Determination of flavors and their metabolites in e-liquids and biological fluids
Identification of biomarkers of exposure to prominent e-cigarette flavors

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Abstract

Almost all e-cigarette liquids (e-liquids) contain **flavor chemicals**. Although the majority of the flavors used are `generally regarded as safe´ (GRAS) food additives, very little is known about their **metabolic fate after inhalation**. Thus, concerns are raised by regulatory authorities on the potential health risks caused by flavor additives in e-cigarettes. As a pre-requisite for further toxicological evaluation of this new product category, **novel analytical methods** for the determination of the most prominent flavor compounds are needed to investigate the uptake into the human body via the route of inhalation.
We **developed** and **validated bioanalytical methods** for the **quantification of menthol**, a frequently used flavor additive, and its major metabolite, **para-menthane-3,8-diol (M-I)** in various human body fluids by means of **Headspace(HS)-SPME-GC-MS** and **LC-MS/MS**, respectively. Moreover, a **multi-analyte method** based on **HS-SPME-GC-MS** was developed for the **simultaneous determination of 12 most abundant flavors** in e-liquids. The straightforward sample preparation procedure allows a **time- and cost-efficient screening** of those compounds in clinical studies. Here, the analytical methods for the quantification of flavor additives are presented and their potential as **biomarkers of exposure to e-cigarettes** are discussed.

1. HS-SPME-GC-MS analysis of menthol

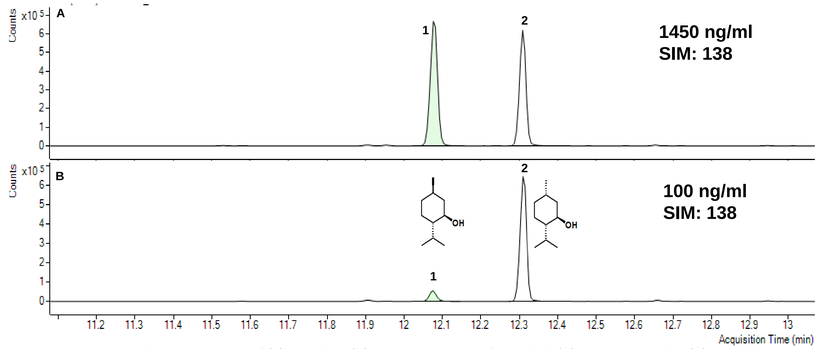

Fig. 1: HS-SPME-GC-MS analysis of (L)-menthol (**1**): Discrimination of menthol (**1**) and isomenthol (**2**) Chromatograms of rat plasma sample containing 1450 ng/ml menthol (A) and 100 ng/ml menthol (B); EI (70eV) GC: DB-1701

- ✓ Robust method for menthol determination in various body fluids validated according to FDA guidelines[1]
- ✓ Calibration range: 7 – 14,000 ng/ml (urine); 100 – 2,500 ng/ml (plasma)
- ✓ Applicable for various matrices: Plasma, urine, cell medium
- ✓ Discrimination of the 4 stereoisomers of menthol

2. Determination of the primary menthol metabolite M-I using LC-MS/MS

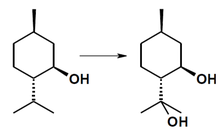

Fig. 2: Conversion of (L)-menthol to its primary metabolite **trans-para-menthane-3,8-diol (M-I)** by CYP2A6 mediated hydroxylation

- ✓ Validation according to FDA guidelines[1]
- ✓ Robust, precise and accurate
- ✓ Range: 5 – 1,000 ng/ml
- ✓ Matrices: Plasma, cell medium
- ✓ Baseline separation between cis- and trans-form of M-I
- ✓ High throughput

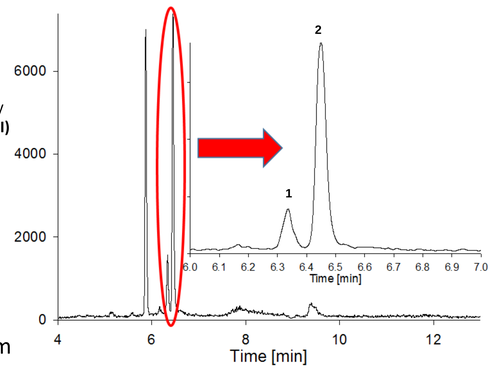

Fig. 3: Chromatogram of a rat plasma sample containing M-I (329 ng/ml). Baseline separation of **cis-M-I** (IS; **1**) and **trans-M-I** (**2**) LC: Kinetex PFP, 2.6µ

3. Quantification of flavors by means of a targeted HS-SPME-GC-MS (Flavoromics)

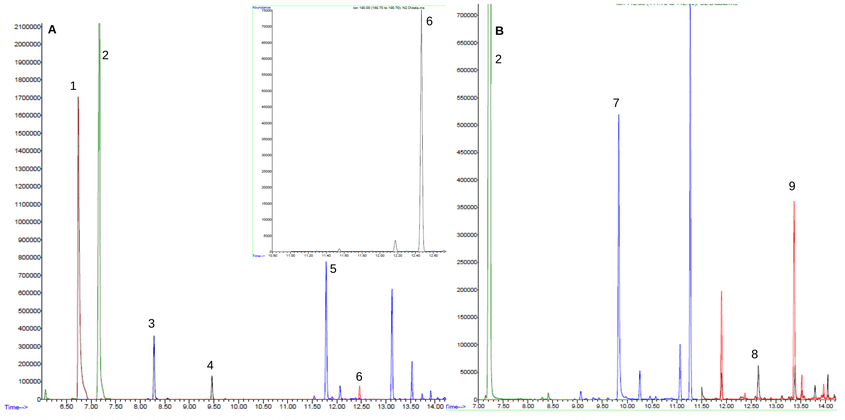

Fig. 4: HS-SPME-GC-MS analysis of 12 flavors chemicals after neutral extraction (A) and acidic extraction (B).

Tab. 1: Concentrations (ng/mg liquid) of 4 flavors detected in two e-liquids (liq. 1 w/o nic; liq. 2 with nic)

E-Liquid	Flavor 2	Flavor 4	Flavor 7	Flavor 8
Liquid 1 -Nic	1152	0.2	2348	<LOD
Liquid 2 +Nic	<LOD	2.8	52.2	89.4

Conclusion

- 3 different analytical methods for quantification of 12 flavors for characterization of e-liquid ingredients and their metabolism
- Excellent results concerning accuracy, precision and sensitivity
- Methods applicable for various body fluids, *in vitro* assays (cell media), as well as e-liquids (flavoromics)
- Multi-analyte method by HS-SPME-GC-MS covers a wide range of flavors: Up to now 12 flavors of differing physicochemical properties included in this targeted approach
- Straightforward sample preparation with high throughput

➡ time- and cost-efficient screening in clinical studies
➡ relevant data for regulatory purposes

Outlook

- Screening of various e-liquids from the German market by means of the flavoromics approach
- Implementation of further relevant flavors into the targeted analysis and expansion of ABF´s Flavoromics library
- Determination of flavors in different body fluids
- Detailed investigation of their metabolic fate

References:
[1] Food and Drug Administration (FDA) (2001): Guidance for Industry - Bioanalytical Method Validation. Vol. 2014, pp. 1-22.
[2] Hutzler, C., *et al.* (2014) Chemical hazards present in liquids and vapors of electronic cigarettes. *Archives of Toxicology*, 88(7), 1295-308.
[3] Merckel, C., *et al.* (2006) Application of headspace solid phase microextraction to qualitative and quantitative analysis of tobacco additives in cigarettes. *J. Chromatogr. A.*, (1116) 10-19.

- HS-SPME-GC-MS analysis for quantification of 12 most abundant flavor agents in e-liquids[2], e.g. vanillin, ethyl maltol, menthol, piperonal, linalool, eugenol, citral
- Extraction procedure developed based on Merckel *et al.*[3]
  - neutral extraction of 9 flavors (Fig. 4A)
  - acidic extraction of 3 further analytes (Fig. 4B)

➢ 4 different flavors quantified in the liquids: 3-methyl-1,2-cyclopentane-dione **2**, menthol **4**, ethyl maltol **7**, and vanillin **8** (Tab. 1)

- ✓ Automated SPME for high-throughput targeted screening of e-liquids
- ✓ Low background and high selectivity due to Headspace injection
- ✓ “Flavoromics” approach will be extended to human body fluids, esp. urine
- ✓ Constant expansion of flavor library