Characterization and In Vitro Testing of Whole Smoke Condensates from Combustible Cigarettes

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Introduction & Objectives

INTRODUCTION

Health Canada (HC) has promulgated methods for in vitro testing with separately collected gas and particulate phases of mainstream (MS) cigarette smoke for cytotoxicity and genotoxicity assessment. Under these conditions, the extracts have consistently shown cytotoxicity and genotoxicity, however the suggested methods may have some limitations:

- Partial evaluation of toxicological effect of the whole smoke
  - Gas/vapor phase (GVP) is only evaluated for cytotoxicity
  - Use of aqueous impinger solvent (i.e. phosphate buffered saline (PBS) limits the trapping efficiency; mainly the water-soluble GVP constituents are evaluated in cytotoxicity assay
- Stability concerns of the GVP fraction (even if evaluated within 60 min of collection) of MS smoke, which limits long term storage and requires fresh collection at each testing

Herein, we tested an alternative whole smoke collection procedure, which intended to capture both gas and particulate phases of MS smoke (Whole Smoke Condensates or "condensates") using an organic solvent, that may have some advantages over the HC method.

OBJECTIVES

- To collect whole MS cigarette smoke as condensates and perform analytical comparison of selected constituents to those in MS smoke
- To test biological activity of condensate using a battery of standard in vitro toxicity assays

Methods

Test Articles

Reference cigarette 3R4F (University of Kentucky)

Whole Smoke (WS) Condensates

MS smoke was generated according to Health Canada Method T-115 (55 mL puff volume, 30-second interval, 2-second duration with 100% of the ventilation holes blocked, using sine wave profile) on a rotary smoking machine. Total particulate matter (TPM) from 20 cigarettes was collected on a conditioned 92 mm Cambridge Filter Pad (CFP) connected in series to an impinger filled with 30 mL USP-grade ethanol, cooled in an ice water bath.

The CFP was extracted with impinger contents and then filtered using sterile cheesecloth to produce the condensate (final concentration of 28.1 mg TPM/mL in ethanol). Selected smoke constituents (nicotine, volatile organic compounds (VOCs) (1,3-butadiene, isoprene, acrylonitrile, benzene and toluene) and carbonyls (formaldehyde, acetaldehyde, crotonaldehyde and acrolein) were measured immediately after collection and at several time points during 8 weeks storage (-70° C) to track its stability (Exception: Time 0 analytical data was not available for VOCs and so data from 7 day old condensate was used for all comparisons). To determine the trapping efficiency of the method, analytical comparison was made with MS smoke yields measured using standard methods.

The condensates were subjected to in vitro assays within 48-72 hrs of collection. Ethanol was used as the vehicle control.

Neutral Red Uptake (NRU) Assay

BALB/c 3T3 cells were incubated either in presence of the vehicle control or increasing concentrations of condensate for ~48 hrs according to OECD 129. The maximum concentration of the condensates was up to 0.5% (v/v).

Salmonella Mutagenicity (Ames) Assay

The condensate was tested in five *Salmonella typhimurium* strains: TA1537, TA98, TA100, TA1535 and TA102 according to OECD 471. Cytotoxicity was checked to set the testing concentration, with the maximum concentration tested up to 100 µL/plate. The testing was performed in triplicate in presence and absence of metabolic activation (S9).

In Vitro Micronucleus (MNvit) Assay Using TK6 Cells

The condensate was evaluated for micronucleus induction according to OECD 487 in TK6 cells during short (4 hrs) incubations with and without S9 followed by an extended recovery of 40 hrs, and long (27 hrs) incubations without S9. Cytotoxicity was checked to set the testing concentration, with the maximum concentration tested up to 1 % (v/v).

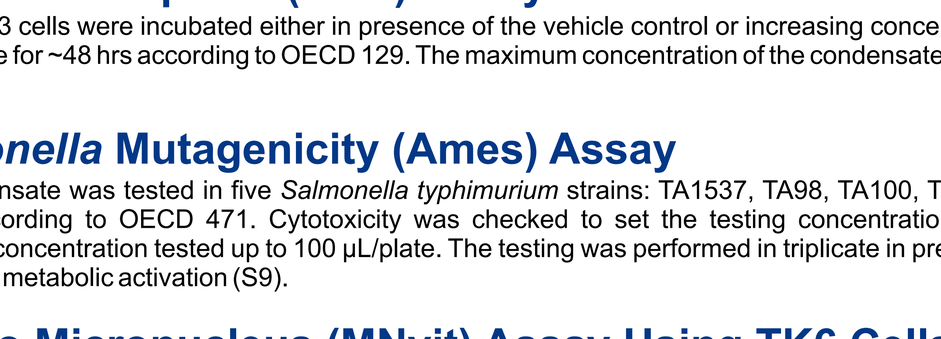
All in vitro studies were conducted at Charles River Laboratories, Skokie, IL.

Results

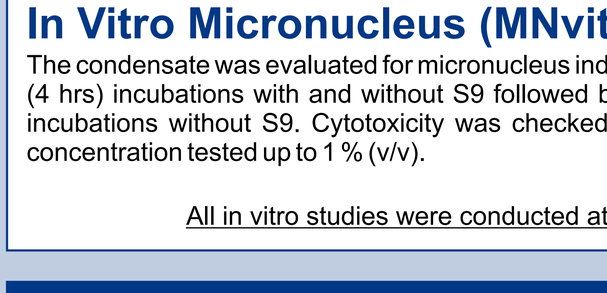
FIGURE 1.

Analytical Comparison between condensates and MS smoke.

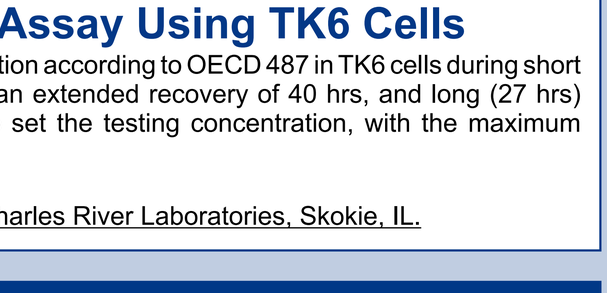
Data expressed as mean quantity of analyte per cigarette in condensate (N=1 sample prepared for in vitro studies) or mean ± SD for MS smoke (N=5 independent smoking sessions).



Carbonyl Compounds



Volatile Organic Compounds



References

- Health Canada Neutral Red Uptake Assay for Mainstream Tobacco Smoke 2004. Official method T-502, Version 1.1, pp. 1–18
- Nara H., et al. (2013). Exper. Toxicol. Pathol. 65; 683-688.
- Bruennemann, K.D. et al. (1990) Carcinogenesis (11) 10; 1863-1868.
- Health Canada Official Method T-115: December 1999. Determination of "Tar", Nicotine and Carbon Monoxide in Mainstream Tobacco Smoke.
- Rickert ,W.S. et al., (2007) Toxicological Sciences, 96(2); 285-293.
- OECD. (2010) OECD Guidance Document on Using Cytotoxicity Tests to Estimate Starting Doses for Acute Oral Systemic Toxicity Tests.
- OECD. (1997) OECD Guideline for Testing Chemicals Test Guideline 471, Bacterial Reverse Mutation Test.
- OECD. (2016) OECD Guideline for Testing Chemicals Test Guideline 487, In vitro Mammalian Cell Micronucleus Test.

This poster may be accessed at www.altria.com/ALCS-Science

Results

TABLE 1.

Trapping Efficiency of condensates in comparison to MS smoke

Constituents	MS Smoke	Condensate	Trapping Efficiency (%)
TPM (mg)	47.84	42.15	-
Nicotine (% TPM)	4.32	4.67	108.9%
VOCs			
1,3-butadiene (% TPM)	0.2	0.03	16.3%
Acrylonitrile (% TPM)	0.06	0.07	111.9%
Benzene (% TPM)	0.21	0.25	118.7%
Isoprene (% TPM)	2.05	0.83	40.3%
Toluene (% TPM)	0.38	0.51	134.2%
Carbonyls			
Acetaldehyde (µg/cig)	1813.66	971.15	53.6%
Acrolein (µg/cig)	169	59.77	35.4%
Crotonaldehyde (µg/cig)	60.62	49.01	80.9%
Formaldehyde (µg/cig)	86.51	74.24	85.8%

- % of TPM is calculated using (Qty of constituents in mg × 100)/(Qty of TPM in mg)
- Trapping efficiency is calculated using (Qty in condensate × 100)/(Qty in MS smoke)

TABLE 2.

Stability: Relative percentage of selected constituents in the condensates over 8 weeks of storage

Constituents	Nicotine	Acetaldehyde	Acrolein	Crotonaldehyde	Formaldehyde
% Remaining after 8 weeks Storage	98.6	96.2	65.9	115.0	102.6

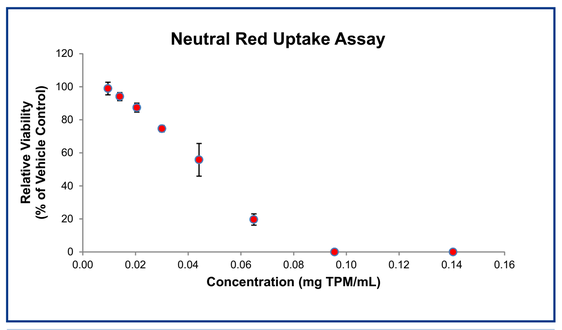
Constituents	1,3-butadiene	Acrylonitrile	Benzene	Isoprene	Toluene
% Remaining after 8 weeks Storage	85.5	98.8	101.0	141.5	98.2

- All selected MS smoke constituents were detected in condensates
- Trapping efficiency in the condensate varied, ranging ~16 to >100% for selected constituents
- Condensates were overall stable (>85%) for selected constituents, with an exception-acrolein (~66%)

NRU Assay

FIGURE 2.

Cytotoxicity - NRU Assay

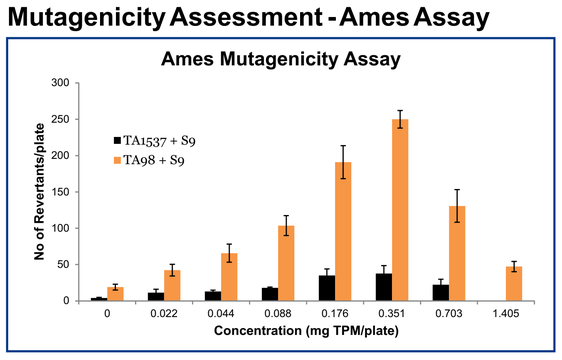


WS condensate from 3R4F show cytotoxicity ($1/EC_{50}$: 20.83 mL/mg TPM)

Ames Assay

FIGURE 3.

Mutagenicity Assessment - Ames Assay

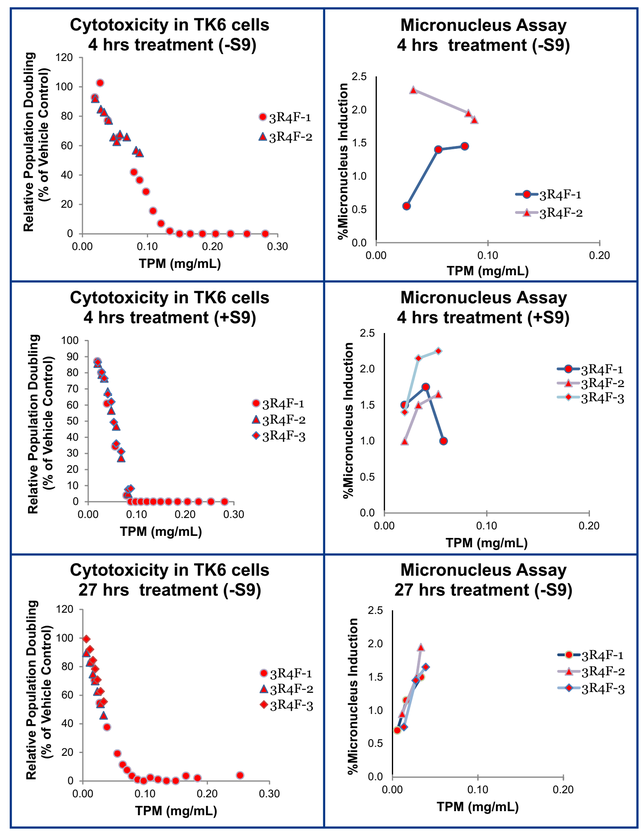


Concentration dependent increase in spontaneous revertants for strains TA 98 and TA1537 in presence of metabolic activation relative to vehicle control.

In Vitro Micronucleus Assay

FIGURE 4.

Cytotoxicity / Genotoxicity - MNvit Assay



Concentration dependent decrease in viability and weak but reproducible and significant (both statistically and for trend) induction in micronucleus relative to vehicle control.

Summary

The alternate whole smoke collection method offers improvements over the HC methods, including:

1. Provides a single sample containing constituents from both particulate and gas phase.
2. Allows in vitro screening of combined phases of MS smoke in the same experiment.
3. Allows standard analytical characterization of extracts intended for in vitro evaluation.
4. Samples can be stored for longer periods with retention of majority of constituents.

Consideration: Although we demonstrated presence of all selected volatile organic constituents of GVP with ethanol, due to limited analytical data available in literature from the HC method, the trapping efficiency estimated here with the alternate method is regarded preliminary. Additional work that directly compares trapping efficiency of HC and alternate methods using a wide set of MS smoke constituents is desirable.