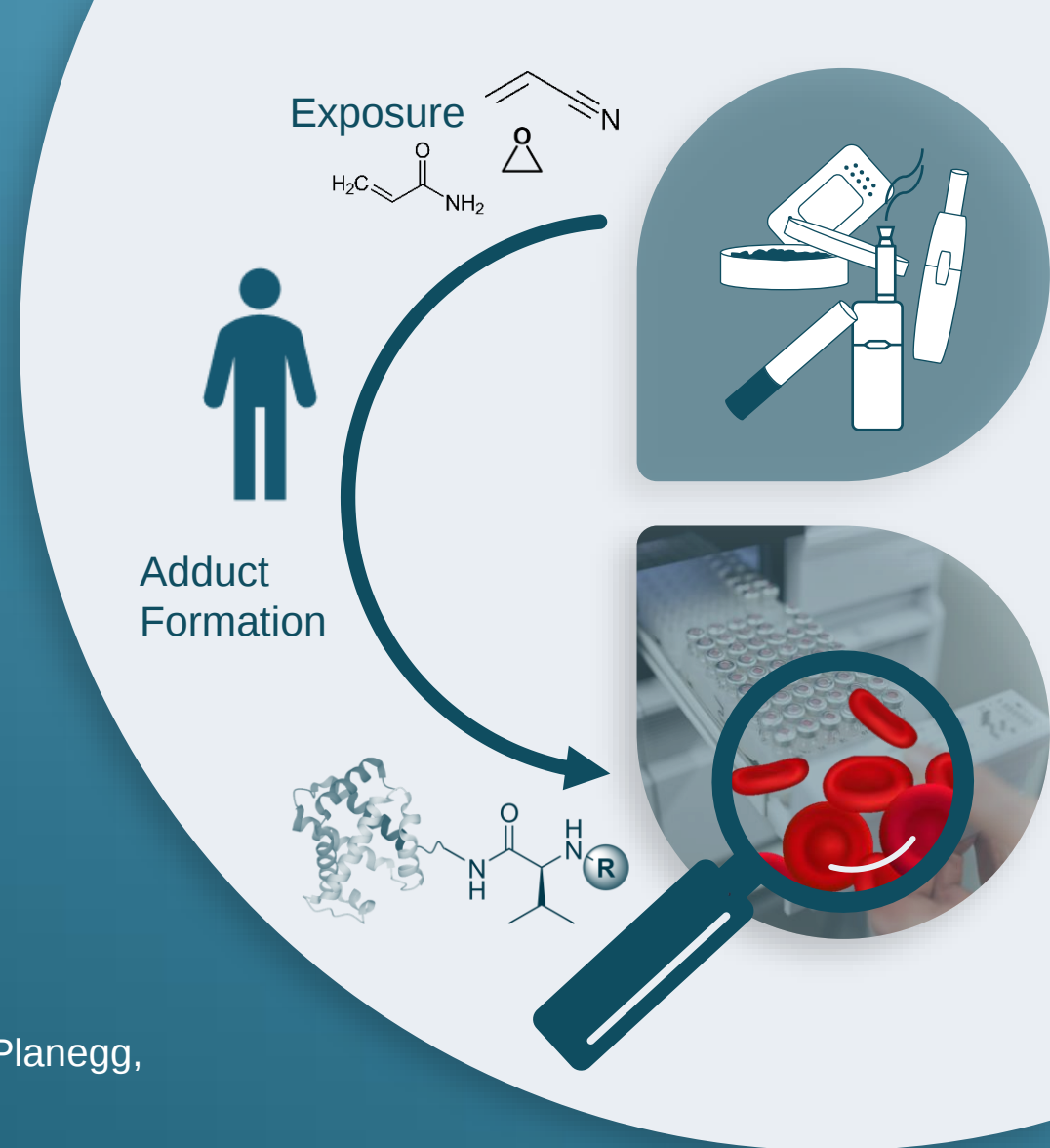


Identification of use-specific hemoglobin adduct patterns for different tobacco/nicotine product user groups by non-targeted GC-MS/MS analysis

M. Scherer, F. Pilz, T. Burkhardt, G. Scherer, N. Pluym

Analytisch-Biologisches Forschungslabor GmbH, Semmelweisstr. 5, 82152 Planegg, Germany

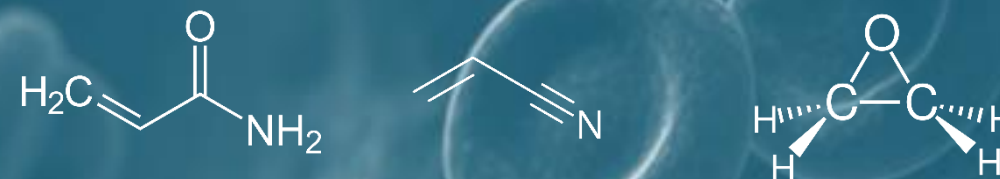


Introduction

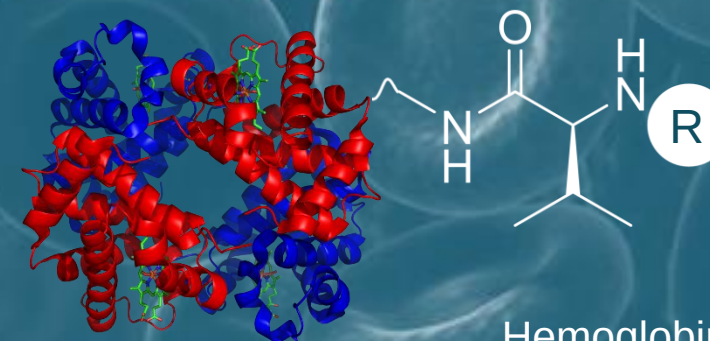
- **Biomarkers of exposure** commonly monitored in urine
→ short half-life
- **Hemoglobin (Hb)**: Life-time of ~ 120 days
→ Formation of adducts with electrophilic compounds
- **Electrophilic compounds**: formed *in vivo* by the metabolism of molecules derived from endogenous (e.g., oxidative stress) and exogenous (e.g., diet, smoking) sources
 - Modification of nucleophilic sites on DNA and functional proteins (e.g., HSA and Hb)
 - Increased cancer risk
- Chemically stable adducts accumulate during exposure

Potential **biomarker** for monitoring
long-term *in vivo* exposure

Electrophilic compounds



Formation of hemoglobin adducts



Hemoglobin with N-terminal adduct

Introduction

- **Biomarkers of exposure** commonly monitored in urine
→ short half-life
- **Hemoglobin (Hb)**: Life-time of ~ 120 days
→ Formation of adducts with electrophilic compounds
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Potential **biomarker** for monitoring
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REQUIREMENT

Identification of product use-specific
hemoglobin-adducts



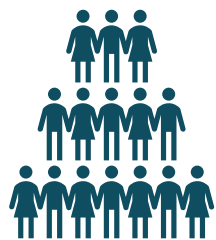
Risk evaluation



Compliance

Clinical Study – Study Design

Clinical Study with 5 different nicotine product user groups and a non-user group (control)

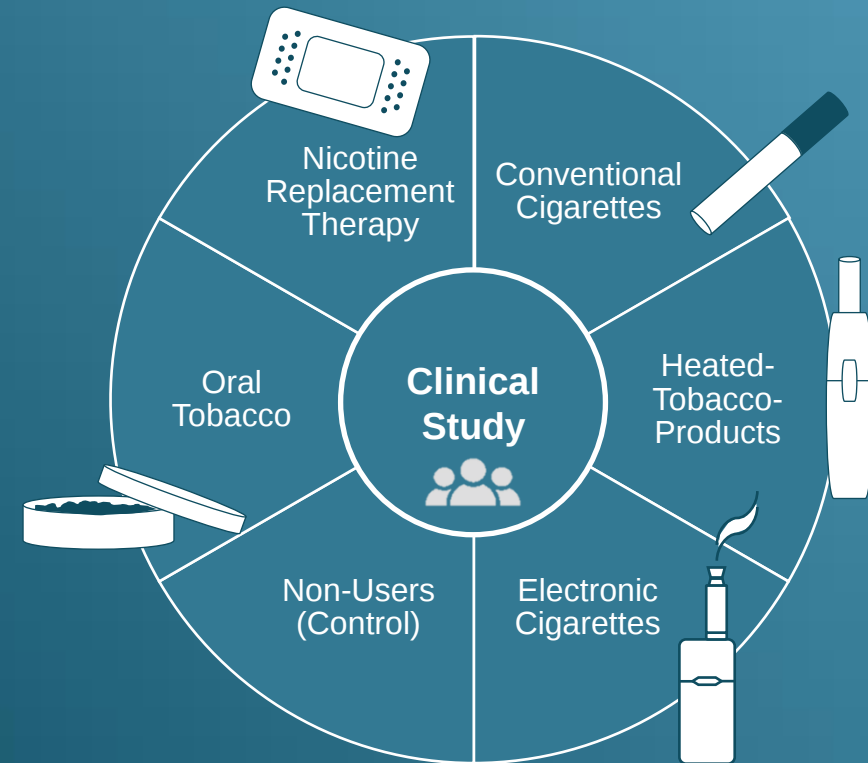


60 healthy volunteers

- Males and Females
- Aged 19 – 65 years
- BMI: 18 – 33 kg / m²

GENERAL STUDY OBJECTIVE

Identify biomarkers or biomarker patterns capable to discriminate between different nicotine product user groups and non-users.



10 subjects per group

Day 1

Day 2

Day 3

Analytical Strategy

Bioanalytical methods

Holistic



Introduction

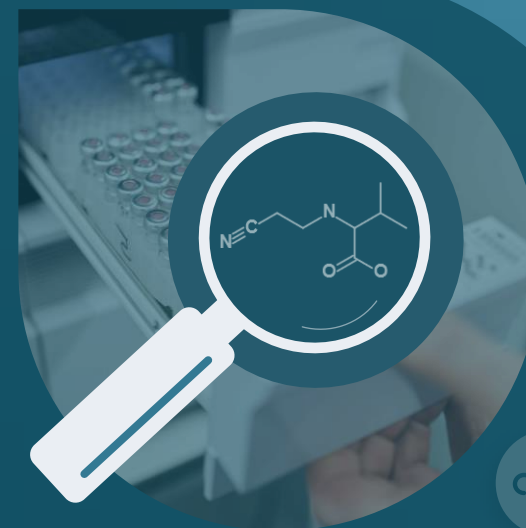
Study Design & Method

Results & Discussion

Conclusion

We have a problem !

Screening
Hemoglobin adducts
for specific product categories



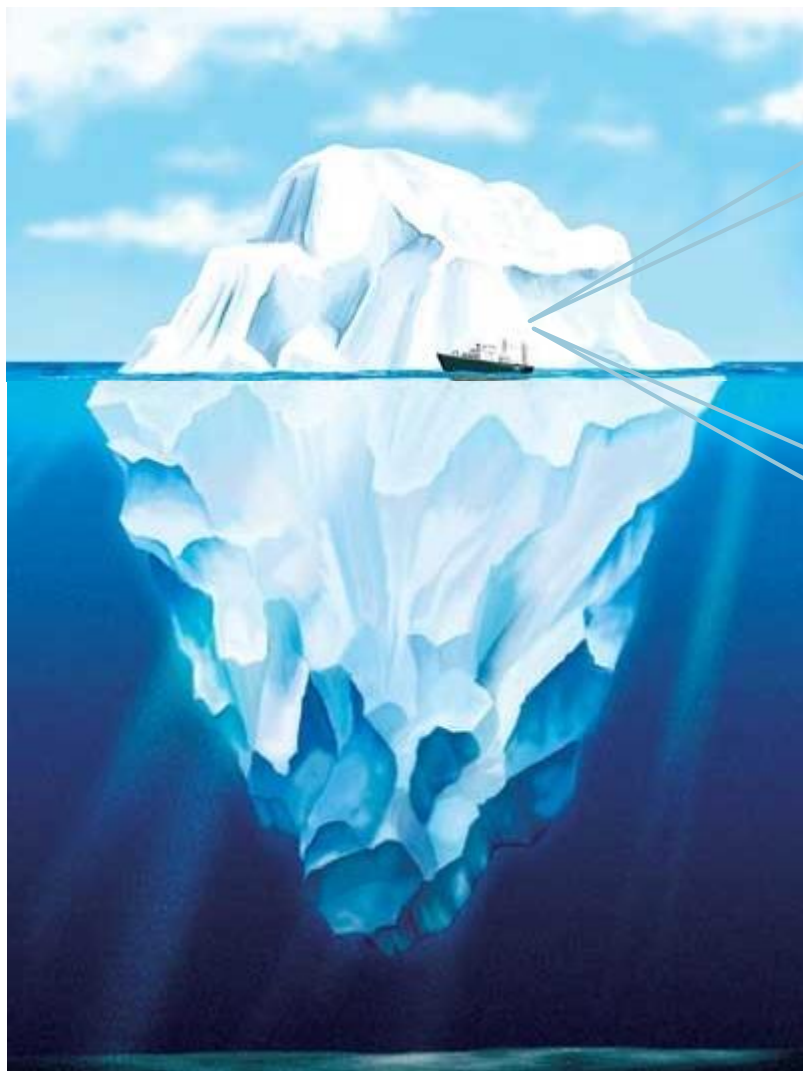
abf

Analytical Strategy

Bioanalytical methods

Targeted

Holistic



Introduction

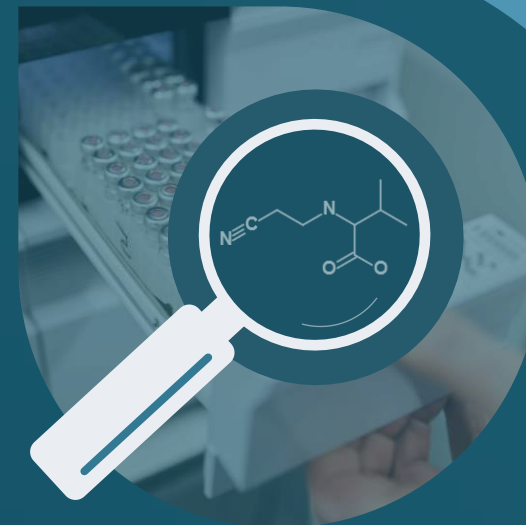
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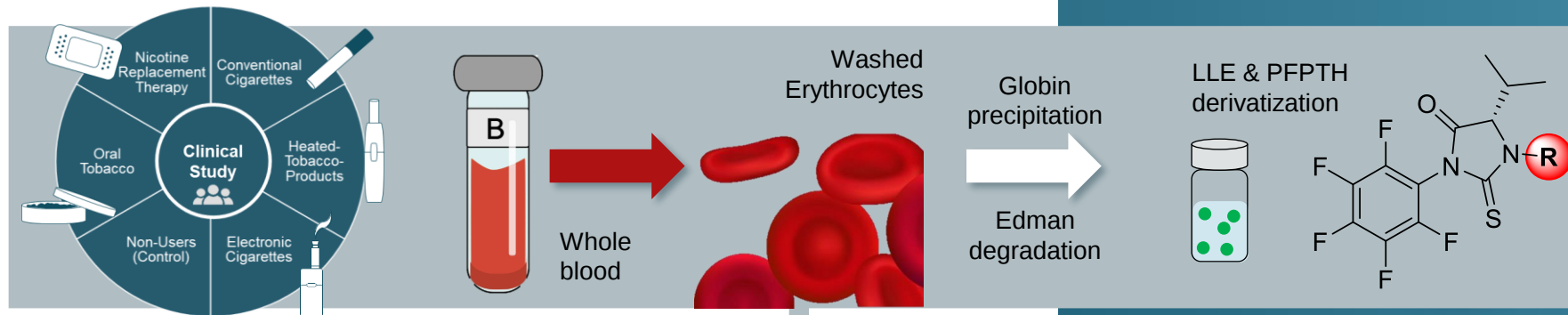
Can we
identify the
root cause?

Targeted quantitative profiles



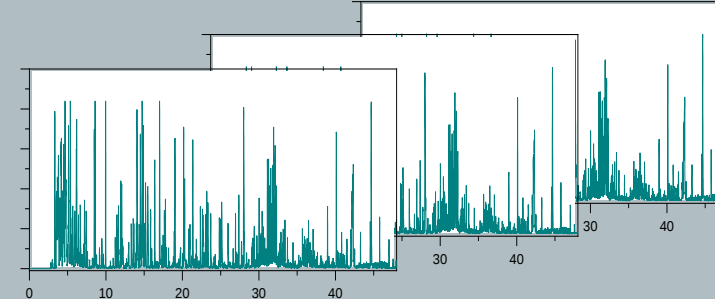
abf

Non-targeted screening – Workflow

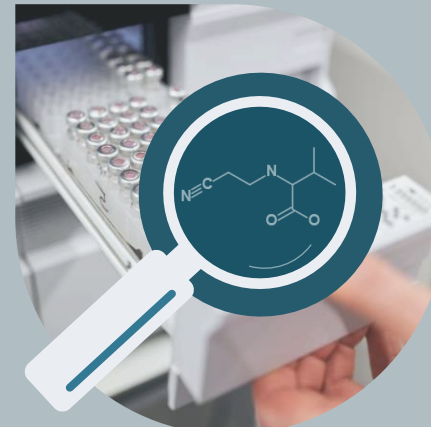
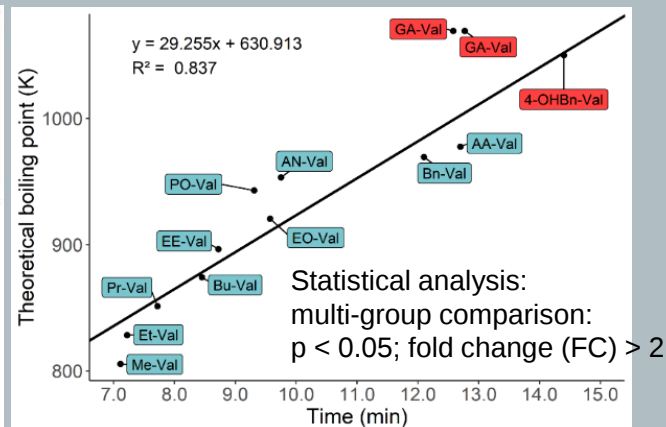
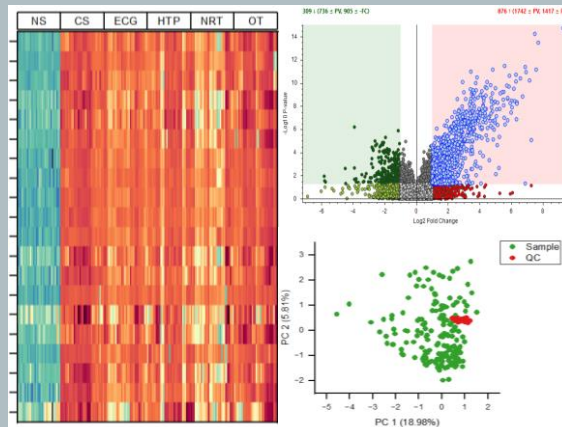


Sampling &
Sample Preparation

GC-MS/MS
Shimadzu TQ8050



Sample Analysis



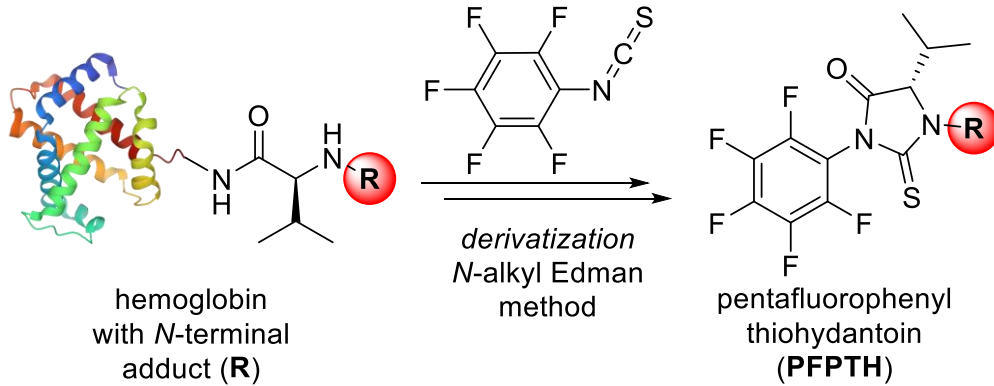
Data Processing
Statistical Analysis
Compound Identification

Non-Target Screening

Derivatization of Hb with pentafluorophenyl isothiocyanate (PFPITC)

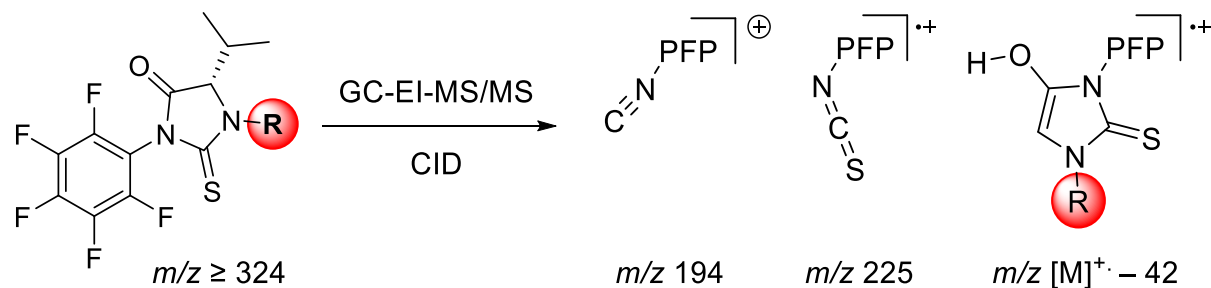


Formation of small molecules (PFPTH) for analysis

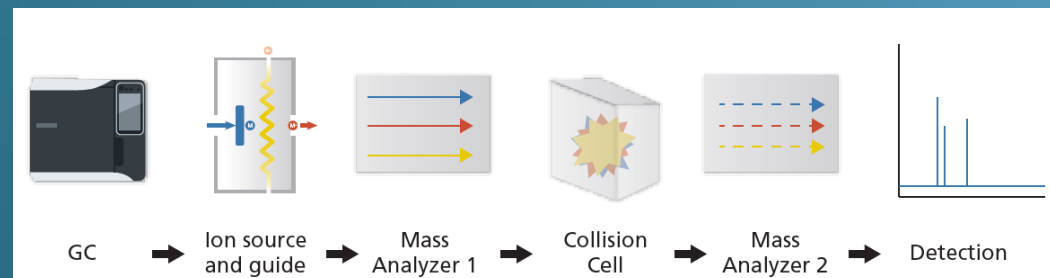


Non-Target Screening

- PFPTH derivatives of N-terminal Hb adducts show a similar fragmentation pattern in MS/MS



- MRM screening approach uses the structural relation and similar core structure of PFPTHs
- Screening range in Q1: m/z 338 – 487 (multiple injections)

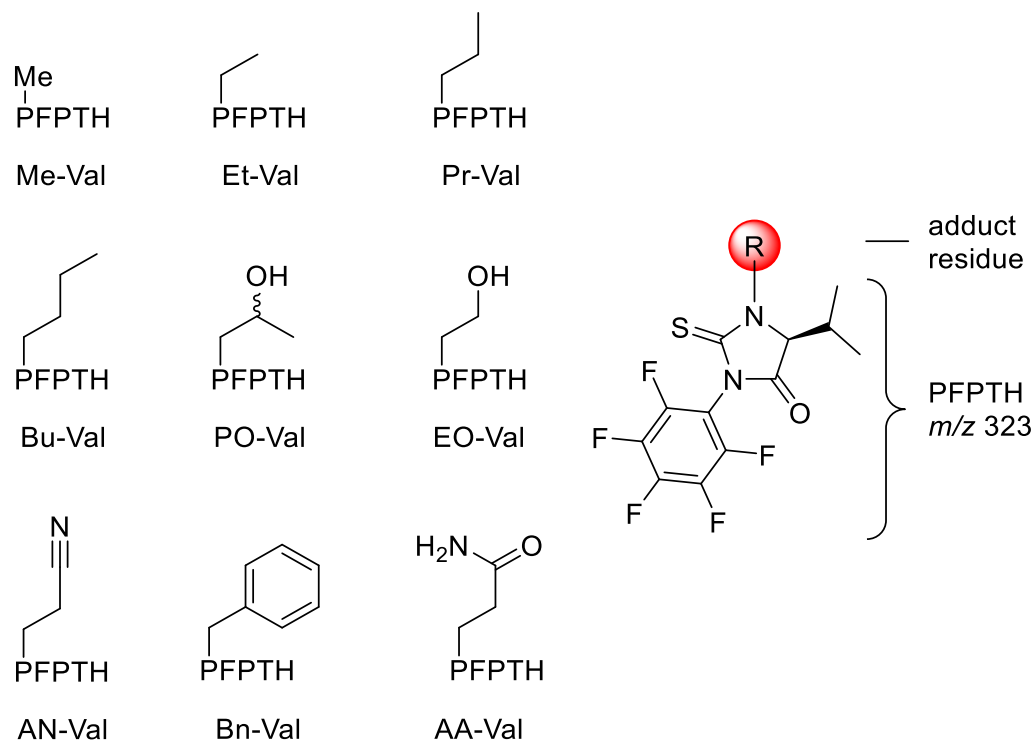


MRM method used to screen for N-terminal Hb adducts

Q1: Parent ion		Q3: Fragments	
$[M]^+$	$[M]^+ - 42$	225	194
338	296	225	194
339	297	225	194
340	298	225	194
⋮	⋮	⋮	⋮
487	445	225	194

Method Validation

9 compounds evaluated:



→ Cover the expected retention time (RT), polarity and mass range

Results

Repeatability (CV, N = 5)

4.6 – 13.1 %

Precision Intraday (CV, N = 3) Interday (CV, N = 4)

1.4 – 19.0 %

6.6 – 19.0 %

Accuracy Intraday (N = 3) Interday (N = 4)

82.6 – 107.9 %

89.2 – 117.8 %

Carry over

0.0 – 0.3 %

Linearity (R², Range: 15, 60, 120 – 3600 pmol/g Gb)

≥ 0.9903

Post-preparative stability (room temperature, 5 d)

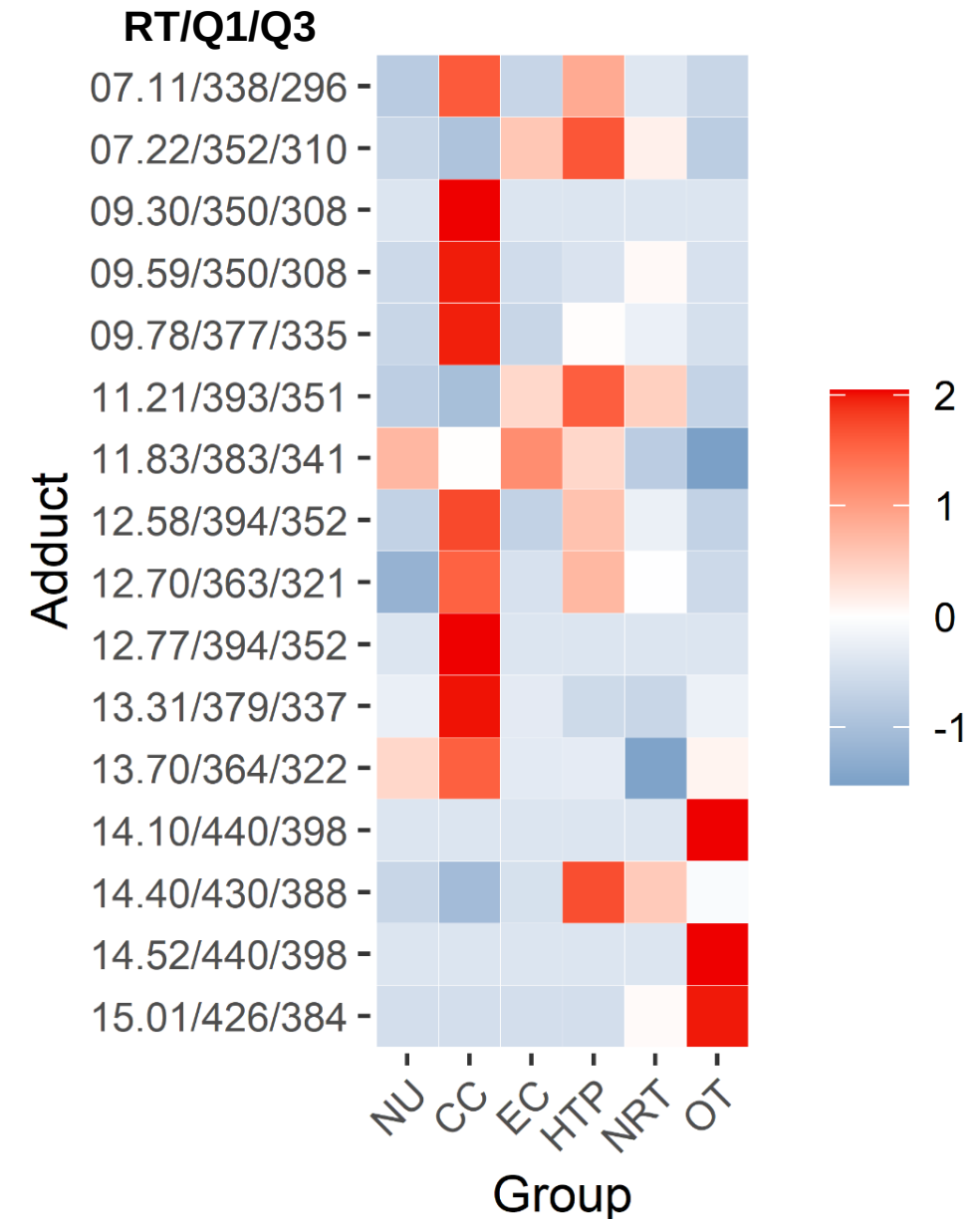
76.8 – 123.6 %

Non-Target Screening

- 13 adducts showed significant differences among groups ($p < 0.05$)
- 3 more adducts determined by fragmentation pattern ($p > 0.05$)

Identification based on...

- Adductome data**
 - Parent ions
 - Retention time
 - Isotopic distribution
- Literature review** and **database search** on exposure from tobacco/nicotine products and previously reported adducts



Identification Strategy

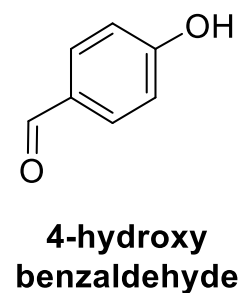
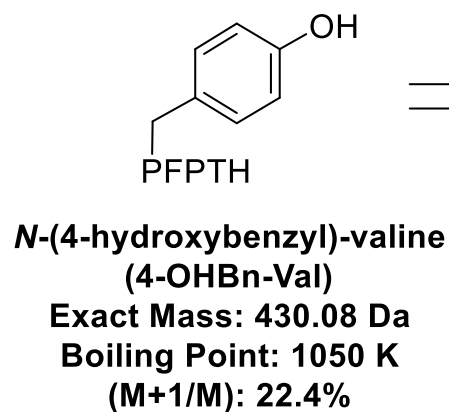
Adduct at 14.40 min

RT (min)	Parent Ion (m/z)	Relative Intensities of Mass Transitions (%) ^a		
		194	225	precursor ion -42
14.40	430	39.6 (55.7)	35.9 (26.6)	100.0
	431	14.9 (12.6)	-	22.4 (20.6)

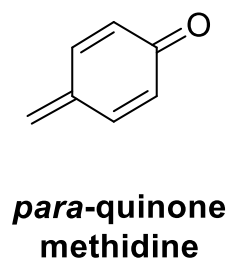
^aRSDs in percent in brackets.

Potential Adduct

Potential Precursor



or

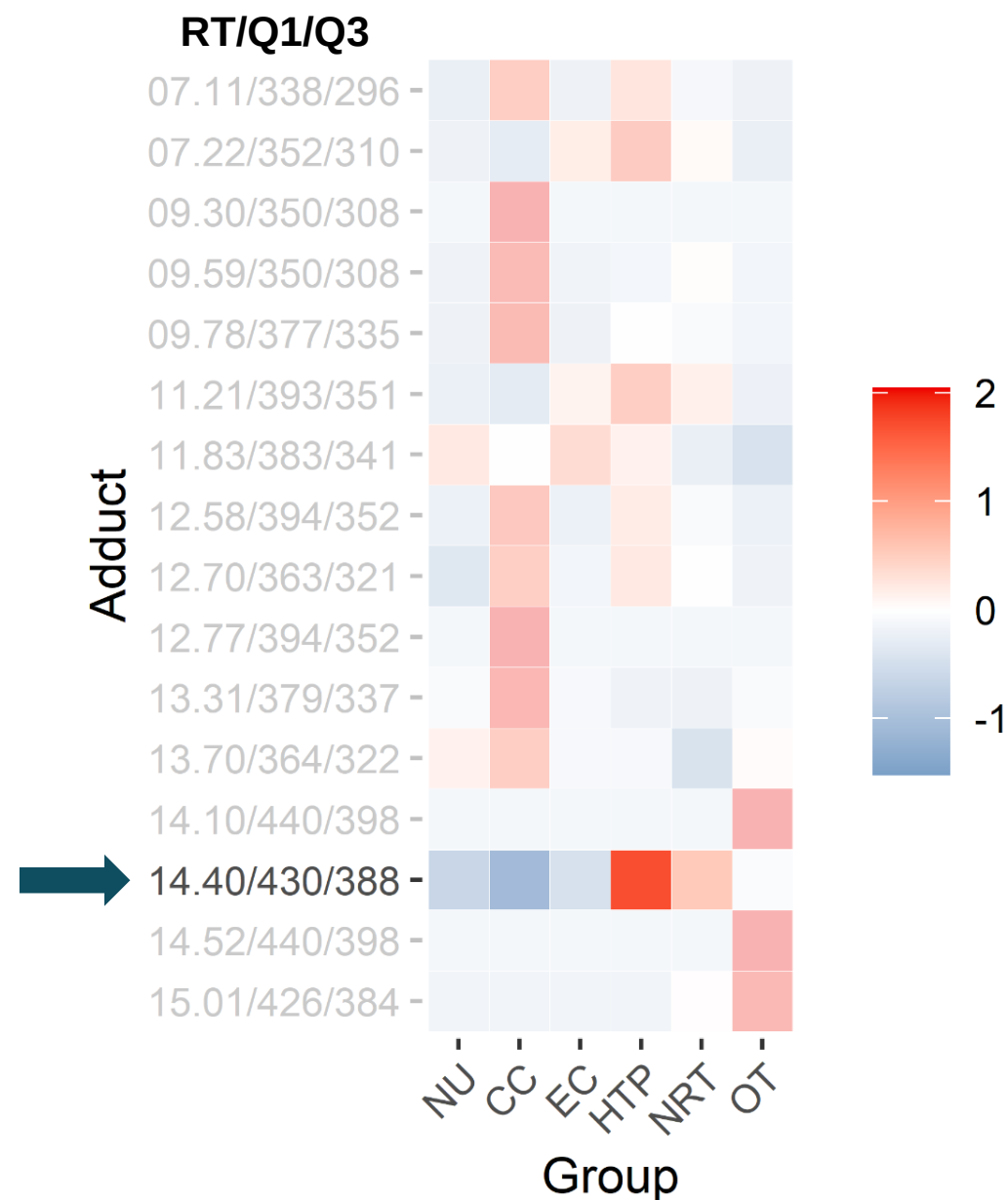


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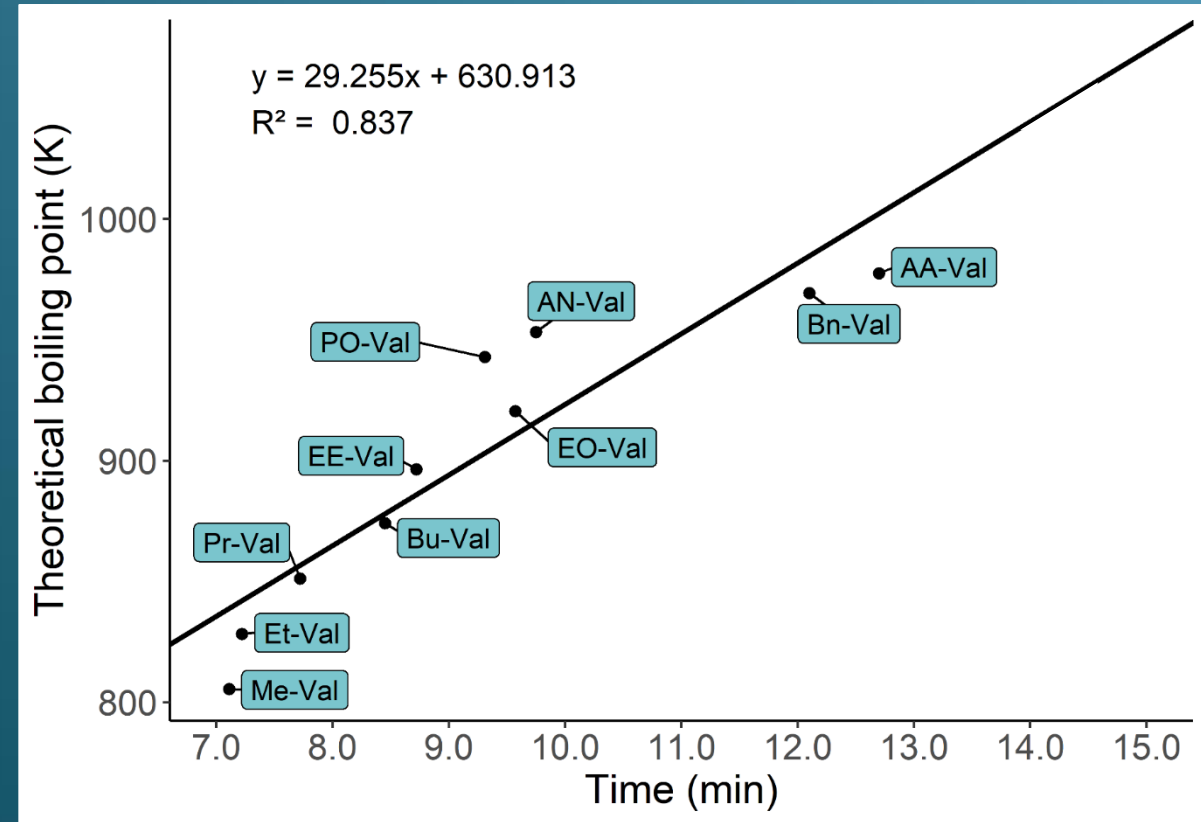
Conclusion



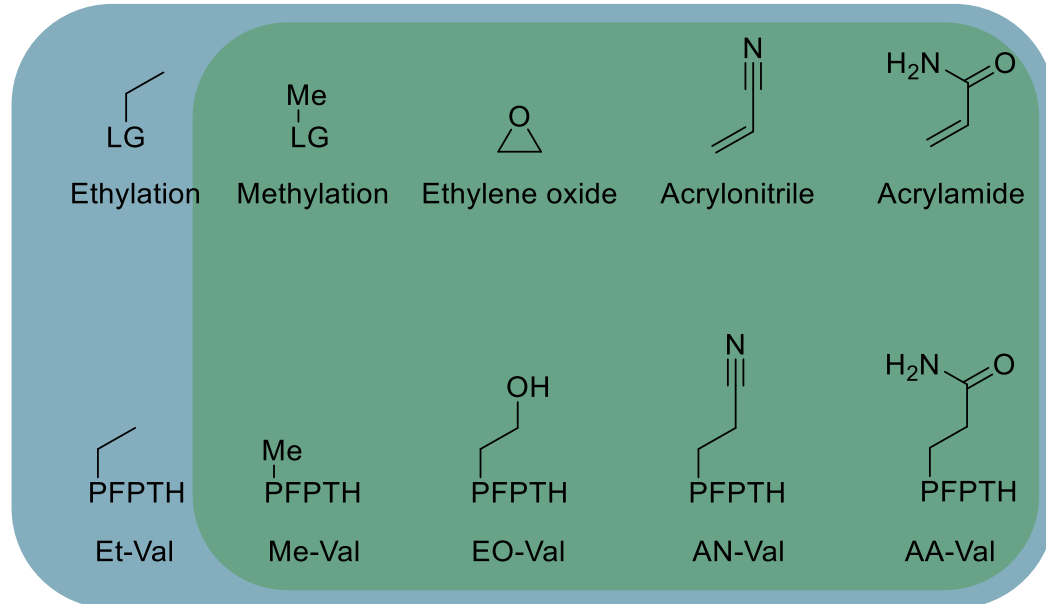
Identification Strategy

Boiling Point Model

Hb adducts used to generate a linear model to predict boiling points of unknowns based on the observed retention times (RTs)

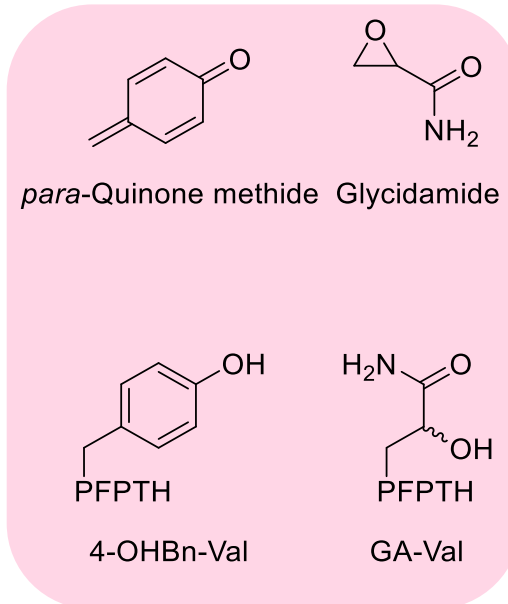


Identified Hb Adducts



Identified via a **modified dipeptide standard**

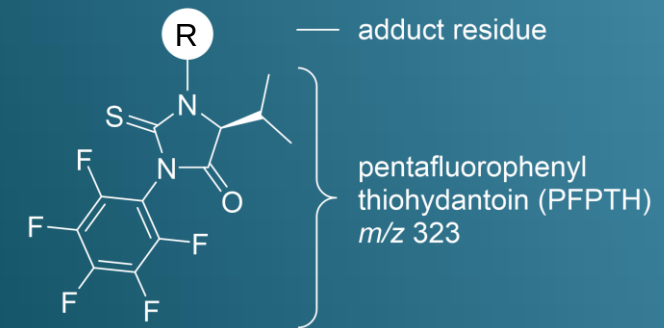
Identified via the **incubation with deuterated electrophiles**



Identified via the **incubation with electrophiles**

Precursor

Hb Adduct

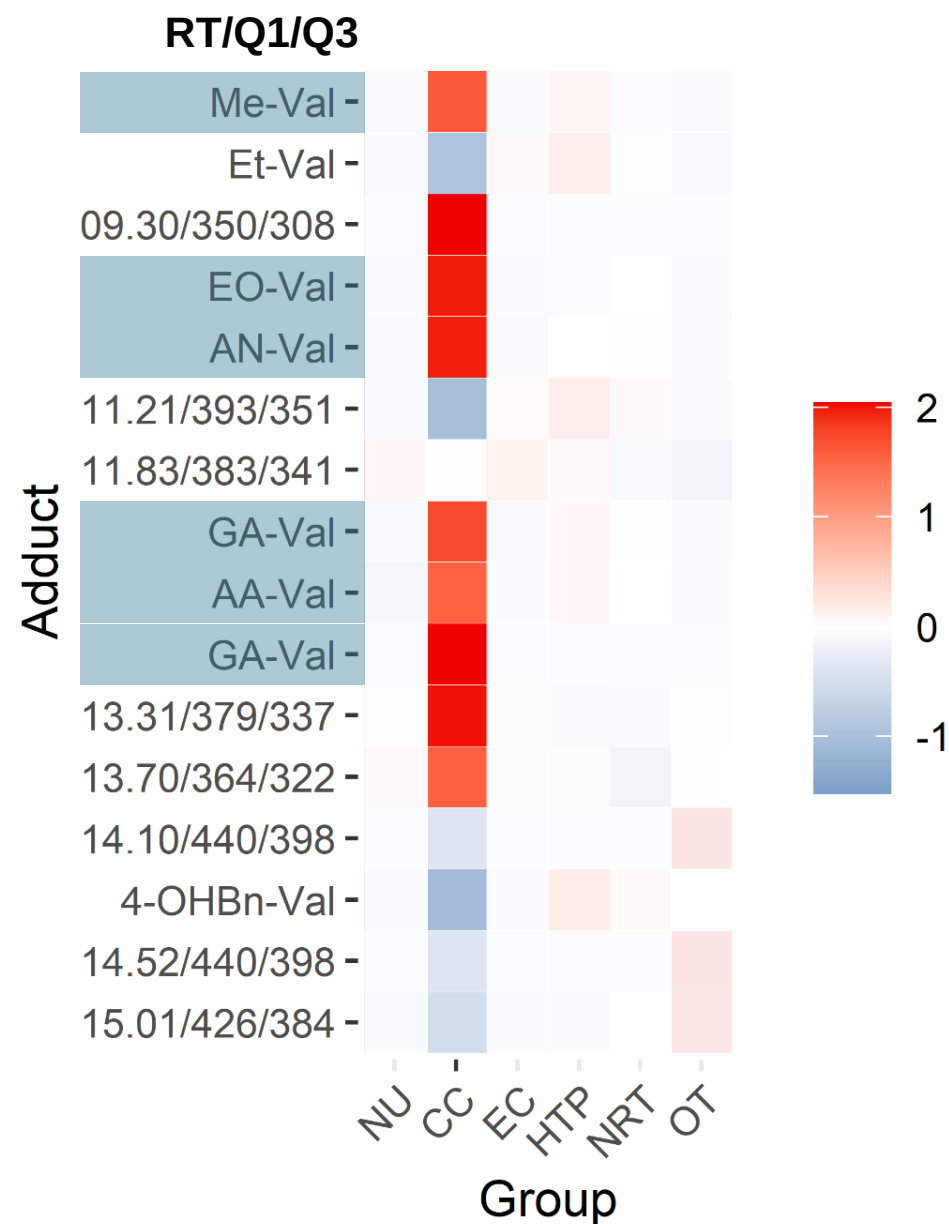


LG - leaving group

Identified Hb Adducts

Cigarette Smokers (CC):

High levels of Me-Val, EO-Val, AN-Val, AA-Val and its metabolite GA-Val



Identified Hb Adducts

Cigarette Smokers (CC):

High levels of Me-Val, EO-Val, AN-Val, AA-Val and its metabolite GA-Val

Users of Heated Tobacco Products (HTP):

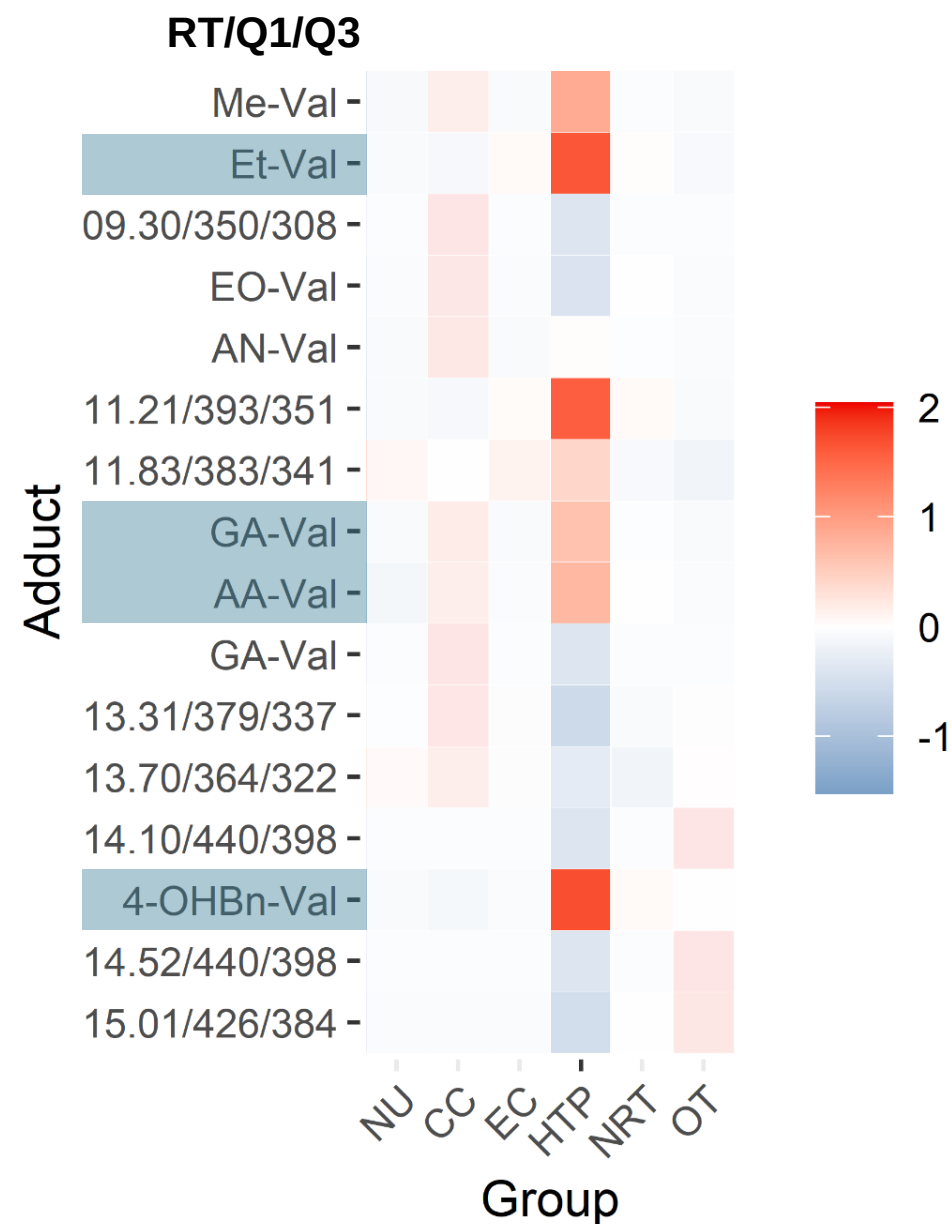
Significantly elevated levels of Et-Val, AA-Val and GA-Val as well as 4-OHBn-Val

Introduction

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Identified Hb Adducts

Cigarette Smokers (CC):

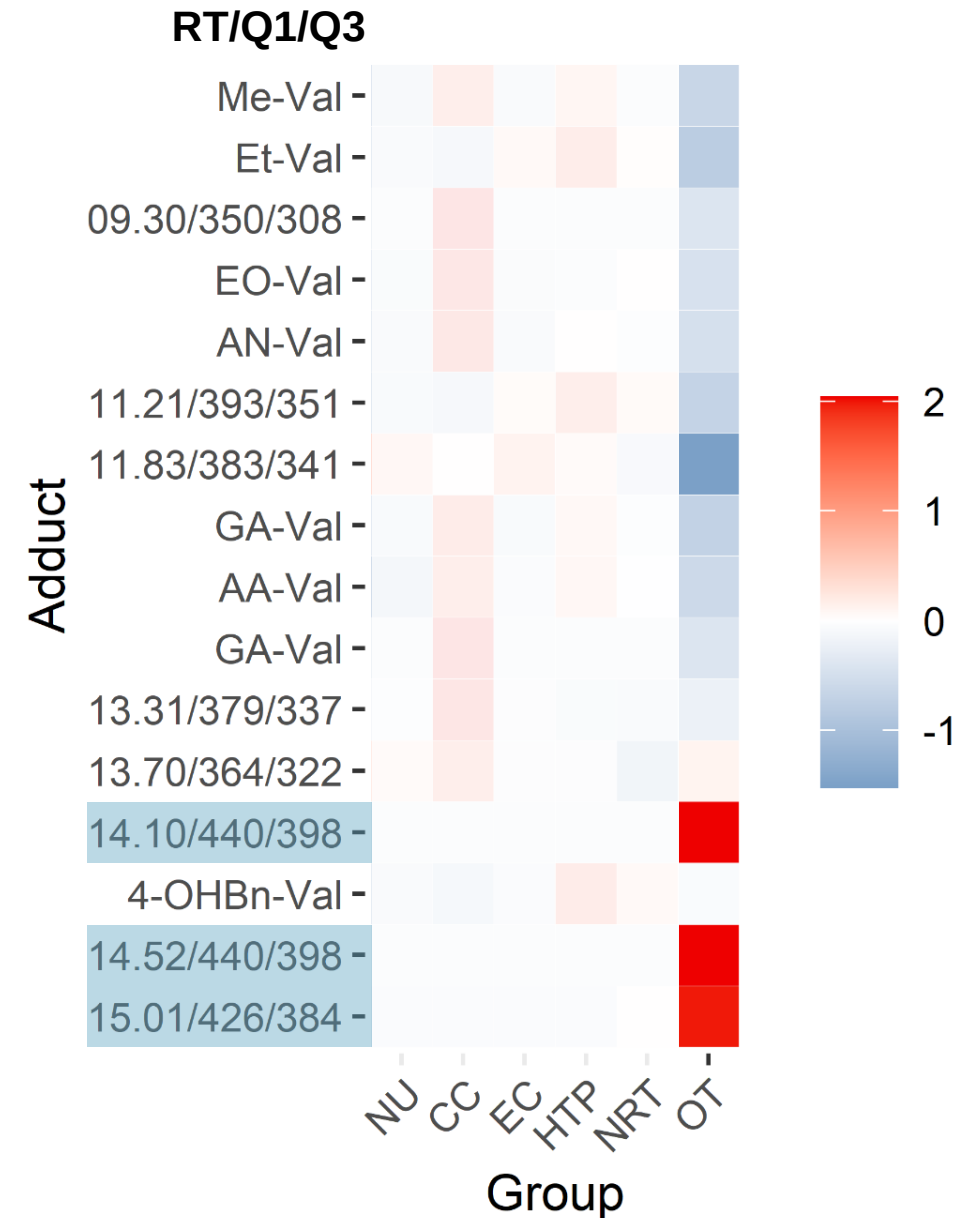
High levels of Me-Val, EO-Val, AN-Val, AA-Val and its metabolite GA-Val

Users of Heated Tobacco Products (HTP):

Significantly elevated levels of Et-Val, AA-Val and GA-Val as well as 4-OHBn-Val

Users of Oral Tobacco (OT):

Elevated levels of unknown adducts at high RTs



Identified Hb Adducts

Cigarette Smokers (CC):

High levels of Me-Val, EO-Val, AN-Val, AA-Val and its metabolite GA-Val

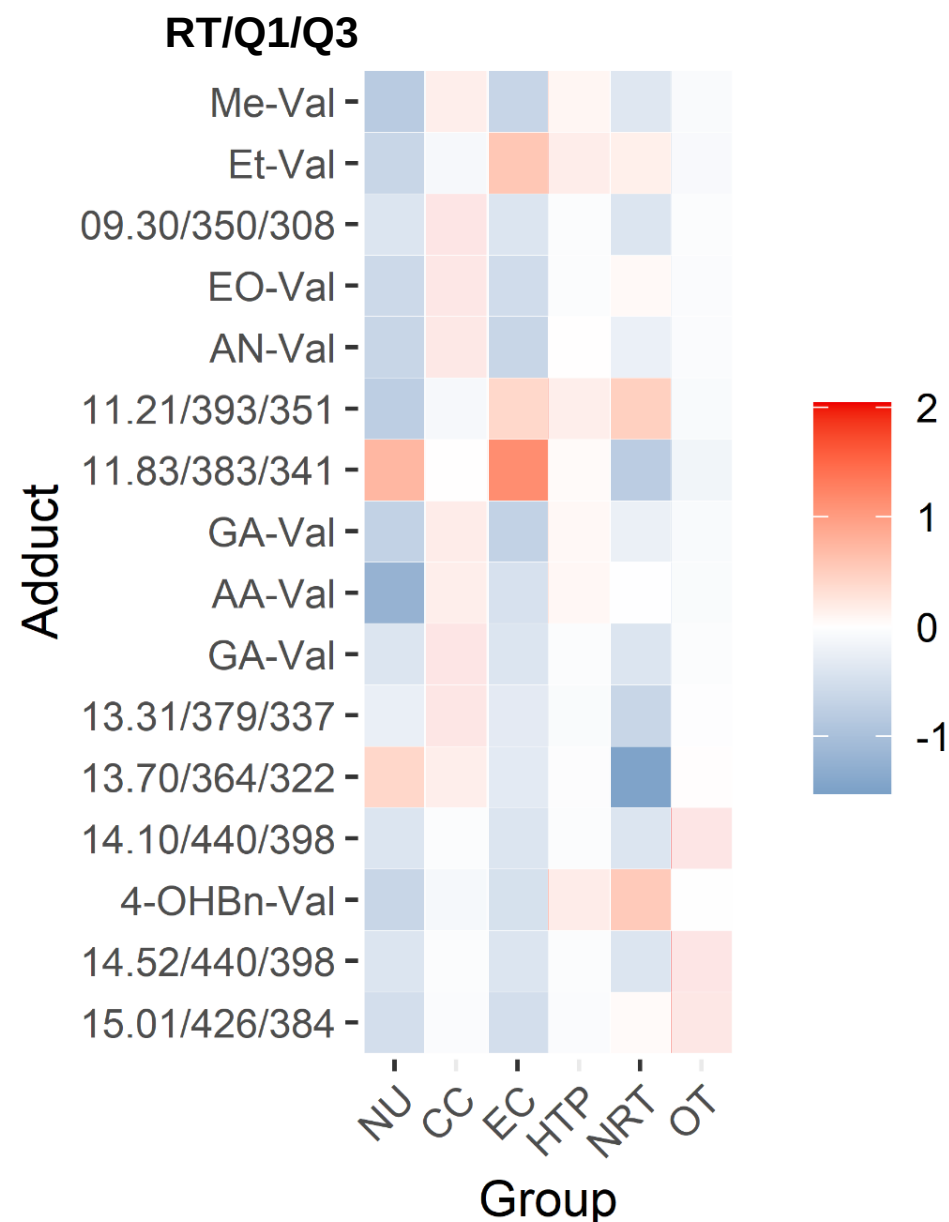
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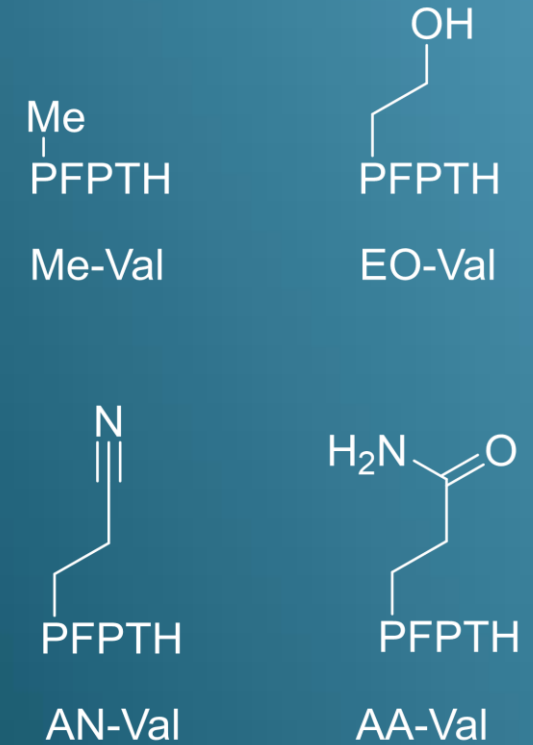
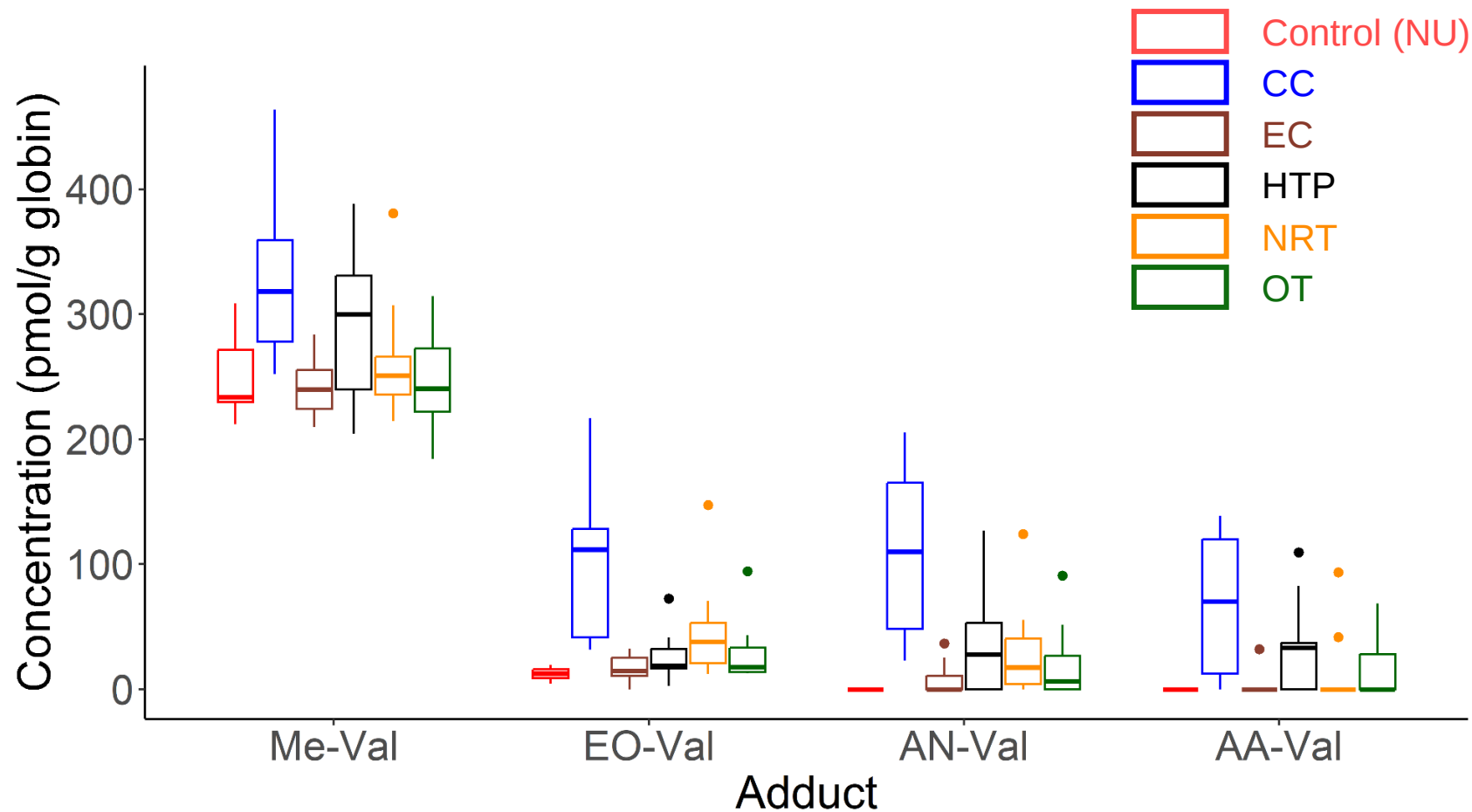
Users of Oral Tobacco (OT):

Elevated levels of unknown adducts at high RTs

The adduct profile of e-cigarette (EC), nicotine replacement therapy (NRT) users, and the control group (NU) is not significantly different

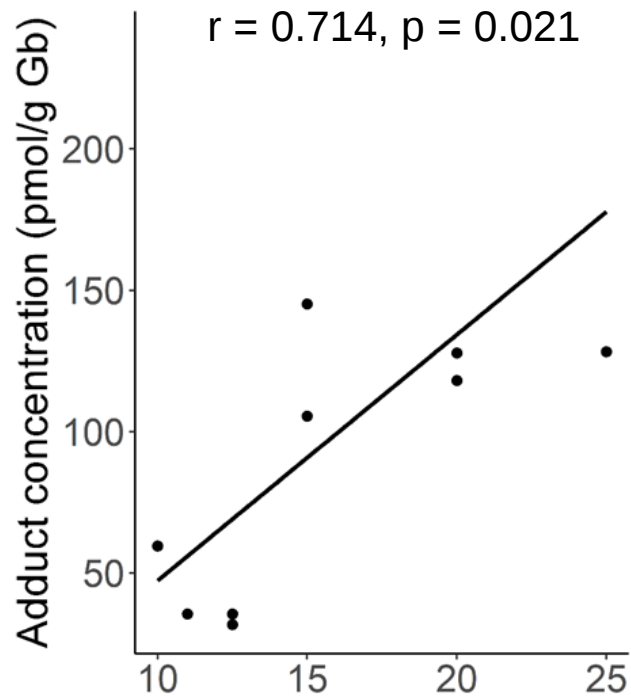


Quantification of Me-Val, EO-Val, AN-Val and AA-Val

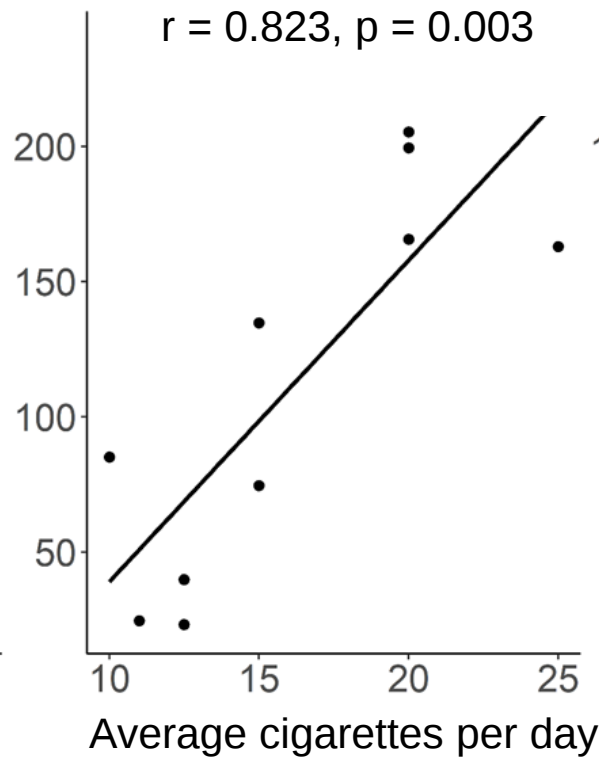


Correlation between Adduct Levels and Cigarette Consumption in Smokers

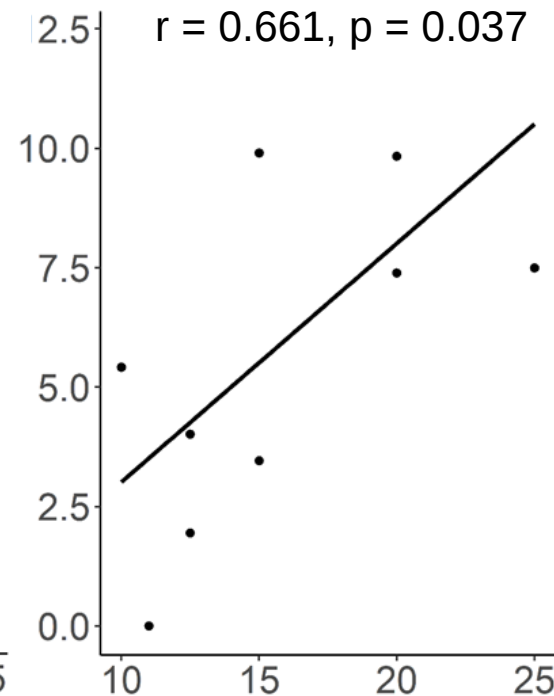
EO-Val



AN-Val



GA-Val (sum)



For Smokers (CC)

Significant moderate to strong **correlation** between selected adducts and cigarette consumption ($p < 0.05$)

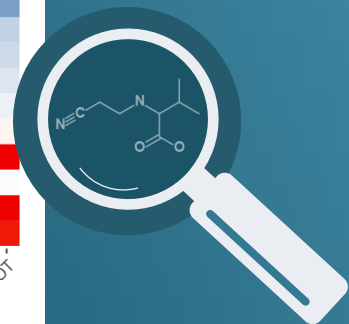
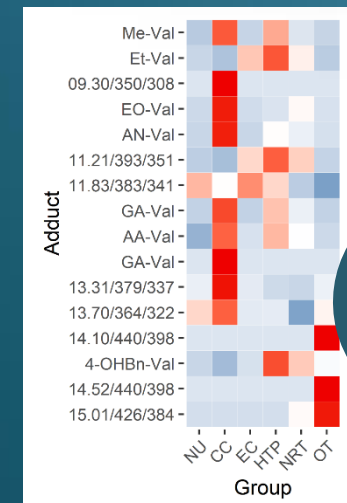
Conclusion

Method Development & Validation

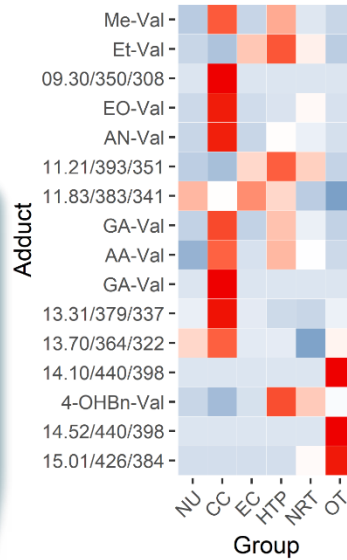
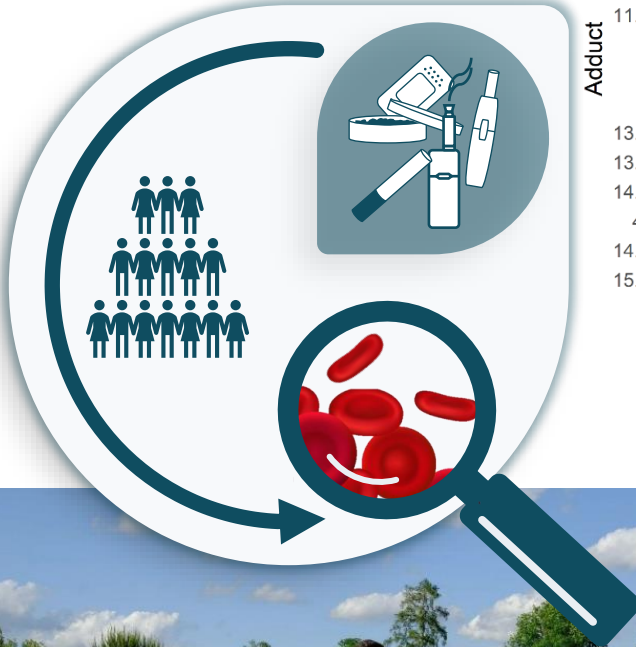
- Successful **development** of a **non-targeted GC-MS/MS method** for the analysis, evaluation and identification of hemoglobin adducts
- Successful **method validation** with 9 representative standards
- Development of a targeted method Hb-adduct method for quantification

Application of the Method & Identification

- Detection of **13 adducts** showing **significant differences** between the different nicotine user groups
- **Identification** of features using modified dipeptides and incubation experiments
- **Smokers (CC)** exhibited the **highest exposure to electrophiles** and showed good correlation with cigarette consumption for EO-Val, AN-Val and GA-Val
- Users of **HTPs** show elevated levels of Et-Val, AA-Val, GA-Val and 4-OHBn-Val



Acknowledgment



ABF Team
Fabian Pilz
Dr. N. Pluym
Dr. Therese Burkhardt
Prof. Dr. Gerhard Scherer



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