Methods for Analysis of Sweeteners and Flavor Components in Nicotine Pouches and Saliva Samples

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Abstract

Modern oral nicotine products are becoming increasingly popular alternatives to smoking. However, the palatability is largely dependent on added flavorants which may have high chemical diversity with regard to analytical methods development. Furthermore, in clinical studies the measurement of remnant flavors in used products and in saliva during use are often critical for understanding the bioavailability of product components and the overall expected usage timeframe. In this study, a comprehensive strategy was developed and validated to quantify two non-volatile artificial sweeteners plus fourteen volatile flavor components in unused and used smokeless pouch products as well as in saliva for application in support of clinical trials. Analysis of artificial sweeteners required two independent LC-ESI-MS/MS injections using reversed phase and HILIC separation prepared from a single aqueous extract. The volatile flavor components were analyzed by GC-MS using an ethanol-based extract. Each method developed in this study demonstrates analytical characteristics well suited for use in clinical trials in both matrices including low saliva volumes required, low or sub ppm limits of quantification (0.025 µg/mL for sweeteners and <3 µg/mL for flavorants). The ranges of applicability for the methods are on the order of 100-fold for sweeteners and 400-fold for flavorants (i.e., up to 2.5 µg/mL and 750 µg/mL per saliva sample, respectively). Linearity (R2 ≥0.996 for sweeteners and ≥0.998 for flavorants), accuracy, precision, and specificity all met validation criteria. The development process, methodology, and exemplary validation results will be discussed.

Instruments

- **LC-ESI-MS/MS (Waters-Xevo Acquity TQ MS)**
  - Column (NH2-50, 150x2mm; 5µm) for **Xylitol**
  - Flow rate: 0.25 mL/min; Gradient: 85% A for 3 min then to 75% A at 3.1 min and hold for 5 min
  - Solvents: Acetonitrile (ACN) “A”, Water “B”
  - Column (XDB-C8, 150x3.0mm; 3.5µm) for **Ace-K**
  - Solvents: 0.1% AA in Water “A”, 0.1% AA in Methanol “B”
  - Flow rate: 0.25 mL/min; Gradient: 70% A for 1 min then to 5% A over 5 min and hold for 5 min
- **GC-MS (Agilent-7890B/5977B)**
  - Injector 250 °C
  - Column: DB-WAX (30 m X0.25 mm X 0.5 µm)
  - Column flow 1 mL/Min
  - Oven program: 50 °C for 2 min, 100 °C/min ramp to 140 °C , 2 °C/min to 155 °C, 35 °C min ramp to 260 °C and a hold of 6 min. Total run time 19.4 min

Sample Preparation

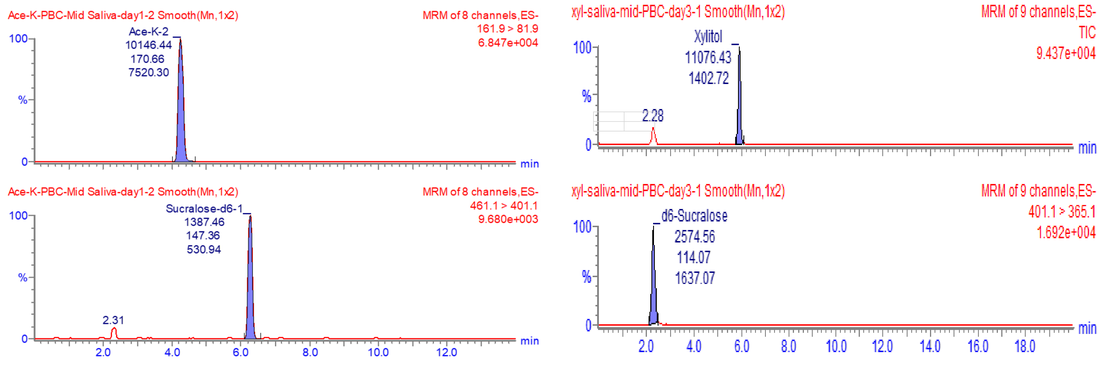
GCMS: Saliva: a 1 :25 dilution into ethanol containing an internal standard added (Tridecane) and mixed well

Tobacco pouches: Pouches are cut and placed in falcon tube with 25 ml of ethanol containing an internal standard. Sample is vortex, sonicated and centrifuged.

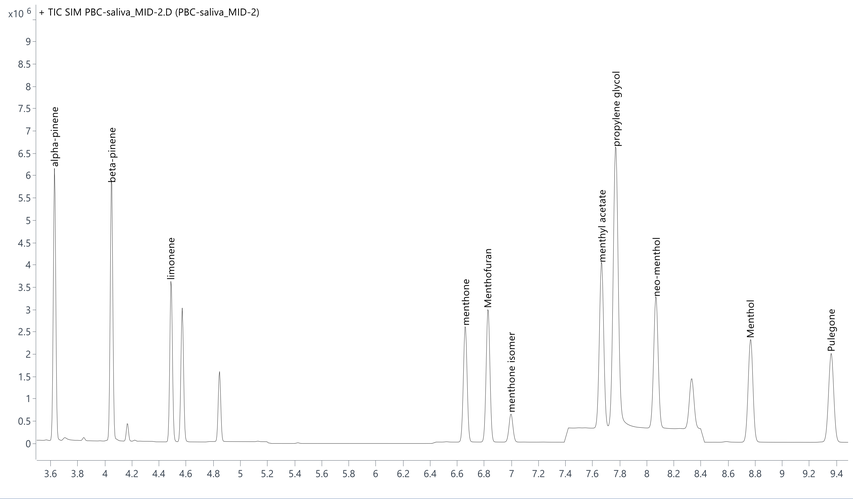
LC-ESI-MS/MS: Saliva: a 1:5 dilution into water containing internal standard (Sucralose-d6)

Tobacco pouches: Pouches are cut and placed in falcon tube with 25 mL of water. The samples are shaken for 1 hour then syringe filtered. If the sample is analyzed for Xylitol, the extract is diluted 1:50 with 80:20 ACN/water containing an internal standard (Sucralose-d6) and mixed well. If the sample is analyzed for Ace-K, the extract is diluted 1:5 with water containing an internal standard (Sucralose-d6) and mixed well.

LC-ESI-MS/MS Chromatograms



GC/MS Chromatogram



Target Ions		
Compounds	RT (min)	Quantifier (m/z)
α-Pinene	~ 3.63	93
β-Pinene	~4.07	93
Limonene	~4.50	68
Eucalyptol	~4.57	93
L-Menthone*	~6.66	139
Methofuran	~6.83	108
Menthone isomer	~7.00	139
Menthyl acetate	~7.67	138
Propylene glycol	~7.80	61
Neo-menthol	~8.08	95
Menthol	~8.79	71
Pulegone	~9.38	81
Tridecane (ISTD)	~4.85	71

Total Ion Chromatogram (TIC) of a mid-level fortified saliva sample acquired in SIM mode

Results

➢ Saliva matrix

	Compounds	% Recovery	LOD (ng/mL)	LOQ (ng/mL)	R^2 value
GCMS	α-Pinene	93.9	563	1875	0.999
	β-Pinene	100.0	563	1875	0.999
	Limonene	98.5	563	1875	0.999
	Eucalyptol	101.7	563	1875	0.999
	L-Menthone*	104.6	458	1525	0.999
	Methofuran	105.4	450	1500	0.999
	Menthone isomer	106.2	105	350	0.999
	Menthyl acetate	107.2	563	1875	0.999
	Propylene glycol	98.4	2813	9375	0.998
	Neo-menthol	106.7	563	1875	0.999
	Menthol	103.3	563	1875	0.999
	Pulegone	105.4	900	3000	0.999
	Compounds	% Recovery	LOD (ng/mL)	LOQ (ng/mL)	R^2 value
LC-QQQ	Xylitol	103	8.00	25.0	0.997
	Ace-K	97.8	8.00	25.0	0.998

➢ Nicotine Pouches

	Compounds	% Recovery	LOD (ng/item)	LOQ (ng/item)	R^2 value
GCMS	α-Pinene	87.2	563	1875	0.999
	β-Pinene	92.7	563	1875	0.999
	Limonene	91.8	563	1875	0.999
	Eucalyptol	92.7	563	1875	0.999
	L-Menthone*	NR	458	1525	0.999
	Methofuran	100.7	450	1500	0.999
	Menthone isomer	NR	105	350	0.999
	Menthyl acetate	95.6	563	1875	0.999
	Propylene glycol	101.0	2813	9375	0.998
	Neo-menthol	100.9	563	1875	0.999
	Menthol	NR	563	1875	0.999
	Pulegone	99.6	900	3000	0.999
	Compounds	% Recovery	LOD (ng/item)	LOQ (ng/item)	R^2 value
LC-QQQ	Xylitol	93.2	1.88	6.25	0.997
	Ace-K	102	0.188	0.625	0.998

NR= Not reported (high background levels)

- The analytical standard menthone is the sum of two isomers. The menthone isomer eluting after L-menthone is semi-quantitated by interpolating its response (Internal Standard method) in the L-menthone calibration curve.
- Recovery values based on the average (n=9) of mid-level fortified samples obtained during three separate days.
- R2 values based on the average of minimum three calibration curves injected on three separate days.

Summary & Conclusions

We have been able to develop and validate both a GC/MS and two LC-ESI-MS/MS methods for twelve flavoring compounds along with two artificial sweeteners. The three methods obtained low limits of quantitation, excellent recoveries from matrixes and were linear over 3 orders of magnitude in saliva and pouches. Therefore, we feel that these methods are well suited for clinical trials.