Noninvasive Nicotine Quantitation and Flavor Differentiation of Nicotine Pouches Using Raman Spectroscopy: A Direct, Accurate, and Preparation-Free Approach

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Introduction

Nicotine Pouch Products

Growing category
in the smoke-free market

Tobacco-leaf-free pouches,
containing either synthetic or tobacco-derived nicotine

They come in **various nicotine levels and flavors**

Quantitative Analysis of Nicotine in Nicotine Pouch Products

Various methods have been developed:
GC-FID | GC-MS | UPLC-MS/MS[1] | UPLC-UV

CORESTA standardized method (CRM #62):
GC-FID

Provide accurate and repeatable measurements

Require laboratory set-up and time consuming

Raman Spectroscopy

- Non-invasive and direct through-pouch measurement
- Rapid analysis (~50 seconds per replicate)
- Nicotine quantitation and flavor differentiation

[1]Aldeek et al. (2023) ACS Omega 8, 31256−31264
CORESTA=Cooperation Centre for Scientific Research Relative to Tobacco; CRM=CORESTA recommended method; GC-FID=Gas chromatography flame ionization detector; GC-MS=Gas chromatography-mass spectrometry; OTDN=Oral tobacco-derived nicotine; UPLC-MS/MS=ultra-performance liquid chromatography-tandem mass spectrometry; UPLC-UV=ultra-performance liquid chromatography ultraviolet detection CRM. No. 62, Determination of Nicotine in Tobacco and Tobacco Products by Gas Chromatographic Analysis, 3rd ed; CORESTA, 2021

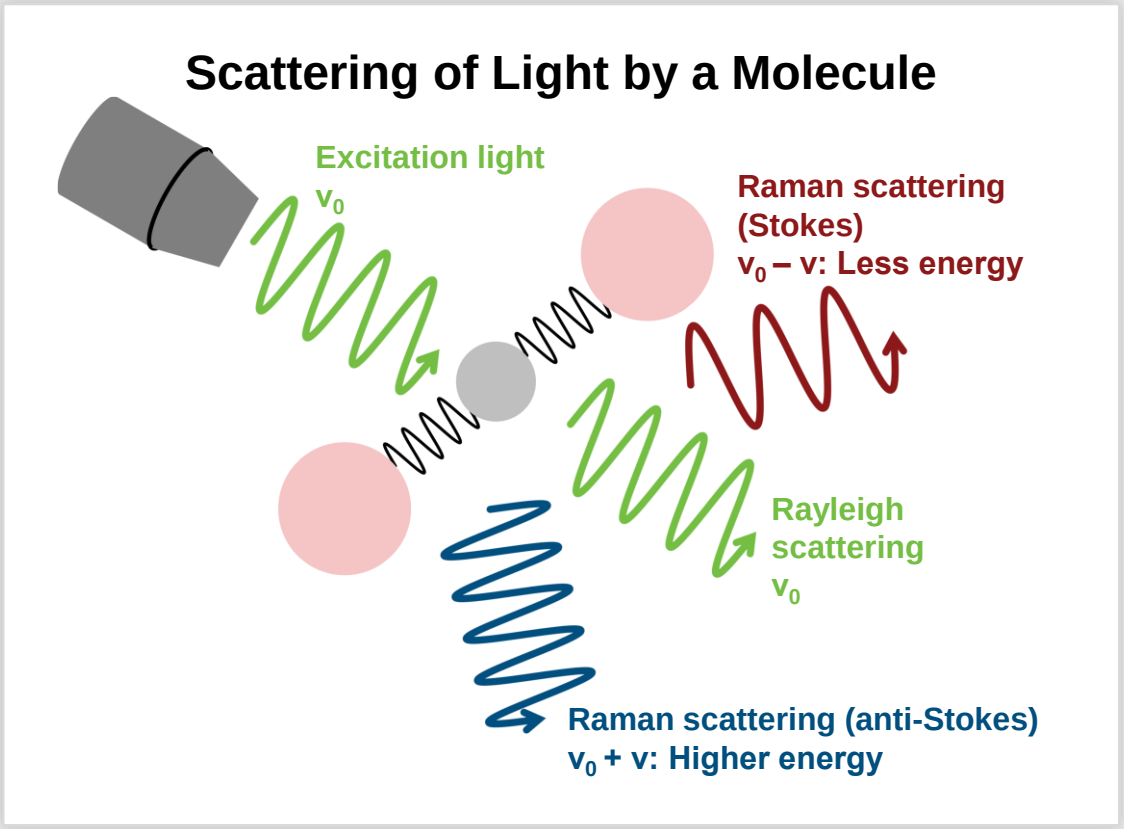
Objective

Simple and Rapid Method for Determination of Nicotine

	GC-FID Method	Raman Spectroscopy
Proximity	Offline analysis at distant laboratories	Online/At-line analysis at the manufacturing site
Solvent Preparation	Acid/Base, Organic standard reagents	None
Nicotine Extraction Time[1]	2 hours	None
Run Time[2]	~12 minutes	50 seconds
Consumables	• Column • Carrier gases • GC autosampler syringes • Inlet septa • Inlet liner • O-ring for inlet liner • GC vials and caps • Gas-tight syringes	None

[1]Nicotine Extraction time for OTDN pouch product (OTDN=Oral tobacco-derived nicotine).
[2]Measurement run time per sample.

Raman Spectroscopy



Energy Difference: Raman Shift

- Information on the vibrational modes of the sample
- Each peak in the Raman spectrum → A particular vibrational mode unique to the molecule (chemical bonds, functional groups)

Flexibility in Instrumental Configuration:

Flexible excitation systems

- Microscope
- Fiber optics
- Wide area illumination

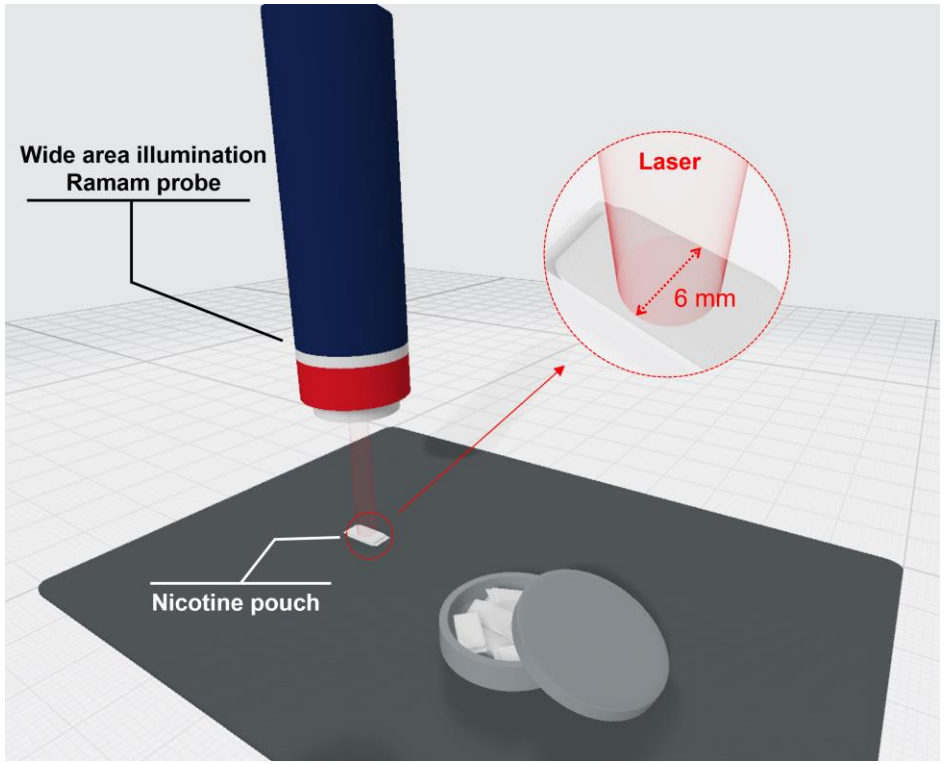
Customization of detector location

- Backscattering
- Spatially offset Raman spectroscopy (SORS)
- Transmission

Beneficial for "through package analysis"

[1]Kim *et al*. (2008) J. Pharm. Biomed. Anal. *48*, 592-597; [2]Kim *et al*. (2007). Anal. Chim. Acta. *587*(2), 200-207; [3]Eliasson *et al*. (2007) *Anal. Chem. 79*, 1696-1701; [4]Bloomfield *et al*. (2013) *J. Pharm. Biomed. Anal. 76*, 65-69

Raman Spectral Acquisition



Wide Area Illumination (WAI) Scheme

- Laser illumination diameter: 6 mm (28.3 mm² area)
- Focal plane distance: 254 mm
- Excitation Laser: 785 nm laser (400 mW)

Raman Spectral Acquisition

- Spectral resolution: 4 cm^{-1}
- Exposure time: 5 seconds
- Scan: 10 repeating scans
- **50 second total acquisition time per pouch**

Data Processing

- Asymmetric Least Squares smoothing (AsLS): Remove baseline variation and fluorescence background
- Normalization: Scaling characteristic variables

Nicotine Pouch Samples

Total: 1800 pouches

5 Flavor variants

- Original
- Mint
- Wintergreen
- Citrus
- Berry

3 Nicotine levels

- 2 mg/pouch
  120 pouches
- 4 mg/pouch
  120 pouches
- 8 mg/pouch
  120 pouches

360 Pouches per flavor

Training Set: 180 pouches

2 mg	2 mg	2 mg
20 pouches	20 pouches	20 pouches
4 mg	4 mg	4 mg
20 pouches	20 pouches	20 pouches
8 mg	8 mg	8 mg
20 pouches	20 pouches	20 pouches

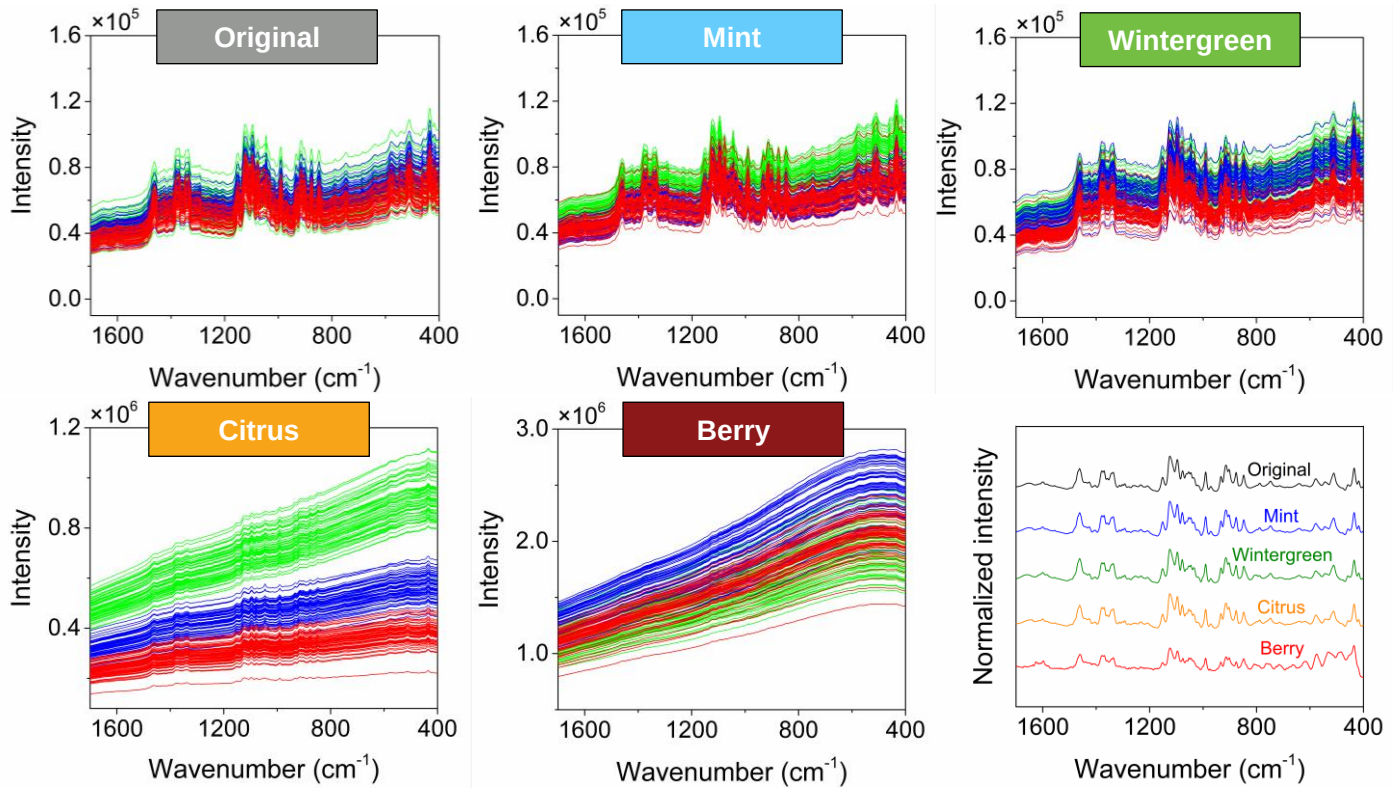
Training set: 900 pouches

Prediction Set: 180 pouches

2 mg	2 mg	2 mg
20 pouches	20 pouches	20 pouches
4 mg	4 mg	4 mg
20 pouches	20 pouches	20 pouches
8 mg	8 mg	8 mg
20 pouches	20 pouches	20 pouches

Prediction set: 900 pouches

Raman Spectral Features of Nicotine Pouches with Five Different Flavors



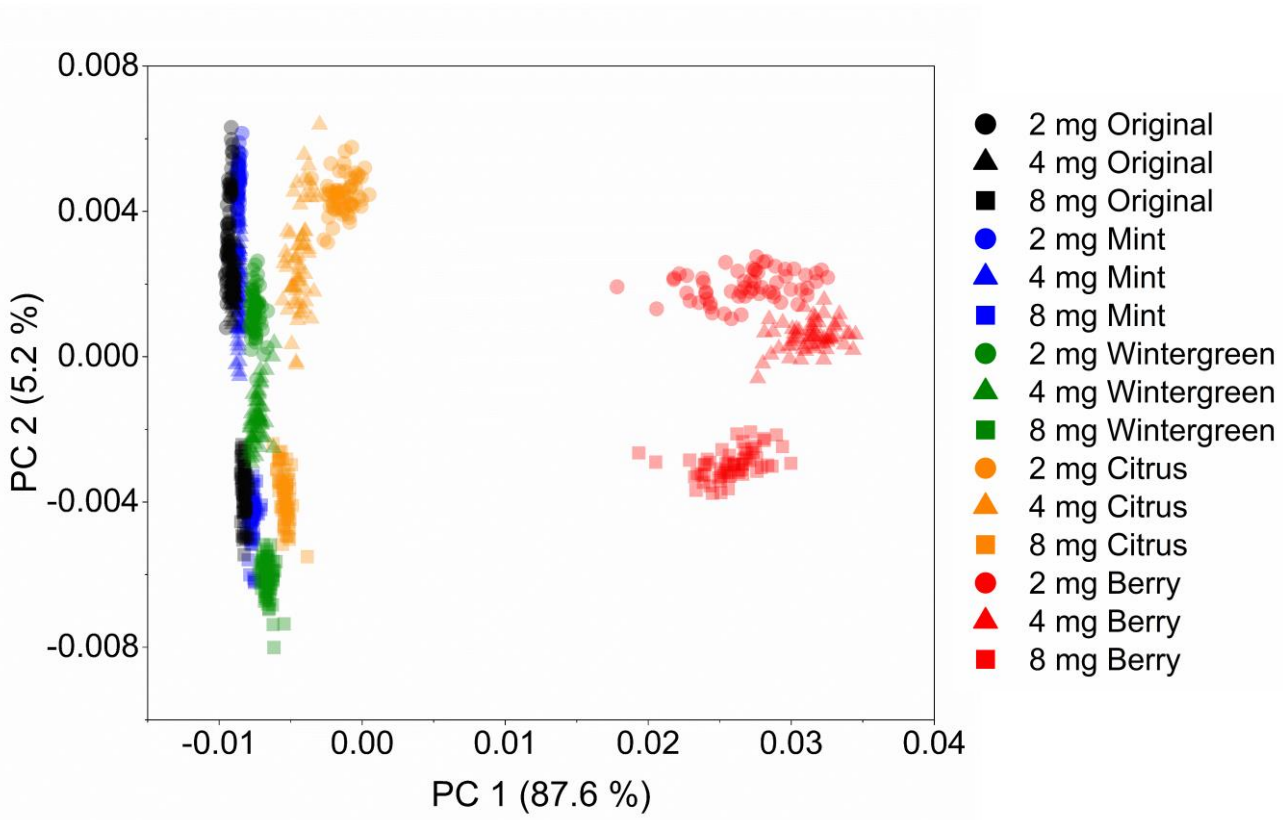
Raw Raman Spectra Collected from the Training Set

- **180 spectra per flavor**
- **2 mg in green, 4 mg in blue, 8 mg in red**
- No additional preparation steps

FLUORESCENCE BACKGROUND
in Citrus and Berry nicotine pouches

Average normalized spectra for each flavor group EFFECTIVELY REDUCE the fluorescence background

Principal Component Analysis (PCA) of The Training Set



Distribution of the 1st and 2nd Principal Component (PC) scores

Two distinct clusters on the PC domain

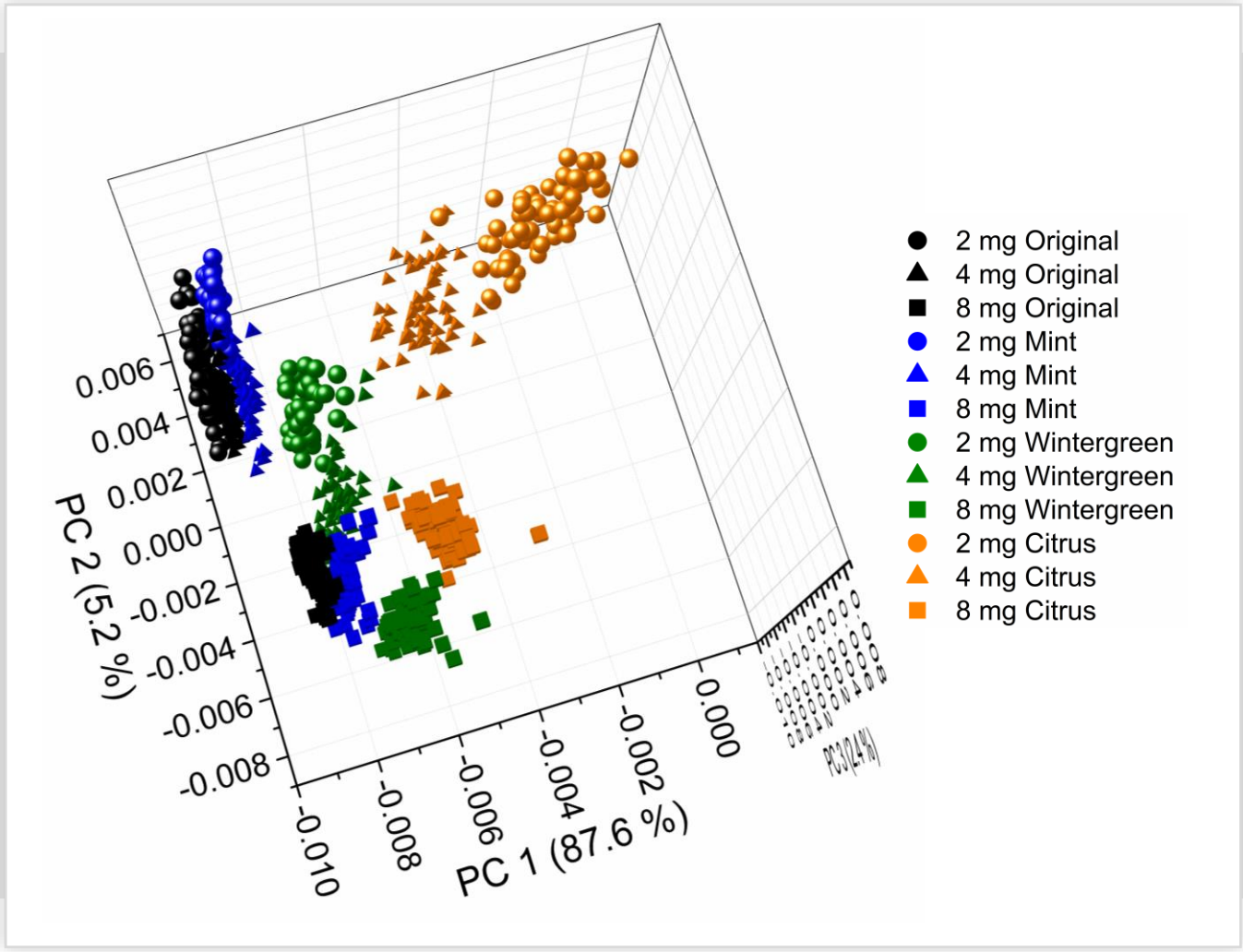
NICOTINE POUCHES

LEFT:
- **Original (black)**
- **Mint (blue)**
- **Wintergreen (green)**
- **Citrus (orange)**

RIGHT:
- **Berry (red)**

- **Different spectral features of Berry nicotine pouches in the 700-400 cm-^{1} range**
- **Sub-groups corresponding to the different nicotine levels are identifiable within the Berry nicotine pouch cluster**

Principal Component Analysis (PCA) of The Training Set



Three-dimensional domain of 1st, 2nd, and 3rd PCs

NICOTINE POUCHES:
Original, Mint, Wintergreen, and Citrus

Within the clusters of Citrus (orange) and Wintergreen (green) nicotine pouches:

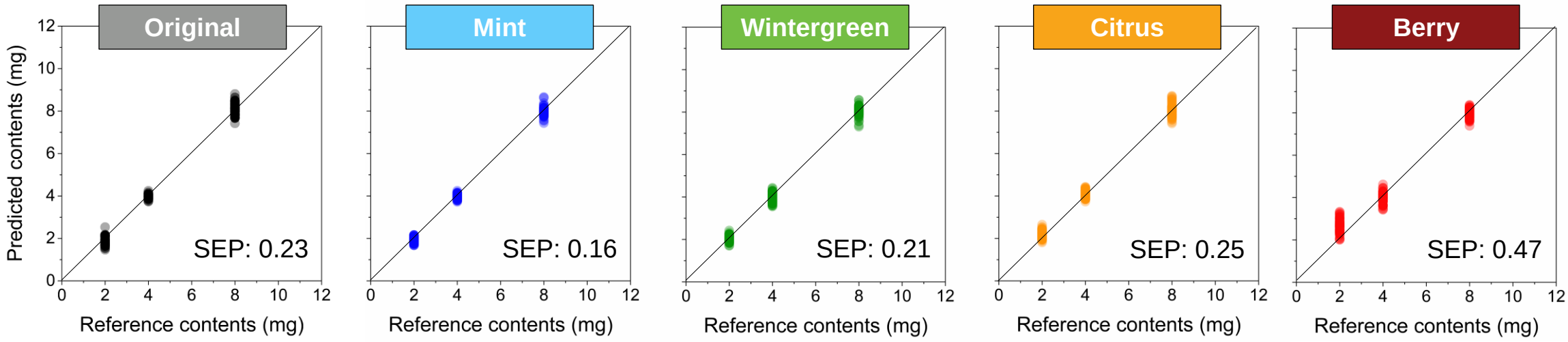
- **Sub-groups corresponding to the nicotine levels of 2 mg (circles), 4 mg (triangles), and 8 mg (squares) can be distinguished**

Within the clusters of the Original and Mint nicotine pouches:

- **8 mg nicotine pouches (squares) formed isolated sub-groups**

The spectral features of the nicotine pouch products are sufficiently descriptive to differentiate their flavors and quantify the nicotine levels

Quantitative Determination of Nicotine Content in Nicotine Pouches



Partial Least Squares (PLS) Regression

- Individual PLS models for each flavor group using the training sets
- The reference values for PLS → The labeled nicotine contents (i.e., 2, 4, and 8 mg per pouch)
- Cross-validated by leave-one-batch-out cross-validation

Prediction of Nicotine Content

- Predict the nicotine content of the corresponding nicotine pouches in the prediction set → Standard errors of prediction (SEPs) were assessed
- Original, Mint, Wintergreen, and Citrus SEPs: 0.16 to 0.25 mg
- Berry SEP: 0.47 mg

Residual Fluorescence Interference in 2 mg Nicotine Pouches

- In the case of 2 mg Berry and Citrus pouches, the estimation of nicotine contents deviates upward from the diagonal line representing 100% prediction accuracy

Quantitative Determination of Nicotine Content in Nicotine Pouches

Flavor	Nicotine Content (mg)	Number of Factors	SECV (mg)	SEP (mg)	Percent (%) Accuracy
Original	2	5	0.21	0.23	93.1
	4				99.4
	8				101.3
Mint	2	6	0.18	0.16	95.7
	4				99.8
	8				99.9
Wintergreen	2	4	0.21	0.21	102.5
	4				99.0
	8				100.5
Citrus	2	5	0.20	0.25	108.8
	4				102.3
	8				100.6
Berry	2	4	0.26	0.47	**133.0**
	4				100.2
	8				99.4

Accuracy is based on the labeled nicotine contents, Factor: Compressed summaries of the spectral features most related to the concentration, SECV: Standard Error of Cross Validation, SEP: Standard Error of Prediction

Flavor Discrimination of Nicotine Pouches

k-Nearest Neighbor (*k*-NN)

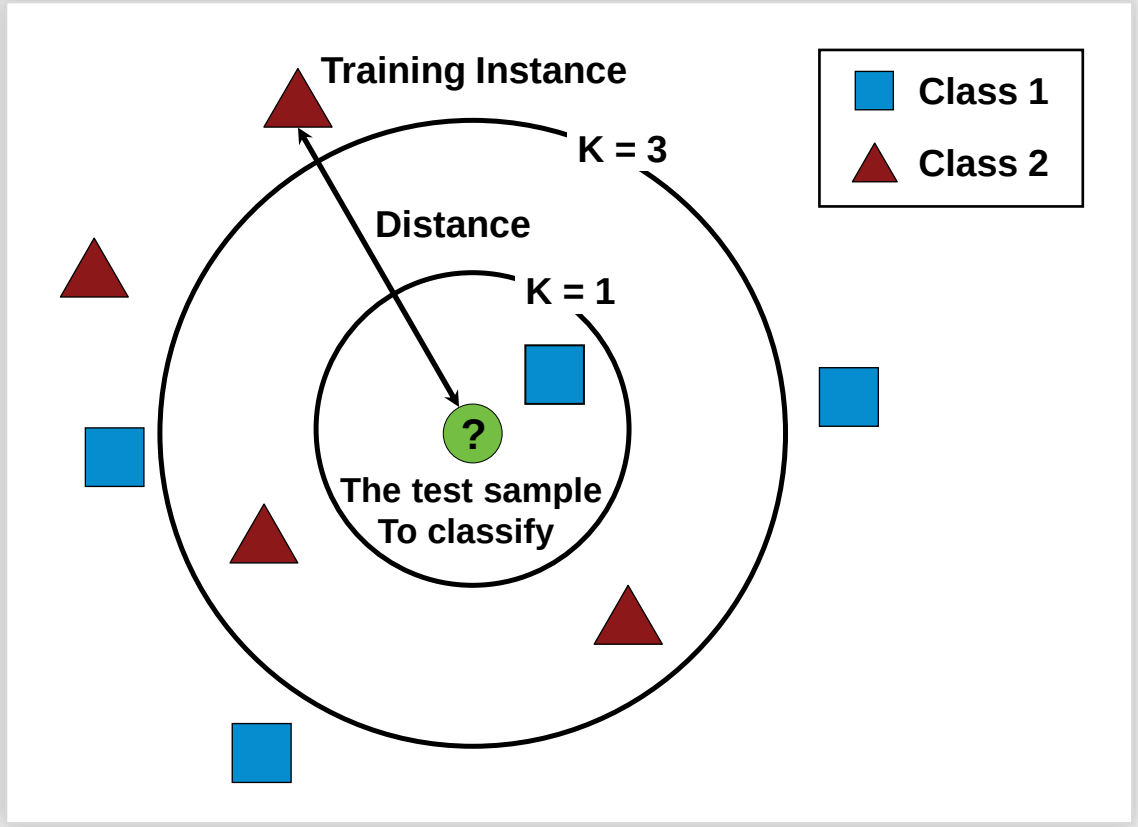
- Simple machine learning method used for classification
- Supervised learning: Training examples with a class label (e.g., flavor)
- Finding the "*k*" closest data points (neighbors) to predict an unlabeled data point
- *k* → Number of data points to include
- **Closeness:** A distance metric that measures similarity between data points

Flavor Discrimination

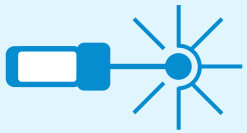
- *k*-NN model: *k* = 3, Euclidean distance metric

The discrimination accuracy was 100%

All flavor groups of the nicotine pouches were correctly identified



Conclusion



Raman Spectroscopy

DEMONSTRATED

a rapid, direct, and non-invasive technique for characterizing nicotine pouch products

Through-pouch measurements using the WAI scheme
effectively captured representative Raman spectra of the pouch products

Accurate nicotine quantification and flavor classification
were accomplished using chemometric tools

Fluorescence background
decreased prediction accuracy of nicotine content of 2 mg Berry product

Ways to address:

Employing longer-wavelength laser excitation	Developing more sophisticated algorithms to suppress fluorescence background	Using time-gated Raman spectroscopy

Rapid, direct, and non-invasive characterization of nicotine pouch products
for routine testing and quick assessment during product development

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