Structural Grouping and Preclinical Characterization of Flavor Mixtures Used in E-vapor Products

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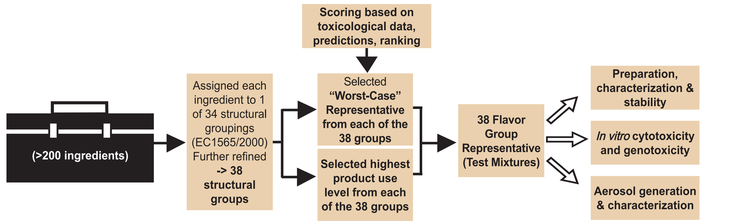
ABSTRACT

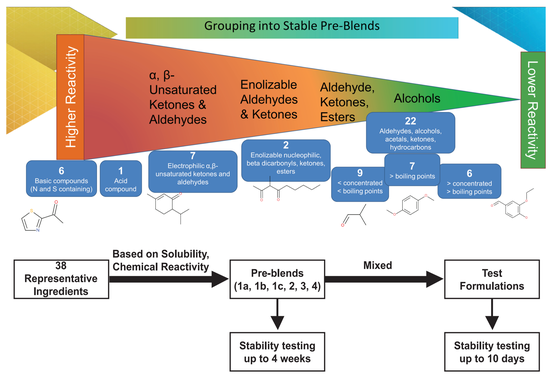
Many flavor compounds used in e-liquids are generally recognized as safe (GRAS) for oral consumption, however, the respiratory effects of most flavors are unknown. Preclinical inhalation studies can provide toxicity hazard data to assess the inhalation risk of flavors in e-vapor aerosols. Considering the number of available flavors and the numerous potential flavor combinations, toxicity testing of each individual compound or formulation may not be always feasible. Therefore, we used a structural grouping approach to select representative compounds and formulate e-liquid flavor mixtures that may reflect over 200 flavors commonly used in e-liquid formulations. Flavors were first grouped into 38 structurally distinct groups and representatives from each group were selected based on toxicological endpoints. The selected flavors were prepared into a total of 6 concentrates (pre-blends) based on their physicochemical properties. Pre-blends were then mixed into the final e-liquid test formulations (total flavor loads up to 18% w/w) and tested for stability. The pre-blends and test formulation (e-liquid) were screened for biological activity using *in vitro* testing: genotoxicity (Ames and micronucleus [MN]) and cytotoxicity (Neutral Red Uptake [NRU]). The test formulations were negative in genotoxicity (Ames and MN) assays but were cytotoxic in all three assays. Cytotoxicity assessment of pre-blends indicated that certain flavors may contribute more to cytotoxicity of test formulations than other flavors. Additionally, to confirm flavor transfer, aerosols from test formulations were generated using a capillary aerosol generator and all monitored flavors were found in the aerosol. PG, glycerin, and nicotine content, as well as pH of the aerosol, were comparable with those of the e-liquid, and particle size was within respirable range (MMAD~1 µm, GSD< 2). Altogether, this structural grouping approach can be used for selection and characterization of representative flavor mixtures that could support product development with respect to selection of flavor ingredients.

METHODS

Structural Grouping & Flavor Representative Selections for Preclinical Testing: Representative flavors were selected based on the approach in EC regulation no. 1565/2000. Briefly, a toxicological review of 246 flavors was conducted based on available data (e.g., acute and repeated dose toxicity, *in vitro* and *in vivo* genotoxicity, developmental/reproductive toxicity, irritation/sensitization, and carcinogenicity). In case of data gaps, *in silico* predictions such as Cramer classification and TOPKAT (predictive software) were used. Both experimental and predicted data were used to select 38 flavors (flavor group representative), which were mixed to create the test formulation.

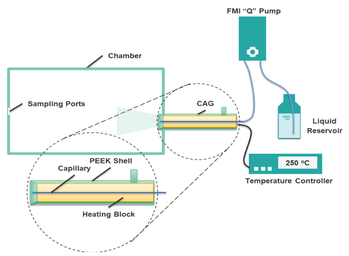


Test Formulation Preparation & Stability Characterization: The 38 flavors were sub-divided (based on solubility and chemical reactivity) to make a total of 6 pre-blends. These pre-blends were mixed to make the test formulations (38 flavor mixtures [up to 18%], with & without nicotine 2%, and carriers [PG/VG/water]). Stability of pre-blends (for up to 1 month) and test formulations (for up to 10 days) were tested using GC/MS under room temperature and refrigerated conditions.



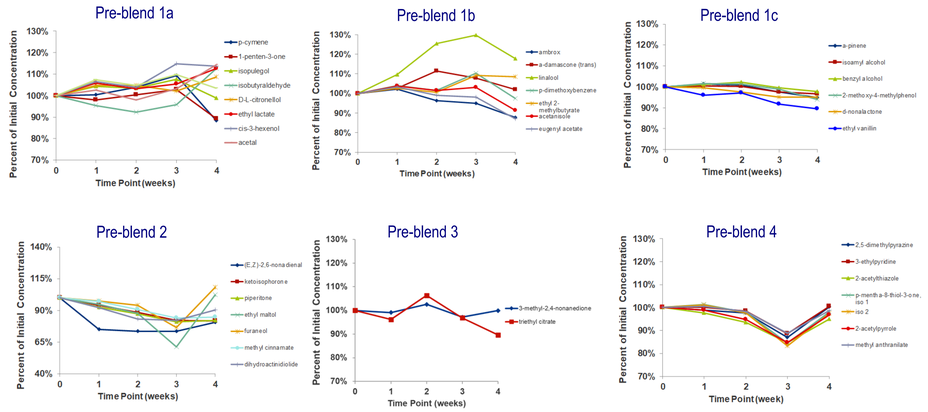
***In Vitro* Cytotoxicity and Genotoxicity:** Pre-blends and test formulations were subjected to the standard CORESTA battery of *in vitro* cytotoxicity (Neutral Red Uptake [NRU]) and genotoxicity (Ames and micronucleus [MN]) assays.

Aerosol Characterization: Test formulation aerosol characterization-Test formulations were aerosolized by a capillary aerosol generator (CAG) at ~250C. Aerosols were collected with a Cambridge filter pad followed by a liquid impinger containing ethanol for flavor analysis. The aerosol mass was determined gravimetrically. Flavors were analyzed with a GC/MS method, as well as the major carrier ingredient (PG and VG), nicotine, and selected carbonyls. Aerosol pH and the particle size were also measured.



RESULTS

Pre-blends (Refrigerated) Stability Characterization (up to 4 weeks)



Test Formulation (Refrigerated) Stability Characterization (up to 11 days)

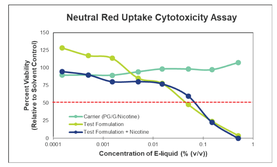
Test Formulation **with** Nicotine

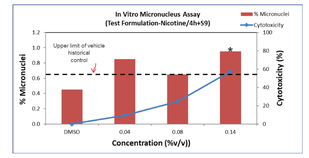
	T0	T1 – 1 day	T2 – 7 days (± 1 day)	T3 – 11 days (± 1 day)
Pre-blend 1A Compounds				
p-cymene	100%	97%	96%	97%
1-penten-3-one	100%	93%	56%	45%
Isopulegol	100%	95%	93%	94%
Isobutyraldehyde	100%	88%	84%	91%
Citronellol, D-L-	100%	96%	90%	91%
Ethyl lactate	100%	96%	90%	94%
Cis-3-hexenol	100%	97%	96%	93%
Acetal	100%	111%	106%	107%
2-methyl-4-phenyl-2-butanol	100%	97%	98%	97%
Pre-blend 1B Compounds				
Ambrox (Cetalox©)	100%	98%	95%	94%
a-damascone (trans)	100%	96%	90%	89%
Linalool	100%	90%	83%	81%
p-dimethoxybenzene	100%	96%	96%	94%
Ethyl 2-methylbutyrate	100%	107%	106%	114%
Acetanisole	100%	94%	92%	89%
Eugenyl acetate	100%	98%	97%	95%
Pre-blend 1C Compounds				
a-pinene	100%	103%	109%	105%
Isoamyl alcohol	100%	101%	104%	104%
Benzyl alcohol	100%	101%	104%	105%
2-methoxy-4-methylphenol	100%	101%	107%	106%
d-nonalactone	100%	99%	99%	99%
Ethyl vanillin	100%	101%	106%	107%

	T0	T1 – 1 day	T2 – 7 days (± 1 day)	T3 – 11 days (± 1 day)
Pre-blend 2 Compounds				
(E,Z)-2,6-nonadienal	100%	94%	89%	79%
Ketoisophorone	100%	100%	104%	104%
Piperitone	100%	100%	106%	106%
Ethyl maltol	100%	100%	111%	106%
Furaneol	100%	96%	93%	86%
Methyl cinnamate	100%	101%	107%	106%
Dihydroactinidiolide	100%	101%	106%	106%
Pre-blend 3 Compounds				
3-methyl-2,4-nonanedione	100%	102%	105%	104%
Triethyl citrate	100%	103%	109%	110%
Pre-blend 4 Compounds				
2,5-dimethylpyrazine	100%	101%	106%	105%
3-ethylpyridine	100%	101%	106%	105%
2-acetylthiazole	100%	101%	108%	105%
p-mentha-8-thiol-3-one	100%	88%	73%	70%
2-acetylpyrrole	100%	102%	106%	106%
Methyl anthranilate	100%	98%	96%	92%
Additional Compounds				
2-methylbutyric acid	100%	99%	107%	100%
Nicotine	100%	97%	115%	102%

Pre-blends & Test Formulations-*In Vitro* Assays

Groups		OECD Assays: Mutagenicity (Ames Assay)	Genotoxicity (Micronucleus)	Cytotoxicity (NRU Assay)
Carrier	Carrier	Negative	Negative	Negative
Test Formulations	High Flavor Only (No Nicotine)	Negative	Equivocal	Cytotoxic
	High Flavor + Nicotine	Negative	Negative	Cytotoxic





Groups		OECD Assays: Mutagenicity (Ames Assay)	Genotoxicity (Micronucleus)	Cytotoxicity (NRU Assay)
Pre-Blends	Pre-blend-1a	Negative	Negative	Cytotoxic
	Pre-blend 1b	Negative	Positive	Cytotoxic
	Pre-blend 1c	Negative	Negative	Cytotoxic
	Pre-blend 2	Negative	Negative	Cytotoxic
	Pre-blend 3	Negative	Negative	Negative
	Pre-blend 4	Negative	Negative	Cytotoxic

Test formulations (with or without nicotine) are cytotoxic per NRU assay. Few individual flavors in pre-blends may contribute to cytotoxicity potential (1a: isopulegol; 2: furaneol, ethyl maltol).

Aerosol Generation and Characterization

(A) Aerosol size distribution measured using a cascade impactor.

	Test Formulation w/ Nicotine (n = 4)	Test Formulation w/o Nicotine (n = 4)
MMAD (µm)	0.97 ± 0.07	1.23 ± 0.06
GSD	1.77 ± 0.18	1.82 ± 0.13

(B) Analytical characterization of liquid and aerosol generated from test formulations

	Test Formulation w/ Nicotine (N = 3)			Test Formulation w/o Nicotine (N = 3)		
Analyte	**Liquid**	**Aerosol**	**Transfer[b]**	**Liquid**	**Aerosol**	**Transfer[b]**
Aerosol Mass (mg)	NA	98.1±2.0	NA	NA	108.2±1.8	NA
Ethanol (mg/g)	20.44±0.13	BLOQ	NA	20.19±0.23	BLOQ	NA
Glycerin (mg/g)	144.3±0.3	146.2±2.1[a]	101%	146.1±0.5	147.1±3.1[a]	101%
Nicotine (mg/g)	20.21±0.17	20.61±0.25[a]	102%	ND	ND	NA
PG (mg/g)	580.6±2.14	611.2±14.2[a]	105%	625.3±0.99	656.3±26.5[a]	105%
Water (mg/g)	63.11±0.89	79.90±2.37[a]	127%[c]	55.81±0.71	73.81±0.71[a]	132%[c]

[a] The values were normalized by the collected aerosol mass.
[b] The transfer was calculated as Transfer (%) = $\frac{Concentration\ in\ Aerosol\ (\frac{mg}{g})}{Concentration\ in\ E\text{-}liquid\ (\frac{mg}{g})} \times 100\%$.
[c] Water exceeded 100% by a wide margin due to the hygroscopicity of PG and Glycerin.
NA = not applied; ND = not detected; BLOQ = below the limit of quantification.

(C) pH of test formulations & generated aerosols

	Test Formulation w/o Nic	Test Formulation w/ Nic
Liquid pH (n = 3)	4.6	7.7
Aerosol pH (n = 3)	4.7	7.6

pH adjustment in the formulation?

	Test Formulation w/o Nic (pH adj. w/ NaOH)
Liquid pH (n = 1)	7.1
Aerosol pH (n=3)	4.6

Always characterize the test atmosphere for confirmation.

SUMMARY/CONCLUSIONS

- Structural grouping approach allows a representative e-formulation mixture that covers >200 flavors for preclinical characterization and toxicity testing. This framework for pre-clinical characterization of flavor mixtures can be used for selection and characterization of flavors in e-vapor products.
- Pre-blends were stable for up to 4 weeks and the final test formulations were stable for 3 days (with nicotine) and 10 days (without nicotine) under refrigerated conditions. The use of pre-blends substantially simplify the repeated preparation and characterization necessary for long-term testing.
- Test mixtures (with and without nicotine) were cytotoxic in NRU assay, negative in Ames mutagenicity assay. In the *in vitro* MN genotoxicity assay, the test formulation with nicotine was negative; the test formulation without nicotine provided equivocal results. Similar to the cytotoxicity of the test mixture, most pre-blends except pre-blend 3 were cytotoxic in the NRU assay.
- PG, glycerin, and nicotine content, as well as the pH of the aerosol, were comparable with those of the test formulations. Test formulation without nicotine has pH of ~4: when an attempt was made to adjust the formulation pH (using NaOH), the pH adjustment did not transfer to aerosol pH.
- Flavor transfer from the formulation to aerosols was confirmed. The particle size for both test formulations were in the respirable range (MMAD<1.6 µm, GSD<2) for rodents.

STRENGTHS AND LIMITATIONS

Strengths:

- Structural grouping approach allows generation of "toolbox of flavors" with the indication of inhalation safety levels, that can be used in the development and biological assessment of e-vapor flavor mixtures. By supporting individual flavor usage levels, this potentially reduces the need for individual flavor animal testing.
- Use of pre-blends as part of test formulation preparation reduces and simplifies the preparation and characterization time, especially in support of long-term high-volume (*in vivo*) inhalation studies.

Limitations:

- This approach is based on assumption, based on available information, that the flavor group representative (FGR) is the most toxic in the group and all flavors in the same group can be used at the cleared FGR concentration. Based on lack of inhalation data, some prediction was based on *in silico* data, which needs to be verified experimentally.
- We did not include complex flavors (naturals, extracts) that are commonly used in some e-vapor products.
- Combinatorial responses among flavors such as synergism, potentiation, or antagonism may affect overall toxicological outcomes.