Non-targeted Analysis (NTA) of Flavors in Aged e-Liquid Formulations: Case Study

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Abstract

e-Liquid formulations are typically composed of carriers (propylene glycol [PG], vegetable glycerin [VG]), nicotine, and varying levels of flavor mixtures. Prior to pre-clinical toxicological assessment of e-liquid and e-vapor inhalation studies, test material characterization and stability evaluation are critical to confirm the composition is consistent throughout the testing period. As part of pre-work for a long-term inhalation study, we first investigated prototype e-liquid formulations containing flavor mixtures (approx. 18% w/w) for daily room temperature exposure use and short term refrigerated shelf life. The formulations were sealed in amber glass vials with minimal headspace and analyzed after storing under refrigerated and ambient conditions. The formulation with nicotine showed minor changes after 3 days, while the formulation without nicotine remained unchanged throughout the 10 day assessment. Therefore, use of the formulations for subsequent inhalation studies would be limited to these maximum lengths of time. Secondary, we analytically evaluated the aged formulations using non-targeted analysis (NTA) to evaluate potential byproducts after an exaggerated long-term storage up to 2 years. In general, regardless of the presence of nicotine, the majority of flavor compounds in e-liquids remained unchanged at ambient storage conditions for 2 years. For the flavors that did not meet the study criteria (>80% target concentration), byproducts identified from NTA included, for example, low molecular weight flavor compounds (e.g., 1-penten-3-one), sulfur containing compounds (e.g., p-mentha-8-thiol-3-one) and compounds containing ester functionality (e.g., eugenyl acetate). In summary, test materials such as e-liquid formulations may change over time and intended ingredients, as well as unintended byproducts, should be characterized based on study design. This is critical for study planning and accurate interpretation of subsequent "dose-response" assessments of biological testing.

Methods

- Test e-Liquid Formulation Compositions

  Table 1 shows the key compositions of the test e-liquid formulations, with and without nicotine. Note, the test formulations contain approximately 18% flavor (non-menthol, w/w) which is regarded as substantially higher than the flavor loads in typical commercial e-liquid formulations.

- e-Liquid Flavor Assessment Study Design

  Formulations were prepared fresh and sealed in amber glass vials with minimal headspace and for one time use for flavor analysis. They were stored in two temperature conditions: 1) Room temperature at 20°C to 25°C in cabinet without ambient light, or 2) Refrigerated storage at 4°C ± 4°C.

  Study questions:
  - Is the test formulation changing over time at room temperature during a typical all-day daily exposure (8 hours)?
  - Can the test materials (Test, Base, Carrier) shelf life be prolonged if stored in refrigerated conditions? An extended shelf life would limit the number of frequent e-liquid preparations and analytical characterizations.
  - For aged e-liquid formulations, what reactions or byproducts can be characterized using NTA?

- e-Liquid Flavor Characterization

  Solvents Used for Dilution of Formulations: Dichloromethane (DCM) and DCM:Methanol (80:20 v/v)

  Standards: Purchased commercially from Sigma Aldrich, Vigon International, Excellentia International, Berjé, Synerzine and TCI (minimum food grade purity)

  Internal Standards: Tricosane-d48 and Isophorone-d8

  Instrumentation – Gas Chromatography-Mass Spectrometry (GC-MS):

  - Agilent 7890B (6890N) with 5977A (5997) Mass Selective Detector – Column Restek Stabilwax® with Integra-guard column, 30m L x 0.25mm ID x 0.25µm
  - Agilent 7890A with Agilent 7200 Q-ToF-MS – Column J&W DB-624UI column, 30m L x 0.25mm ID x 1.4µm
  - Thermo Scientific Q Exactive GC Orbitrap with Trace 1300 GC – Column Restek Stabilwax® with Integra-guard column, 30m L x 0.25mm ID x 0.25µm

  Short Term Flavor Assessment: Selective ion monitoring (SIM) mode was used for short term flavor assessment due to overlapping peaks, in which one target ion and at least one qualifier ion were monitored for each flavor using positive electron ionization (EI+). Full scan mode was utilized in all instruments for the investigation of new compounds. The target acceptance criteria for was ± 20% of the initial measured concentration.

  Long Term Flavor Assessment: Full scan mode was utilized, but without SIM, and semi-quantitative characterization was conducted. NTA was performed by using similar methods on GC-MSD and GC Orbitrap that allowed peaks to be identified by retention time, in which thermoscientifc software xcalibur generated a molecular formula from the molecular ion identified using chemical ionization (CI) on the GC Orbitrap. The long-term (2 years) flavor assessment data are reported as is, as the percentages of the theoretical (added flavor amount) instead of the initial (T0) measured concentration.

References

- Ehman et al. Preclinical testing of flavors in e-vapor products – Part 1: Selection of representative flavor mixtures for toxicological evaluations using a structural grouping approach. The 73rd Tobacco Science Research Conference (TSRC), Leesburg, VA, September 2019.
- Smith et al. Preclinical testing of flavors in e-vapor products – Part 2: Preparation and Stability Characterization of Representative Flavor Mixtures. The 73rd Tobacco Science Research Conference (TSRC), Leesburg, VA, September 2019.
- Kumar et al. Selection and preclinical characterization of flavor mixtures using structural grouping. The 26th Society for Research on Nicotine and Tobacco (SRNT) Annual Meeting, New Orleans, LA, March 2020.

Results - Short Term Flavor Assessment

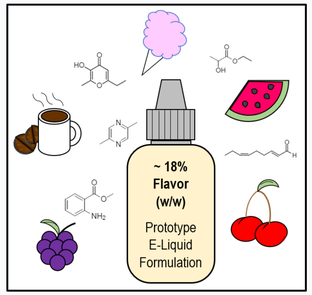


Table 1. Test e-Liquid Formulations (%, w/w)

Major Ingredients	with Nicotine	without Nicotine
Propylene Glycol (PG)	56.8 %	58.4 %
Vegetable Glycerin (VG)	14.2 %	14.6 %
Water	5.9 %	5.9 %
Ethanol	2.5 %	2.5 %
Nicotine	2.0 %	0.0%
Flavor mixtures	18.6 %	18.6%

Table 2. Room Temperature Exposure Assessment (0 to 8 hrs.)

Evaluation of Test Article Flavors during Daily Usage

With Nicotine

Group #	Flavor Compound	T0	4 hrs. (± 1 hr.)	8 hrs. (± 1 hr.)
1	acetal	100%	104%	114%
2	isobutyraldehyde	100%	NA	101%
3	isoamyl alcohol	100%	107%	101%
4	2-methylbutyric acid	100%	NA	NA
5	ethyl 2-methylbutyrate	100%	104%	100%
6	(E,Z)-2,6-nonadienal	100%	100%	98%
7	citronellol, D-L-	100%	101%	101%
8	cis-3-hexenol	100%	103%	110%
9	isopulegol	100%	95%	105%
10	1-penten-3-one	100%	99%	96%
11	linalool	100%	99%	99%
12	a-damascone (trans)	100%	100%	112%
13	piperitone	100%	102%	104%
14	d-nonalactone	100%	100%	97%
15	ethyl lactate	100%	105%	113%
16	triethyl citrate	100%	92%	88%
17	3-methyl-2,4-nonanedione	100%	NA	NA
18	dihydroactinidiolide	100%	94%	96%
19	ethyl maltol	100%	101%	118%
20	furaneol	100%	102%	107%
21	2-methyl-4-phenyl-2-butanol	100%	99%	99%
22	ambrox (Cetalox®)	100%	105%	94%
23	eugenyl acetate	100%	104%	105%
24	p-mentha-8-thiol-3-one	100%	96%	95%
25	acetanisole	100%	105%	112%
26	methyl cinnamate	100%	113%	112%
27	ethyl vanillin	100%	100%	100%
28	benzyl alcohol	100%	100%	108%
29	2,5-dimethylpyrazine	100%	100%	101%
30	2-methoxy-4-methylphenol	100%	98%	100%
31	p-dimethoxybenzene	100%	106%	107%
32	methyl anthranilate	100%	98%	101%
33	3-ethylpyridine	100%	104%	112%
34	2-acetylpyrrole	100%	103%	111%
35	2-acetylthiazole	100%	102%	114%
36	ketoisophorone	100%	99%	105%
37	a-pinene	100%	101%	92%
38	p-cymene	100%	104%	104%

Without Nicotine

Group #	Flavor Compound	T0	4 hrs. (± 1 hr.)	8 hrs. (± 1 hr.)
1	acetal	100%	100%	102%
2	isobutyraldehyde	100%	NA	103%
3	isoamyl alcohol	100%	99%	99%
4	2-methylbutyric acid	100%	NA	NA
5	ethyl 2-methylbutyrate	100%	99%	103%
6	(E,Z)-2,6-nonadienal	100%	91%	89%
7	citronellol, D-L-	100%	102%	97%
8	cis-3-hexenol	100%	97%	101%
9	isopulegol	100%	102%	102%
10	1-penten-3-one	100%	103%	94%
11	linalool	100%	100%	104%
12	a-damascone (trans)	100%	101%	103%
13	piperitone	100%	98%	102%
14	d-nonalactone	100%	102%	105%
15	ethyl lactate	100%	104%	110%
16	triethyl citrate	100%	99%	99%
17	3-methyl-2,4-nonanedione	100%	NA	NA
18	dihydroactinidiolide	100%	94%	95%
19	ethyl maltol	100%	101%	110%
20	furaneol	100%	106%	102%
21	2-methyl-4-phenyl-2-butanol	100%	104%	105%
22	ambrox (Cetalox®)	100%	104%	103%
23	eugenyl acetate	100%	102%	102%
24	p-mentha-8-thiol-3-one	100%	107%	99%
25	acetanisole	100%	101%	104%
26	methyl cinnamate	100%	103%	103%
27	ethyl vanillin	100%	101%	110%
28	benzyl alcohol	100%	98%	107%
29	2,5-dimethylpyrazine	100%	102%	102%
30	2-methoxy-4-methylphenol	100%	103%	104%
31	p-dimethoxybenzene	100%	103%	104%
32	methyl anthranilate	100%	95%	110%
33	3-ethylpyridine	100%	101%	108%
34	2-acetylpyrrole	100%	105%	109%
35	2-acetylthiazole	100%	104%	112%
36	ketoisophorone	100%	99%	93%
37	a-pinene	100%	99%	96%
38	p-cymene	100%	98%	96%

Outcome Summary

All flavors in test e-Liquid formulations, with and without nicotine, remained unchanged up to 8 hrs. at room temperature.

Table 3. Refrigerated Shelf Life Assessment (Up to 12 Days)

Establish "Use By" Date for Test Article Flavors for Repeated Exposures

With Nicotine

Group #	Flavor Compound	T0	T1 - 1 day	T2 - 7 days (± 1 day)	T3 - 11 days (± 1 day)
1	acetal	100%	111%	106%	107%
2	isobutyraldehyde	100%	88%	94%	91%
3	isoamyl alcohol	100%	101%	104%	104%
4	2-methylbutyric acid	100%	99%	107%	105%
5	ethyl 2-methylbutyrate	100%	107%	106%	114%
6	(E,Z)-2,6-nonadienal	100%	94%	89%	79%
7	citronellol, D-L-	100%	96%	90%	91%
8	cis-3-hexenol	100%	97%	98%	99%
9	isopulegol	100%	95%	93%	94%
10	1-penten-3-one	100%	93%	58%	45%
11	linalool	100%	96%	82%	81%
12	a-damascone (trans)	100%	97%	97%	97%
13	piperitone	100%	100%	100%	100%
14	d-nonalactone	100%	99%	99%	99%
15	ethyl lactate	100%	98%	97%	94%
16	triethyl citrate	100%	103%	109%	113%
17	3-methyl-2,4-nonanedione	100%	102%	103%	104%
18	dihydroactinidiolide	100%	101%	105%	106%
19	ethyl maltol	100%	100%	111%	109%
20	furaneol	100%	96%	93%	86%
21	2-methyl-4-phenyl-2-butanol	100%	97%	98%	97%
22	ambrox (Cetalox®)	100%	98%	95%	94%
23	eugenyl acetate	100%	99%	97%	95%
24	p-mentha-8-thiol-3-one	100%	88%	73%	70%
25	acetanisole	100%	98%	97%	99%
26	methyl cinnamate	100%	101%	107%	108%
27	ethyl vanillin	100%	101%	106%	107%
28	benzyl alcohol	100%	101%	104%	105%
29	2,5-dimethylpyrazine	100%	101%	103%	105%
30	2-methoxy-4-methylphenol	100%	101%	107%	108%
31	p-dimethoxybenzene	100%	99%	96%	94%
32	methyl anthranilate	100%	98%	96%	92%
33	3-ethylpyridine	100%	101%	106%	108%
34	2-acetylpyrrole	100%	102%	106%	108%
35	2-acetylthiazole	100%	103%	108%	105%
36	ketoisophorone	100%	100%	104%	104%
37	a-pinene	100%	103%	99%	103%
38	p-cymene	100%	97%	96%	97%

Without Nicotine

Group #	Flavor Compound	T0	T1 - 1 day	T2 - 7 days (± 1 day)	T3 - 11 days (± 1 day)
1	acetal	100%	102%	107%	95%
2	isobutyraldehyde	100%	108%	102%	88%
3	isoamyl alcohol	100%	98%	99%	96%
4	2-methylbutyric acid	100%	98%	97%	97%
5	ethyl 2-methylbutyrate	100%	100%	104%	105%
6	(E,Z)-2,6-nonadienal	100%	98%	99%	92%
7	citronellol, D-L-	100%	100%	91%	82%
8	cis-3-hexenol	100%	99%	101%	87%
9	isopulegol	100%	103%	104%	88%
10	1-penten-3-one	100%	99%	92%	80%
11	linalool	100%	93%	90%	86%
12	a-damascone (trans)	100%	100%	97%	97%
13	piperitone	100%	97%	97%	97%
14	d-nonalactone	100%	98%	102%	96%
15	ethyl lactate	100%	95%	98%	92%
16	triethyl citrate	100%	102%	114%	108%
17	3-methyl-2,4-nonanedione	100%	100%	100%	101%
18	dihydroactinidiolide	100%	99%	100%	97%
19	ethyl maltol	100%	102%	110%	104%
20	furaneol	100%	97%	102%	96%
21	2-methyl-4-phenyl-2-butanol	100%	99%	99%	98%
22	ambrox (Cetalox®)	100%	99%	96%	95%
23	eugenyl acetate	100%	97%	97%	94%
24	p-mentha-8-thiol-3-one	100%	99%	92%	92%
25	acetanisole	100%	95%	98%	96%
26	methyl cinnamate	100%	97%	103%	98%
27	ethyl vanillin	100%	96%	105%	100%
28	benzyl alcohol	100%	97%	101%	97%
29	2,5-dimethylpyrazine	100%	97%	97%	97%
30	2-methoxy-4-methylphenol	100%	99%	103%	98%
31	p-dimethoxybenzene	100%	96%	93%	92%
32	methyl anthranilate	100%	97%	92%	92%
33	3-ethylpyridine	100%	99%	99%	98%
34	2-acetylpyrrole	100%	98%	98%	98%
35	2-acetylthiazole	100%	97%	97%	97%
36	ketoisophorone	100%	97%	101%	97%
37	a-pinene	100%	101%	105%	100%
38	p-cymene	100%	102%	104%	94%

Outcome Summary

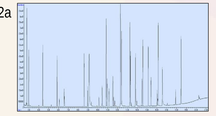
Test e-Liquid formulation with nicotine showed minor changes over the course of 3 days, while the formulation without nicotine remained unchanged for up to 10 days. This suggests that the formulation (with nicotine) should be prepared at least twice/week for long-term studies.

Results - Long Term Flavor Assessment

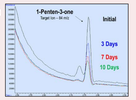
Figure 2. Non-targeted Analysis (NTA) Workflow

1) Investigate GC-MS data for formation of new compounds or potential byproducts with respect to time.

Figure 2a shows the total ion chromatogram for the Test e-liquid formulation with nicotine via GC-MS (EI+) full scan mode.



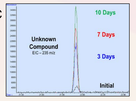
2b



2) A flavor ingredient decreases with time.

Figure 2b shows the extracted ion chromatogram (EIC: 84 m/z) for 1-penten-3-one (compound #10) in the test e-liquid formulation with nicotine.

2c



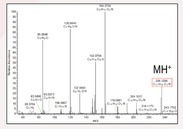
3) A new unknown compound increases with time.

Figure 2c shows the EIC (235 m/z) for the molecular ion of the new peak in the test e-liquid formulation with nicotine.

4) Identification of Molecular Ion & Software Predicted Molecular Formula

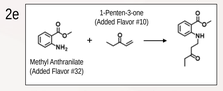
Figure 2d shows the mass spectrum of the unknown compound peak via GC-HRMS with CI mode.

2d



Unknown Compound
M^+ = 235.1208 m/z
Formula = C13H17NO3

2e



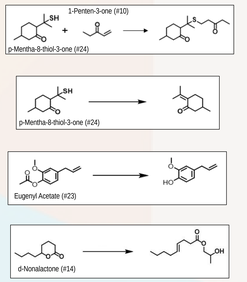
5) Propose Structure Based on Fragment Pattern Obtained from GC-HRMS in EI+ mode

Figure 2e shows the reaction product of two flavor compounds.

Table 4. Exaggerated Long-Term Flavor Assessment at Room Temperature (2 years)

Group #	Flavor Compound	With Nicotine	Without Nicotine
1	acetal	40 - 80%	40 - 80%
2	isobutyraldehyde	40 - 80%	40 - 80%
3	isoamyl alcohol	> 80%	> 80%
4	2-methylbutyric acid	Method Limitation	
5	ethyl 2-methylbutyrate	> 80%	> 80%
6	(E,Z)-2,6-nonadienal	Method Limitation	
7	citronellol, D-L-	> 80%	> 80%
8	cis-3-hexenol	> 80%	> 80%
9	isopulegol	> 80%	> 80%
10	1-penten-3-one	< 40%	< 40%
11	linalool	> 80%	> 80%
12	a-damascone (trans)	> 80%	> 80%
13	piperitone	> 80%	> 80%
14	d-nonalactone	< 40%	< 40%
15	ethyl lactate	40 - 80%	< 40%
16	triethyl citrate	> 80%	< 40%
17	3-methyl-2,4-nonanedione	Method Limitation	
18	dihydroactinidiolide	> 80%	> 80%
19	ethyl maltol	> 80%	> 80%
20	furaneol	Method Limitation	
21	2-methyl-4-phenyl-2-butanol	> 80%	> 80%
22	ambrox (Cetalox®)	> 80%	> 80%
23	eugenyl acetate	40 - 80%	< 40%
24	p-mentha-8-thiol-3-one	< 40%	< 40%
25	acetanisole	> 80%	> 80%
26	methyl cinnamate	> 80%	> 80%
27	ethyl vanillin	> 80%	> 80%
28	benzyl alcohol	> 80%	> 80%
29	2,5-dimethylpyrazine	> 80%	> 80%
30	2-methoxy-4-methylphenol	> 80%	> 80%
31	p-dimethoxybenzene	> 80%	> 80%
32	methyl anthranilate	> 80%	> 80%
33	3-ethylpyridine	> 80%	> 80%
34	2-acetylpyrrole	> 80%	> 80%
35	2-acetylthiazole	40 - 80%	40 - 80%
36	ketoisophorone	40 - 80%	40 - 80%
37	a-pinene	40 - 80%	> 80%
38	p-cymene	40 - 80%	> 80%

Figure 3. Additional Proposed Reactions



Outcome Summary

The long-term flavor assessment testing confirms that some flavors in e-liquids degrade after exaggerated storage (2 years, room temperature).

- With Nicotine – 25 out of 33 measured concentrations were >80% of target.
- Without Nicotine – 23 out of 33 measured concentrations were >80% of target.

In general, the following flavor characteristics did not withstand 2 years of storage:

- Low molecular weight compounds
  isobutyraldehyde, 1-penten-3-one, acetal, ethyl lactate
- Sulfur containing compounds
  2-acetylthiazole, p-Mentha-8-thiol-3-one
- Compounds containing ester functionality
  ethyl lactate, eugenyl acetate, δ-nonalactone, triethyl citrate

Summary

- Test e-liquid formulations (containing 38 unique flavor compounds), with and without nicotine, remained unchanged at room temperature for up to 8 hours, allowing typical daily use for nonclinical studies.
- Consistency of the e-liquid formulation was longer under the refrigerated conditions: the test formulation with nicotine remained unchanged for 3 days and the formulation without nicotine for 10 days. Once confirmed, the longer stability of test materials can reduce the number of formulation preparations and repetitive analytical characterizations.
- The long-term testing confirms that some flavors in e-liquids degrade, or react, after an exaggerated storage condition (2 years, room temperature) causing levels to decline below 80% target. Examples include low molecular weight flavor compounds (e.g., 1-penten-3-one), sulfur containing compounds (e.g., p-mentha-8-thiol-3-one) and compounds containing ester functionality (e.g., eugenyl acetate).
- NTA allowed for identification of flavor reaction products that demonstrated its utility and importance in both short term and long term stability studies.