Prediction of Analyte Yields, Mutagenicity, and Cytotoxicity of Mainstream Tobacco Smoke from Tobacco Blends

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Abstract

The purpose of this study was to determine if analyte yields, mutagenicity, and in-vitro cytotoxicity of two blended experimental cigarettes containing multiple types of tobacco could be predicted from the response of experimental cigarettes containing a single tobacco type represented within each blended experimental cigarette. Select mainstream tobacco smoke (MS) analytes were collected using the International Organization for Standardization puff regimen and measured. Spontaneous mutant frequency was determined using a mouse lymphoma assay. The number of revertant colonies was determined via the Salmonella reverse mutation assay. Cytotoxicity ($1/EC_{50}$) of gas vapor phase (GVP) and total particulate matter (TPM) from MS was determined using the neutral red uptake assay. Under the assumption of linear blending, the predicted result for a tobacco blend is based on the weighted average of the responses of the experimental cigarettes containing the corresponding single types of tobacco that constitute the blend. Accurate predictions were considered to be those with values ±10% of measured value. Accurate Predictions for 60–82% of selected mainstream analytes were made for Blend 1 experimental cigarettes (35.41% Bright: 22.96% Burley: 14.63% Oriental: 27% Reconstituted Leaf Domestic Bright) and Blend 2 experimental cigarettes (35.41% Bright: 22.96% Burley: 14.63% Oriental: 27% Expanded Tobacco). Accurate predictions for spontaneous mutation frequency were made for Blends 1 and 2 experimental cigarettes. The number of revertant colonies was not consistently predicted with accuracy for Blend 1 or 2 experimental cigarettes. However, $1/EC_{50}$ could be accurately predicted for GVP in Blend 1 and 2 experimental cigarettes, but TPM could only be accurately predicted for Blend 2 experimental cigarettes. In conclusion, predictions via linear blending were not sufficient to replace measurement of analyte yields, mutagenicity or cytotoxicity. However, linear blending may serve as an additional screening tool when toxicological and mutagenic responses of experimental or blended tobacco cigarettes of known tobacco composition must be evaluated.

Methods

Analyte yields from MS were measured, mutagenicity assays (MLA and *Salmonella* reverse mutation assay) and an in-vitro cytotoxicity assay (NRU) were conducted on two sets of experimental cigarettes. Experimental cigarettes in Set 1 comprised individual tobacco types or a mix of tobaccos in the U.S., except for Oriental tobacco which was sourced internationally. Experimental cigarettes in Set 2 comprised individual types or a mix of tobaccos which were sourced internationally, except for the expanded tobacco (ET), which was predominantly US-sourced. A University of Kentucky reference cigarette 1R4F (Tobacco and Health Research, Lexington, KY) was used as an internal assay standard.

Analytical Chemistry

MS analytes were collected from each experimental cigarette and quantified using validated analytical methods. Since the experimental cigarettes tested yielded statistically significantly different amounts of water, the analytical chemistry, mutagenicity, and cytotoxicity results were reported on a per mg tar basis. Smoke analyte yield predictions were done on a per cigarette basis to determine analyte yields in Blend 1 and Blend 2 experimental cigarettes.

In-Vitro Mutagenicity: Mouse Lymphoma Assay

The mouse lymphoma TK assay (MLA) was conducted in general compliance with Organization for Economic Cooperation and Development (OECD) guideline 476.[1] Mouse lymphoma cells were treated with MSC for 4 h at 37 °C, with/without metabolic activation (Aroclor-induced rat liver S9). Positive (-S9 = Methyl methanesulfonate; +S9 = benzo[a]pyrene) and negative controls (DMSO) were used in each assay. Experimental cigarettes were ranked on spontaneous mutant frequency on a per cigarette basis. The concentration for the effect level of three times the spontaneous mutant frequency (C_{3B}) was calculated from the dose response curve. Higher C_{3B} values are associated with decreased spontaneous mutant frequency, and lower C_{3B} values are associated with increased spontaneous mutant frequency. Spontaneous mutant frequency predictions, using C_{3B} values (95% confidence limits), were done on Blend 1 and Blend 2 experimental cigarettes on a per cigarette basis.

Experimental Cigarettes

Description of experimental cigarettes used in this study

Set 1	Tobacco content				
Tobacco type	Bright	Burley	Oriental	RLDBright	Blend 1
Percentage of tobacco	100%	100%	100%	50%RLD; 50%Bright	Bright;Burley;Oriental;RLD[a]
Source	USA	USA	International	USA	USA
Set 2					
Tobacco type	Bright	Burley	Oriental	ET	Blend 2
Percentage of tobacco	100%	100%	100%	100%	Bright;Burley;Oriental;ET[b]
Source	International	International	International	USA	International + USA

ET, expanded tobacco; RLDBright, reconstituted leaf domestic Bright
[a]Blend 1: Bright(35.41%)+Burley(22.96%)+Oriental (14.63%) +RLD (27%)
[b]Blend 2: Bright(35.41%)+Burley(22.96%)+Oriental (14.63%) +ET (27%)

Methods

In-Vitro Mutagenicity: *Salmonella* Mutagenicity

The *Salmonella typhimurium* reverse mutation assay was conducted in general compliance with OECD guideline no. 471.[2] To measure number of revertant colonies, TA98 and TA100 strains were treated with MSC (three replicates from each of two separate MSC collections/assay) in the presence or absence of S9 for 44–72 h at 36–37 °C. Positive controls (TA98+S9 = 2-amino anthracene; TA98-S9 = daunomycin; TA100+S9 = 2-amino anthracene; TA100-S9 = methyl methanesulfonate) and negative controls (DMSO) were used in each assay. For each assay, the genotype of each strain was confirmed, and number of revertant colonies was measured in response to MSC on a per mg of dry condensate basis. The specific number of revertant colonies induced by MSC was measured over a range of non-toxic dose levels, with the slopes of regression lines compared to determine mutagenic response to MSC.[3] Experimental cigarettes were ranked by number of revertant colonies on a per mg dry condensate basis.[4] Number of induced revertant colonies was predicted using the weighted average of the tobacco components for Blend 1 and Blend 2 experimental cigarettes on a per mg dry condensate basis.

In-Vitro Cytotoxicity: NRU Assay

TPM and GVP fractions of MSC from each experimental cigarette were added separately to mouse embryo Balb/c 3T3 cells, obtained from the American Type Culture Collection (Manassas, VA). To determine $1/EC_{50}$ (the reciprocal of the concentration that reduces the number of viable cells by 50% as compared with the control), 24 h post-seeding, cells were treated with TPM, GVP or a positive control (acrolein) dissolved in media for 24 h. After 24 h, media containing TPM, GVP or positive control were replaced with media containing neutral red. After 3 h, cells were washed in PBS, and the neutral red taken up by the cells was extracted. Optical density was read at 540 nm. Three separate batches of TPM or GVP were collected and assayed independently. Experimental cigarettes were expressed as $1/EC_{50}$ for TPM and GVP fractions on a per mg tar basis. Reciprocal EC_{50} predictions using linear blend calculations were done for Blend 1 and Blend 2 experimental cigarettes on a per mg tar basis.

Evaluation: Weighted Average of Components Assumption

The predicted result for a tobacco blend is based on the weighted average of the responses of the experimental cigarettes containing the corresponding single types of tobacco that constitute the blend.

The weighted average assumption was applied to analyte yields above the limit of quantitation, mutagenicity (MLA and *Salmonella* reverse mutation assay) and in-vitro cytotoxicity. The weighted average for Blend 1 experimental cigarette was calculated as follows:

Bright * 0.3541 + Burley * 0.2296 + Oriental * 0.1463 + 0.27 * 2 * (RLDBright – 0.5 * Bright) =
Bright * 0.0841 + Burley * 0.2296 + Oriental * 0.1463 + RLDBright * 0.54.

Similarly the weighted average for the Blend 2 experimental cigarette was calculated as follows:

Bright * 0.3541 + Burley * 0.2296 + Oriental * 0.1463 + ET * 0.27

Results

Set 1 C_{3B} from MLA Assay

S9	Cigarette	C_{3B}
No	Bright	36.9
	Burley	55.8
	Oriental	35.5
	RLDBright	39.4
	Blend1	40.8
	Predicted	42.4
	% Error	3.8%

Set 2 C_{3B} from MLA Assay

S9	Cigarette	C_{3B}
No	Bright	37.0
	Burley	52.5
	Oriental	37.0
	ET	43.2
	Blend2	42.0
	Predicted	42.2
	% Error	0.4%

Set 1 C_{3B} from MLA Assay

S9	Cigarette	C_{3B}
Yes	Bright	141.4
	Burley	184.3
	Oriental	126.2
	RLDBright	198.9
	Blend1	172.6
	Predicted	180.0
	% Error	4.3%

Set 2 C_{3B} from MLA Assay

S9	Cigarette	C_{3B}
Yes	Bright	110
	Burley	163
	Oriental	133
	ET	180
	Blend2	158
	Predicted	144
	% Error	-8.5%

Salmonella Reverse Mutation Assay Results (per mg dry condensate)

Set 1				Set 2			
S9	Strain	Cigarette	Mean	S9	Strain	Cigarette	Mean
Yes	TA98	Bright	3470	Yes	TA98	Bright	3670
		Burley	5809			Burley	5867
		Oriental	2167			Oriental	3159
		RLDBright	3501			ET	2637
		Blend 1	3993			Blend 2	4282
		Predicted	3833			Predicted	3821
		% Error	-4.0%			% Error	-10.8%
Yes	TA100	Bright	1721	Yes	TA100	Bright	2019
		Burley	2793			Burley	2988
		Oriental	1386			Oriental	1529
		RLDBright	1624			ET	1162
		Blend 1	1989			Blend 2	2001
		Predicted	1865			Predicted	1938
		% Error	-6.2%			% Error	-3.1%

Results

Measured analyte yield values together with the predicted value assuming weighted average. Red values indicated predicted values greater than ±10% from measured values.

	Set 1							Set 2						
Analyte	Bright Meas.	Burley Meas.	Oriental Meas.	RLDBright Meas.	Blend 1 Meas.	Predicted Wtd Ave	Difference Pred - Meas. % Diff	Bright Meas.	Burley Meas.	Oriental Meas.	ET Meas.	Blend 2 Meas.	Predicted Wtd Ave	Difference Pred - Meas. % Diff
TPM (mg/cig)	29.3	22.2	32.5	16.6	20.2	21.3	5.3%	24.0	15.8	26.4	14.3	19.1	19.8	3.9%
Tar (mg/cig)	22.9	16.8	26.2	13.0	15.7	16.6	6.0%	19.4	12.6	21.9	11.2	15.3	16.0	4.5%
Nicotine (mg/cig)	2.77	2.24	1.33	1.05	1.44	1.51	4.8%	2.77	1.8	1.86	1.29	1.93	2.01	4.4%
Water (mg/cig)	3.64	3.17	4.98	2.61	3.05	3.17	4.0%	1.79	1.41	2.65	1.83	1.82	1.84	1.1%
Carbon monoxide (mg/cig)	15.7	15.8	16.9	14.4	15.5	15.2	-2.0%	11.3	10.0	13.0	10.0	11.0	10.9	-1.0%
1,3-Butadiene (µg/cig)	47.8	45.4	52.5	34.8	44.9	40.9	-8.9%	48.2	36	60.8	52.3	47	48.3	2.9%
Isoprene (µg/cig)	708	479	444	336	448	416	-7.2%	664	464	404	623	561	569	1.4%
Formaldehyde (µg/cig)	50.9	14.6	83.7	67.0	43.3	56.1	29.5%	39.7	29	48.3	49.1	40.5	41.0	1.3%
Acetaldehyde (µg/cig)	825	679	740	718	737	721	-2.1%	737	639	721	588	732	672	-8.2%
Acrolein (µg/cig)	88.7	60.3	89.7	77.7	78.3	76.4	-2.4%	54.7	42.6	62.1	61.6	64.9	54.9	-15.5%
Propionaldehyde (µg/cig)	63.6	49.9	63.1	55.5	56.9	56.0	-1.6%	71.5	52.3	77.5	52.5	67.8	62.8	-7.3%
Acrylonitrile (µg/cig)	15.2	20.7	15.7	10.3	14.3	13.9	-2.9%	16.1	17.0	16.6	11.9	15.2	15.2	0.3%
Hydrogen cyanide (µg/cig)	177	150	150	116	143	134	-6.4%	180	152	209	90	197	154	-22.1%
2-Nitropropane (µg/cig)	7.4	18.2	6.7	8.8	10.1	10.5	4.8%	9.3	19.0	12.7	9.9	12.8	12.2	-5.0%
o-Toluidine (ng/cig)	126	160	105	55	74	92	24.9%	114	130	78.3	42.8	81.5	93.2	14.4%
o-Anisidine (ng/cig)	5.59	5.05	2.05	2.95	3.22	3.52	9.4%	Not Measured						
2-Naphthylamine (ng/cig)	11.5	15.7	8.0	4.9	6.9	8.4	21.6%	12.2	11.0	8.1	4.4	9.7	9.2	-4.7%
4-Aminobiphenyl (ng/cig)	1.99	3.83	1.43	1.03	1.43	1.8	26.7%	2.3	3.47	1.92	1.09	2.23	2.19	-2.0%
Vinyl chloride (µg/cig)	27.3	27.4	40.5	27.1	31.2	29.1	-6.6%	All values below LOQ (<19.8)						
Nitrogen oxides (mg/cig)	144	457	151	232	287	264	-7.9%	0.146	0.265	0.151	0.089	0.171	0.159	-7.2%
Benzene (µg/cig)	53.1	49.1	73.8	36.0	46.1	46.0	-0.3%	47.3	44.1	56.9	34.0	45.7	44.4	-2.9%
Toluene (µg/cig)	84.3	81.3	130.5	57.1	72.8	75.7	4.0%	76.5	76.7	86.9	44.0	69.2	69.3	0.1%
NNN (ng/cig)	103	543	32	264	234	281	19.9%	43.5	336	28.6	51.0	131	111	-15.6%
NNK (ng/cig)	190	159	<12 #	179	224	151	-32.6%	51.5	55.4	< 17.6 #	59.1	51.7	49.5	-4.3%
Phenols (µg/cig)	62.9	41.7	61.7	19.3	27.4	34.3	25.2%	71.3	39.2	57.6	16.6	44.5	47.2	6.0%
Catechol (µg/cig)	139	56	185	64	72	86	19.7%	122	52.2	113	42.9	83.3	83.3	0.0%
Benz[a]anthracene (ng/cig)	35.8	18.1	60.2	15.1	18.6	24.1	29.7%	23.4	12.5	25.1	9.48	16.8	17.4	3.5%
Benzo[b]fluoranthene (ng/cig)	15.3	10.2	22.2	8.2	9.3	11.3	21.6%	19.3	10.2	21.8	7.04	11.9	14.3	19.9%
Benzo[k]fluoranthene (ng/cig)	3.50	1.65	5.65	2.04	2.23	2.60	16.7%	Not Measured						
Benzo[j]fluoranthene (ng/cig)	7.53	4.19	10.68	3.86	4.30	5.24	21.9%	Not Measured						
Benzo[a]pyrene (ng/cig)	17.6	8.8	28.6	8.3	9.7	12.2	25.4%	12.2	5.2	11.5	4.0	7.8	8.3	6.6%
Indeno[1,2,3-cd]pyrene (ng/cig)	7.53	3.94	12.82	3.98	4.64	5.56	19.9%	3.03	0.81	2.35	< 0.261 #	1.39	1.67	20.3%
Dibenz[a,h]anthracene (ng/cig)	1.89	0.94	4.91	0.98	1.22	1.62	33.0%	Not Measured						

The < LOQ value was replaced with the LOQ for the purposes of the predicted Blend 1 and 2 values

Cytotoxicity ($1/EC_{50}$) determined by NRU (per mg tar)

	GVP		TPM	
Cigarette	Set 1	Set 2	Set 1	Set 2
Bright	4.2	3.4	10.0	11.5
Burley	2.9	2.2	10.6	10.9
Oriental	5.1	3.6	10.2	11.7
RLDBright	6.6	–	10.4	–
ET	–	5.8	–	9.8
Blend	5.4	4.2	9.6	11.8
Predicted	5.3	3.8	10.4	10.9
% Error	-1.3%	-9.5%	8.2%	-7.4%

Cytotoxicity ($1/EC_{50}$) determined by NRU (per cigarette)

	GVP		TPM	
Cigarette	Set 1	Set 2	Set 1	Set 2
Bright	115	88	272	298
Burley	59	37	220	184
Oriental	144	104	291	338
RLDBright	116	–	182	–
ET	–	85	–	144
Blend	109	86	191	242
Predicted	107	78	214	236
% Error	-1.9%	-9.2%	12.0%	-2.4%

Summary

There were mixed results regarding whether the blended cigarette values could be well predicted by the simple weighted average of the individual component values.

- The biological assays were generally well predicted, though they did not show a large difference between tobacco types.
- Many of the compounds in smoke, such as tar, nicotine, and carbon monoxide, are well predicted by the simple additive model.
- Some of the trace level constituents, such as the polyaromatic hydrocarbons, had differences from the predicted value of more than 10%, likely due to their low levels.
- Some compounds, such as NNN, NNK and formaldehyde, were not well predicted by the simple additive model, perhaps due to the complex nature of their formation mechanisms.

Note that different crop year tobaccos could perform differently, because of natural variation in agricultural products.

References

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