (Bond Elut Plexa PCX)
Chromatography	Column 1) ACE® Phenyl 50 x 3.0 mm, 5 µm Methanol gradient 2) Zorbax Bonus-RP 150 x 4.6 mm, 5 µm Methanol gradient	Zorbax SB-Phenyl 50 x 4.6 mm, 3.5 µm Methanol gradient
Acquisition Time	1) 6.0 minutes 2) 7.0 minutes	8.5 minutes
Detector	API 4000™	API 5000™

## SENSITIVITY AND SELECTIVITY

Non-smoker urine lots exhibiting common isobaric peaks were selected to demonstrate successful separation of analytes and internal standards away from potential interferences for appropriate selectivity. The chromatography of nicotine, cotinine, *trans*-3'-hydroxycotinine, and respective glucuronides (with ISTDs) is derived from a single matrix effect/selectivity urine lot in the validation study.

From a method development run, chromatography of norcotine and norcotinine (with ISTDs) was taken from one urine lot, nicotine-*N'*-oxide (with ISTD) was from a 2<sup>nd</sup> lot, and cotinine-*N'*-oxide (with ISTD) was from a 3<sup>rd</sup> lot.



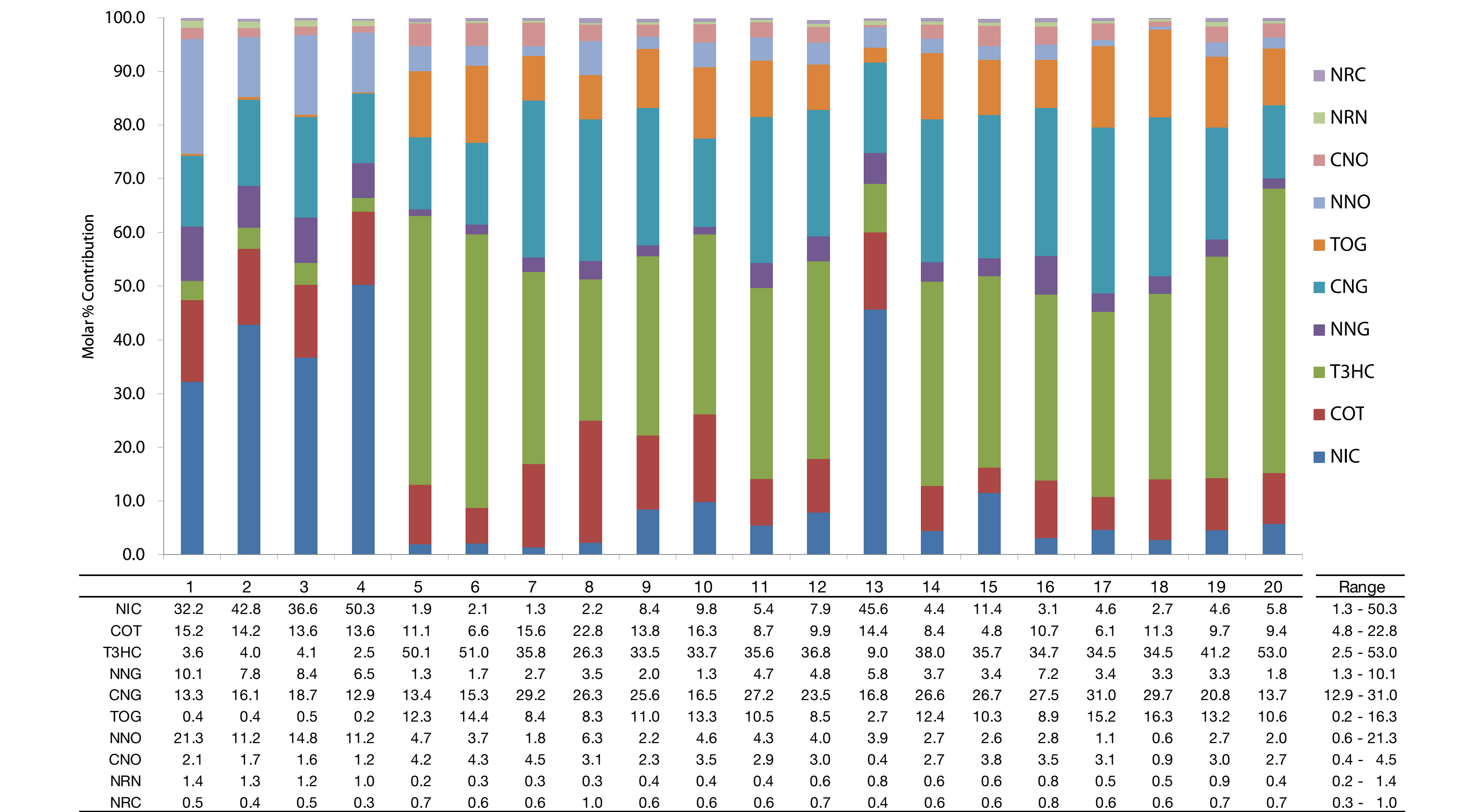
LLOQ (red) and Double Blank (blue) Extracts of Non-Smokers' Urine

### Mean Concentrations (n=2) in ng/mL of Nicotine Plus Nine Metabolites in 20 Lots of Smokers' Urine (Spot Collections) from 14 Donors

Donor:	A	A	A	A	B	B	C	D	E	F	G	H	I	J	K	K	L	L	M	N	Range	Max/Min
Lot:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		
NIC	2010	1690	1540	1550	124	238	22.7	103	289	346	908	1580	2450	470	4340	434	293	51.3	677	1420	22.7	- 4340
COT	1030	606	620	457	777	829	290	1170	518	626	1570	2170	843	964	2000	1610	427	229	1560	2520	229	- 2520
T3HC	266	185	206	92.3	3850	6940	729	1470	1370	1410	7050	8760	572	4780	16100	5710	2620	763	7240	15500	92.3	- 16100
NNG	1320	639	736	420	175	415	98.4	343	143	94.1	1620	2000	652	820	2670	2100	458	127	1010	952	94.1	- 2670
CNG	1800	1380	1710	866	1880	3810	1090	2700	1920	1270	9860	10300	1960	6130	22100	8300	4300	1200	6710	7330	866	- 22100
TOG	54.9	33.8	47.7	14.4	1800	3750	327	893	864	1070	3980	3860	334	2990	8890	2820	2200	691	4470	5940	14.4	- 8890
NNO	1460	487	683	380	335	464	33.3	324	85.2	179	789	891	229	310	1080	427	77.3	12.5	434	538	12.5	- 1460
CNO	158	77.5	80.3	43.1	324	584	91.8	174	95.0	146	576	707	24.8	338	1720	573	232	20.7	520	792	20.7	- 1720
NRN	78.0	46.2	44.4	29.2	13.6	36.2	5.08	15.0	12.2	13.0	60.0	108	39.5	59.7	214	96.7	27.2	7.81	117	100	5.08	- 214
NRC	31.1	17.3	20.4	9.22	45.8	72.9	11.0	47.3	22.2	20.8	108	138	23.0	68.9	243	112	41.1	10.3	110	177	9.22	- 243

*red italics* = extrapolated value above accepted range (AAR) or below limit of quantification (BLQ)

### Molar % Contribution of Individual Analytes to Nic+9 as Representing Total Nicotine Equivalents in 20 Lots of Smokers' Urine



%Deviation of Molar % Contribution Versus Nic+9 as Representing Total Nicotine Equivalents in 20 Lots of Smokers' Urine	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Range
Nic+5	33.7	17.4	22.2	15.9	11.1	9.8	7.6	11.9	6.0	10.1	8.6	9.7	5.8	7.0	8.3	8.5	5.3	1.8	7.8	6.3	1.8 - 33.7
Nic+6*	4.1	3.8	3.2	2.7	5.6	5.5	6.0	4.7	3.4	4.8	3.8	5.1	1.8	4.0	5.4	5.3	4.2	1.8	4.8	4.1	1.8 - 6.0

\*addition of nicotine-*N'*-oxide as the 6<sup>th</sup> metabolite

## SUMMARY & CONCLUSIONS

- A method with FDA-compliant selectivity for the analysis of Nic+5 has been validated. A method for the remaining 4 analytes of Nic+9 (i.e. norcotine/norcotinine/nicotine-*N'*-oxide/cotinine-*N'*-oxide) is in progress.
- Achievement of full selectivity, as appropriate for FDA-regulated bioanalysis, may require use of separate extraction and/or chromatography procedures for a large panel of analytes.
- In a panel of 20 smoker urine lots:
  - Analyte concentrations varied diversely, with expression of *trans*-3'-hydroxycotinine *O*-glucuronide over the broadest range.
  - Metabolic profiles (i.e. molar % contribution to total nicotine equivalents) varied significantly between lots from some different donors, but in general appeared relatively consistent between different lots from a single donor.
  - Norcotine and norcotinine had overall the lowest contributions to total nicotine.
  - Addition of just nicotine-*N'*-oxide to the panel of Nic+5 analytes (i.e. "Nic+6") makes a high impact for closer approximation to Nic+9 as representing total nicotine equivalents.

## ACKNOWLEDGEMENTS

Thank you to Marzuki Mohamed for his assistance with preparation of samples for this poster and Marc Moussallie for helpful discussion.