NNN – a suitable biomarker of exposure from smoke and new nicotine delivery products?

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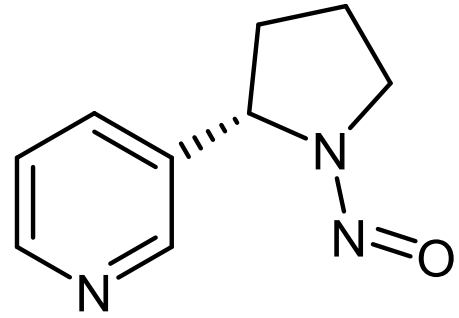
N-Nitrosonornicotine

- NNN (like all other TSNAs) is mainly formed during the curing, aging, and processing of tobacco
- NNN: Group 1 carcinogen: „carcinogenic in humans“ (IARC)
- Measurement of NNN within clinical studies to assess risk profile and toxicology of new nicotine delivery products like e-cigarettes, smokeless tobacco or heat-not-burn products
- Determination in urine (total NNN) as biomarker of exposure specific to the uptake of NNN



Urinary NNN concentrations by product use group

- Highest burden in smokers (CC) and smokeless tobacco users
- Reduced exposure was observed in vapers (EC) and HTP users
- Exposure depends on the product characteristics
- Mean concentration ranges in urine:

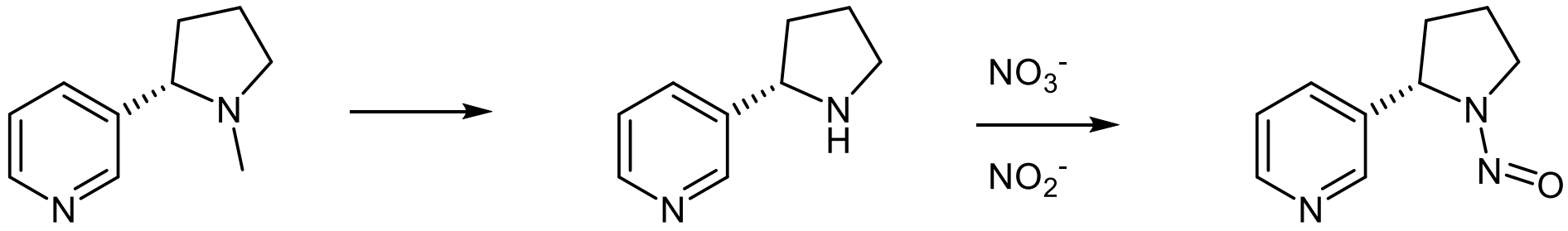
Smokers of conventional cigs :	5 – 25 pg/mL
Smokeless tobacco users :	2 – 13 pg/mL
HTP users:	0.5 – 3 pg/mL
E-Cigarette users:	0.3 – 1.5 pg/mL
Non-smokers :	0.0 – 0.3 pg/mL

Gale 2018; Goniewicz 2018; Gregg 2013; Haziza 2016; Kavvadias 2009; Krautter 2014; O'Connell 2016; Round 2015; Round 2018; Shepperd 2015

Artefactual formation of NNN

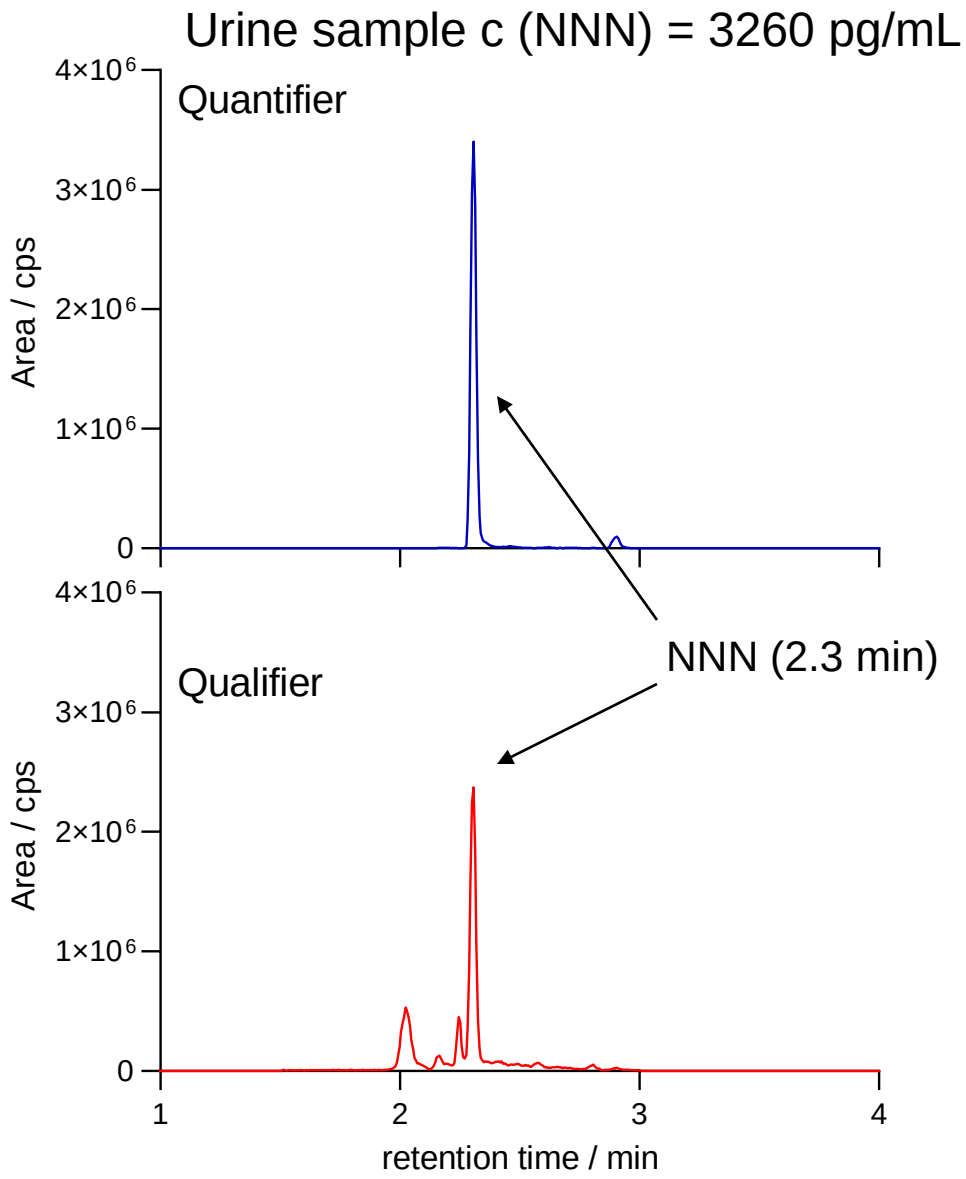
- Plausible concentrations in urine could usually not exceed 50 pg/mL
- Occasionally much higher urinary levels >>100 pg/mL were observed

- Artefactual formation caused by reaction of nornicotine with nitrosating agents already shown in the literature



Nicotine Nornicotine NNN

Peak specificity



- Several mass transitions are detected in MS/MS for NNN
- Specific and most sensitive mass transition is used for quantification (Quantifier)
- Specific and second most sensitive mass transition is used for confirmation (Qualifier)
- Quan-Qual ratios in study samples with very high NNN levels confirm presence of NNN at implausibly high amounts (most likely not caused by interfering peaks)

Artefactual formation of NNN in urine
Incubation Study

- Aim of our study:

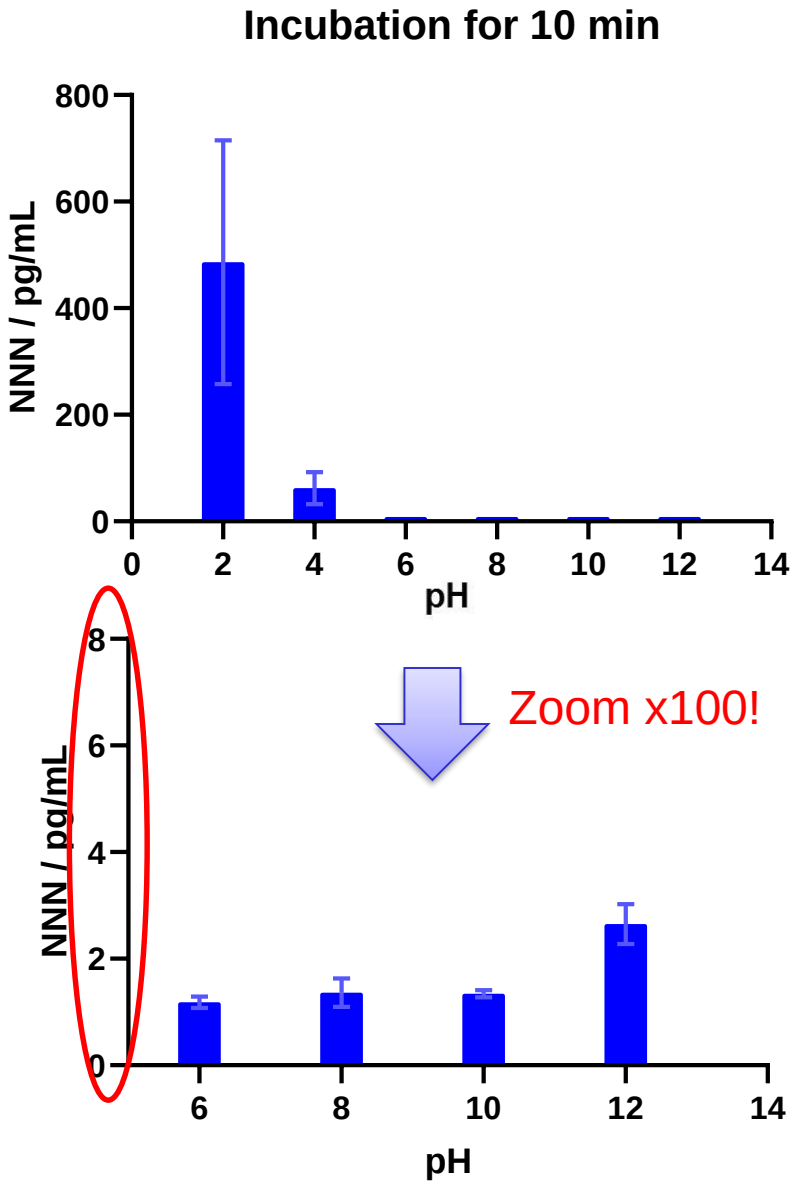
  Investigate how the formation of NNN is triggered and how fast NNN can be formed in urine

- Experiment:

  Incubation of precursors nornicotine, nitrite/nitrate in non-smoker urine by varying

  - Precursor amounts: nitrite/nitrate: 20-50 µg/mL; Nornic: 50-500 ng/mL
  - pH (2 – 12)
  - Storage time (10 min, 24h, 48h)
  - Storage temperature (-80°C – 37°C)

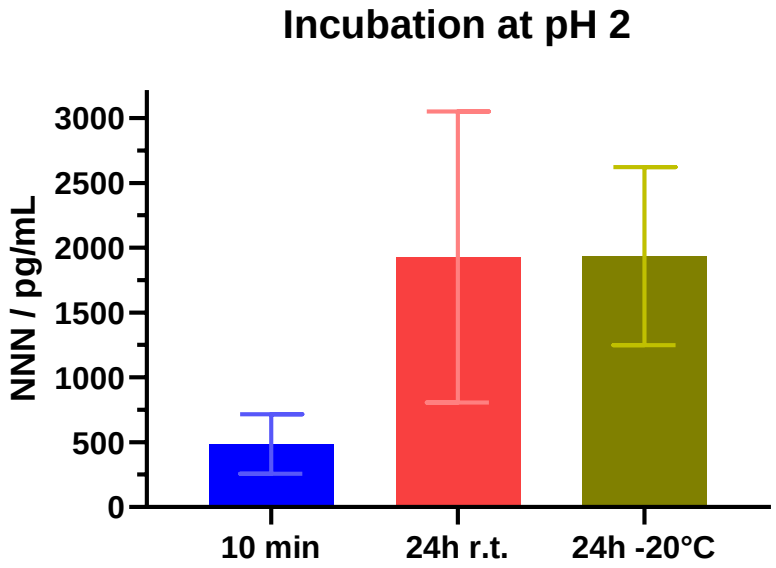
Artefactual formation of NNN in urine
Results

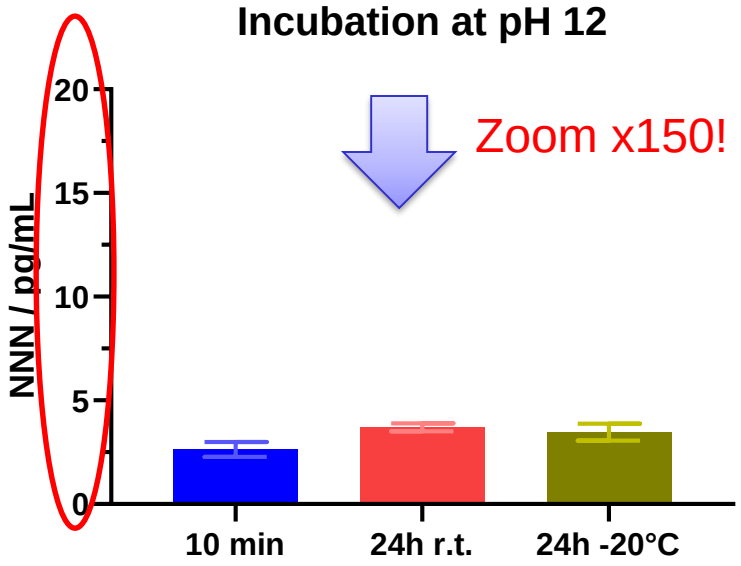


- Urine pH adjusted; each pH in triplicates
- Nornic (500 ng/mL) + NO_2^-/NO_3^- (50 µg/mL)
- Immediate centrifugation (10 min) and further purification (SPE)
- LC-MS/MS analysis (LLOQ: 0.5 pg/mL)

➢ Very rapid formation of NNN at acidic pH
➢ pH ≥ 6: low conversion despite high amounts of precursors

Artefactual formation of NNN in urine
Results

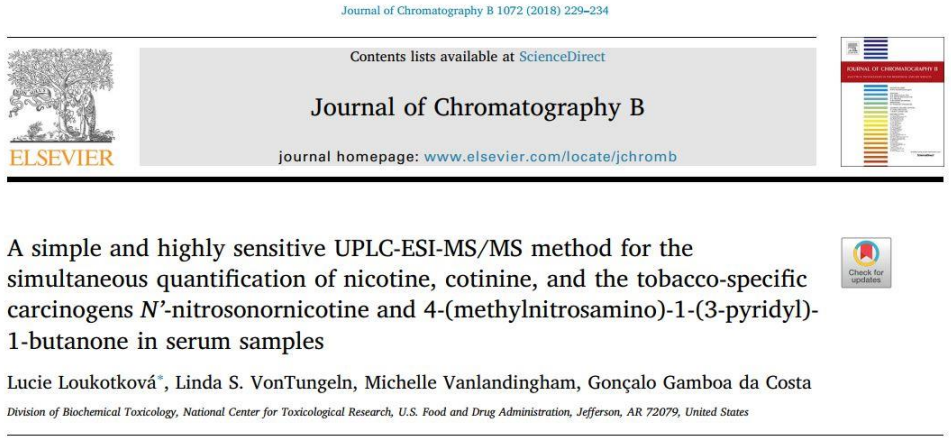




- At acidic conditions further increase after 24hours, even in frozen samples (-20 °C)
- No further formation over time at alkaline pH
- Urinary pH is of decisive importance for NNN formation from NorNic and nitrite/nitrate
- Reaction appears very fast → endogenous formation seems likely

NNN in plasma (?)

- Urinary pH variation:
  healthy subjects: pH 6.5 – 7.5 <-> disease states (urinary tract infection, kidney, diabetes): pH 5.0 – 5.5
- ➢ Formation to a significant extent already below pH 6.0
- ➢ High NNN levels may occur due to the presence of the precursors esp. at acidic pH rather than NNN uptake from the product (e.g. e-cigarette)
- ➢ Blood pH much less variable compared to urine (blood: 7.37-7.43) → less artefactual formation?
- ➢ Development of a sensitive and high-throughput LC-MS/MS method in plasma based on a recently published method by the FDA (Loukotkova 2018, JCB)



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A simple and highly sensitive UPLC-ESI-MS/MS method for the simultaneous quantification of nicotine, cotinine, and the tobacco-specific carcinogens *N'*-nitrosonornicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in serum samples

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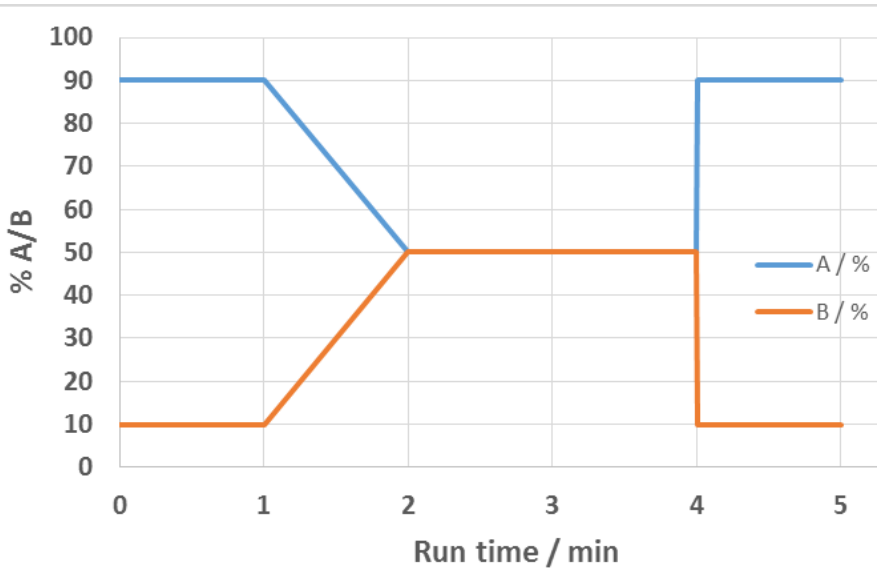
NNN in plasma
LC-MS/MS method

Sample preparation:

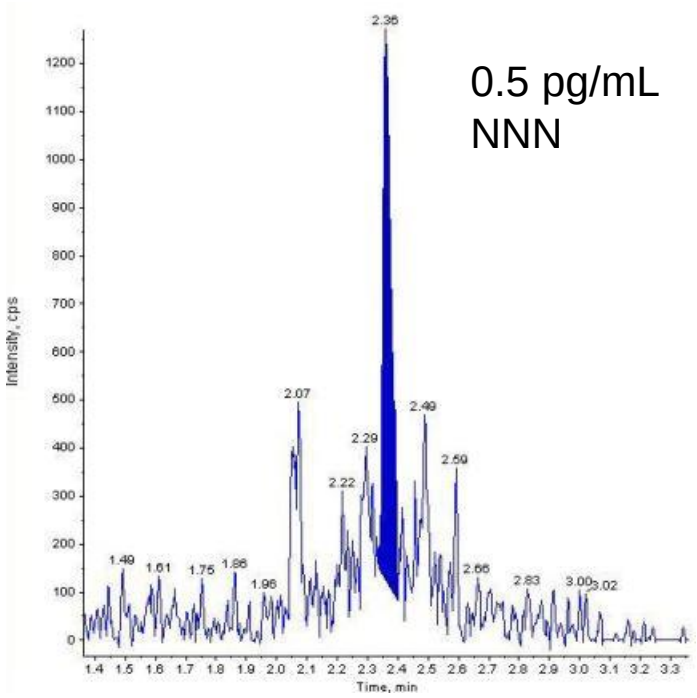
- LLE of 1 mL plasma with methyl *tert*-butyl ether (MTBE)
- Organic residue acidified with 4M formic acid and re-extracted with MTBE
- Evaporation of aqueous phase and reconstitution in water/methanol

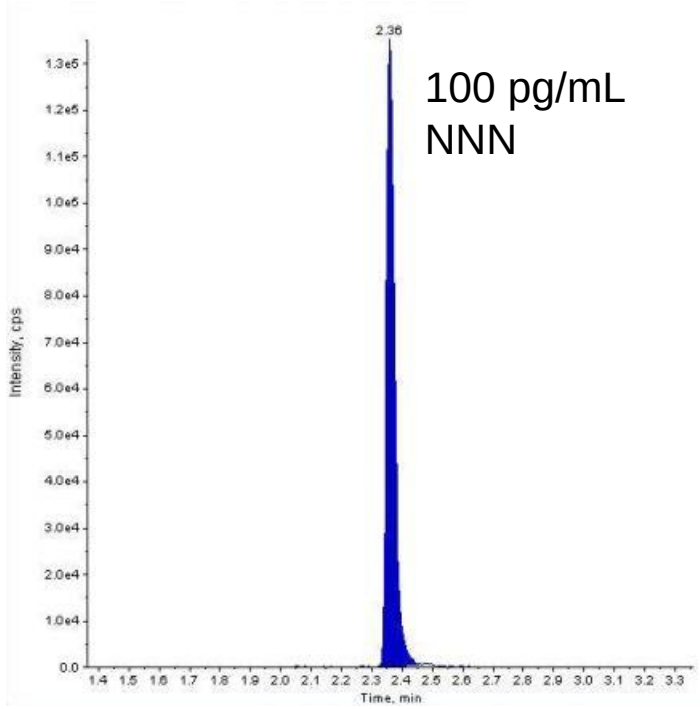
LC-MS/MS analysis:

- Gradient elution with H_2O+ 0.1% NH_4OAc (A) / ACN+0.1% FA (B) on UPLC column Acquity HSS T3 1.8µ (100x2.1mm)
- Run time: 5 min
- ESI positive
- MRM: 178 -> 148 (Quan); 178 -> 120 (Qual)



NNN in plasma
Results from pilot study (5 smokers / 5 non-smokers)





Sample No	NNN / pg/mL
Smoker 1	5.3
Smoker 2	6.9
Smoker 3	3.8
Smoker 4	4.0
Smoker 5	1.4

NNN in non-smokers: <LOD (N=5)

Method validated according to FDA Guidelines
LLOQ: 0.5 pg/mL
ULOQ: 1000 pg/mL

Summary

- Formation of NNN in urine from its precursors nornicotine and nitrite/nitrate
- High NNN concentrations at low pHs (2 – 6) already after 10 min, increasing over time
- Formation significantly reduced at neutral and basic conditions

➢ pH is of decisive importance for NNN formation in urine

➢ Acidic urinary pH in disease states like diabetes may trigger artefactual NNN formation

➢ Rapid formation in incubation experiments may indicate endogenous formation

- New method based on LC-MS/MS was developed for plasma NNN
- Method is also suitable for saliva
- High sensitivity, broad linear range and high throughput → suitable for large PK studies
- Suitability shown in a first small pilot (N=5 smokers; all > LLOQ)

Is NNN a suitable biomarker of exposure for new nicotine delivery products?

- Endogenous formation shown for saliva[1] and indicated in our study for urine
- Formation has various contributors

➢ Detailed characterization of the collected urine and subject habits in the clinic helpful (pH, nitrite, dietary habits, disease state)

- NNN analysis in saliva and/or urine add important data for risk assessment of new product categories – even if no or only very low levels of NNN present in the product
  But: NNN levels in saliva/urine do not necessarily reflect the specific uptake from the product; Urinary NNN may even increase over longer storage periods and falsify results
- Blood pH much less variable compared to urine (blood: 7.37-7.43; urine: 5.0-7.5)

➢ Determination of NNN in plasma to explore suitability as a biomarker for the <u>product-specific</u> exposure to NNN

[1] Bustamante et al 2018: Presence of the Carcinogen N′-Nitrosonornicotine in Saliva of E-cigarette Users

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