Optimization of Extraction and In Vitro Evaluation of Market Nicotine Gums

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Background and Purpose

The European Pharmacopoeia describes the use of mastication apparatuses to simulate chewing for in vitro dissolution testing of gum products. While these apparatuses can also be used to prepare test material for in vitro toxicity testing, they lack throughput and scalability.
The present feasibility study aimed to optimize preparation of extracts from nicotine gum products in a scalable and reproducible manner for subsequent in vitro toxicological assessment.

Materials and Methods

Test Articles: Three commercial nicotine gum products (MP-1, MP-2 and MP-3, with nicotine strengths ranging from 2 to 4 mg per piece) were used for in this study.

Extract Preparation: Sample preparation of gum products in artificial saliva was done using the optimized method (Table 1).
Briefly, frozen (-80 ℃) commercial gum product was ground (3-cycles alternating hit and cut at 4000 rpm and 7000 rpm, respectively, for 15 seconds each). The ground gum was then added to a suitably sized sealable vessel and 10mL of artificial saliva was added for each unit of ground gum (1 gum/10mL). The gum solution was vortexed then shaken on an orbital shaker at 200 rpm for 3 hours. The resulting extract was centrifuged at 4000 rpm and the supernatant was filtered twice (0.2 µm) for sterility. The final extracts were evaluated for nicotine recovery relative to the product label's nicotine and expressed as %Recovery. Mastication method[1] was used for comparison.
Additionally, assay specific media (artificial saliva[2] for the Ames, DMEM for cytotoxicity and RPMI for genotoxicity) were used for in vitro testing.

Table 1: Evaluated Method

Method	Pieces of Gum	Volume (mL)	Time (hr)
Mastication	**1**	**40**	**1**
Optimized	1	10	3

Combustible Tobacco Product (1R6F Cigarettes) Collection: Cigarette smoke condensate was collected in ethanol, using ISO intense (ISO 20778[3]) puffing regimen[4].

In Vitro Assessment:
Mutagenicity (Ames): OECD 471 Bacterial Reverse Mutation Assay: Ames Preincubation method, strains TA98, TA100, TA102, TA1535 and TA1537 ± S9
Genotoxicity (Micronucleus-MN): OECD 487 Mammalian human TK6 micronucleus assay: Flow-based analysis, 4h±S9 and 22h-S9
Cytotoxicity (Neutral Red Uptake-NRU): ISO-10993-5/OECD 129 Mammalian mouse 3T3 cell viability: NRU, 48h treatment

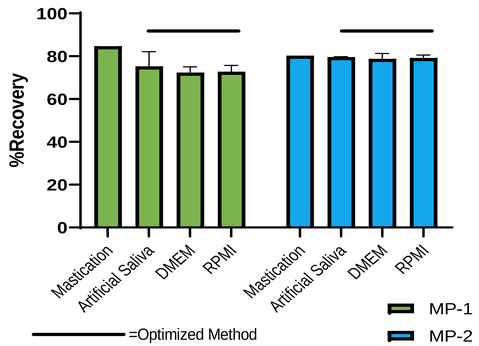
Strengths & Limitations

Strengths:
1) The optimized method is transferable and highly reproducible.
2) Nicotine recovery from the optimal method was comparable to those obtained from mastication.
3) The resulting extract was suitable for in vitro evaluation and contained >10-fold higher nicotine concentrations than cigarette condensate.

Limitations:
1) Method was optimized based on only nicotine extracted, extraction of other components was not evaluated.
2) The dosing concentrations were limited by osmolality of the extracts.

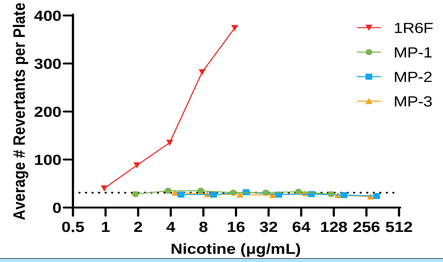
Results

Figure 1. Nicotine Recovery with Optimized Extraction Method and Mastication. Comparison of mastication method to the optimized method in different media. Nicotine recovery from two market products (MP-1 and MP-2) were evaluated using mastication of one piece of gum in 40mL artificial saliva and compared to the optimized method (one piece of gum extracted in 10 mL of either artificial saliva or cell culture media). The optimized method provided the highest %recovery.



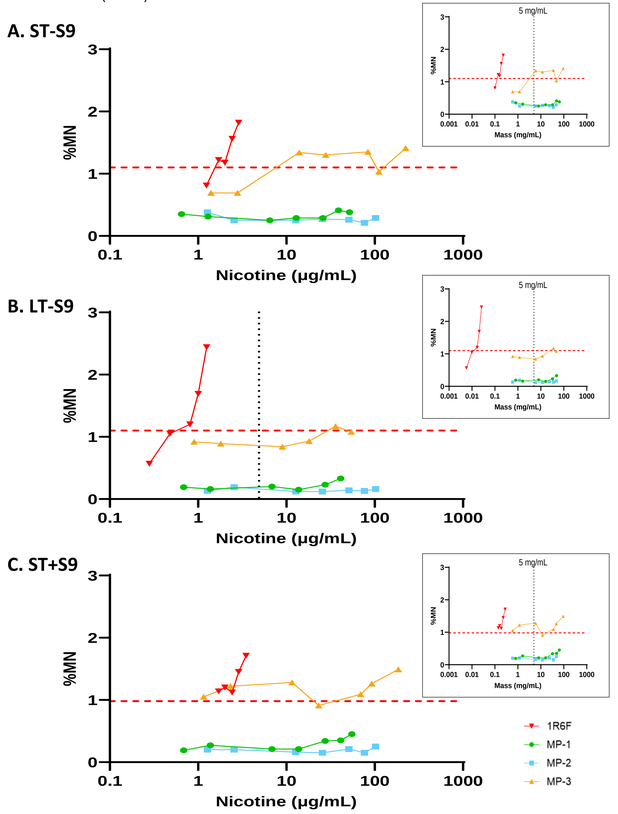
- Mastication data of MP-1 and MP-2 (84.7% and 80.3% respectively; n=1) is comparable to similar products evaluated previously (Aldeek et al).
- When extracted in artificial saliva, comparable nicotine recoveries were observed between mastication (84.7 and 80.3 for MP-1 and MP-2 respectively) and optimized method (75.3±6.80 and 79.6±0.20; n=6 for MP-1 and MP-2 respectively).
- Utilizing the optimized extraction method in 2 different cell culture media (DMEM and RPMI) exhibited similar nicotine recoveries as those conducted in artificial saliva.
- The optimized extraction method was selected for further evaluation due to its comparable nicotine %recovery to mastication, relatively short duration and requirement of minimal specialized equipment.

Figure 2. Ames Assay. Gum extracts prepared in artificial saliva using the optimal method were applied in the Ames assay. Strain TA98+S9 shown.



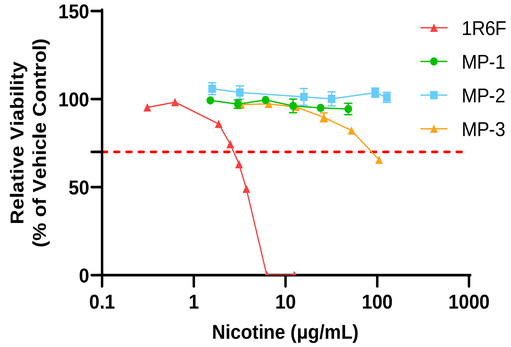
Mutagenicity
- 1R6F is mutagenic in strain TA98 with metabolic activation (and in strain TA1537+S9, not shown).
- All 3 market gum product extracts were non-mutagenic in all strains as evaluated under OECD 471 (TA98, TA100, TA1535, TA1537 and TA102) both with and without metabolic activation), even when tested at 7.8 to 20-fold higher than 1R6F cigarette.

Figure 3. In vitro Micronucleus Assay Normalized to Nicotine and Mass (insert). Gum extracts were prepared directly into RPMI for application up to 80% w/v to the cells. Osmolality limited evaluated doses to 40% w/v or less. Cytotoxicity was evaluated using relative increase in cell counts (RICC). A. 4-hour without metabolic activation (ST-S9) B. 22-hour without metabolic activation (LT-S9) and C. 4-hour with metabolic activation (ST+S9).



Genotoxicity
- 1R6F is genotoxic at <0.5 µg/mL nicotine.
- MP-1 and MP-2 are non-genotoxic.
- MP-3 is positive for genotoxicity.
  - Statistically significant positive responses observed at > 5 mg/mL mass (ST+S9).
- Gum extracts were evaluated at nicotine concentrations 16- to 53-fold higher than 1R6F cigarette.

Figure 4. Neutral Red Uptake Assay in BALB/c 3T3 Cells. Gum extracts were prepared directly into DMEM for application up to 80% w/v to the cells. Osmolality limited evaluated doses to 40% w/v or less.



Cytotoxicity
- 1R6F is cytotoxic (IC_{50} <5 µg/mL nicotine)
- MP-1 and MP-2 are non-cytotoxic, however, MP-3 exhibited cytotoxicity (Viability <70% as per ISO-10993-5/OECD 129) at the highest dose (65% viability).
- Gum extracts were evaluated at nicotine concentrations 6.5 to 17-fold higher than 1R6F cigarette.

Conclusion

- The optimized extraction method for in vitro testing provided comparable nicotine recovery to mastication
- In vitro testing of nicotine gum products demonstrate substantially lower biological activity than cigarette smoke

The optimized extraction method provides a high-throughput and scalable alternative to mastication and is suitable for in vitro toxicological assessment of nicotine-gum products.

References

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2. German Institute for Standardization, Determination Of The Colourfastness Of Articles For Common Use - Part 1: Test With Artificial Saliva. 2002, DIN V 53160-1 2002-10.
3. ISO 20778 (2018). Cigarettes - Routine analytical cigarette smoking machine - Definitions and standard conditions with an intense smoking regime.
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Acknowledgements
1. In vitro testing of 1R6F cigarette condensate was conducted at Charles River Laboratories, Skokie, IL.
2. Collection of 1R6F cigarette condensate; extraction, nicotine assessment and in vitro testing of gum products conducted at McKinney Specialty Labs, Richmond, VA.

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