In-depth characterization of chemical differences between heat-not-burn tobacco products and cigarettes using LC-HRAM-MS-based non-targeted differential screening (NTDS)

Daniel Arndt, Christian Wachsmuth, Christoph Buchholz, Mark Bentley

PMI R&D, Philip Morris Products S.A., Quai Jeanrenaud 5, CH-2000 Neuchâtel, Switzerland

Overview

Novel aspect: LC-HRAM-MS-based NTDS applied for the aerosol characterization of differences between a Tobacco Heating System (commercialized under the *IQOS*® brand name) and a reference cigarette (3R4F)[1] by means of a generic compound identification approach and an empirically developed mathematical model.

Results:
Using a reporting threshold of ≥ 100 ng/stick, approximately 2,500 compounds were elevated in cigarette smoke compared with *IQOS* aerosol. In contrast, only 177 compounds were identified in *IQOS* aerosol, 13 of which were significantly more abundant in *IQOS* aerosol generated under HCI smoking regime[2] compared to cigarette smoke. No compounds unique to *IQOS* aerosol were observed.

Introduction and Objectives

Quantitative (targeted) analysis for 54 harmful or potentially harmful constituents (HPHC) is routinely performed to evaluate product emissions. In addition, non-targeted differential screening (NTDS) based on liquid chromatography–high-resolution accurate mass spectrometry (LC-HRAM-MS) is employed as a key methodology for the characterization of differences in chemical composition between two samples. Using an unbiased approach, the NTDS workflow is based on comprehensive chemical characterization without predefined target compounds and identifies differences by considering the relative abundance of all detected constituents as well as a semiquantitative estimate of absolute abundance. Hence, it is able to identify differences beyond those limited to a set of 54 HPHCs.

Goal:
- To cover the broadest possible range of chemical classes amenable to liquid chromatographic separation for the comparison of *IQOS* aerosol and 3R4F cigarette smoke
- To achieve semiautomated confirmation of structural proposals
- To identify major differences between 3R4F cigarette smoke and *IQOS* aerosol

Workflow

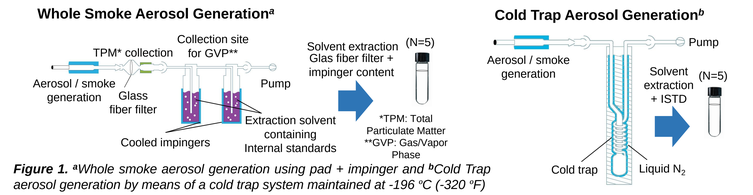
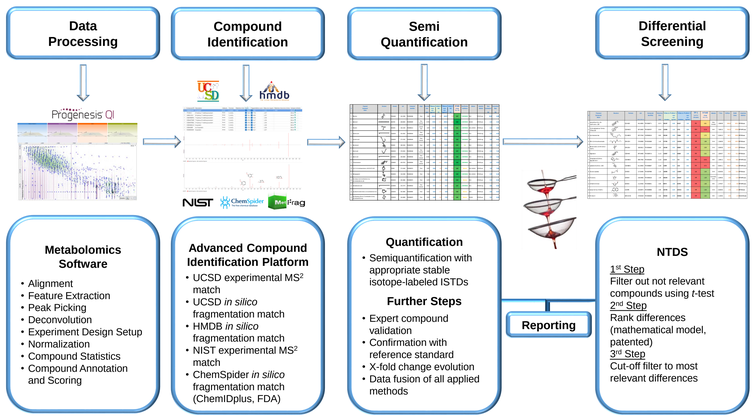


Figure 1. *[a]Whole smoke aerosol generation using pad + impinger and [b]Cold Trap aerosol generation by means of a cold trap system maintained at -196 °C (-320 °F)*



Analytical Methods

- **RP separation:**
  Hypersil GOLD™ column 150 × 2.1 mm i.d., 1.9 µm
  RP-HESI(+) & RP-APCI(+):
  MP A: 10 mM NH_4Ac in water, MP B: 1 mM NH_4Ac in MeOH
  Internal Standard: D8-Isophorone ($C_9H_6D_8O$)
  RP-HESI(-): MP A: 1 mM NH_4F in water, MP B: MeOH
  Internal Standard: D19-Decanoic acid ($C_{10}HD_{19}O_2$)

- **HILIC separation:**
  HILIC-HESI(+):
  Accucore™ HILIC column 150 × 2.1 mm i.d., 2.6 µm
  MP A: 10 mM NH_4Ac in water MP B: 10 mM NH_4Ac in ACN
  Internal Standard: D4-Myosmine ($C_9H_6D_4N_2$)

- **Mass Spectrometry:**
- Q Exactive™ Hybrid Quadrupole Orbitrap MS (Thermo Scientific)
- HRAM full-scan MS at 70.000 (FWHM) over *m/z* 80 – 800
- Data-dependent MS^2 Top3 of each scan at 17.500 (FWHM)
- Stepped normalized collision energies (S-NCE) of 25, 50, and 75 eV; Isolation window 1 Da
- Vaporizer temperature, capillary temperature, spray voltage, sheath gas, and auxiliary gas were set at 350˚C, 380˚C, ±3.00 kV, 60, and 20 arbitrary units, respectively, for HESI modes
- Vaporizer temperature, capillary temperature, discharge current, sheath gas, and auxiliary gas were set at 450˚C, 380˚C, 5.0 µA, 50, and 5 arbitrary units, respectively, for APCI mode
- Column oven at 50 ˚C
- Injection volume of 1.5 µL

Time [min]	A [%]	B [%]	Flow [µL/min]
0	85	15	400
7.00	10	90	400
12.80	0	100	400
18.00	0	100	400
18.10	85	15	400
20.00	85	15	400

Time [min]	A [%]	B [%]	Flow [µL/min]
0	2	98	500
7.00	25	75	500
8.00	2	98	500
15.00	2	98	500

Results

Chromatographic separation and compound identification
Non-targeted screening revealed the presence of 177 compounds (using a semiquantitative threshold of 100 ng/stick) in *IQOS* aerosol across all analytical methods, whereas approximately 2,500 compounds were present in 3R4F-derived cigarette smoke. In addition to the non-targeted methods with complementary separation and ionization modes, a high coverage of chemical space was achieved due to the employed complementary compound ID strategies (querying of multiple databases, comparison of both *in silico*-predicted and reference MS^2 spectra). The majority of identified compounds were present in UCSD[3] (in-house database). The remaining part of the compounds could be identified by means of *in silico* prediction of MS^2 spectra based on HMDB 4.0[4] and Chemspider using data sources of ChemIDplus and FDA databases as well as MS^2 spectral match using NIST MS/MS library.

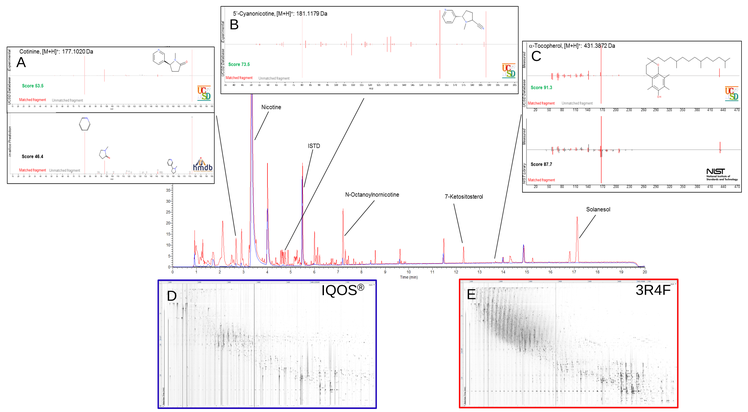


Figure 2. *Overlaid base peak chromatograms of 3R4F-derived smoke (red) and IQOS aerosol (blue) acquired in RP-HESI(+). Mass spectra for (**A**) cotinine, (**B**) 5'cyanonicotine, and (**C**) α-tocopherol are given as examples of different applied compound ID strategies. Heatmaps of IQOS aerosol (**D**) compared with 3R4F smoke (**E**) leading to ~90% less complexity for IQOS aerosol .*

NTDS[5]
In order to identify compounds that exhibit significant different, a two-tailed distributed heteroscedastic Student's *t*-test (2 groups, 5 replicates = 10 observations) was initially performed. Compounds yielded with $p > 0.05$ were discarded from further analysis.
To consider the relevance of each finding, compounds were ranked according to the relative difference in abundance (x-fold change) and the semiquantitatively estimated absolute abundance based on peak area ratios between the analyte and the assigned internal standard with known concentration. The sorting of obviously different compounds (variables) by their relevance was done by applying an empirically developed formula (RANK)[6] on the *t*-test filtered data sets.

The RANK formula mathematically combines two criteria:
1. Abundance of the variable (Average Concentration for a pre-defined group [µg/item])
2. Relative difference of the variable ("Effect" in %)

$$\%Effect = \frac{Ly-Lx}{Ly+Lx} * 100, \quad Index = \frac{\%Effect^3}{1000}, \quad RANK = \frac{Index \times (Lx+Ly)}{2}$$

Equation 1. *Lx is the measured average concentration for sample group x to be compared with sample group y, and Ly is the measured average concentration for sample group y to be compared with sample group x.*

Identified compounds significant elevated in *IQOS* aerosol vs. 3R4F-derived smoke

#	Proposed Compound Name[a]	CAS	Formula	Compound Identifier	Identification Confidence[b]	Identification Score[b]	Fragmentation Score[b]	Δm[c]	Isotope Similarity	IQOS® mean conc.	IQOS® RSD[d]	3R4F mean conc.	3R4F RSD[d]	X-fold change[e]	p-Value	RANK[f] Value
								[ppm]	[%]	[µg/item]	[%]	[µg/item]	[%]	IQOS>3R4F		
1	Lanost-8-en-3-ol, 24-methylene-, (3beta)	6890-88-6	C31H52O	PMI0006771	High	46.8	40.3	-0.13	93.91	6.30	20.8	1.61	9.54	3.92	1.1E-09	825
2	12,14-Labdadiene-7,8-diol, (8a,12E)	na[g]	C20H34O2	PMI0005787	High	54.4	75.0	-0.51	97.57	1.43	15.3	0.064	17.36	22.3	7.4E-13	571
3	Isolinderanolide	139559-06-1	C21H36O3	HMDB38105	High	45.4	31.5	-0.87	96.31	4.99	16.2	1.85	5.45	2.70	0.00	331
4	Ethyl 2,4-dioxohexanoate	13246-52-1	C8H12O4	PMI0010568	Medium	45.3	27.9	-0.21	98.66	6.73	22.8	3.57	4.53	1.89	1.3E-06	150
5	Benzoic acid, 2,5-dihydroxy-methyl	96937-49-4	C9H10O4	PMI0004649	Medium	41.8	10.9	-0.36	98.48	4.55	19.6	2.18	4.61	2.09	5.4E-08	148
6	Ergosterol	57-87-4	C28H44O	PMI0006710	High	50.6	59.6	0.27	93.55	3.18	20.8	1.58	4.80	2.02	1.8E-07	91.2
7	Ethyl linoleate	544-35-4	C20H36O2	PMI0007484	Confirmed	61.4	83.7	-1.49	97.32	0.135	16.2	0.008	41.06	16.9	1.1E-12	50.2
8	Labdane-8,15-diol, (13S)	10267-21-7	C20H38O2	PMI0008387	High	49.1	52.1	-1.39	95.25	0.143	20.8	0.015	23.78	9.75	8.8E-11	42.6
9	2H-Pyran-2-one, tetrahydro-5-hydroxy	33691-73-5	C5H8O3	PMI0003015	Confirmed	54.6	75.6	2.79	98.95	4.45	17.3	3.11	7.58	1.43	6.6E-06	21.4
10	Pyranone	28564-83-2	C6H8O4	PMI0000228	Confirmed	55.8	63.2	-0.48	98.32	6.54	14.4	5.07	6.44	1.29	2.3E-05	12.0
11	5-Methylfurfural	620-02-0	C6H6O2	PMI0000001	Confirmed	55.0	63.6	2.71	99.54	0.995	16.2	0.632	16.49	1.58	1.5E-07	9.07
12	Isoquinoline, 3-methyl	1125-80-0	C10H9N	PMI0003968	Medium	43.5	28.2	-0.26	89.41	6.29	13.6	4.99	8.30	1.26	3.4E-05	8.73
13	Pyridoxin	65-23-6	C8H11NO3	PMI0002009	Medium	44.7	25.7	-0.51	98.22	0.699	14.9	0.526	6.32	1.33	1.1E-05	1.73

Table 1. *[a]Compounds are sorted in descending order of RANK[f] values; [b]Confidence levels: dark green, confirmed - retention time, MS^2 mass spectra within specified tolerance ranges in comparison to an injected reference standard; light green, high - score > 50 or score > 45 and fragmentation score > 45; orange, medium - score < 45 or score between 45-50 and frag. score < 45; [c]Δm, difference between experimental and theoretical mass; [d]RSD, relative standard deviation (N = 15 total observations from three sample replicates that were injected fivefold); [e]X-fold change, compound evolution of IQOS > 3R4F; [f]RANK value, outcome of the applied difference evaluation, as higher the value as more relevant the difference; [g]na, not available. Data as reported to FDA on December 8, 2017, as part of the Modified Risk Tobacco Product Application.*

In total, only 13 compounds were evaluated as being elevated in *IQOS* aerosol compared with cigarette smoke. No compounds unique to *IQOS* aerosol were present. In contrast, approximately 2,500 compounds were elevated in cigarette smoke compared with *IQOS* aerosol. An investigation of the possible source of the constituents indicated that the majority of constituents identified as significantly higher in *IQOS* aerosol derived from differences in tobacco variety and plant secondary metabolites.

Conclusions

- The application of a generic compound identification approach enabled the identification of unexpected compounds, demonstrating the versatility of our NTDS workflow for the analysis of different matrices.
- In total, only 13 constituents were identified significantly higher in *IQOS* aerosol compared with 3R4F derived, whereas approximately 2,500 compounds were elevated in 3R4F smoke.
- In-depth characterization of chemical differences between a heat-not-burn tobacco product and cigarettes using LC-HRAM-MS-based NTDS could be demonstrated.

References

[1] Roemer, E, et al., *Contributions to Tobacco Research,* **2012**, 25, 316
[2] Official Method T-115, Determination of "Tar", Nicotine and Carbon Monoxide in Mainstream Tobacco Smoke, *Department of Health,* Canada, **1999**
[3] Martin, E.; Monge, A.; Duret, J. A.; et al., *J Cheminform,* **2012**, 4, 11.
[4] Wishart, D. S.; Feunang, Y. D.; Marcu, A.; et al., *Nucleic Acids Res,* **2018**, 46, D608.
[5] Arndt, D; Knorr, A; Bentley, M., *Researchgate*, **2016**, DOI10.13140/RG.2.1.2352.0889
[6] Knorr, A., International Patent WO 2013098169 A1, PCT/EP2012/076244, ***2013***.Jul 4.

Competing Financial Interest
Philip Morris International is the sole source of funding and sponsor of this project

PMI SCIENCE
PHILIP MORRIS INTERNATIONAL