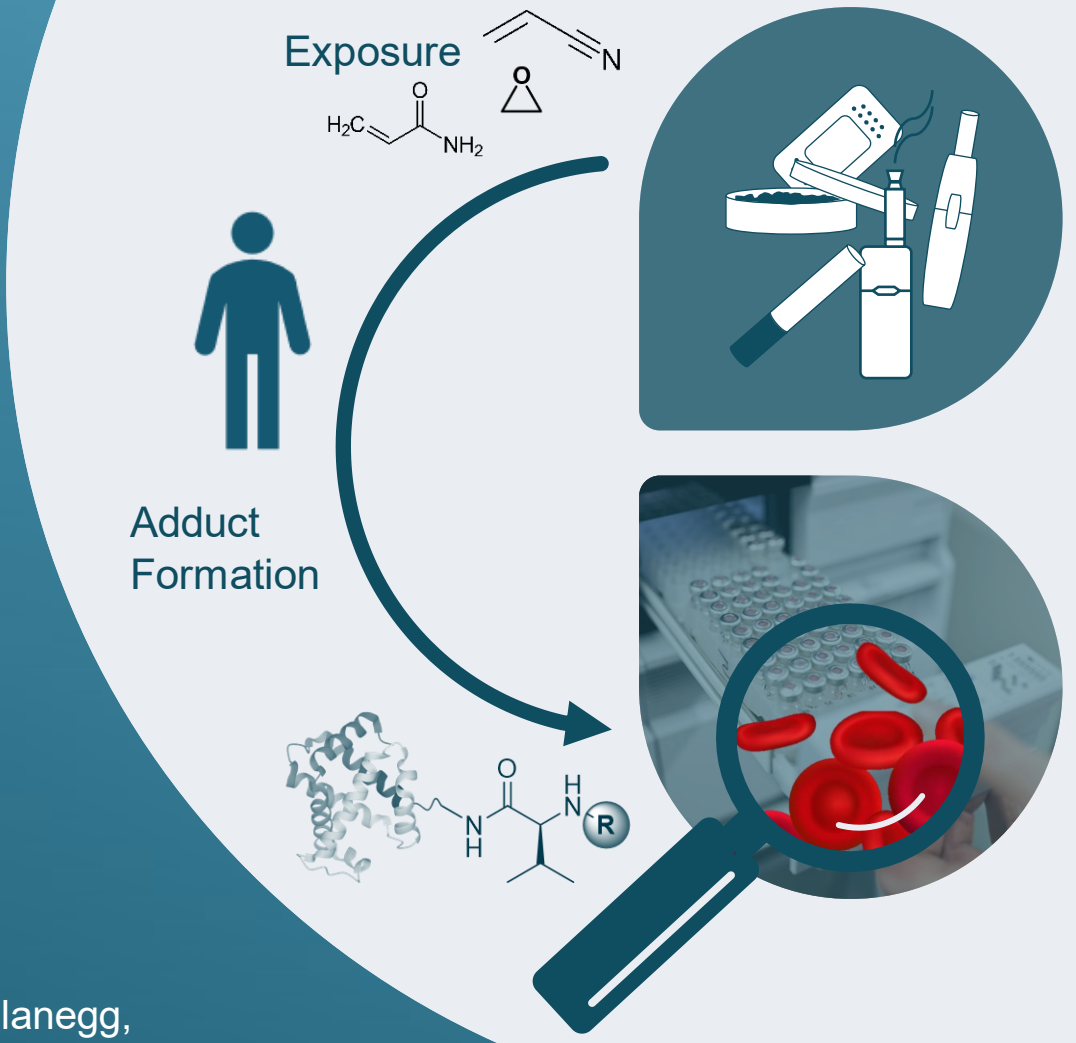
Identification of use-specific hemoglobin adduct patterns for different tobacco/nicotine product user groups by non-targeted GC-MS/MS analysis



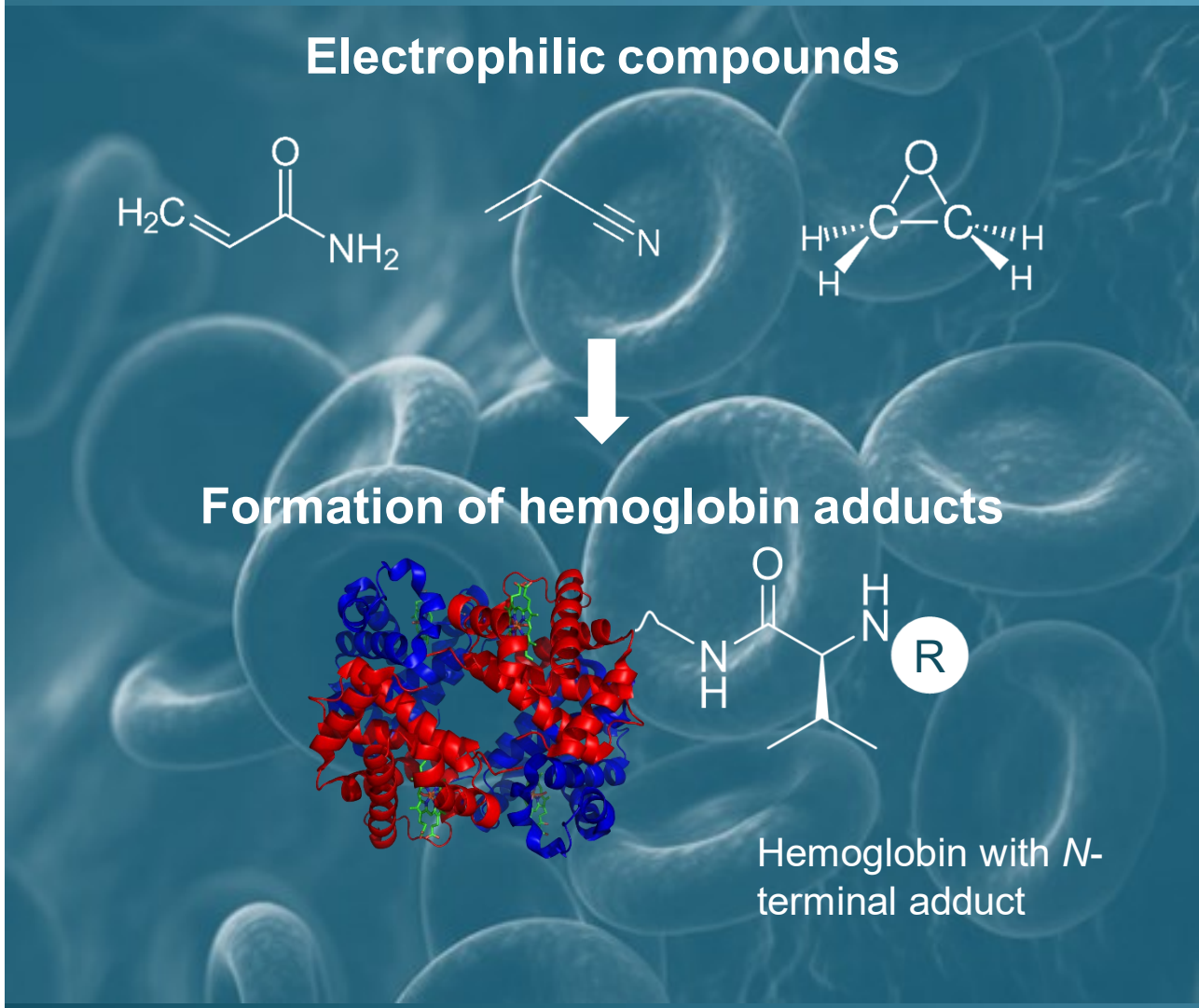
M. Scherer, F. Pilz, T. Burkhardt, G. Scherer, N. Pluym

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



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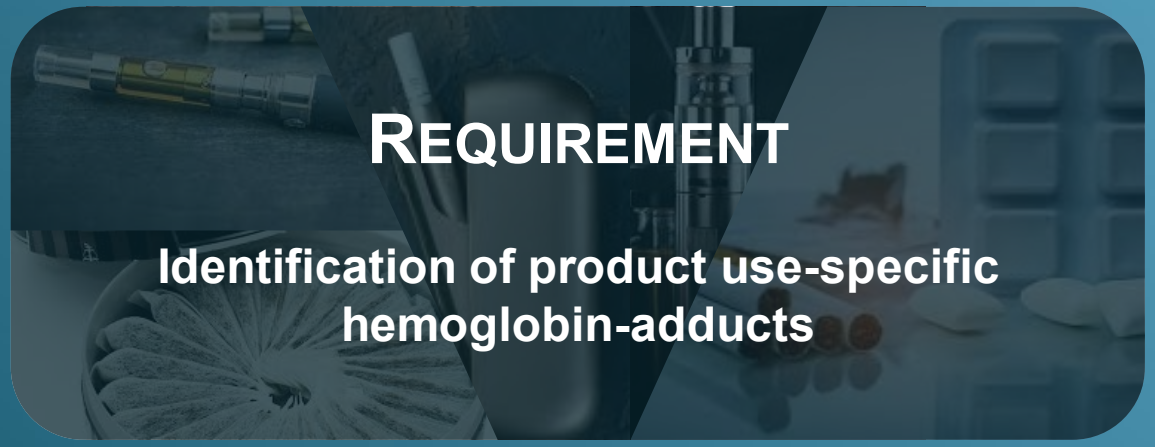


Risk evaluation

+

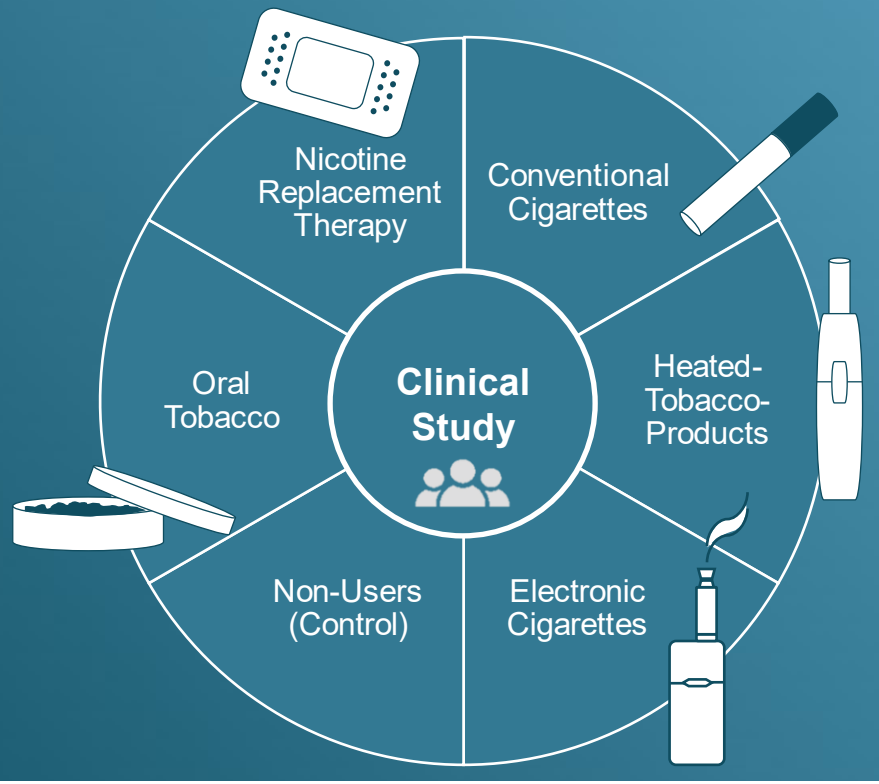
Compliance

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 / m^2



10 subjects per group

Day 1 → Day 2 → Day 3

GENERAL STUDY OBJECTIVE

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

Sibul F, et al.: Identification of biomarkers specific to five different nicotine product user groups: Study protocol of a controlled clinical trial. *Contemporary clinical trials communications* 2021, 22:100794.

Analytical Strategy

Bioanalytical methods

Holistic

We have a problem !

Screening
Hemoglobin adducts
for specific product categories

Analytical Strategy

Bioanalytical methods

Holistic

Targeted

We have a problem !

Screening
Hemoglobin adducts
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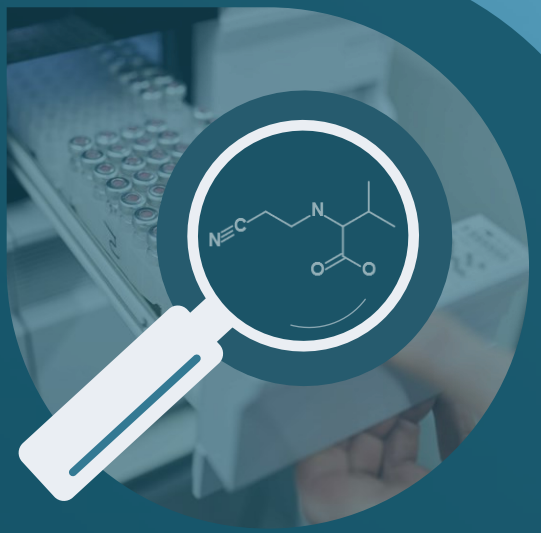
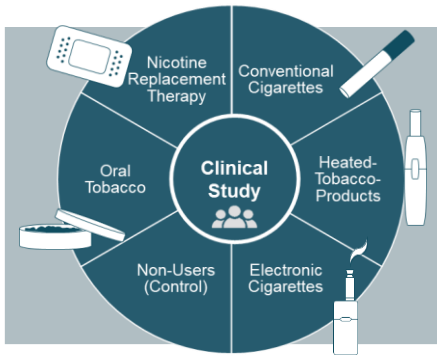
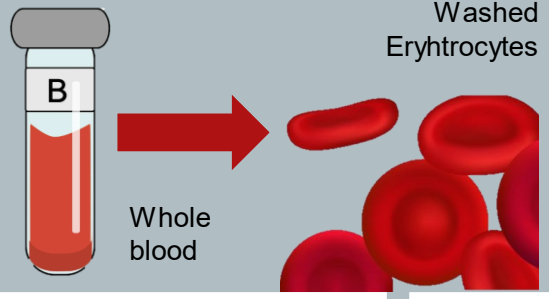


Can we identify the root cause?

Targeted quantitative profiles

Non-targeted screening – Workflow

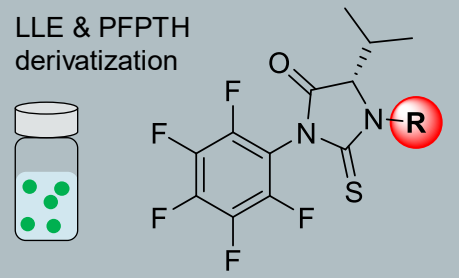




Globin precipitation

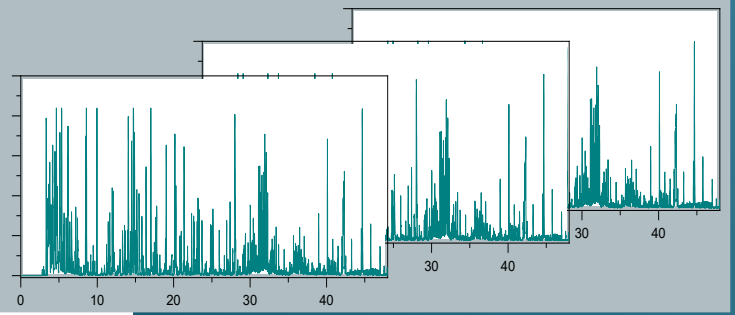
Edman degradation

LLE & PFPTH derivatization

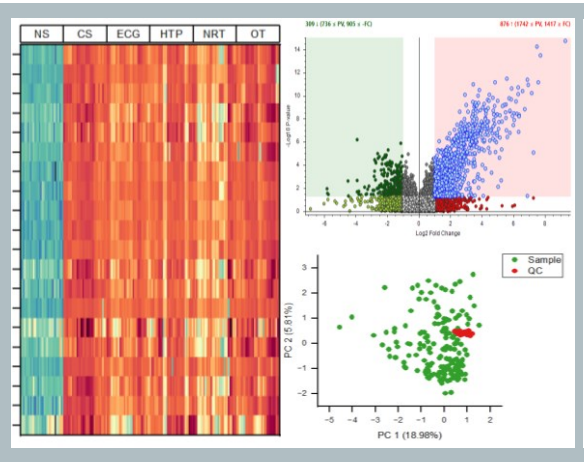


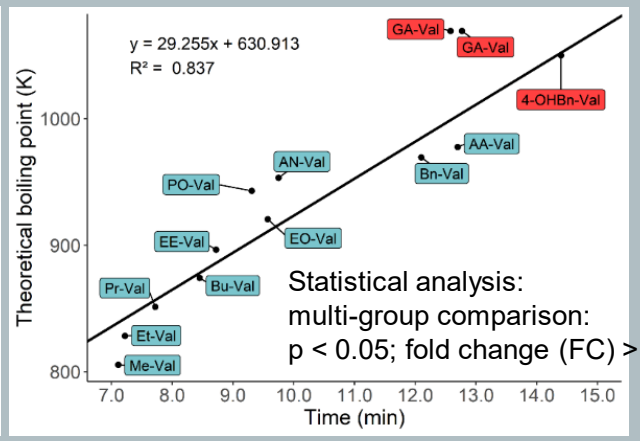
Sampling & Sample Preparation

GC-MS/MS
Shimadzu TQ8050

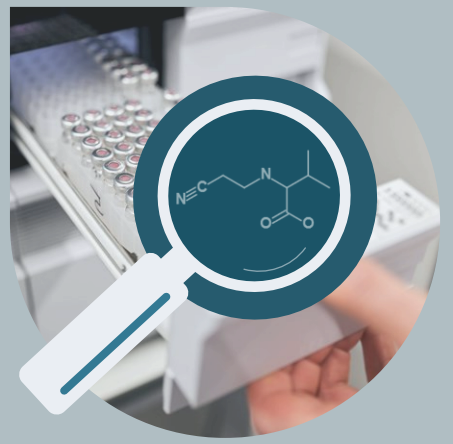


Sample Analysis





Statistical analysis:
multi-group comparison:
$p < 0.05$; fold change (FC) > 2

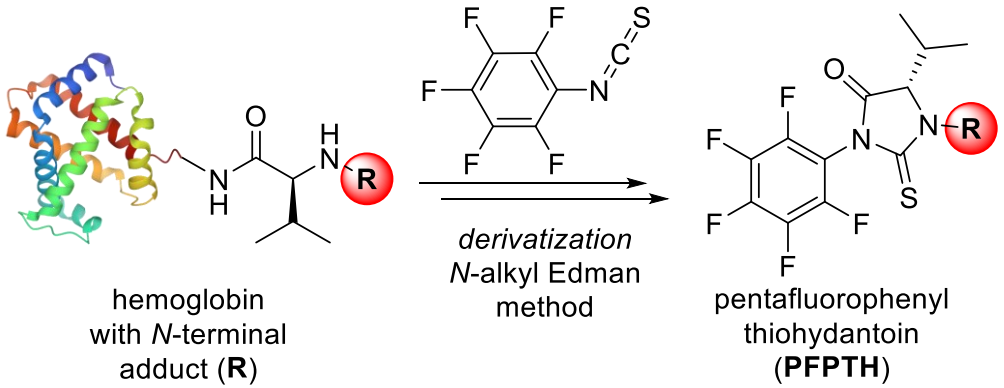


Data Processing
Statistical Analysis
Compound Identification

Non-Target Screening

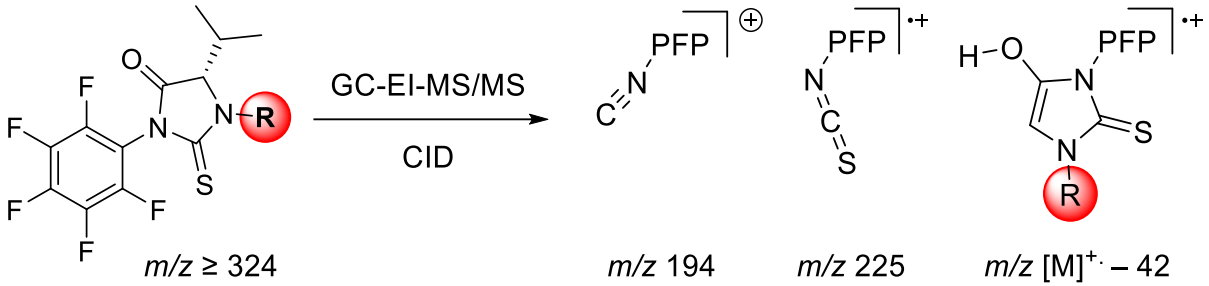
Derivatization of Hb with pentafluorophenyl isothiocyanate (PFPITC)

Formation of small molecules (PFPTH) for analysis

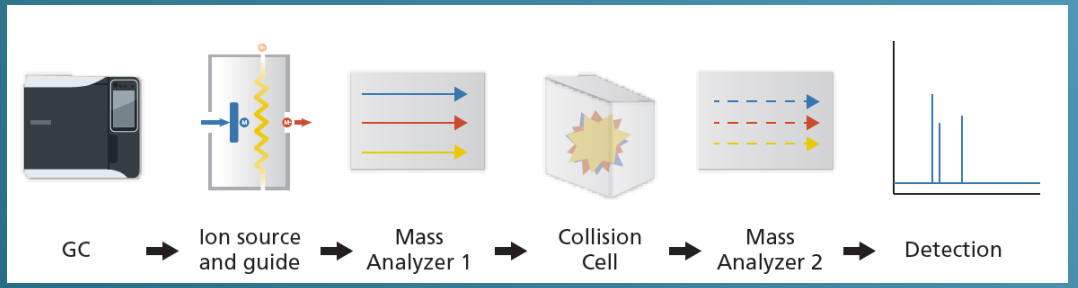


Non-Target Screening

- 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)

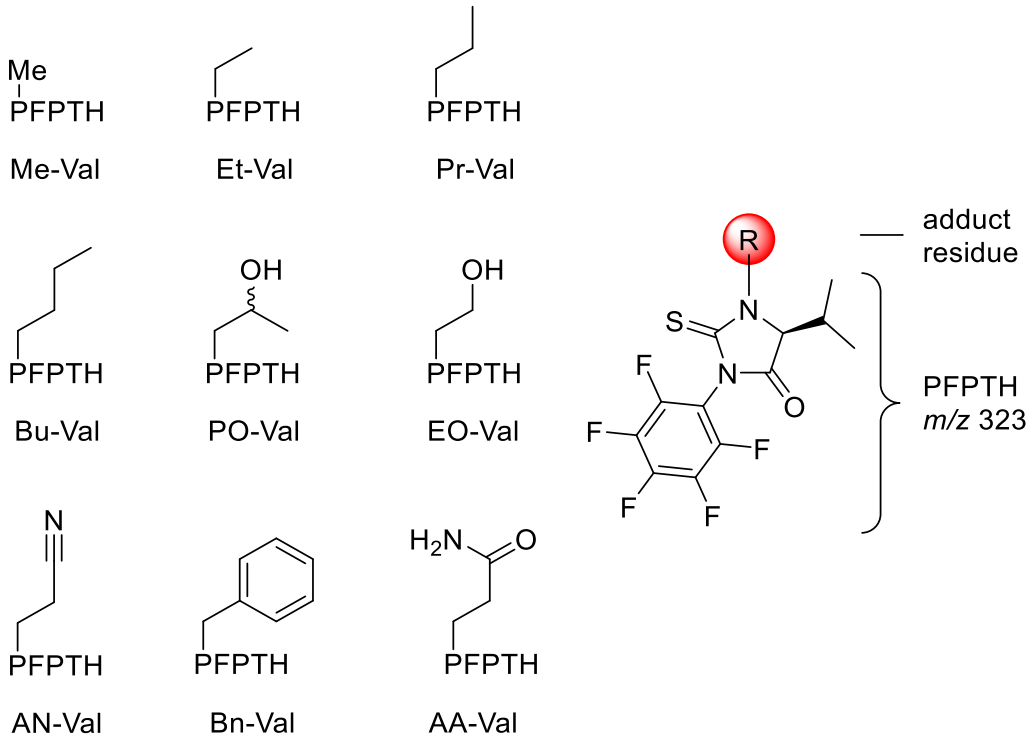


MRM method used to screen for *N*-terminal Hb adducts

Q1: Parent ion	Q3: Fragments		
$[M]^{+\cdot}$	$[M]^{+\cdot}$-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

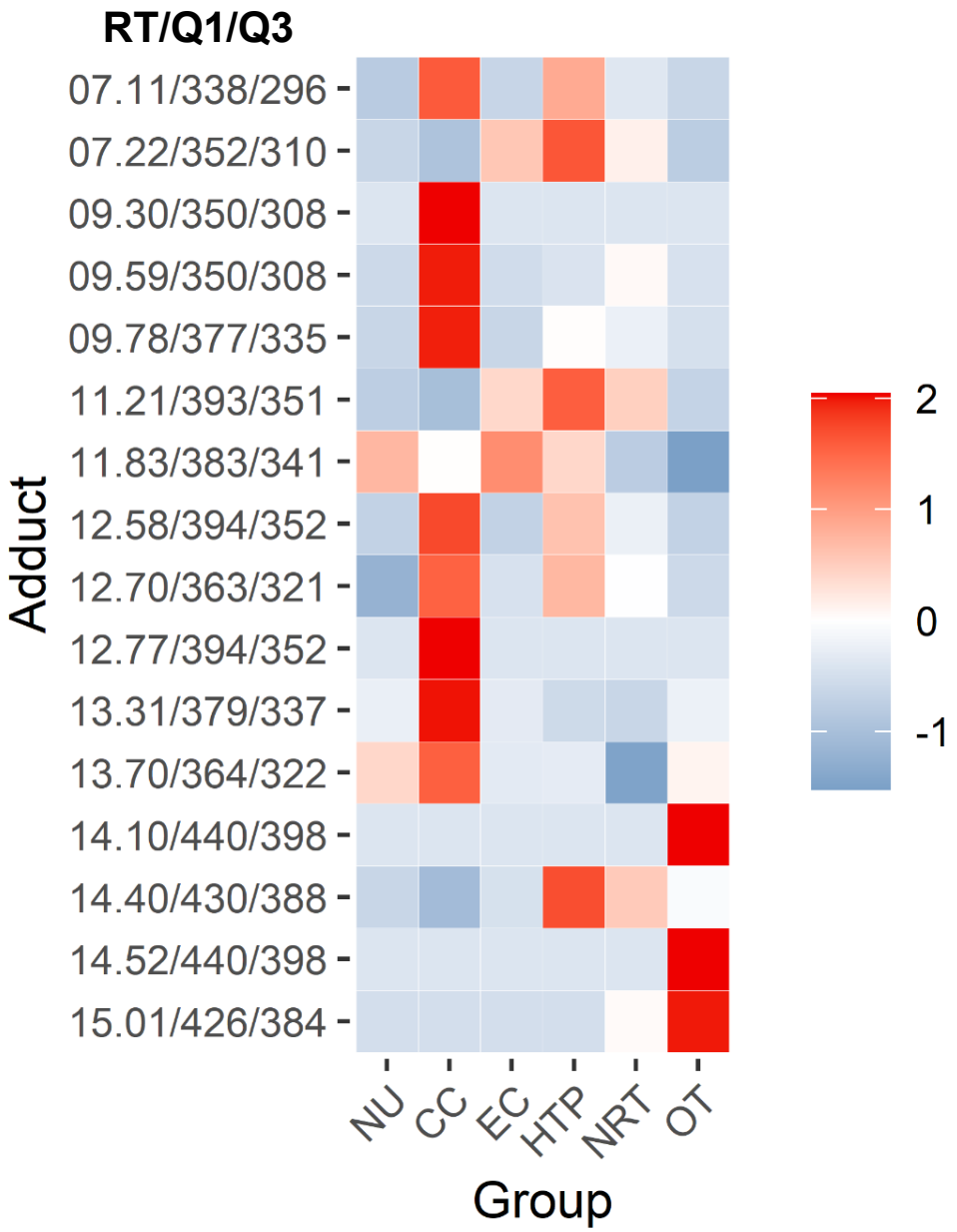
Repeatability (CV, N = 5)	4.6 – 13.1 %
Precision **Intraday** (CV, N = 3) **Interday** (CV, N = 4)	 1.4 – 19.0 % 6.6 – 19.0 %
Accuracy **Intraday** (N = 3) **Interday** (N = 4)	 82.6 – 107.9 % 89.2 – 117.8 %
Carry over	0.0 – 0.3 %
Linearity (R^2, Range: 15, 60, 120 – 3600 pmol/g Gb)	≥ 0.9903
Post-preparative 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…

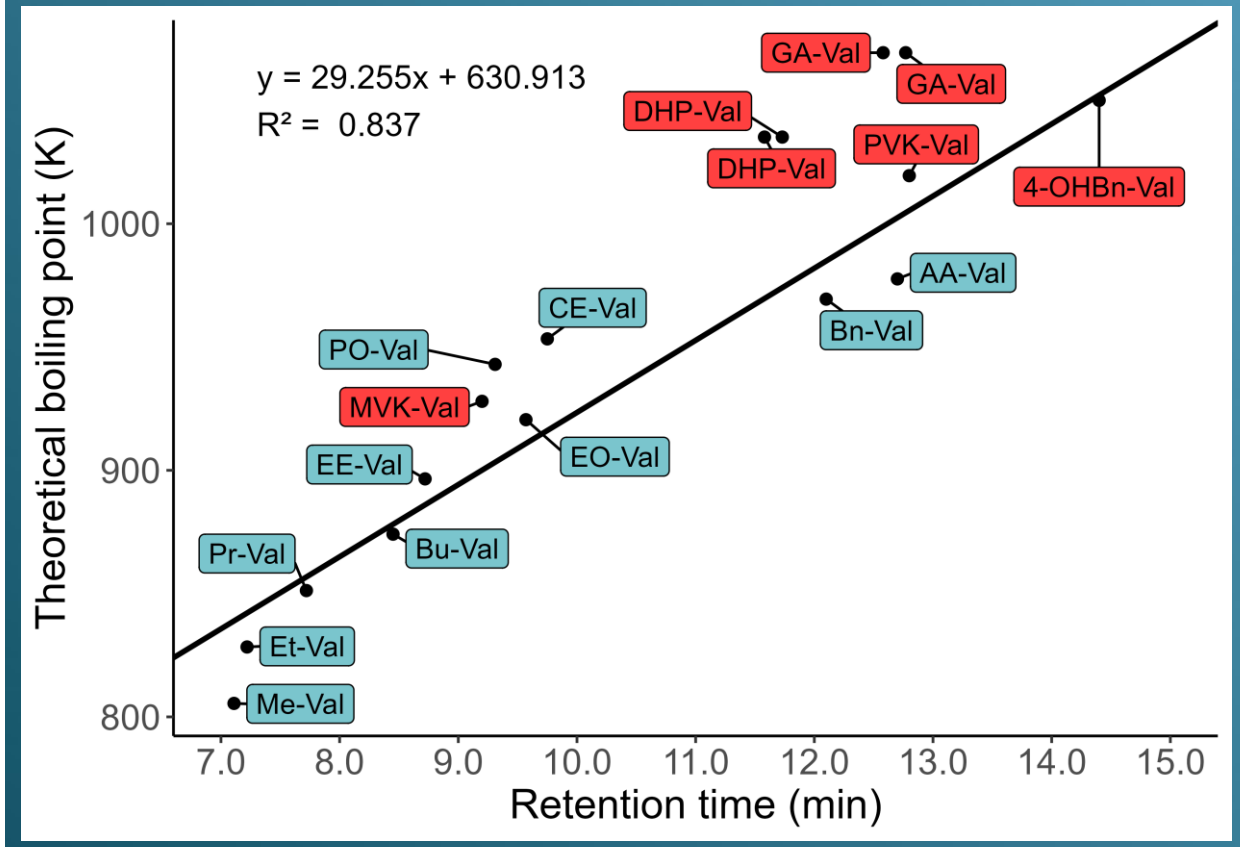
- **Adductome data**
  Parent ions
  Retention time
  Isotopic distribution
- **Literature review** and **database search** on exposure from tobacco/nicotine products and previously reported adducts



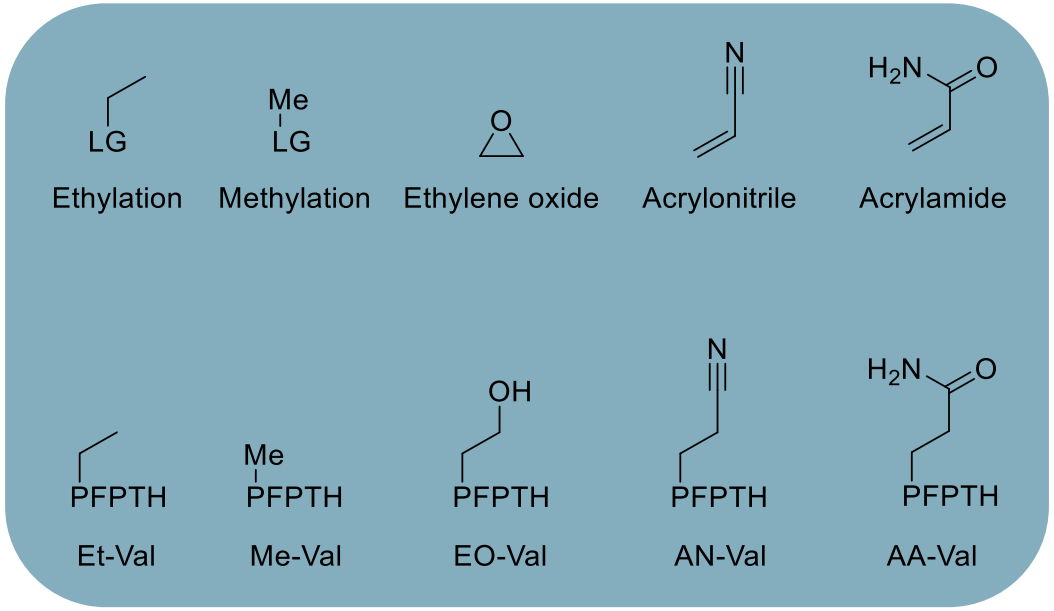
Identification Strategy

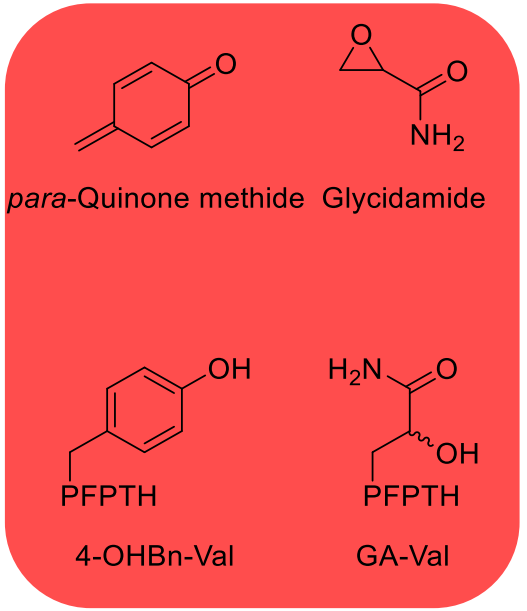
Boiling Point Model

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



Identified Hb Adducts



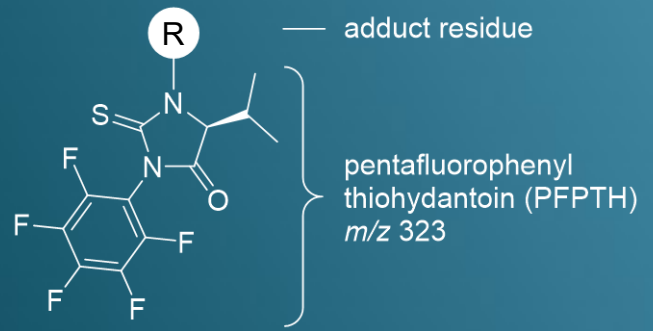


Identified via a **modified dipeptide standard**

Identified via the **incubation** with **electrophiles**

Precursor

Hb Adduct

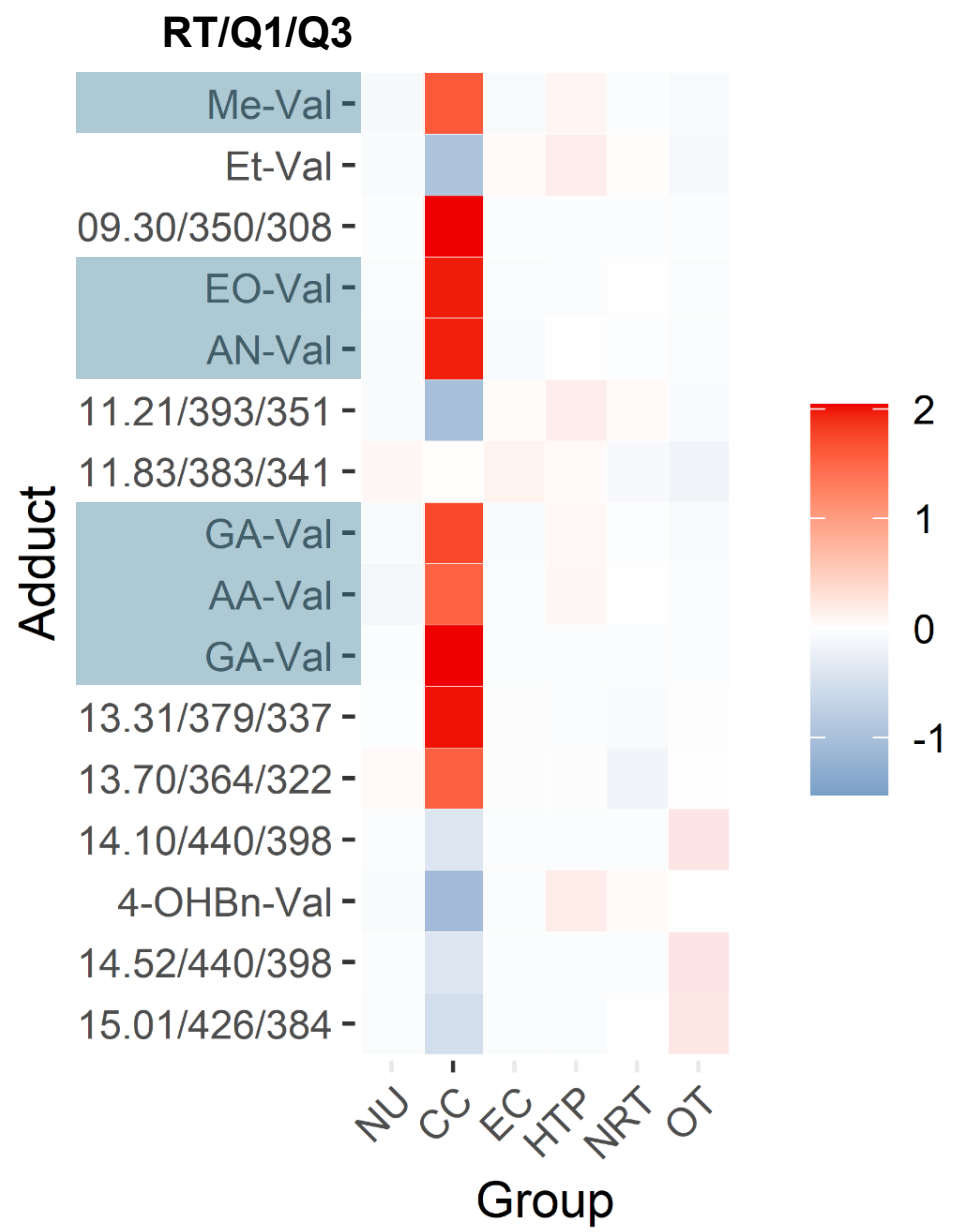


LG - leaving group

Identified Hb Adducts

Cigarette Smokers (CC):

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



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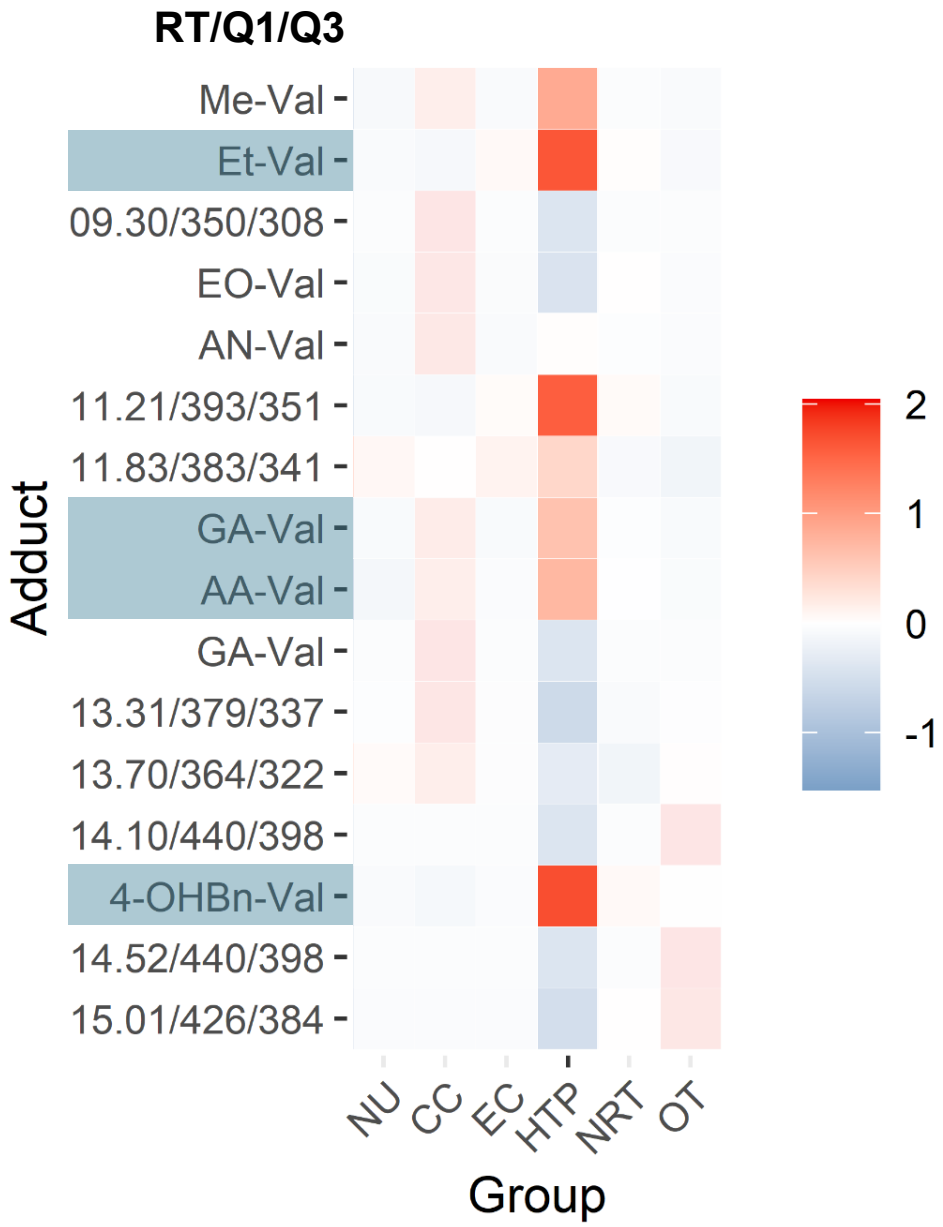
Users of Heated Tobacco Products (HTP):

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



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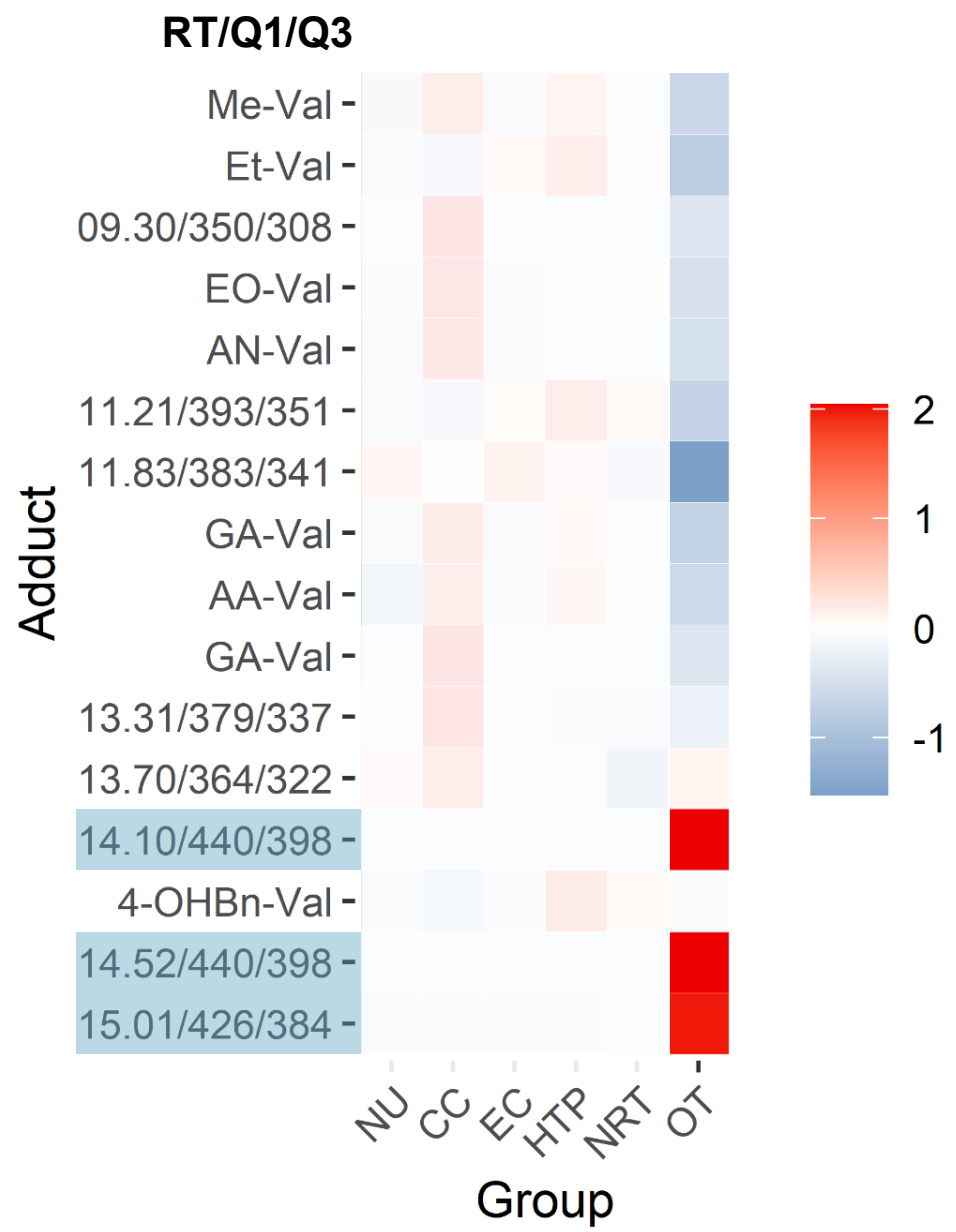
Elevated levels of unknown adducts at high RTs



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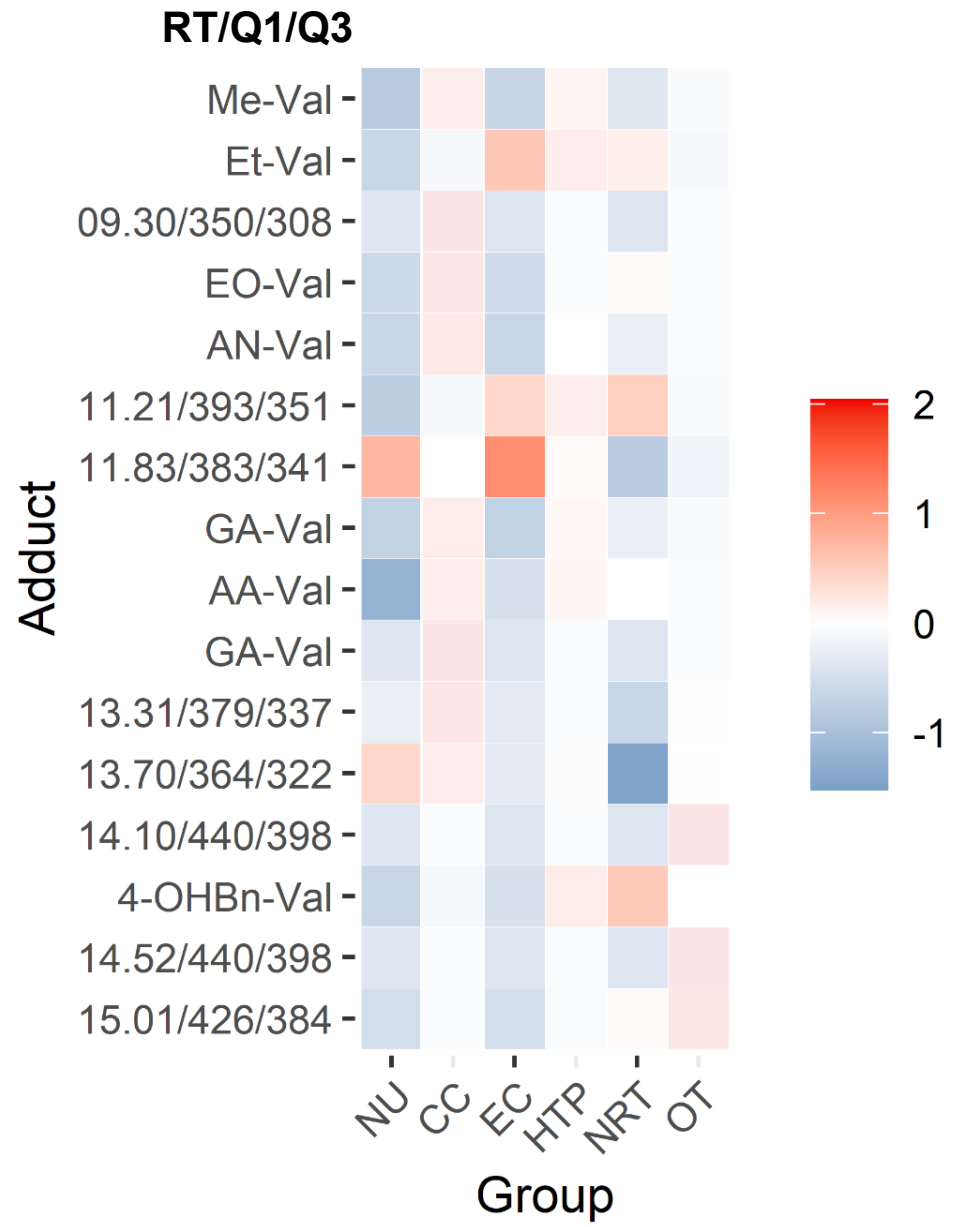
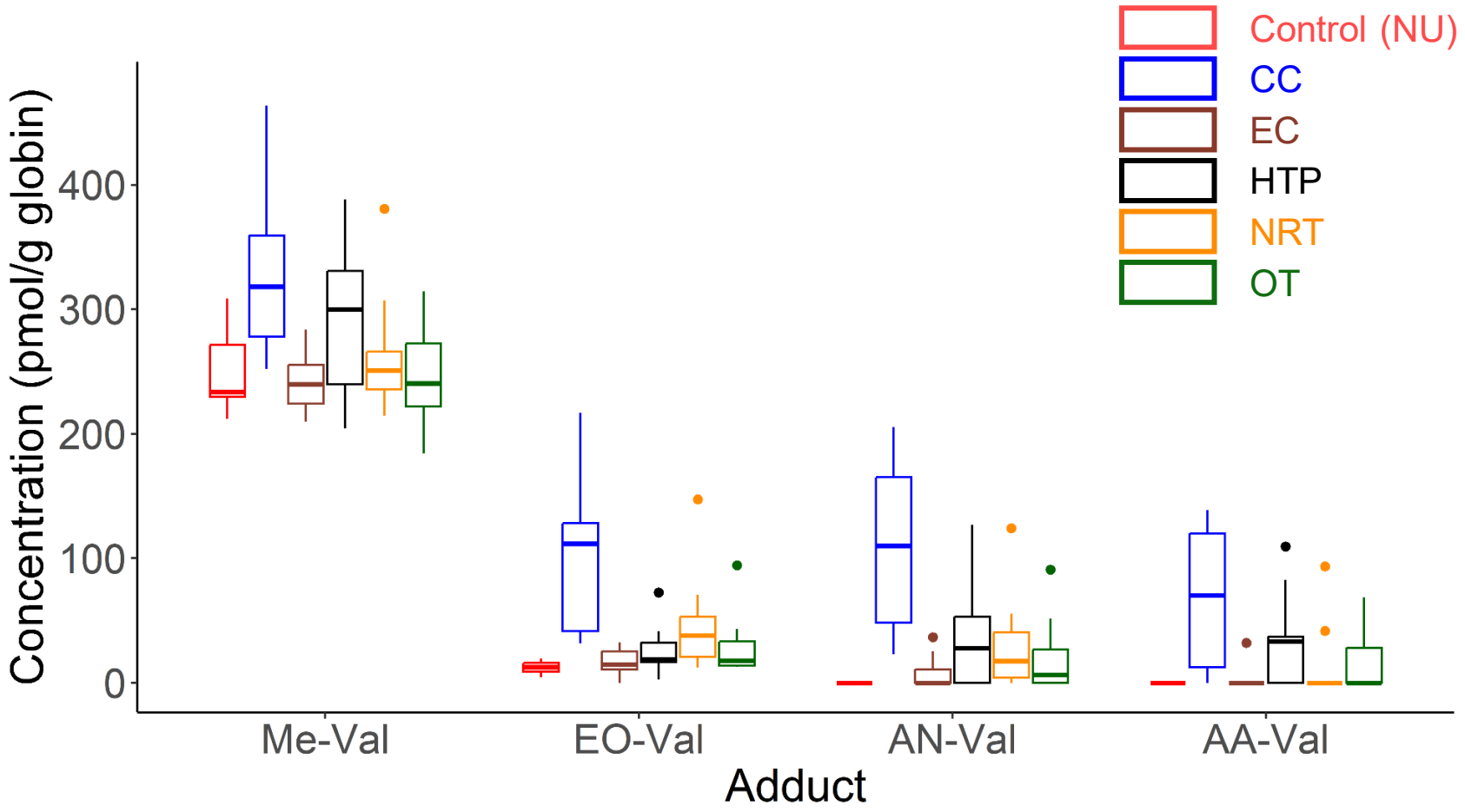
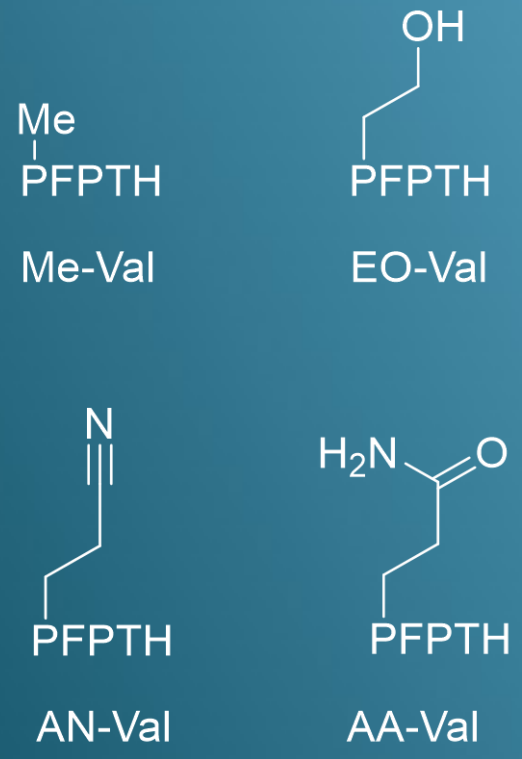
The adduct profile of e-cigarette (**EC**), nicotine replacement therapy (**NRT**) users, and the control group (**NU**) is not significantly different



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



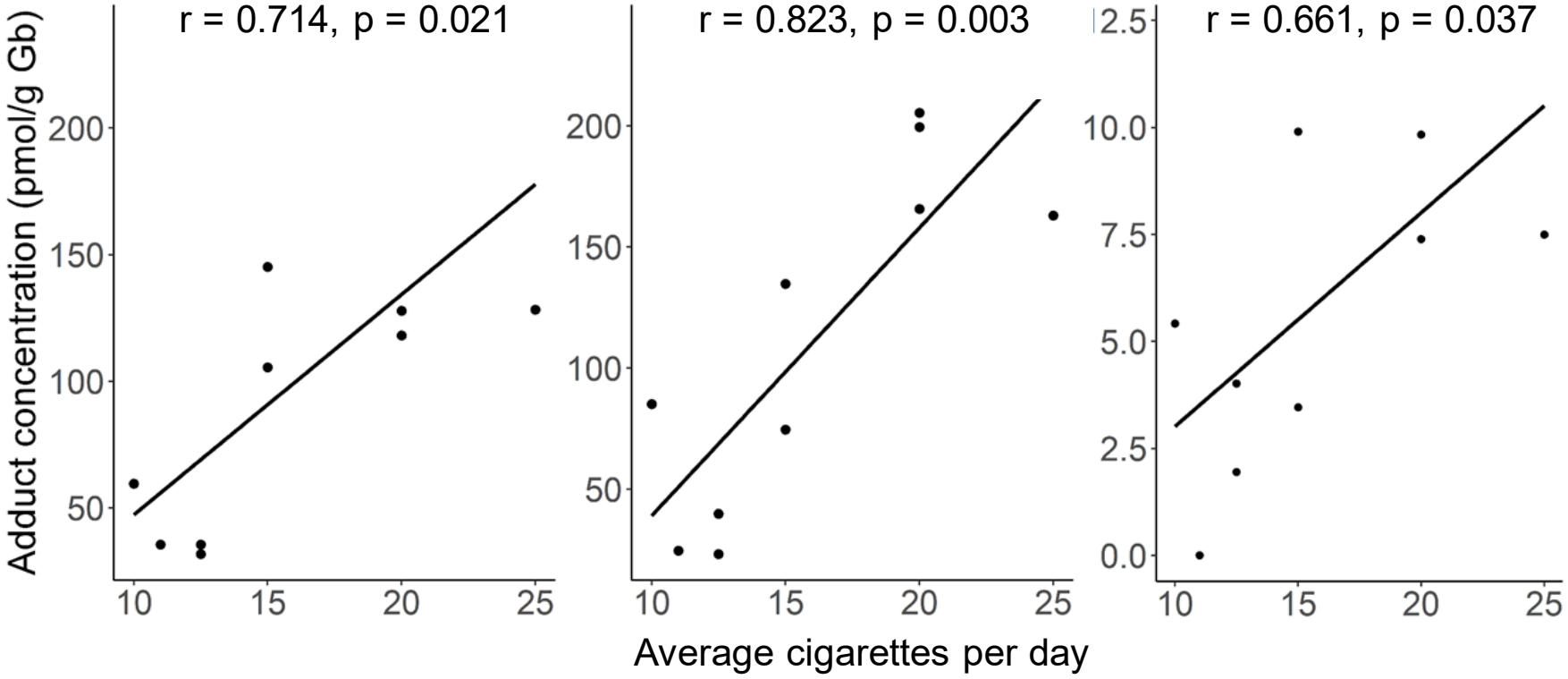


Correlation between Adduct Levels and Cigarette Consumption in Smokers

EO-Val

AN-Val

GA-Val (sum)



For Smokers (CC)

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

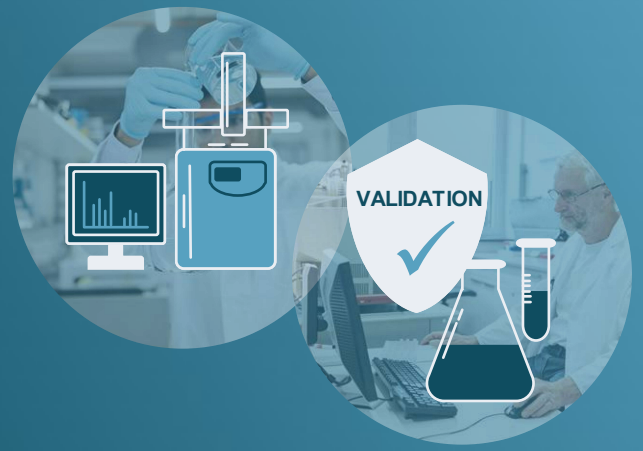
Conclusion

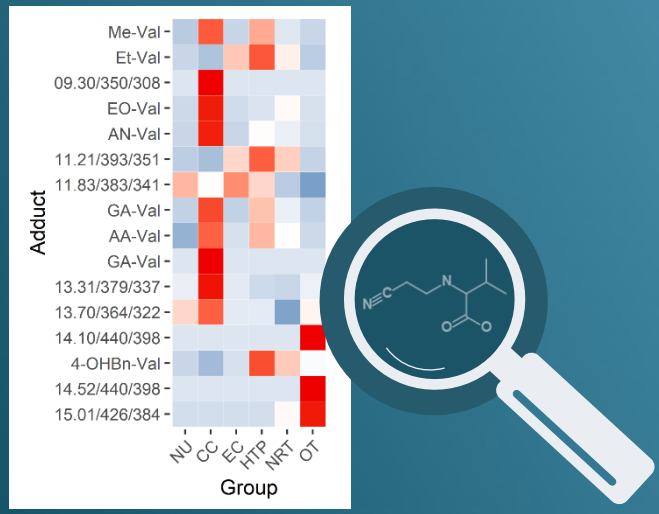
Method Development & Validation

- 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

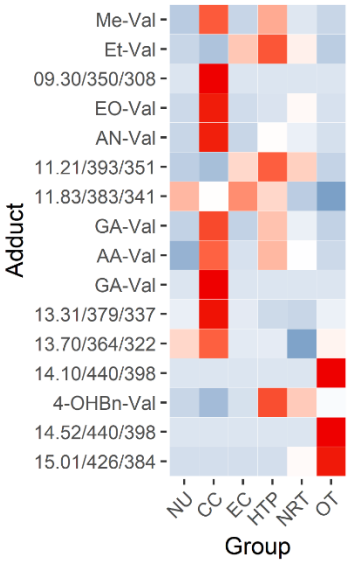
Application of the Method & Identification

- 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

FOUNDATION FOR A SMOKE-FREE WORLD

Funding:

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