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Assessment of priority tobacco additives per the requirements in the EU Tobacco Products Directive (2014/40/EU): Part 2: Smoke chemistry and *in vitro* toxicology

Regina Stabbert[a,*], David Ghosh[b], Anna Clarke[c], Jacqueline Miller[d], Jane Collard[e], Ian Crooks[e], Donatien Tafin Djoko[a], Sylvain Larroque[d], Guy Jaccard[a], Ewald Roemer[f], Chul-Hoon Park[g], Marco Esposito[a]

[a] *Philip Morris International, Philip Morris Products SA, Rue des Usines 90, 2000, Neuchâtel, Switzerland*
[b] *Philip Morris International, Philip Morris International Management SA, Avenue de Rhodanie 50, 1001, Lausanne, Switzerland*
[c] *Imperial Tobacco, Winterstoke Road, Bristol, BS32LL, United Kingdom*
[d] *Japan Tobacco International SA, Rue Kazem Radjavi 8, 1202, Geneva, Switzerland*
[e] *British American Tobacco, Research & Development, Regents Park Road, Southampton, SO158TL, United Kingdom*
[f] *TobToxConsulting, Imp. Blanchet-Dailleres 9, 1585, Cotterd, Switzerland*
[g] *KT&G Research Institute, 30, Gajeong-ro, Yuseong-gu, Daejeon, 34128, Republic of Korea*

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ABSTRACT

This publication is part of a series of three publications and describes the non-clinical assessment performed to fulfill the regulatory requirement per Art. 6 (2) of the EU Tobacco Products Directive 2014/40/EU under which Member States shall require manufacturers and importers of cigarettes and Roll Your Own tobacco containing an additive that is included in the priority list established by Commission Implementing Decision (EU) 2016/787 to carry out comprehensive studies (European Comission, 2016). This publication contains the results of a literature search, comprehensive smoke chemistry, additive transfer, and *in vitro* toxicity studies for the 13 priority additives (carob bean extract, cocoa powder, fenugreek extract, fig juice concentrate, geraniol, glycerol, guaiacol, guar gum, liquorice extract powder, maltol, *l*-menthol (synthetic), propylene glycol, and sorbitol) commissioned by the members of the Priority Additives Tobacco Consortium to independent Contract Research Organizations. Comparisons of the 39 World Health Organisation smoke emissions in smoke from cigarettes with and without priority additives identified some differences that, with few exceptions, were minor and well within the inherent variability of the analytical method observed for the 3R4F monitor cigarette. Most differences were not statistically significant and did not show consistent additive-related increases or decreases. However, test cigarettes with guar gum showed a statistically significant, additive-related increase in formaldehyde and cadmium; test cigarettes with sorbitol showed a statistically significant, additive-related increase in formaldehyde and acrolein; test cigarettes with glycerol showed a statistically significant, additive-related decrease in phenols, benzo[*a*]pyrene and *N*-nitrosoanabasine; and test cigarettes with propylene glycol showed a statistically significant, additive-related decrease in phenol and *m* + *p*-cresols. These changes were not observed when the additives were tested as a mixture. None of the increases or decreases in smoke chemistry translated into changes in the *in vitro* toxicity. Comparisons of the *in vitro* toxicity of smoke from cigarettes with and without priority additives gave some differences that were minor, well within the inherent variability of the assays, not statistically significant, and did not show consistent additive-related increases or decreases. Thus, it can be concluded that the addition of priority additives had no effect on the *in vitro* toxicity of the cigarette smoke. The results obtained in our studies are consistent with those in scientific literature.

1. Introduction

This publication, along with two others, which are part of the same assessment program (McEwan et al., 2019; Simms et al., 2019), presents the results of comprehensive smoke chemistry and *in vitro* toxicity studies for 13 tobacco additives. These additives have been listed by the

* Corresponding author.
E-mail address: Regina.Stabbert@pmi.com (R. Stabbert).

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Abbreviations and glossary

ATSDR Agency for Toxic Substances and Disease Registry
B[*a*]P Benzo[*a*]pyrene
CAS Chemical Abstract Service (division of the American Chemical Society)
Cast Sheet Specific type of reconstituted tobacco
CIR Cosmetic Ingredient Review
CD 3R4F monitor cigarette variability
CHO Chinese Hamster Ovary
CLP Classification, Labelling and Packaging
CMR Carcinogenic, Mutagenic, Reprotoxic (Classification according to EU CLP Regulation 1272/2008)
CO carbon monoxide
CRO independent Contract Research Organisation
DMSO dimethylsulphoxide
ECHA European Chemicals Agency
EFSA European Food Safety Authority
EU European Union
FEMA Flavoring Extract Manufacturer' Association
FDA Food and Drug Agency of the USA
GC-FID gas chromatography-flame ionization detector
GC/MS gas chromatography - mass spectrometry
GLP Good Laboratory Practices
GMO Genetically modified organism
GVP Gas Vapor Phase
HPLC High Pressure Liquid Chromatography
HCI Health Canada Intense smoking condition
IC_{50} Half maximal Inhibitory Concentration, a quantitative measure that indicates how much of a substance is needed to inhibit a given biological process, e.g., the concentration of TPM that reduces the number of viable cells due to cell death and decreased proliferation to half of that found in the control without TPM exposure
ISO International Organisation for Standardization
JECFA Joint FAO/WHO Expert Committee on Food Additives
*iv*MN *in vitro* Micronucleus test; a genotoxicity test recommended in nearly all regulatory test batteries
monitor cigarette A cigarette produced in large quantities with minimal production variation; provided to interested researchers as a utility to compare their results of analyses on this cigarette type with those of other laboratories (in several scientific publications also referred to as Standard Reference Cigarette). In the studies conducted for the purpose of this report, the 3R4F Kentucky Reference cigarette served as the monitor cigarette
MS Mainstream Smoke
NFDPM Nicotine Free Dry Particulate Matter (TPM where the amount of nicotine and water has been subtracted mathematically)
NNK 4-(N-nitrosomethylamino)-1-(3-pyridyl)-1-butanone
NNN N-nitrosonornicotine
NAB N-Nitrosoanabasine
NRU Neutral Red Uptake assay
NTP National Toxicology Program
OECD Organisation for Economic Co-operation and Development
PBS Phosphate-Buffered Saline
Ph. Eur European Pharmacopeia
REACH Registration, Evaluation, Authorization and Restriction of Chemicals
Reconstituted Tobacco Tobacco product that makes use of, e.g., stems and broken bits (fines) where, as in a paper-mill, the tobacco after grinding and mixing with water and a binder (e.g. guar gum) is converted into a paper-like material, that can be processed like normal tobacco lamina
reference cigarette The comparative study approach compares two types of cigarettes that differ only in one aspect: a cigarette with and a cigarette without the addition of the additive. The reference cigarette is the additive-free cigarette with identical tobacco blend and cigarette construction
SCHEER Scientific Committee on Health, Environmental and Emerging Risks
test cigarette The comparative study approach compares two types of cigarettes that differ only in one aspect: a cigarette with and a cigarette without the addition of the additive. The test cigarette is the cigarette with the additive.
Top flavor Top flavors are added at the end of tobacco processing to the cut tobacco as a mixture of volatile flavors to provide the final taste of the cigarette smoke according to the preference of the individual smoker
TPD Tobacco Products Directive
TPM Total Particulate Mass
USP/NF United States Pharmacopeia (USP)-National Formulary (NF)
UVCB Unknown or Variable composition, Complex reaction product or Biological material
WHO World Health Organisation

European Commission in the Priority List established by Commission Implementing Decision (EU) 2016/787 (European Commission, 2016). Manufacturers and importers of cigarettes and Roll Your Own tobacco containing an additive that is included in this Priority List are required to carry out comprehensive studies to support their continued use in the EU. These studies shall examine for each of these priority additives whether it

> "(a) **contributes to the toxicity** or addictiveness of the products concerned, **and whether this has the effect of increasing the toxicity** or addictiveness of any of the products concerned to a significant or measurable degree;
>
> (b) results in a characterising flavour;
>
> (c) facilitates inhalation or nicotine uptake; or
>
> (d) **leads to the formation of substances that have CMR properties,**[1] the quantities thereof, and whether **this has the effect of increasing the CMR properties** in any of the products concerned to a significant or measurable degree."

The present study addressed requirements (a) and (d) (i.e., smoke chemistry analyses, determinations of ingredient levels in tobacco and their transfer rates into smoke, and *in vitro* toxicity studies of mainstream smoke (MS) from cigarettes with and without additives on a comparative basis); test cigarettes containing one or more priority additives were compared to an additive-free reference cigarette with identical tobacco blend. Further in-depth information on the background of this assessment is presented in the Part 1 of the series of publications (Simms et al., 2019).

[1] carcinogenic, mutagenic or reprotoxic (CMR) properties.

Table 1
Overview of the comprehensive testing program (Part 2) for the priority additives used in cigarettes and Roll Your Own tobacco subject to enhanced reporting obligations.

	Comprehensive literature review	Transfer rates	Smoke chemistry	In-vitro toxicity		
			WHO list of 39 priority emissions	Ames	Neutral Red Uptake	In-vitro Micronucleus
Carob bean extract	✓	✗	✓	✓	✓	✓
Cocoa powder	✓	✓	✓	✓	✓	✓
Fenugreek extract	✓	✗	✓	✓	✓	✓
Fig juice concentrate	✓	✗	✓	✓	✓	✓
Geraniol	✓	✓	✓	✓	✓	✓
Glycerol	✓	✓	✓	✓	✓	✓
Guaiacol	✓	✓	✓	✓	✓	✓
Guar Gum	✓	✗	✓	✓	✓	✓
Liquorice extract powder	✓	✓	✓	✓	✓	✓
Maltol	✓	✓	✓	✓	✓	✓
Menthol	✓	✓	✓	✓	✓	✓
Propylene glycol	✓	✓	✓	✓	✓	✓
Sorbitol	✓	✗	✓	✓	✓	✓
Mix1	not applicable	✗	✓	✓	✓	✓
Mix2	not applicable	✗	✓	✓	✓	✓
Mix3	not applicable	✗	✓	✓	✓	✓

Table 2
Chemical identity and lot specification of priority additives. **Note:** UVCB are substances of Unknown and Variable composition, Complex reaction products or Biological materials (ECHA, 2016). [1] Results of the Constituent of Relevance analysis is provided in Part 1 (Simms et al., 2019).

	CAS No	Purity	Lot Specification	Analysis of Constituents of Relevance[1]
Carob bean extract	84961-45-5	UVCB	Botanical statement; European flavor regulation statement; Food regulation certificate; FEMA GRAS status; GMO free certificate; Heavy metal statement; certificate of analysis;	✓
Cocoa powder	95009-22-6	UVCB	Food grade purity complying with EU regulations; total fibers 32.6% (solubles 4.1%), cocoa butter 10.5%, total carbohydrates 10.2%, starch 9.7%, vitamin E 2.3%, theobromine 2.3%; certificate of analysis; GMO free certificate;	✓
Fenugreek extract	84625-40-1	UVCB	Food grade purity complying with EU regulations; Botanical statement; European flavor regulation statement; FEMA GRAS status; GMO free cerificate; Heavy metal statement; certificate of analysis;	✓
Fig juice concentrate	90028-74-3	UVCB	Botanical statement; European flavor regulation statement; FEMA GRAS status; GMO free certificate; Heavy metal statement; Certificate of analysis;	✓
Geraniol	106-24-1	97.0%	Methyl eugenol-free confirmation by supplier; European flavor regulation statement; Food regulation certificate; FEMA GRAS status; GMO free certificate; Heavy metal statement; Purity statement; synthetic (nature-identical);	✗
Glycerol	56-81-5	99.5%	European Pharmacopeia (Ph. Eur.) grade; vegetable, GMO-free glycerol; Chemical analyses confirmed compliance with the product characteristics including impurities set forth by EP, USP, and E422;	✗
Guaiacol	90-05-1	97.0%	European flavor regulation statement; Food regulation certificate; FEMA GRAS status; GMO free certificate; Heavy metal statement; synthetic (nature-identical); purity statement;	✗
Guar Gum	9000-30-0	UVCB	Certificate of analysis; Food grade statement;	✓
Liquorice extract powder	68916-91-6	UVCB	Analytical report; product specification; Concerning microorganisms, Ochratoxin A and pesticides the material complied with EU regulations for food.	✓
Maltol	118-71-8	99.0%	European flavor regulation statement; Food regulation certificate; FEMA GRAS status; GMO free certificate; Heavy metal statement; synthetic (nature-identical); purity statement;	✗
/-Menthol	2216-51-5	> 99.9%	synthetic; purity and residual solvents statement; Chemical analyses confirmed compliance with the product characteristics including impurities set forth by US FEMA and FDA GRA	✗
Propylene glycol	57-55-6	>99.9 %	Certificate of analysis; meets specification of USP39/NF34 ; chemical analyses confirmed compliance with the product characteristics including impurities set forth by Ph. Eur. and USP/NF	✗
Sorbitol	50-70-4	77.7%	food grade purity; compliance with all pertinent EU regulations laying down specifications for food additives regarding, e.g., their purity and quality, chemical contamination, and bacterial contamination	✗

2. Materials and methods

2.1. Tobacco additives

The priority list (European Commission, 2016/787/EU, 2016) specifies 15 additives subject to enhanced reporting obligations. Two of them are not covered by this publication, diacetyl and titanium dioxide, and the explanation is given in Part 1 of this series (Simms et al., 2019). Thus, 13 additives underwent comprehensive smoke chemistry and *in vitro* toxicology assessment as described in Table 1.

Table 3
Targeted additive levels and, in brackets, concentrations achieved. Percentages are inclusion levels on the tobacco.

Additive	Additive Concentration (%)					
	Low	Max	Max Plus	Mix 1	Mix 2	Mix 3
Carob bean extract	0.2	0.4	0.6	not contained in Mix 1	not contained in Mix 2	not contained in Mix 3
Cocoa powder	0.5 (0.605)	1.0 (1.014)	1.5 (1.440)	not contained in Mix 1	not contained in Mix 2	0.4 (0.24)
Fenugreek extract	0.01	0.02	0.03	0.02	0.02	0.02
Fig juice concentrate	0.025	0.15	0.3	not contained in Mix 1	not contained in Mix 2	0.025
Geraniol	0.015 (0.0151)	0.03 (0.0251)	0.045 (0.0529)	0.03 (0.024)	0.03 (0.027)	0.03 (0.025)
Glycerol	2.5 (2.303)	5.0 (4.325)	6+ (5.974)	1.0 (0.57)	1.5 (0.97)	1.5 (0.93)
Guaiacol	0.0005 (0.000559)	0.0010 (0.000877)	0.0015 (0.001555)	0.001 (0.00047)	0.001 (0.00051)	0.001 (0.00051)
Guar gum	0.5 (0.33–0.55)	1.0 (1.03–1.18)	1.5 (1.44–1.57)	not contained in Mix 1	not contained in Mix 2	1.0
Liquorice extract powder	0.6 (0.42)	1.2 (1.05)	1.8 (1.73)	not contained in Mix 1	not contained in Mix 2	0.8 (0.44)
Maltol	0.005 (0.0045)	0.01 (0.0079)	0.015 (0.0111)	0.01 (0.0042)	0.01 (0.0061)	0.01 (0.0046)
Menthol	0.6 (0.55)	1.2 (1.14)	1.8 (1.73)	not contained in Mix 1	not contained in Mix 2	not contained in Mix 3
Propylene glycol	2.5 (2.05)	5.0 (4.53)	6+ (4.80)	1.0 (1.10)	2.0 (1.57)	1.0 (1.13)
Sorbitol	0.6 (0.65)	1.2 (1.10)	1.8 (1.60)	not contained in Mix 1	2.0 (1.54)	not contained in Mix 3

The suppliers for the additives were BASF, Ludwigshafen, Germany; Cargill Cocoa & Chocolate, Schiphol, Netherlands; Gumix International, Inc., Fort Lee, USA; Hepner & Eschenbrenner GmbH & Co. KG, Hamburg, Germany; Hertz Flavors GmbH & Co. KG, Hamburg, Germany; Takasago Europe GmbH, Zülpich, Germany; and Verbio Vereinigte BioEnergie AG, Zörbig, Germany. Additives were obtained following the same procedure and specifying the same grades as for commercial manufacture. All food-grade ingredients were certified to be compliant with the requirements of the European regulation or equivalent obligations (European Union, 2008) (Table 2). According to the specifications of the suppliers, the chemical composition was typical for the respective additives and also typical for the material delivered to and used by the members of the Tobacco Consortium in their standard manufacturing processes. For additives derived from plant materials, further analysis was performed identifying the constituents of relevance. The constituents of relevance for carob bean extract, cocoa powder, fenugreek extract, fig juice concentrate, guar gum and liquorice extract powder additives were analyzed by two independent Contract Research Organizations (CRO): Yordas Group (formerly REACH Centre) in the UK and UFAG Laboratorien in Switzerland (data is reported in the first paper of this series Simms et al., 2019).

2.2. Experimental cigarettes

The test and additive-free reference cigarettes were manufactured by British American Tobacco in Germany within the parameters of those sold in the EU. The cigarettes were high-speed machine-made as per standard commercial cigarette production, using a typical American blend of tobacco. Further details on the manufacturing of experimental cigarettes is provided in Part 1 (Simms et al., 2019).

Additives were applied to the tobacco blend using the standard process of manufacturing. Three additive levels in the cigarettes were defined based on the Quantity Not Exceeded level, which is the highest concentration used by the original members of the Tobacco Consortium (BAT, Imperial Tobacco, JTI, PMI) in any of their products manufactured for sale in the EU. This concentration is referred as the “Max” level in the studies. In addition, a “Low” and a “Max Plus” level were also applied, corresponding to three application levels for the 13 priority additives, three mix samples and the additive-free reference cigarette, 43 samples in total. The target concentration for the “Low” level was 50% of the “Max” level and the “Max Plus” level was 150% of the “Max” level whenever technically achievable. The additive-containing cigarettes are referred to as test cigarettes; the additive-free cigarette is referred to as the reference cigarette. Additive levels per test cigarette are presented in Table 3.

The 3R4F monitor cigarette was purchased from the University of Kentucky. This cigarette was used in parallel to the additive-free reference and test cigarettes to monitor the performance of the methods/assays. This was done to include an internal control into the study to ensure that the results of the testing are valid and not influenced by potential technical problems (Roemer et al., 2012). In addition, the long-term variability of the 3R4F data at Labstat (Canada) was used to characterize the inherent variability of the methods/assays and the product variability based on manufacturing processes, e.g., small differences in cigarette tobacco weight (Baker et al., 2004a; Belushkin et al., 2015). The 3R4F variability for each analyte and assay response was calculated before starting the study and reported in the corresponding GLP study plans (Table 4, Table 5).

2.3. Smoke generation

Before smoking, the cigarettes were conditioned unpacked at 22 ± 1 °C and 60 ± 3% relative humidity according to ISO Standard 3402 (ISO 3402, 1999) for at least 48 h. Mentholated cigarettes were conditioned in their original sealed polypropylene packages to prevent the loss of menthol.

Smoke generation was carried out on automatic smoking machines according to the smoking parameters and machine specifications set out in ISO Standard 3308 (ISO 3308, 2012) (i.e., puff volume 35 mL, puff interval 60 s, puff duration 2 s, bell-shaped puff profile, and no vent blocking).

Three replicate smoke samples of each of the 42 test cigarettes, additive-free reference cigarettes, and monitor cigarettes were generated on the same day to minimize the impact of confounding factors.

2.4. Smoke chemistry

Smoke chemistry studies were conducted by the independent CRO Labstat in Canada in compliance with the applicable requirements of the Organisation for Economic Co-operation and Development (OECD) Principles of Good Laboratory Practice (GLP) as revised on November 26th, 1997 [C(97)186/Final] and of 21 CFR Part 58 (Code of Federal Regulations, Food and Drug Administration) Good Laboratory Practices for Nonclinical Laboratory Studies as amended on May 21st, 2002. Labstat used validated and standardized methods to determine the 42 emissions in MS generated under the ISO smoking regimen for the test cigarettes containing all three levels of the single additive and additive mixtures. These methods are part of the official Health Canada recommended methods and used for regulatory submissions in Canada. The emissions were compared to the additive-free reference cigarette. All 39 priority emissions, as defined by World Health Organisation (WHO) (WHO Technical report series 989, 2015), plus tar, water, nitric oxide (NO) – thus adding up to 42 emissions – and where demanded menthol, glycerol, and propylene glycol were included (see Table S1).

Table 4
3R4F monitor cigarette variability$_{99\%}$ [%] calculations for MS consituents. **Note:** "n": number of studies.

Health Canada Method	Analysis	Units	Average	Std. Dev.	LOD	LOQ	3R4F Monitor Cigarette Variability (%)
	Ammonia						
T-101	Ammonia	[µg/cig]	10.2	1.1	0.731	2.44	27.5
	Aromatic Amines						
T-102	1-aminonaphthalene	[ng/cig]	15.3	1.6	0.128	0.425	25.1
T-102	2-aminonaphthalene	[ng/cig]	9.81	0.90	0.166	0.552	22.4
T-102	3-aminobiphenyl	[ng/cig]	2.19	0.18	0.021	0.069	19.8
T-102	4-aminobiphenyl	[ng/cig]	1.53	0.11	0.021	0.069	17.9
	Benzo[a]pyrene						
T-103	Benzo[a]pyrene	[ng/cig]	6.28	0.65	0.211	0.704	25.3
	Selected Carbonyls						
T-104	Formaldehyde	[µg/cig]	25.8	2.9	0.361	1.20	27.2
T-104	Acetaldehyde	[µg/cig]	601	47	0.973	3.24	19.1
T-104	Acetone	[µg/cig]	252	21	0.846	2.82	20.0
T-104	Acrolein	[µg/cig]	53.1	5.0	0.713	2.38	22.9
T-104	Propionaldehyde	[µg/cig]	44.3	3.6	1.00	3.34	19.7
T-104	Crotonaldehyde	[µg/cig]	11.7	1.7	0.988	3.29	35.1
T-104	MEK	[µg/cig]	64.5	5.9	1.10	3.66	22.6
T-104	Butyraldehyde	[µg/cig]	30.2	2.8	0.812	2.71	22.8
	Hydrogen Cyanide						
T-107	Total HCN	[µg/cig]	93.9	10.1	0.525	1.75	26.3
	Mercury						
T-108	Mercury	[ng/cig]	2.23	0.26	0.429	1.43	28.8
	Toxic Trace Metals						
T-109	Cadmium	[ng/cig]	27.1	3.8	0.477	1.59	34.5
T-109	Lead	[ng/cig]	8.39	1.72	3.85	12.8	N/A
T-109	Arsenic	[ng/cig]	2.74	0.47	1.12	3.75	N/A
	Oxides of Nitrogen						
T-110	NO	[µg/cig]	201	13	0.810	2.70	15.8
T-110	NOx	[µg/cig]	217	14	2.26	7.52	16.3
	Tobacco Specific Nitrosamines (GC/TEA)						
T-111	NNN	[ng/cig]	113	9	1.49	4.95	19.6
T-111	NAT	[ng/cig]	118	10	1.87	6.25	19.9
T-111	NAB	[ng/cig]	14.6	1.5	0.633	2.11	24.5
T-111	NNK	[ng/cig]	97.3	7.7	3.72	12.4	19.3
	Pyridine, Quinoline and Styrene (Semi-Volatiles)						
T-112	Pyridine	[µg/cig]	8.01	1.37	0.237	0.791	41.9
T-112	Quinoline	[µg/cig]	0.195	0.020	0.007	0.024	25.5
T-112	Styrene	[µg/cig]	5.84	0.97	0.167	0.558	40.8
	Phenolic Compounds						
T-114	Hydroquinone	[µg/cig]	31.7	2.6	0.927	3.09	20.2
T-114	Resorcinol	[µg/cig]	0.622	0.138	0.521	1.74	N/A
T-114	Catechol	[µg/cig]	36.1	3.4	3.15	10.5	22.8
T-114	Phenol	[µg/cig]	6.79	0.97	0.623	2.08	34.9
T-114	m+p Cresols	[µg/cig]	6.03	0.74	0.460	1.53	29.9
T-114	o-Cresol	[µg/cig]	2.15	0.31	0.322	1.07	35.8
	Tar, Nicotine, and Carbon Monoxide						
T-115	Tar	[mg/cig]	8.14	0.45	0.071	0.237	13.6
T-115	Nicotine	[mg/cig]	0.675	0.040	0.001	0.004	14.7
T-115	CO	[mg/cig]	10.3	0.6	0.067	0.223	15.2
T-115	Water	[mg/cig]	0.678	0.173	0.038	0.128	62.4
T-115	Propylene Glycol	[mg/cig]	0.005	0.002	0.002	0.008	N/A
T-115	Menthol	[mg/cig]	0.001	0.003	0.002	0.008	N/A
T-115	Glycerol	[mg/cig]	0.745	0.070	0.014	0.048	23.0
	Selected Volatiles						
T-116	1,3-Butadiene	[µg/cig]	33.7	4.2	0.291	0.971	30.7
T-116	Isoprene	[µg/cig]	302	31	2.66	8.86	24.8
T-116	Acrylonitrile	[µg/cig]	7.70	0.92	0.282	0.939	29.3
T-116	Benzene	[µg/cig]	34.3	3.1	1.39	4.63	22.3
T-116	Toluene	[µg/cig]	58.5	6.2	2.50	8.32	26.0

1- Samples generated under 'ISO' smoking conditions:
35mL puff volume; 60 second interval; 2 second duration; 100% vent blocking

Not quantifiable in Monitor cigarette 3R4F. No Equivalence Range can be calculated.

$$3R4F\ Monitor\ Cigarette\ Variability_{99\%}[\%] = 3 \times \sqrt{2} \times RSD[\%] \times \sqrt{\frac{1}{n}}$$

n = 3

Table 5
3R4F monitor cigarette variability$_{99\%}$ [%] calculations for toxicology test methods statistics.
Note: Based on Labstat's extensive experience with the mandated Health Canada methods, it is Labstast's opinion that the variability of the results generated under the ISO smoking regimen were not significantly different from what has been determined under the Health Canada Intense smoking regimen. "n": number of studies.

Equivalence Range$_{99\%}$ [%] Calculations for Toxicology Test Method Statistics and Analyte Yields from Mainstream TPM[1] of Kentucky Reference 3R4F Cigarettes

Health Canada Method	Assay Control/ Analysis	Units	Average	Std. Dev.	n	LOD	LOQ	Percentage Below LOQ	3R4F Monitor Cigarette Variability (%)*
	Ames Regression Slope								
T-501	TA98 (+S9)	[revertants/mg TPM]	1478	249	250				41.3
T-501	TA98 (-S9)	[revertants/mg TPM]	77.4	30.6	239				96.8
T-501	TA100 (+S9)	[revertants/mg TPM]	577	152	234				64.4
T-501	TA100 (-S9)	[revertants/mg TPM]	240	92	234				94.1
T-501	TA102 (+S9)	[revertants/mg TPM]	258	209	226				not determined
T-501	TA102 (-S9)	[revertants/mg TPM]	79	108	221				not determined
T-501	TA1535 (+S9)	[revertants/mg TPM]	16.1	13.5	228				not determined
T-501	TA1535 (-S9)	[revertants/mg TPM]	9.5	10.0	228				not determined
T-501	TA1537 (+S9)	[revertants/mg TPM]	235	48	229				50.2
T-501	TA1537 (-S9)	[revertants/mg TPM]	23.4	12.6	223				not determined
	NRU IC_{50}								
T-502	Particulate Phase (PP)	[µg TPM/mL DMSO]	90.3	13.7	516				37.1
T-502	Gas/Vapour Phase (GVP)	[µg TPM eq./mL PBS]	133	18	436				33.3
	ivMN Regression Slope								
T-503	Schedule 1	[%MN/(mg TPM/mL)]	5.26	0.33	217				15.3
T-503	Schedule 2	[%MN/(mg TPM/mL)]	3.73	0.34	216				22.6
T-503	Schedule 3	[%MN/(mg TPM/mL)]	13.1	0.9	86				17.7

1- Samples generated under 'Health Canada Intense (HCI)' smoking conditions:
55mL puff volume; 30 second interval; 2 second duration; 100% vent blocking

Ames regression slope for Monitor cigarette 3R4F TPM not significantly different from zero (0).
No Equivalence Range can be calculated.

$$3R4F\ Monitor\ Cigarette\ Variability_{99\%}[\%] = 3 \times \sqrt{2} \times RSD[\%] \times \sqrt{\frac{1}{n}}$$

n = 3

Propylene glycol, menthol, and glycerol were determined in smoke to calculate the corresponding transfer rates.

Chemical analyses were performed according to the methods published by Health Canada, which can be obtained upon request (methods available at https://www.canada.ca/en/health-canada/services/health-concerns/tobacco/legislation/federal-regulations/tobacco-reporting-regulations.html).

Results were calculated on a yield per cigarette basis.

2.4.1. Total particulate matter, water, nicotine, and carbon monoxide

Total particulate matter (TPM), water, nicotine, nicotine-free dry particulate matter (NFDPM or 'tar'), carbon monoxide (CO), and puff number were determined according to Health Canada Method T-115 following ISO 3308 and ISO 4387 (ISO 4387, 2000). Five cigarettes were smoked using a standard 20-port linear smoking machine, equipped with a CO analyzer, onto a glass fiber filter pad. The gas phase was introduced into a non-dispersive infrared analyzer, and the percentage of CO was determined. The pre-weighted pad was re-weighted after sample collection with the difference calculated as TPM. The isopropanol extract of the pad was analyzed for nicotine by gas chromatography with flame ionization detector (GC-FID) and water by a thermal conductivity detector. NFDPM was calculated by subtracting water and nicotine from the TPM result.

2.4.2. Carbonyl compounds

Formaldehyde, acetaldehyde, acetone, acrolein, propionaldehyde, crotonaldehyde, and butyraldehyde were determined according to Health Canada Method T-104. Two cigarettes were smoked using a standard 20-port linear smoking machine into a fritted impinger trap with an acidified solution of 2,4-dinitrophenylhydrazine (DNPH) in acetonitrile. An aliquot of the reacted DNPH-smoke extract was then syringe-filtered and diluted with 1% 2-amino-2-(hydroxymethyl)-1,3-propanediol (Trizma® base) in aqueous acetonitrile. The samples were subjected to reverse-phase high-performance liquid chromatography (HPLC), and the carbonyls were quantified with ultra violet spectroscopic detection.

2.4.3. Phenolic compounds

Hydroquinone, resorcinol, catechol, phenols, m + p-cresols, and o-cresol were determined according to Health Canada Method T-114. Five cigarettes were smoked using a standard 20-port linear smoking machine through a glass fiber filter disc (pad). The pad was then extracted with 1% acetic acid. An aliquot of the TPM extract was syringe filtered, diluted and subjected to reversed-phase gradient HPLC, and the phenolic compounds were quantified with selective fluorescence detection.

2.4.4. Benzo[a]pyrene

Benzo[a]pyrene (B[a]P) was determined according to Health Canada Method T-103. Five cigarettes were smoked using a standard

Table 6
Transfer rates.

No.	Additive	Levels of Additive	Targeted Amount of Tobacco Additive	Achieved Amount of Tobacco Additive	Cig. Tob. Weight [mg/cig]	Amount of Tobacco Additive in Cig. Tob. [mg/cig]	Transfer into MS [µg/cig][b]	Transfer [%]
1	Reference cigarette (without tobacco additives)	–	–	–	622	–	–	–
2	Carob bean extract	Low	0.2000%	[a]	594	–	–	–
3	Carob bean extract	Max	0.4000%	[a]	623	–	–	–
4	Carob bean extract	Max Plus	0.6000%	[a]	604	–	–	–
5	Cocoa Powder	Low	0.5000%	0.6046%	610	0.085 theobromine	3.52 theobromine	4.2
6	Cocoa Powder	Max	1.0000%	1.0141%	619	0.144 theobromine	6.51 theobromine	4.5
7	Cocoa Powder	Max Plus	1.5000%	1.4400%	608	0.201 theobromine	10.21 theobromine	5.1
8	Fenugreek extract	Low	0.0100%	[a]	641	–	–	–
9	Fenugreek extract	Max	0.0200%	[a]	660	–	–	–
10	Fenugreek extract	Max Plus	0.0300%	[a]	649	–	–	–
11	Fig Juice concentrate	Low	0.0250%	[a]	613	–	–	–
12	Fig Juice concentrate	Max	0.1500%	[a]	634	–	–	–
13	Fig Juice concentrate	Max Plus	0.3000%	[a]	607	–	–	–
14	Geraniol	Low	0.0150%	0.0161%	621	0.10	7.83	7.8
15	Geraniol	Max	0.0300%	0.0251%	598	0.15	11.1	7.4
16	Geraniol	Max Plus	0.0450%	0.0529%	614	0.32	24.1	7.5
17	Glycerol	Low	2.5000%	2.303%	627	14.4	657	4.6
18	Glycerol	Max	5.0000%	4.326%	636	27.5	1153	4.2
19	Glycerol	Max Plus	6 + %	5.974%	645	38.5	1730	4.5
20	Guaiacol	Low	0.0005%	0.000559%	612	0.0034	0.25[c]	–[c]
21	Guaiacol	Max	0.0010%	0.000877%	600	0.0053	0.18[c]	–[c]
22	Guaiacol	Max Plus	0.0015%	0.001555%	600	0.0093	0.27[c]	–[c]
23	Guar gum	Low	0.5000%	Estimated[a] between 0.33 and 0.55%	610	2.01 to 3.36	–	–
24	Guar gum	Max	1.0000%	Estimated[a] between 1.03 and 1.18%	601	6.19 to 7.09	–	–
25	Guar gum	Max Plus	1.5000%	Estimated[a] between 1.44 and 1.57%	639	9.20 to 10.03	–	–
26	Liquorice extract powder	Low	0.6000%	0.420%	624	2.62	< 1.0 (glycyrrhizin)	0
27	Liquorice extract powder	Max	1.2000%	1.050%	621	6.52	< 1.0 (glycyrrhizin)	0
28	Liquorice extract powder	Max Plus	1.8000%	1.782%	603	10.75	< 1.0 (glycyrrhizin)	0
29	Maltol	Low	0.0050%	0.0045%	617	0.028	1.2	4.3
30	Maltol	Max	0.0100%	0.0079%	621	0.049	2.6	5.3
31	Maltol	Max Plus	0.0150%	0.0111%	618	0.069	3.6	5.2
32	*l*- Menthol, synthetic	Low	0.6000%	0.552%	606	3.35	400	11.9
33	*l*- Menthol, synthetic	Max	1.2000%	1.142%	650	7.42	750	10.1
34	*l*- Menthol, synthetic	Max Plus	1.8000%	1.730%	625	10.8	1170	10.8
35	Propylene glycol	Low	2.5000%	2.05%	616	12.6	114	0.9
36	Propylene glycol	Max	5.0000%	4.53%	571	25.9	236	0.9
37	Propylene glycol	Max Plus	6 + %	4.80%	617	29.6	286	1.0
38	Sorbitol	Low	0.6000%	0.65%	604	3.93	–	–
39	Sorbitol	Max	1.2000%	1.10%	620	6.82	–	–
40	Sorbitol	Max Plus	1.8000%	1.60%	614	9.82	–	–

[a] No standardized method was available to determine the Achieved Amount of the additive(s).
[b] Background-corrected.
[c] Transfer could not be determined due to a high background level in the additive-free reference cigarette.

20-port linear smoking machine through a glass fiber filter pad. The pad was then extracted with cyclohexane. A portion of this solution was filtered through a PTFE filter. An aliquot was passed through a silica cartridge and NH_2 plus cartridge, in series. The B[*a*]P was eluted with hexane, evaporated under a constant stream of nitrogen to dryness, and reconstituted to a constant volume with acetonitrile. The sample was subjected to reversed-phase HPLC and quantified for B[*a*]P via fluorescence detection.

2.4.5. Aromatic amines

1-aminonaphthalene, 2-aminonaphthalene, 3-aminobiphenyl, and 4-aminobiphenyl were determined according to Health Canada Method T-102. Ten cigarettes were smoked using a standard 20-port rotary smoking machine through a glass fiber filter pad. The pad was quartered and extracted with 5% hydrochloric acid solution. After shaking for 30 min, the contents were filtered into a separatory funnel. The filtrate was washed with dichloromethane, made basic with sodium hydroxide solution and extracted with hexane. The hexane extracts were dried with sodium sulphate, derivatized with pentafluoropropionic acid anhydride and trimethylamine, concentrated by rotary evaporation, passed through a Florisil column, and the amines were quantified using gas chromatography - mass spectrometry (GC/MS) under full scan mode.

2.4.6. Oxides of nitrogen

NO and NO_x were determined according to Health Canada Method T-110. One cigarette was smoked on a standard single-port smoking machine through a glass fiber filter pad. The resulting gas/vapor phase (GVP) was exhausted puff by puff into an evacuated smoke mixing chamber located directly behind the pad. The GVP was mixed and an aliquot of each puff was routed by vacuum through a filter to a dual-channel real-time chemiluminescence nitrogen oxides analyzer where

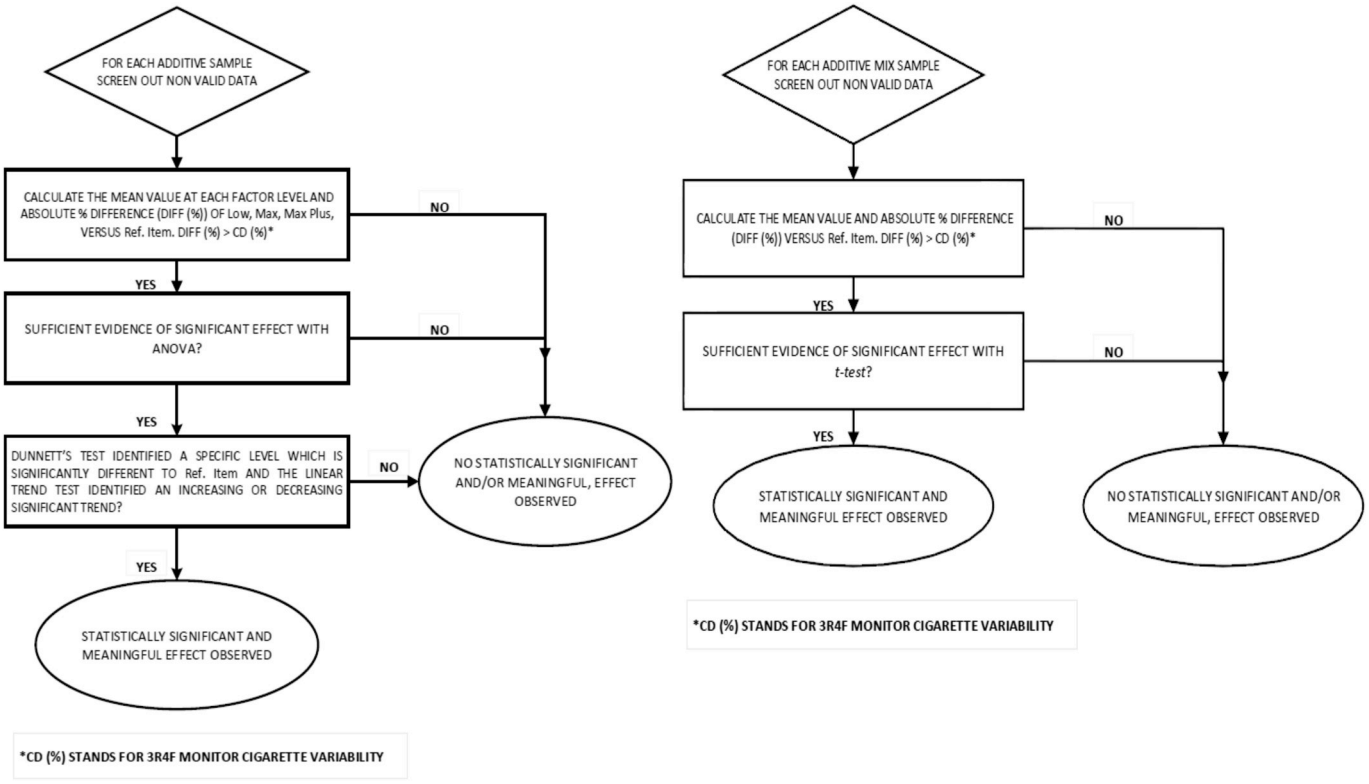

Fig. 1. Assessment rules for each of the 13 additives (left) and the three mixtures (right).

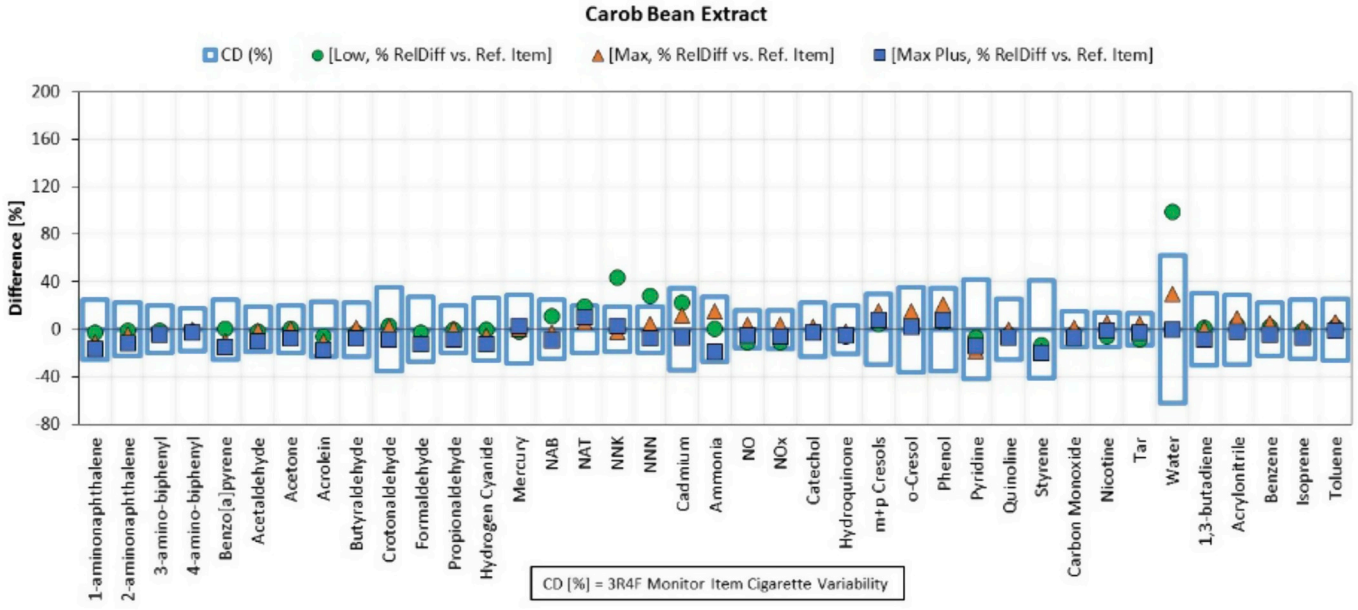

Fig. 2. Relative percentage difference between yields of measured analytes in MS from cigarettes containing Low (0.2%), Max (0.4%), and Max Plus (0.6%) levels of carob bean extract and the corresponding reference item containing no additive.

the gas stream was split immediately into two channels. ln channel A, the sample stream was reacted with ozone and the resultant chemiluminescent emission was directly proportional to the NO concentration and quantified in the sample. ln channel B, the sample stream was chemically reduced first by a catalytic converter and then mixed with ozone in the reaction cell where the resultant chemiluminescent emission was due to NO_x or NO + NO_2. Selective photomultiplier detection monitors the reaction cell gas stream, and the NO and NO_x found in the vapor phase of mainstream tobacco smoke were quantified by external standard calibration.

2.4.7. Hydrogen cyanide

Hydrogen cyanide was determined according to Health Canada Method T-107. Five cigarettes were smoked on a standard linear smoking machine through a glass fiber filter pad with an impinger trap with 0.1 N NaOH directly located behind the pad. The pad was extracted with 0.1 N NaOH and both the extract and the impinger solution were derivatized in an automated continuous flow analyzer with

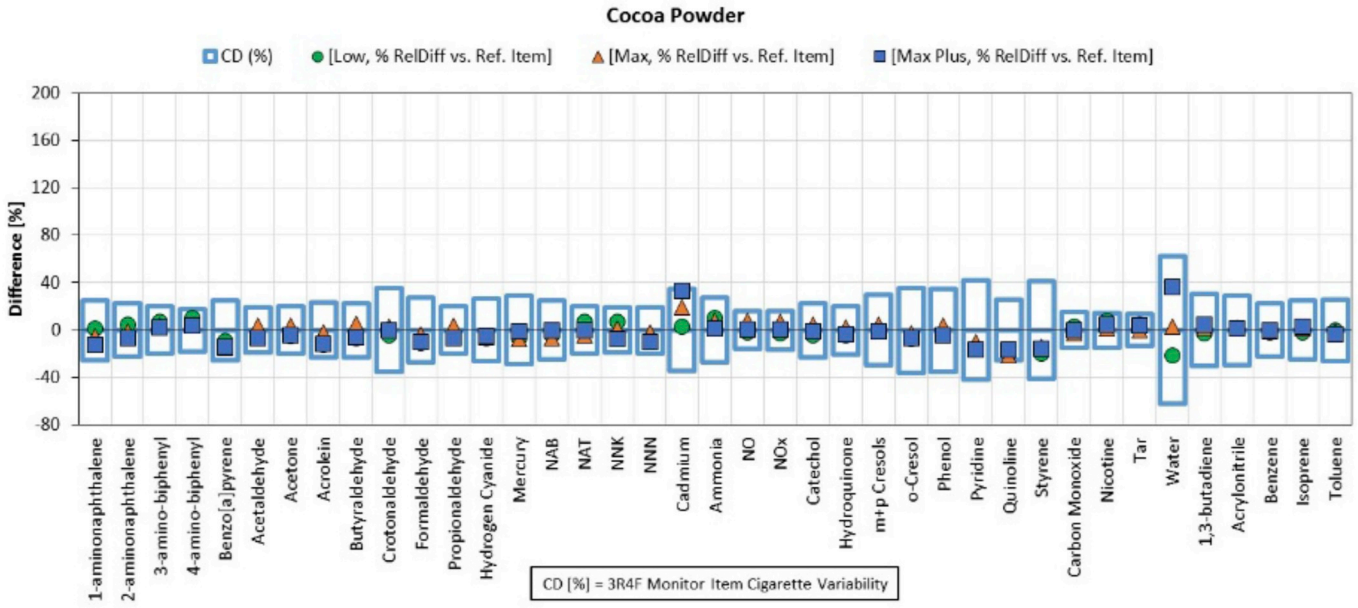

Fig. 3. Relative percentage difference between yields of measured analytes in MS from cigarettes containing Low (0.5%), Max (1.0%), and Max Plus (1.5%) levels of cocoa powder and the corresponding reference item containing no additive.

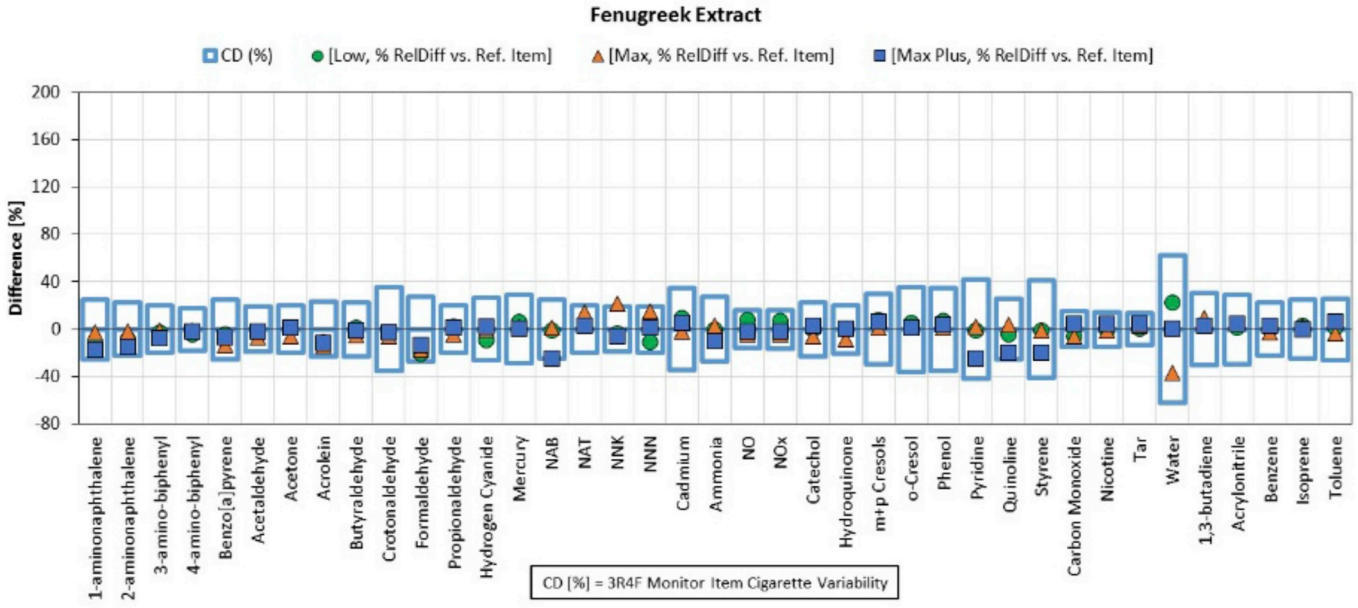

Fig. 4. Relative percentage difference between yields of measured analytes in MS from cigarettes containing Low (0.01%), Max (0.02%), and Max Plus (0.03%) levels of fenugreek extract and the corresponding reference item containing no additive.

chloramine-T, pyridine, and a pyrazolone reagent to a colored complex that was quantified calorimetrically.

2.4.8. Ammonia

Ammonia was determined according to Health Canada Method T-101. Five cigarettes were smoked on a standard rotary smoking machine through a glass fiber filter pad with two impinger traps with 0.1 N sulfuric acid directly located behind the pad. The pad was extracted with the contents of the two impingers. The mixture was then filtered and analyzed by cation exchange chromatography.

2.4.9. Mercury

Mercury was determined according to Health Canada Method T-108. Twenty cigarettes were smoked on a standard rotary smoking machine into two impinger traps containing acidified potassium permanganate solution. The solutions were subjected to microwave digestion. Excess potassium permanganate was reduced with hydroxylamine hydrochloride and made to a final volume of 100 mL. The digestate was then analyzed via cold vapor atomic absorption spectroscopy at 253.7 nm using a continuous flow vapor generator to reduce the divalent mercury to its atomic state with stannous chloride.

2.4.10. Trace elements

Lead, cadmium, and arsenic were determined according to Health Canada Method T-109. Twenty cigarettes were smoked on a standard rotary smoking machine equipped with an electrostatic precipitator (EP) to collect the particulate matter onto a glass EP tube. The TPM was extracted with methanol. The methanol was then evaporated and the

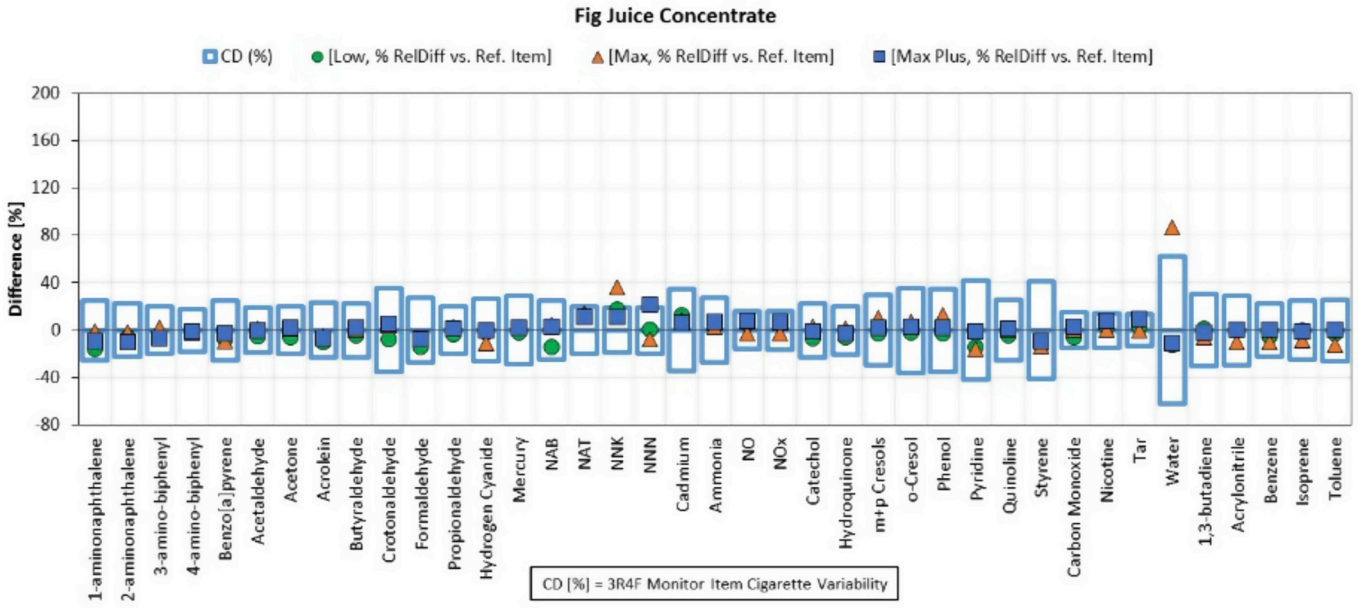

Fig. 5. Relative percentage difference between yields of measured analytes in MS from cigarettes containing Low (0.025%), Max (0.15%), and Max Plus (0.30%) levels of fig juice concentrate and the corresponding reference item containing no additive.

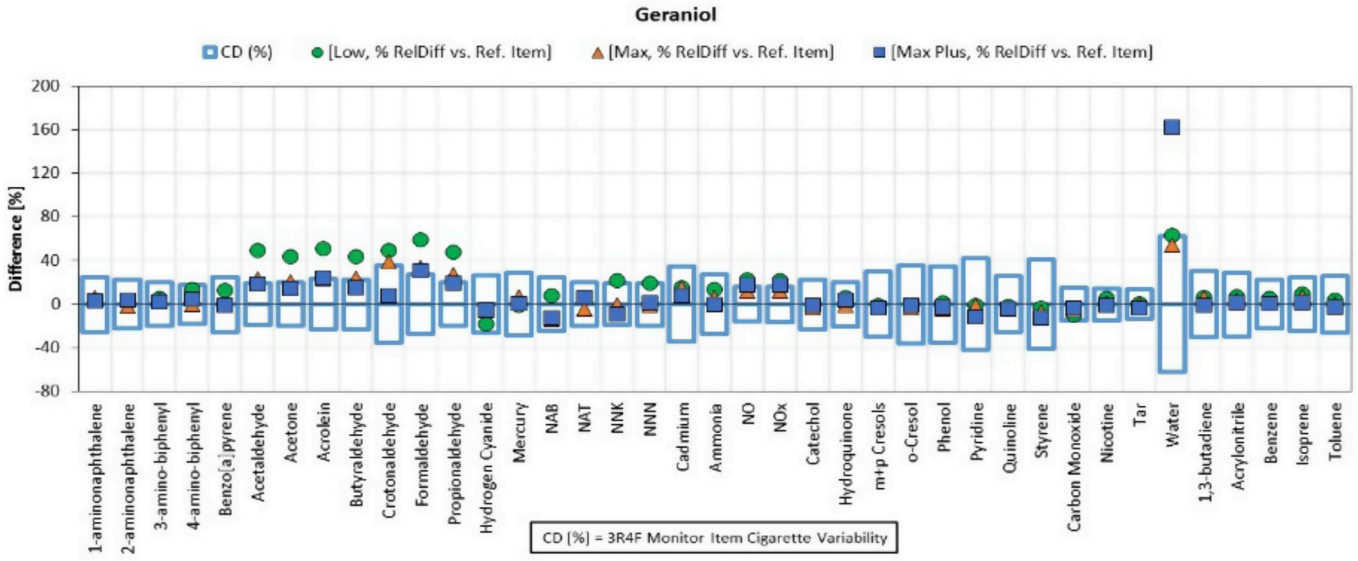

Fig. 6. Relative percentage difference between yields of measured analytes in MS from cigarettes containing Low (0.015%), Max (0.030%), and Max Plus (0.045%) levels of geraniol and the corresponding reference item containing no additive.

remaining sample was subjected to microwave digestion using a mixture of hydrochloric acid, nitric acid and hydrogen peroxide. The gaseous phase metals were trapped by placing an impinger containing a 10% nitric acid solution between the tube and the smoking machine. The impinger solution was added to the same digestion vessel as the TPM. The digest was analyzed by inductively coupled plasma mass spectrometry or inductively coupled argon plasma atomic emission spectrometry.

2.4.11. *Pyridine, quinoline, and styrene*

Pyridine, quinoline, and styrene were determined according to Health Canada Method T-112. Twenty cigarettes were smoked on a standard rotary smoking machine through a glass fiber filter pad with two cryogenic traps with methanol directly located behind the pad. The pad was extracted with the methanol from the two cryogenic traps. An aliquot of the extract was syringe filtered into an auto-sampler vial and analyzed using GC/MS under full scan mode.

2.4.12. *1,3-Butadiene, isoprene, acrylonitrile, benzene, and toluene*

1,3-Butadiene, isoprene, acrylonitrile, benzene, and toluene were determined according to Health Canada Method T-116. Twenty cigarettes were smoked on a standard rotary smoking machine through a glass fiber filter pad with two cryogenic traps with methanol directly located behind the pad. An aliquot of the extract was syringe filtered into an auto-sampler vial and analyzed using GC/MS under full scan mode.

2.4.13. *Tobacco-specific nitrosamines*

N-nitrosonornicotine (NNN), 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK), *N*-nitrosoanatabine (NAT), and *N*-nitrosoanabasine (NAB) were determined according to a laboratory-specific method. Five cigarettes were smoked on a standard linear smoking

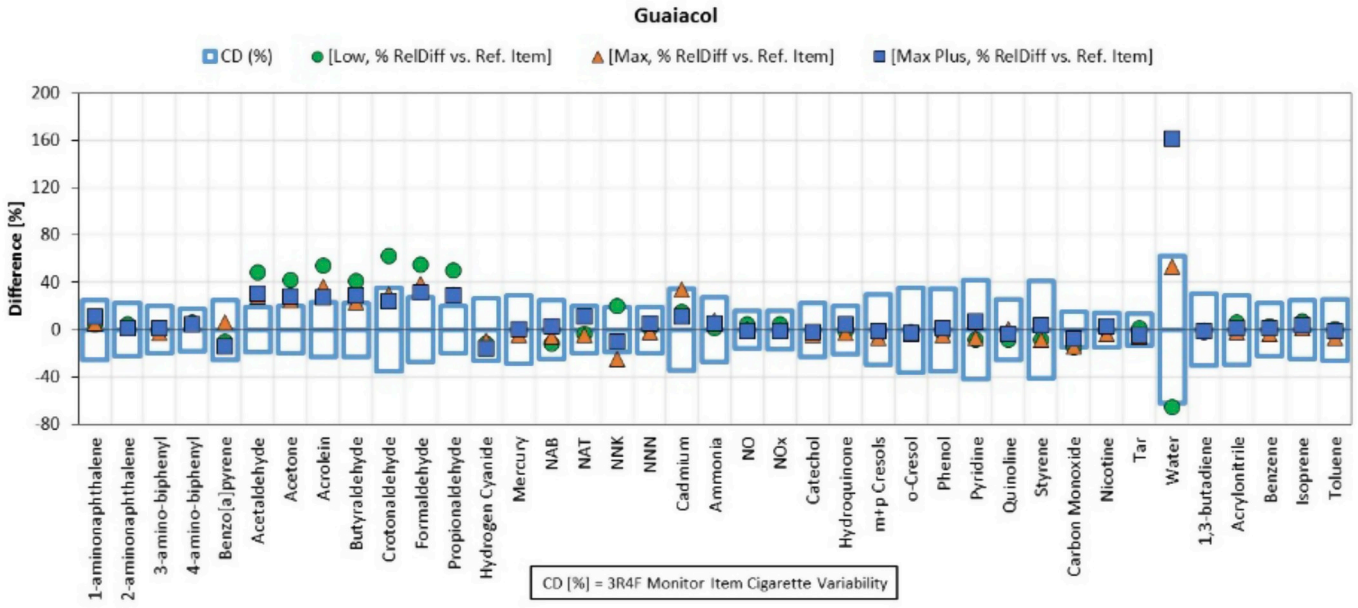

Fig. 7. Relative percentage difference between yields of measured analytes in MS from cigarettes containing Low (0.0005%), Max (0.0010%), and Max Plus (0.0015%) levels of guaiacol and the corresponding reference item containing no additive.

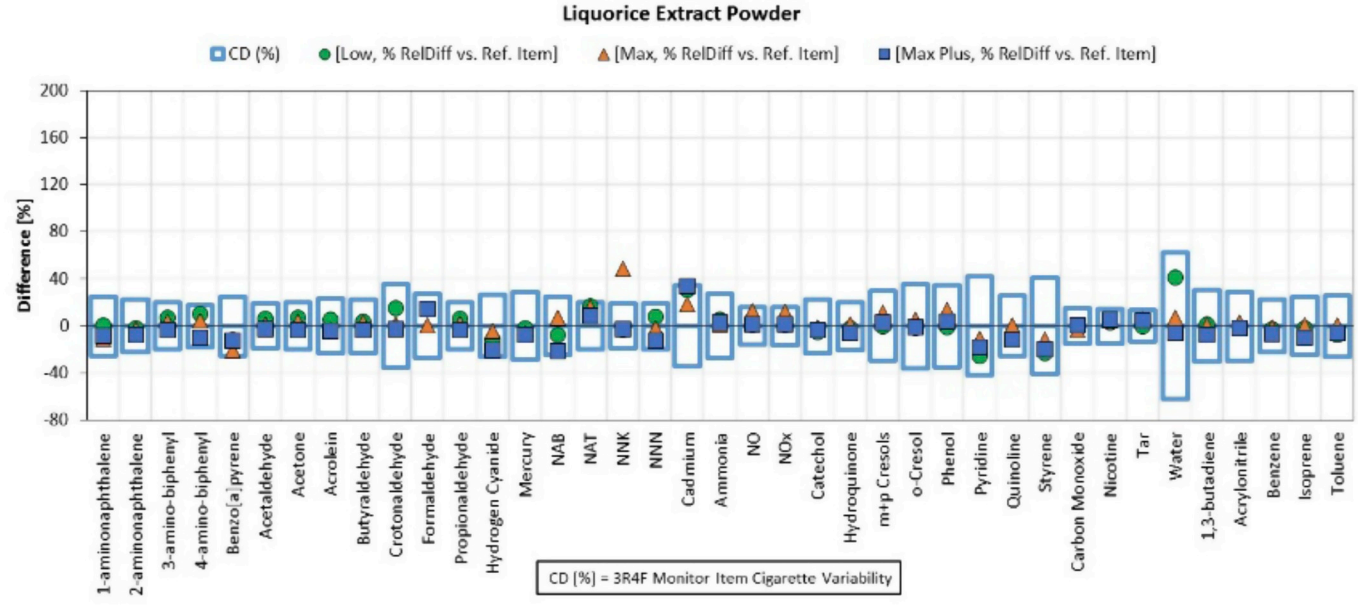

Fig. 8. Relative percentage difference between yields of measured analytes in MS from cigarettes containing Low (0.6%), Max (1.2%), and Max Plus (1.8%) levels of liquorice extract powder and the corresponding reference item containing no additive.

machine through a glass fiber filter pad. The pad was extracted with a 100 mM ammonium acetate solution. The extract was filtered and subject to LC-MS/MS analysis with positive electrospray ionization.

2.4.14. Menthol, glycerol, and propylene glycol in MS

Menthol, glycerol, and propylene glycol were determined according to Health Canada Method T-304/T-115. Five cigarettes were smoked on a standard linear smoking machine through a glass fiber filter pad. The pad was extracted with isopropanol and the extract was analyzed using GC-FID. This method was only applied to MS from test cigarettes containing menthol, glycerol or propylene glycol in order to calculate the transfer of these additives into MS.

2.4.15. Additive transfer rates

Additive concentrations in the tobacco of the cigarettes and in the smoke were determined where analytical methods were available: geraniol, glycerol, guaiacol, maltol, menthol, propylene glycol. For cocoa and liquorice, the lead substances theobromine and glycyrrhizin, respectively, were determined.

The analysis of additives in tobacco was performed by BAT; the analysis of menthol, propylene glycol and glycerol in smoke by Labstat; and the analysis of geraniol, guaiacol, maltol, theobromine, and glycyrrhizin by aromaLab, results of which are presented in Table 6.

2.5. In vitro toxicity

In vitro toxicology assays were conducted by the independent testing

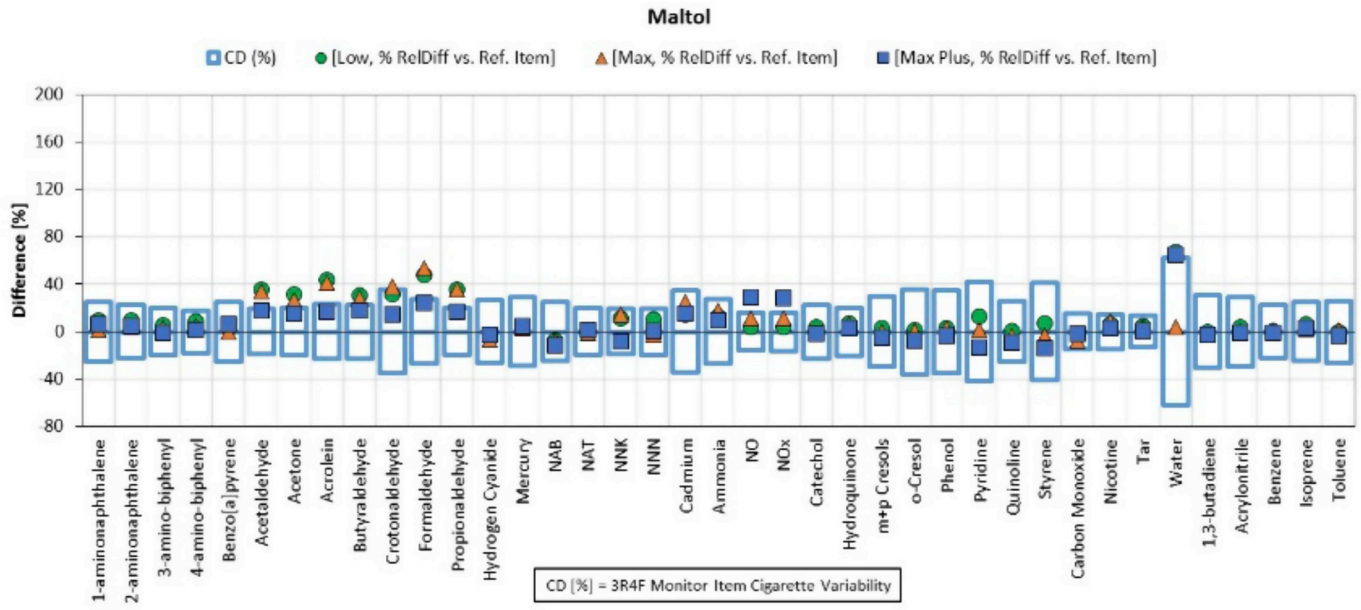

Fig. 9. Relative percentage difference between yields of measured analytes in MS from cigarettes containing Low (0.005%), Max (0.010%), and Max Plus (0.015%) levels of maltol and the corresponding reference item containing no additive.

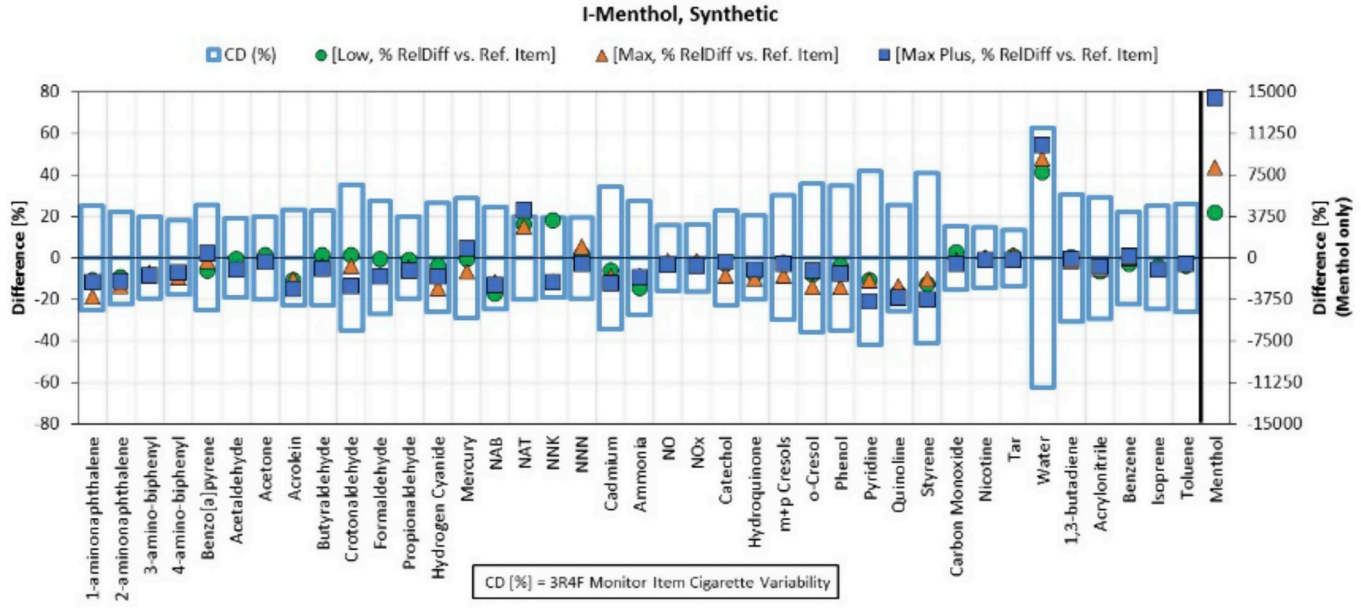

Fig. 10. Relative percentage difference between yields of measured analytes in MS from cigarettes containing Low (0.6%), Max (1.2%), and Max Plus (1.8%) levels of *l*-menthol, synthetic and the corresponding reference item containing no additive.

CRO Labstat in Canada in compliance with the applicable requirements of the Organisation for Economic Co-operation and Development (OECD) Principles of Good Laboratory Practice (GLP) as revised on November 26th, 1997 [C(97)186/Final] and of 21 CFR Part 58 (Code of Federal Regulations, Food and Drug Administration) Good Laboratory Practices for Nonclinical Laboratory Studies as amended on May 21st, 2002. Labstat conducted the *in vitro* studies, in accordance with OECD guidelines, and/or validated, and standardized methods applied by Health Canada for tobacco regulatory reporting purposes. These assays were used to investigate mutagenicity (Ames), cytotoxicity (neutral red uptake (NRU)) and genotoxicity (*in vitro* micronucleus (ivMN)) of MS TPM and cytotoxicity (NRU) of MS GVP generated under the ISO smoking regimen for the reference and test cigarettes containing all three levels of the single additive and the mixtures.

For the mutagenicity and genotoxicity sample collection, MS of 20 cigarettes was trapped onto a glass fiber filter. The TPM trapped on the filter was extracted with dimethylsulphoxide (DMSO) to achieve a target concentration of 10 mg TPM/mL DMSO. For cytotoxicity, MS of 20 cigarettes was passed through a glass fiber filter for TPM collection, and into the cooled impinger containing phosphate-buffered saline (PBS) for GVP collection. Both smoke fractions were prepared to a target concentration of 10 mg TPM/mL DMSO or 10 mg TPM equivalent/mL PBS in the case of GVP and applied to the cells within one hour.

The biological assay responses obtained with the test cigarettes were compared to those of the additive-free reference cigarette when the assay results were considered as valid based on assay-specific acceptance criteria (e.g., evaluation of positive and negative controls, 3R4F monitor cigarette results).

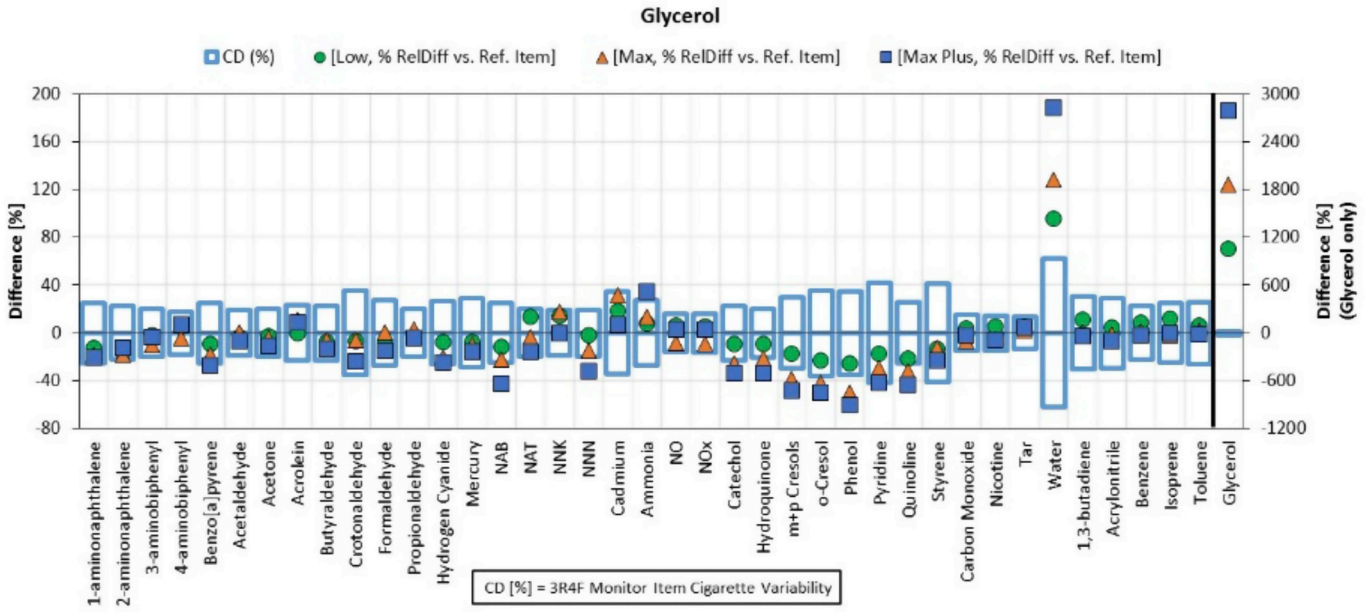

Fig. 11. Relative percentage difference between yields of measured analytes in MS from cigarettes containing Low (2.5%), Max (5.0%), and Max Plus (6 + %) levels of glycerol and the corresponding reference item containing no additive.

2.5.1. Mammalian cell cytotoxicity

The NRU assay on the TPM and GVP of the experimental cigarettes was performed following the requirements of the Health Canada official test method T-502, Second Edition 2004-11-01. In short, Chinese hamster ovary cells (CHO-WBL (IVGT)) obtained from Sigma Aldrich; St. Louis, MO, USA, were cultured in Ham's F-12 media (10% fetal bovine serum, 100 units/mL penicillin, 100µg/mL streptomycin) and exposed for 24 h to a range of concentrations of each TPM and GVP sample. For each sample of the test and additive-free reference cigarette, four replicate 96-well micro titer plates were used, each with eight TPM/GVP concentrations up to 200 µg/mL. Duplicate plates were assayed for the 3R4F monitor cigarette. Each TPM/GVP concentration was replicated six times per micro titer plate. After exposure, the medium was replaced by medium without serum and antibiotics containing the dye neutral red (5 µg/mL). After a three hours incubation period, cells were rinsed and fixed in the wells by a 1% formalin solution. The dye was extracted with a solution of 1% v/v glacial acetic acid, 50% v/v ethanol, and 49% v/v water. The dye concentration, which is directly proportional to the number of viable cells, was determined by the optical density at 540 nm in a microplate reader (BioTek Instruments, Winooski, VT, USA). Sodium lauryl sulphate, 10 µg/mL, was used as the positive control.

The cytotoxic response was characterized as the IC_{50} value (i.e., the concentration that decreased the number of viable cells by 50% relative to the solvent control). Thus, the higher the IC_{50} value, the lower the cytotoxicity of the test substance. The IC_{50} values (mg TPM/mL DMSO and mg TPM equivalent/mL PBS) were calculated from the least square fit of the data to the sigmoid function $y = a/(1 + (x/b)c)$ with x = dose, y = absorbance relative to the solvent control, b = IC_{50}, and a,c = form factors. For each of the three TPM/GVP samples, one IC_{50} was calculated. The mean IC_{50} value was used to characterize each TPM and GVP fraction.

2.5.2. Bacterial mutagenicity

The *Salmonella typhimurium* Reverse Mutation Assay, commonly referred to as the Ames assay, was applied as the plate incorporation version and performed following the requirements of the Health Canada official test method T-501 based on the OECD Guideline No. 471 (1997) with following modification: one replicate testing was performed per day. In short, mutagenicity toward *Salmonella* strains TA98, TA100, TA102, TA1535, and TA1537 was determined in the presence and in the absence of a metabolic activation system consisting of the post-mitochondrial fraction of livers from rats treated with Aroclor 1254 (S9, Molecular Toxicology, Boone, NC, USA). For each sample, eight doses, which were expected to include the linear part of the dose-response curve were prepared and assayed. Each dose was plated in triplicate. For plating, bacteria suspended in culture medium, TPM dissolved in DMSO or DMSO alone, S9 mix or 0.1 mol/L phosphate buffer, pH 7.4 were added to the top agar supplemented with histidine and biotin (0.05 nmol each). The components were mixed and spread evenly on minimal glucose agar plates. After the top agar hardened, the plates were incubated in the dark at 37 ± 1 °C for 48–72 h. The number of His + revertant colonies was determined with an automatic colony counter (aCOLyte 2, SYNBIOSIS, Frederick, MD, USA). Negative and positive strain-specific and S9-specific control substances were assayed concomitantly to check sensitivity and reproducibility.

The mutagenic response was calculated as the slope (revertants/mg TPM) of the linear portion of the Poisson-weighted curve fit of the dose-response. A single slope was calculated for each of the samples.

2.5.3. Mammalian cell genotoxicity

The *iv*MN Assay assay was performed in general accordance with Health Canada Official Method T-503, which is based on the OECD Guideline No. 487 (OECD, 2016). The CHO-WBL cell line purchased from Sigma Aldrich (St. Louis, MO, USA) was used for the assay. The cells were maintained in Ham's nutrient mixture F12 medium (Sigma Aldrich; St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma Aldrich; St. Louis, MO, USA) in a 5% CO_2 incubator at 37 °C ± 2 °C. The TPM fraction was assayed in three treatment schedules: short-term exposure without or with metabolic activation and long-term exposure without metabolic activation. Cell suspension (1 × 105 cells/mL) was pre-incubated for 24 h ± 3 h before treatment. For the short-term exposure, the cell culture was treated with serially diluted test samples for 3 h ± 15 min without or with S9-mix containing Aroclor 1254-induced rat liver homogenate (Molecular Toxicology, Inc.; Boone, NC, USA). After removal of the test sample, the cells were incubated for 27 h ± 1 h. For the long-term exposure, the cells were incubated with test sample for 30 h ± 1 h in the absence of the metabolic activation system. As positive control substances Colchicine (Sigma Aldrich; St. Louis, MO, USA) and mitomycin C (Sigma

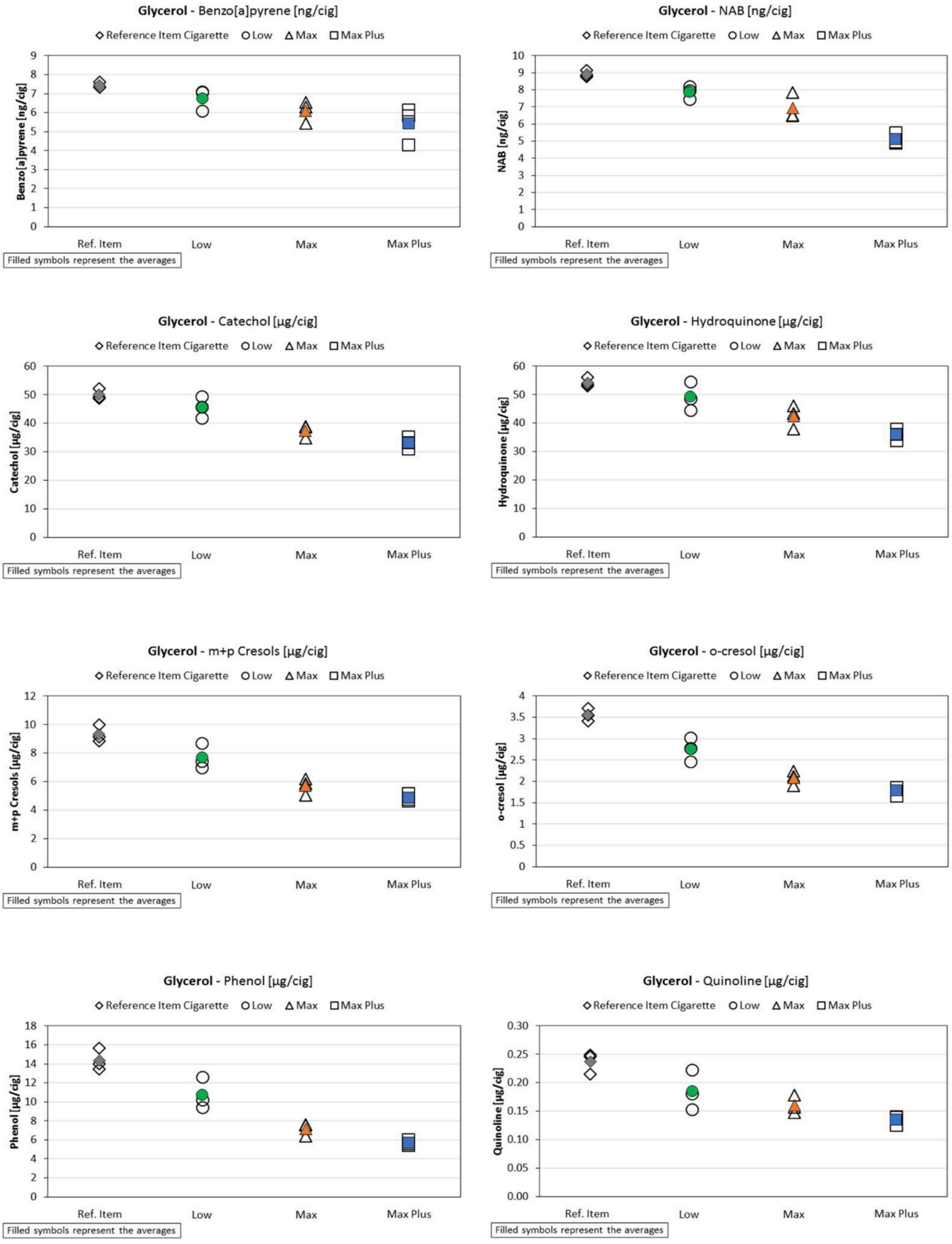

Fig. 12. Statistically significant and meaningful decreases for test cigarettes with glycerol compared to the additive-free reference cigarette.

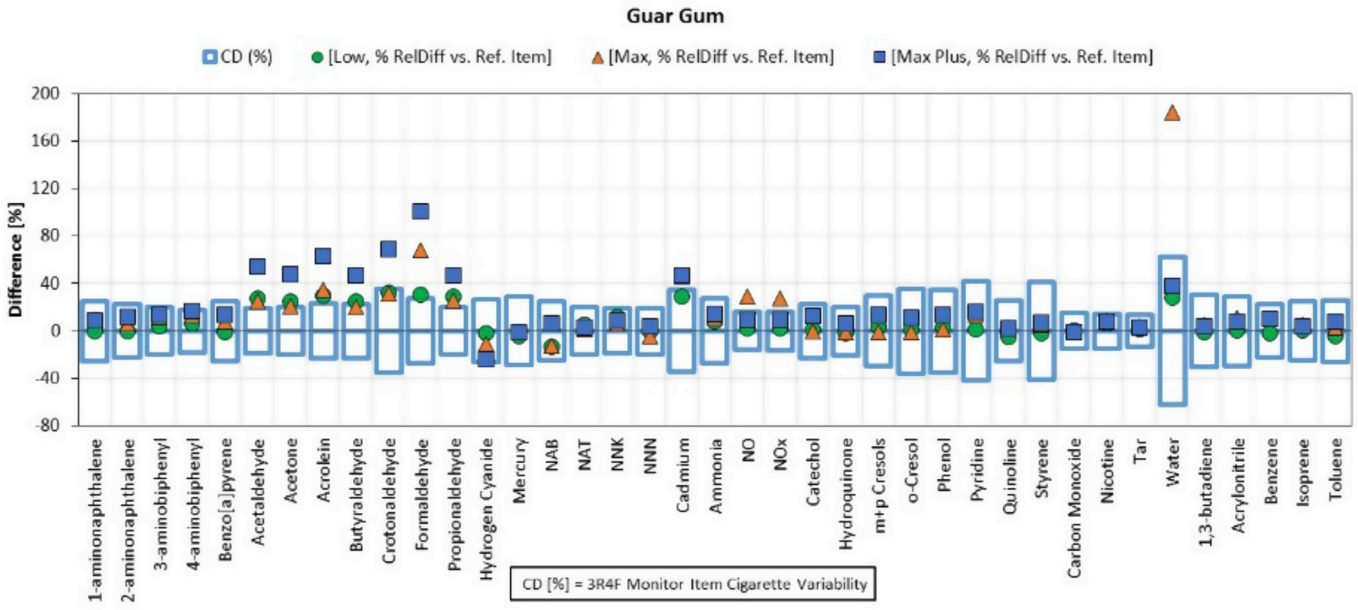

Fig. 13. Relative percentage difference between yields of measured analytes in MS from cigarettes containing Low (0.5%), Max (1.0%), and Max Plus (1.5%) levels of guar gum and the corresponding reference item containing no additive.

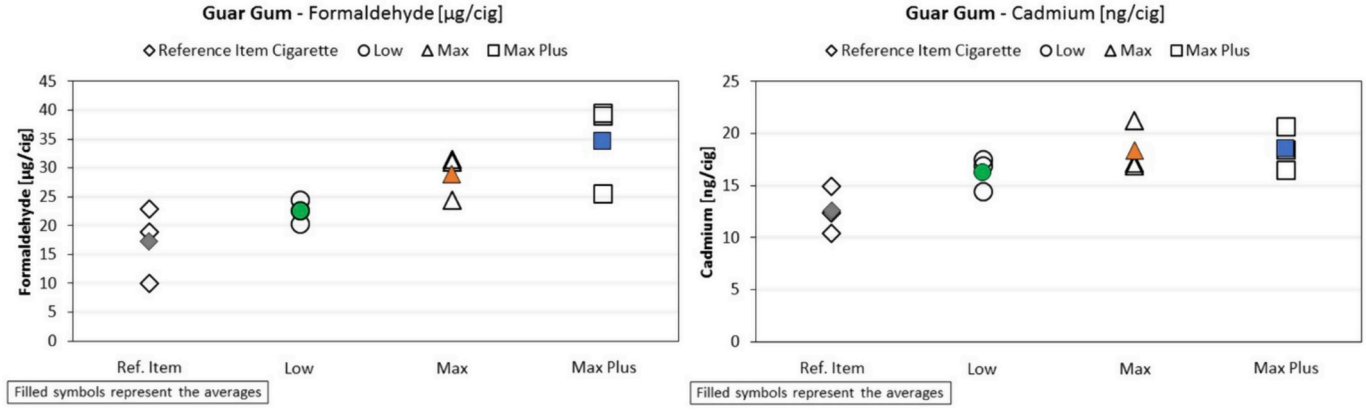

Fig. 14. Statistically significant and meaningful increases for test cigarettes with guar gum compared to the additive-free reference cigarette.

Aldrich; St. Louis, MO, USA) were used in the absence of the metabolic activation system, and cyclophosphamide (Sigma Aldrich; St. Louis, MO, USA) was used in the presence of metabolic activation. DMSO alone was used as solvent control in all experiments. After the incubation period and a recovery period of 27 h ± 1 h the cells were detached and the number of viable cells was counted with a hemocytometer using trypan blue to calculate the relative increase in cell count as a cytotoxicity parameter. The remaining cells were fixed with a solution of glacial acetic acid/methanol (1:3, v/v), placed on glass slides by cytospin centrifugation and stained with acridine orange. All slides were blindly coded and examined manually using fluorescence microscopy. The number of micronucleated cells per 2000 cells (1000 cells/slide, duplicate culture) was scored and the micronucleus (MN) cell frequency (%MN) was calculated. The experiments were conducted twice independently.

For the comparison of genotoxic activity between test samples, linear regression analysis, using the method of ordinary least squares, was performed with data up to a concentration at which the MN frequency was in the linear range. The slope parameter of the function was defined as the genotoxic activity.

2.6. Statistical analyses

Data were characterized by their arithmetic means and standard deviations. For smoke chemistry data, statistical comparisons (see Fig. 1) started with the calculation of the differences between the mean values for each additive level of the test cigarettes and the mean value for the additive-free reference cigarette for each analyte. If the difference exceeded the 3R4F monitor cigarette long-term variability, which served as a measure for the inherent variability of the method (Baker et al., 2004a; Belushkin et al., 2015), it was considered as meaningful. The mean analyte concentrations determined for each test cigarette and additive-free reference were compared using analysis of variance (ANOVA) at $\alpha = 0.05$ with Bonferroni correction for multiple testing. If the ANOVA showed a statistically significant effect between the additive-free reference cigarette and the test cigarette means, comparisons of the mean analyte concentrations among Levels (Low, Max, Max Plus) of each test cigarette with additive to the additive-free reference cigarette were performed using the Dunnett's test (with a family-wise error rate of $\alpha = 0.1$), followed by linear trend analysis. In case of mixtures of additives, the *t*-test was used.

For the *in vitro* assay results, the same principle approach was used.

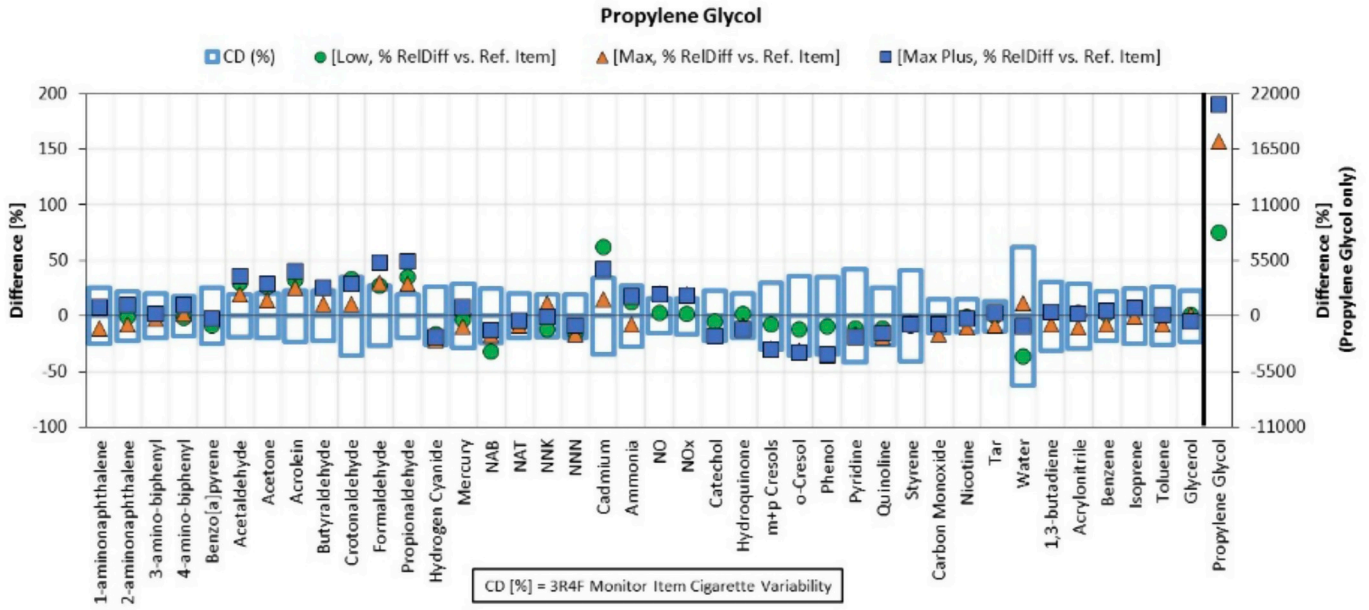

Fig. 15. Relative percentage difference between yields of measured analytes in MS from cigarettes containing Low (2.5%), Max (5.0%), and Max Plus (6 + %) levels of propylene glycol and the corresponding reference item containing no additive.

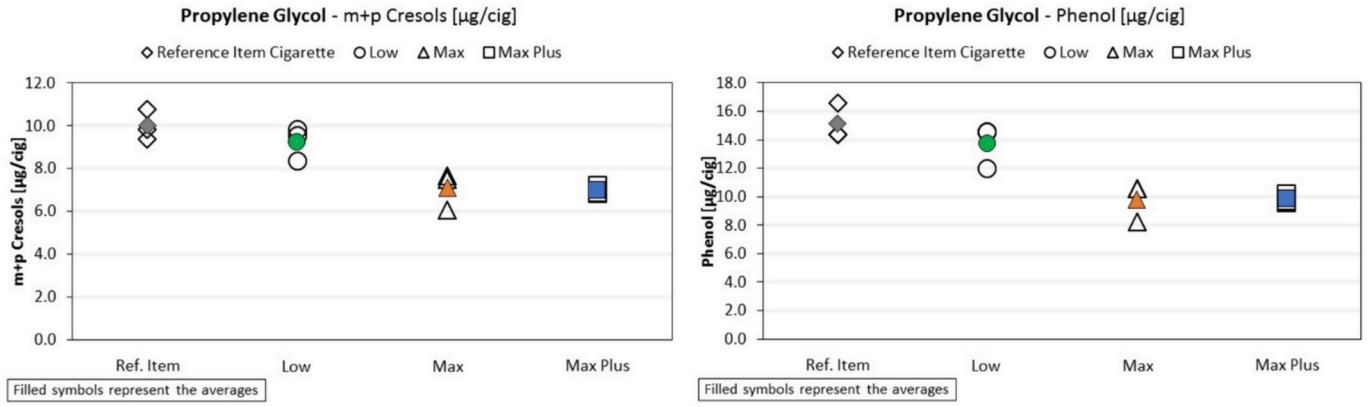

Fig. 16. Statistically significant and meaningful decreases for test cigarettes with propylene glycol compared to the additive-free reference cigarette.

3. Results

3.1. Transfer rates

Transfer rates for cocoa powder, geraniol, glycerol, guaiacol, liquorice extract powder, maltol, *l*-menthol (synthetic) and propylene glycol were calculated from the measured amount in cigarette tobacco and the emissions in MS (Table 6). However, the guaiacol MS yields for the additive-free reference cigarette and the test cigarettes were similar taking into consideration the analytical method variability. Therefore, no reliable transfer rates could be calculated. For liquorice extract powder, glycyrrhizin was below the detection limit in smoke. Therefore, no transfer rates were calculated. For carob bean extract, fenugreek extract, fig juice concentrate, guar gum and sorbitol, no specific transfer markers in smoke are available. Hence, no transfer rates were determined.

3.2. Smoke chemistry

A smoke chemistry study was performed in compliance to GLP requirements by the independent CRO LabStat in Canada analyzing the WHO list of 39 emissions plus glycerol, propylene glycol, menthol, tar, and water contained in MS generated under the ISO smoking regimen for the test cigarettes containing tobacco additives as either a single ingredient using three different levels (i.e. Low, Max, and Max Plus level) or as part of a mixture and for the additive-free reference cigarette. Statistical analysis was used to compare the emissions of the test and additive-free reference cigarettes to each other and to the variability of 3R4F cigarette responses. Analytes not included in statistical analysis, due to non-quantifiable results, were arsenic, lead and resorcinol.

3.2.1. Single additives

3.2.1.1. Carob bean, cocoa, fenugreek, fig, geraniol, guaiacol, liquorice, maltol, and l-menthol. The smoke chemistry study showed no statistically significant and meaningful increases or decreases in any analytes for the test cigarettes containing carob bean, cocoa, fenugreek, fig, geraniol, guaiacol, liquorice, maltol, and *l*-menthol. Beyond the formal assessment that did not identify any statistically significant and consistent additive-level related increases and decreases, there were sporadic cases (e.g., NNK, NNN, and water for carob bean, Fig. 2) in which the relative % difference between the test cigarettes with additive and the additive-free reference cigarette did exceed the inherent variability of the analytical method. However, these

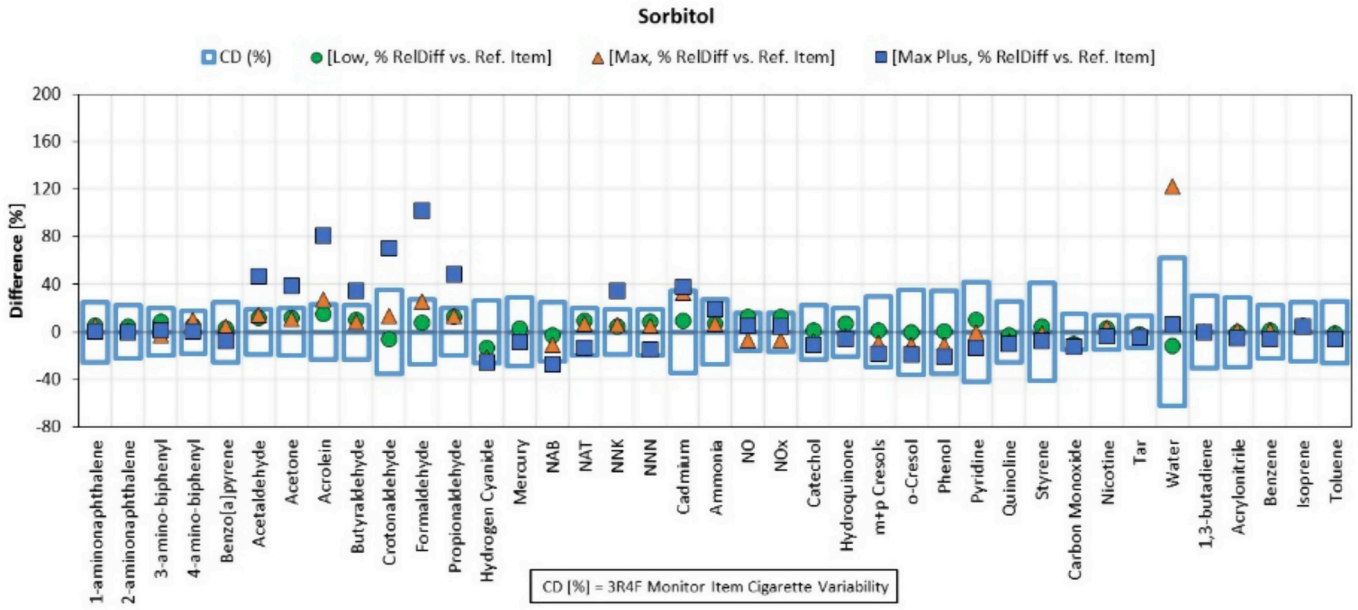

Fig. 17. Relative percentage difference between yields of measured analytes in MS from cigarettes containing Low (0.6%), Max (1.2%), and Max Plus (1.8%) levels of sorbitol and the corresponding reference item containing no additive.

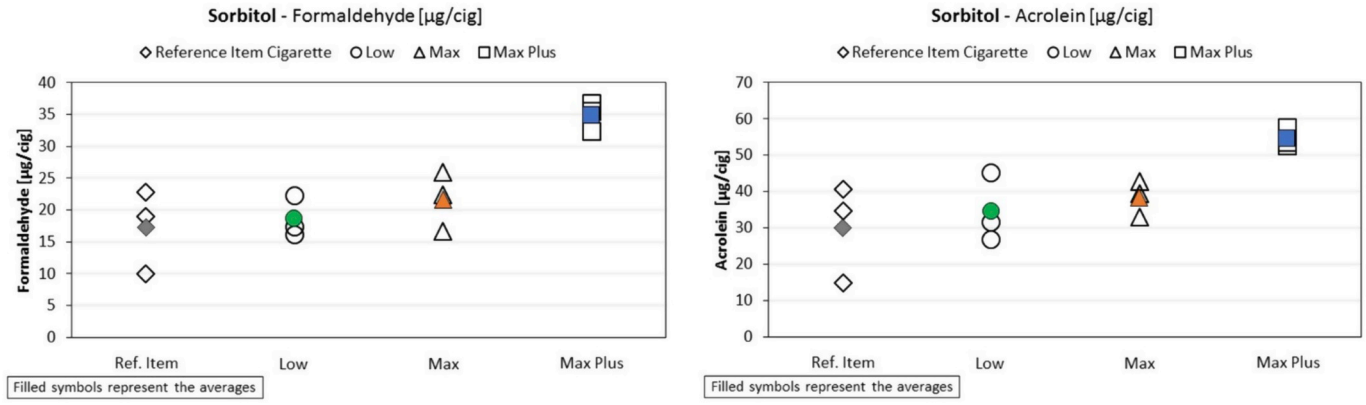

Fig. 18. Statistically significant and meaningful increases for test cigarettes with sorbitol compared to the additive-free reference cigarette.

differences were either not statistically significant or did not show any consistent additive-level increases. The results are shown in Figs. 2–10, and further details are provided in Tables S2–S27 in the Supplementary Material on the journal's website.

3.2.1.2. Glycerol. The smoke chemistry study showed besides the emissions for water no statistically significant and meaningful increases in any analytes for the test cigarettes containing glycerol up to target levels of 6 + % as single additive compared to the additive-free reference cigarette, which were exceeding the 3R4F monitor cigarette variability.

Additionally, the assessment showed statistically significant and consistent additive-level decreases for the emissions of B[*a*]P, NAB, catechol, hydroquinone, *m* + *p*-cresols, *o*-cresol, phenol and quinoline at the "Max" and/or "Max Plus" levels for the test item cigarettes containing glycerol. These decreases exceeded the inherent variability of the analytical method: (−27%) for B[*a*]P and (−43%) for NAB in level Max Plus; (−25%, −34%) for catechol, (−22%, −34%) for hydroquinone, (−39%, −48%) for *m* + *p*-cresols, (−41%, −50%) for *o*-cresol, (−50%, −61%) for phenol and (−32%, −43%) for quinoline in levels Max and Max Plus.

Beyond the statistically significant and consistent additive-level increases and decreases mentioned above, differences in emissions between test and additive-free reference cigarettes exceeded the inherent variability of the analytical method for NNN, ammonia and pyridine (see Fig. 11). However, these differences were either not statistically significant or did not show any consistent additive-level increases.

The results are shown in Figs. 11–12, and further details are provided in Tables S12–S13 in the Supplementary Material on the journal's website.

3.2.1.3. Guar gum. The smoke chemistry study showed statistically significant and meaningful increases for formaldehyde (Max: +67.9%, Max Plus +101%) and cadmium (Max: +46.2%, Max Plus +47.2%) at the Max and Max Plus levels for the test cigarettes containing guar gum as single additive as compared to the additive-free reference cigarette, which were exceeding the 3R4F monitor cigarette variability.

When the cadmium level was analysed in the guar gum sample, a level of 0.005 mg/kg (5 ppb) cadmium was determined. With a transfer rate of 10% (3–10% cadmium transfer for a filter cigarette were previously reported by Piade et al., 2015), less than 0.1% of the cadmium increase determined between the reference and the test cigarettes could be explained by the cadmium impurity of the food-grade guar gum sample.

The differences in emissions between test and additive-free

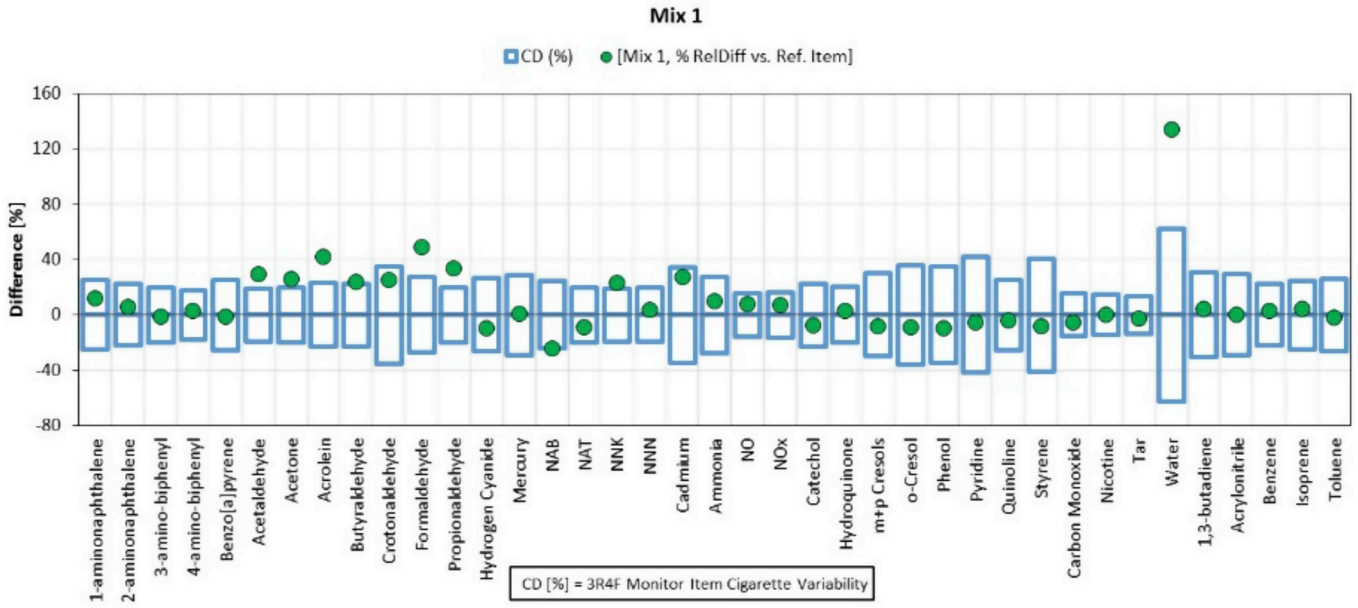

Fig. 19. Relative percentage difference between yields of measured analytes in MS from cigarettes containing Mix 1 (Mix 1: propylene glycol, glycerol, fenugreek extract, geraniol, guaiacol, and maltol) and the corresponding reference cigarette containing no additives.

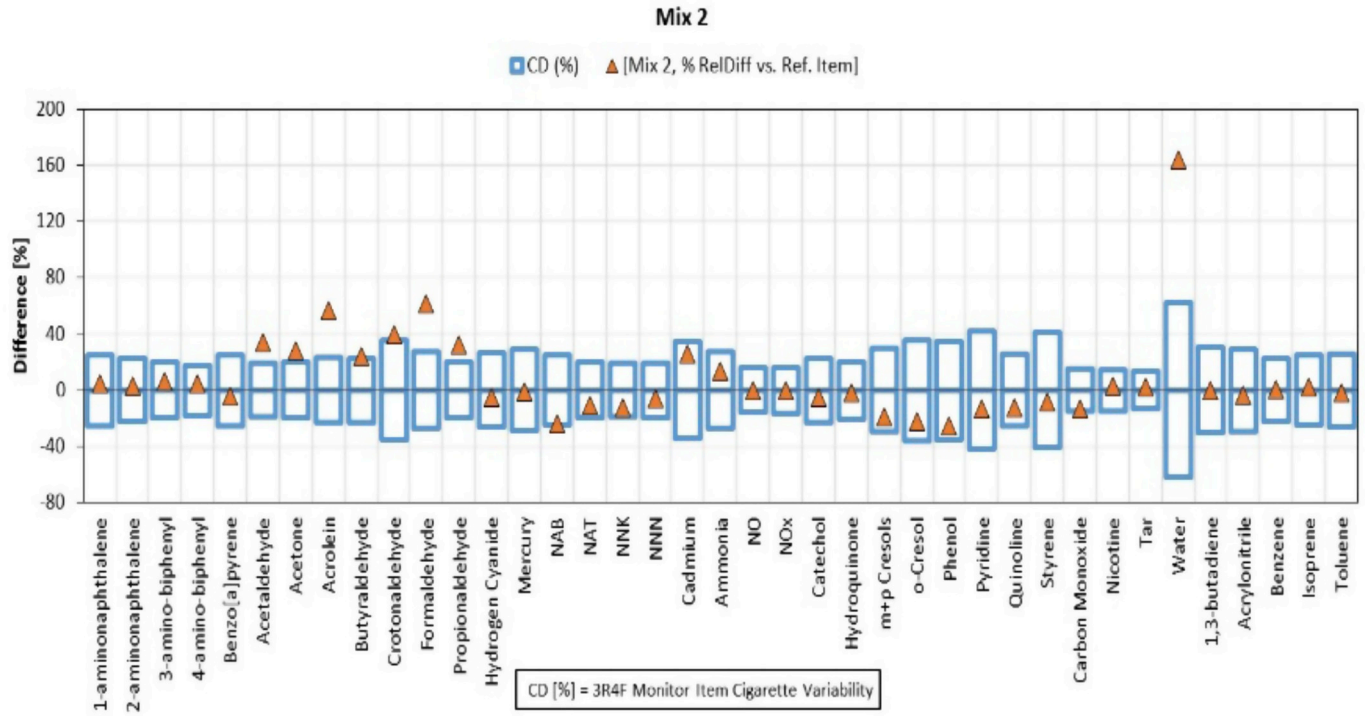

Fig. 20. Relative percentage difference between yields of measured analytes in MS from cigarettes containing Mix 2 (Mix 2: propylene glycol, glycerol, sorbitol, fenugreek extract, geraniol, guaiacol, maltol) and the corresponding reference cigarette containing no additives.

reference cigarettes exceeded also the inherent variability of the analytical method for acetaldehyde, acetone, acrolein (all addition levels), butyraldehyde (Low and Max Plus), crotonaldehyde (Max Plus), propionaldehyde (all addition levels), NO, NOx, and water (Max) (see Fig. 13). However, these differences were either not statistically significant or did not show any consistent additive-level increases.

The results are shown in Figs. 13–14**,** and further details are provided in Tables S16–S17 in the Supplementary Material on the journal's website.

3.2.1.4. Propylene glycol. The smoke chemistry study showed no statistically significant and meaningful increases in any analytes for the test cigarettes containing propylene glycol up to target levels of 6 + % as single additive as compared to the additive-free reference cigarette, which were exceeding the 3R4F monitor cigarette variability.

Statistically significant and consistent additive-level related decreases were observed for *m* + *p*-cresols and phenol at the Max and/or Max Plus levels. These decreases exceed the inherent variability of the analytical method for *m* + *p*-cresols at Max Plus (−30%) and for phenol at Max and Max Plus (−35%, −35%) and were statistically

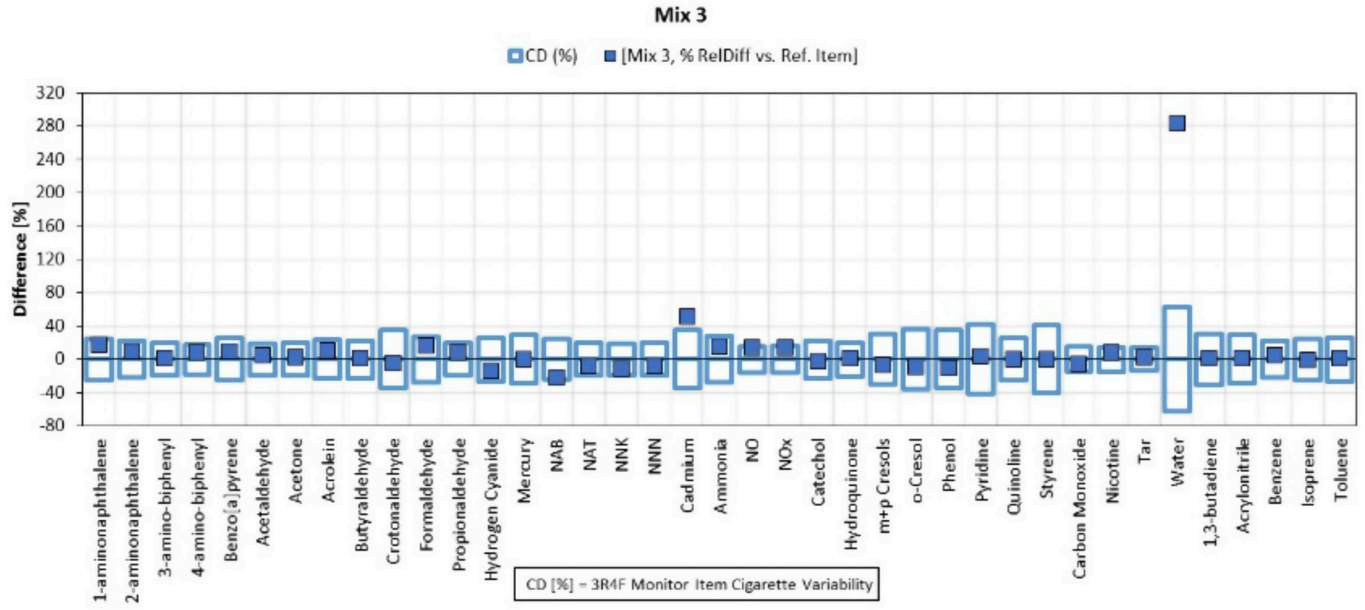

Fig. 21. Relative percentage difference between yields of measured analytes in MS from cigarettes containing Mix 3 (Mix 3: propylene glycol, glycerol, liquorice, cocoa powder, carob bean extract, fig juice concentrate, guar gum plus the following additional top flavors: fenugreek extract, geraniol, guaiacol, maltol) and the corresponding reference item containing no additives.

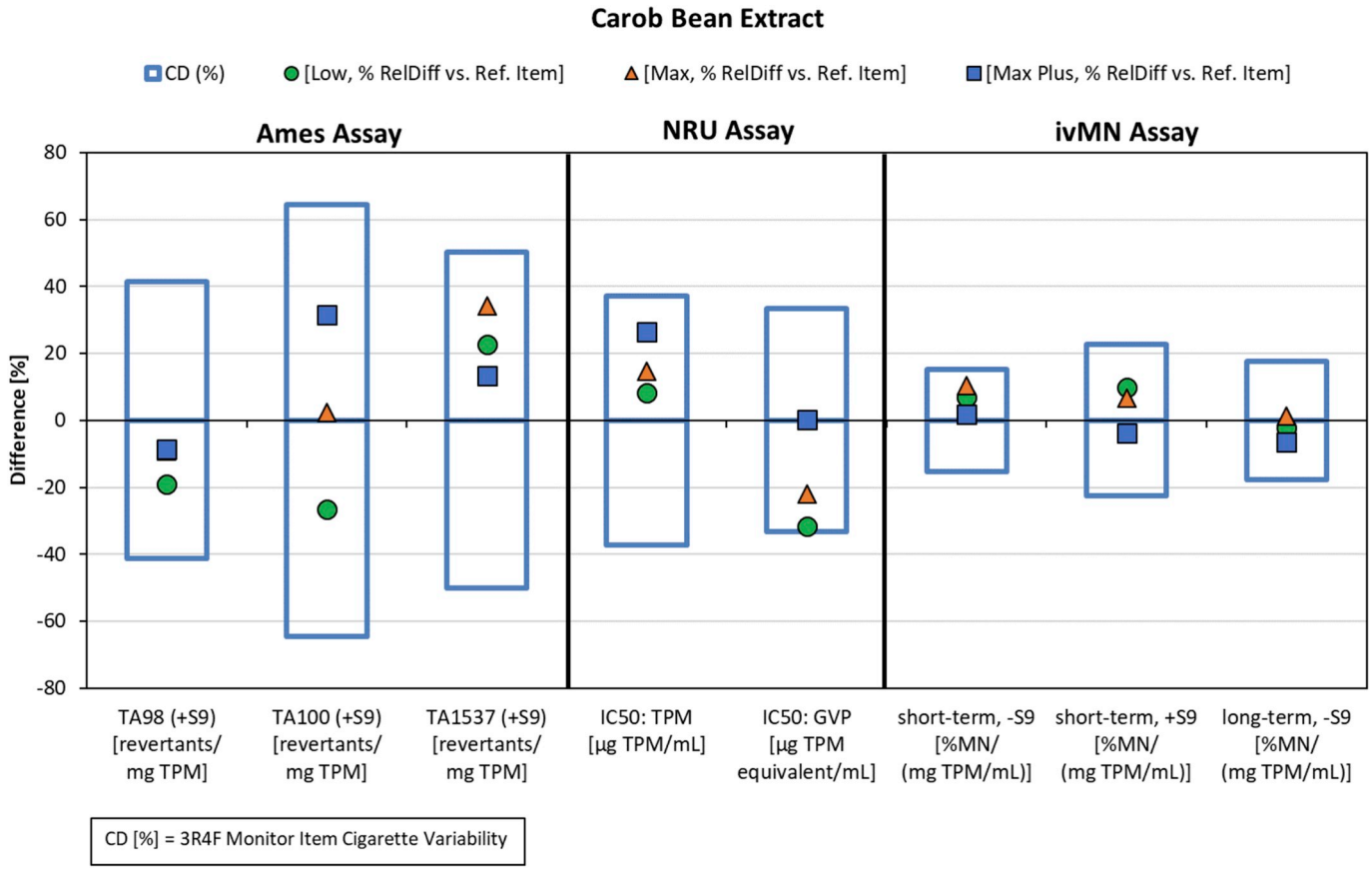

Fig. 22. Relative percentage difference (% RelDiff) between Ames and ivMN assay linear regression slopes and IC_{50} of the NRU assay for TPM/GVP from MS of cigarettes containing Low (0.2%), Max (0.4%), and Max Plus (0.6%) levels of carob bean extract and the corresponding reference cigarette containing no additive compared with the 3R4F monitor cigarette variability.

significant.

In addition, the differences in emissions between test and additive-free reference cigarettes exceeded the inherent variability for acetaldehyde, acetone (Low and Max Plus), acrolein (all levels), butyraldehyde (Low and Max Plus), formaldehyde, propionaldehyde (all addition levels), NAB (Low), cadmium (Low and Max Plus), NO, NOx

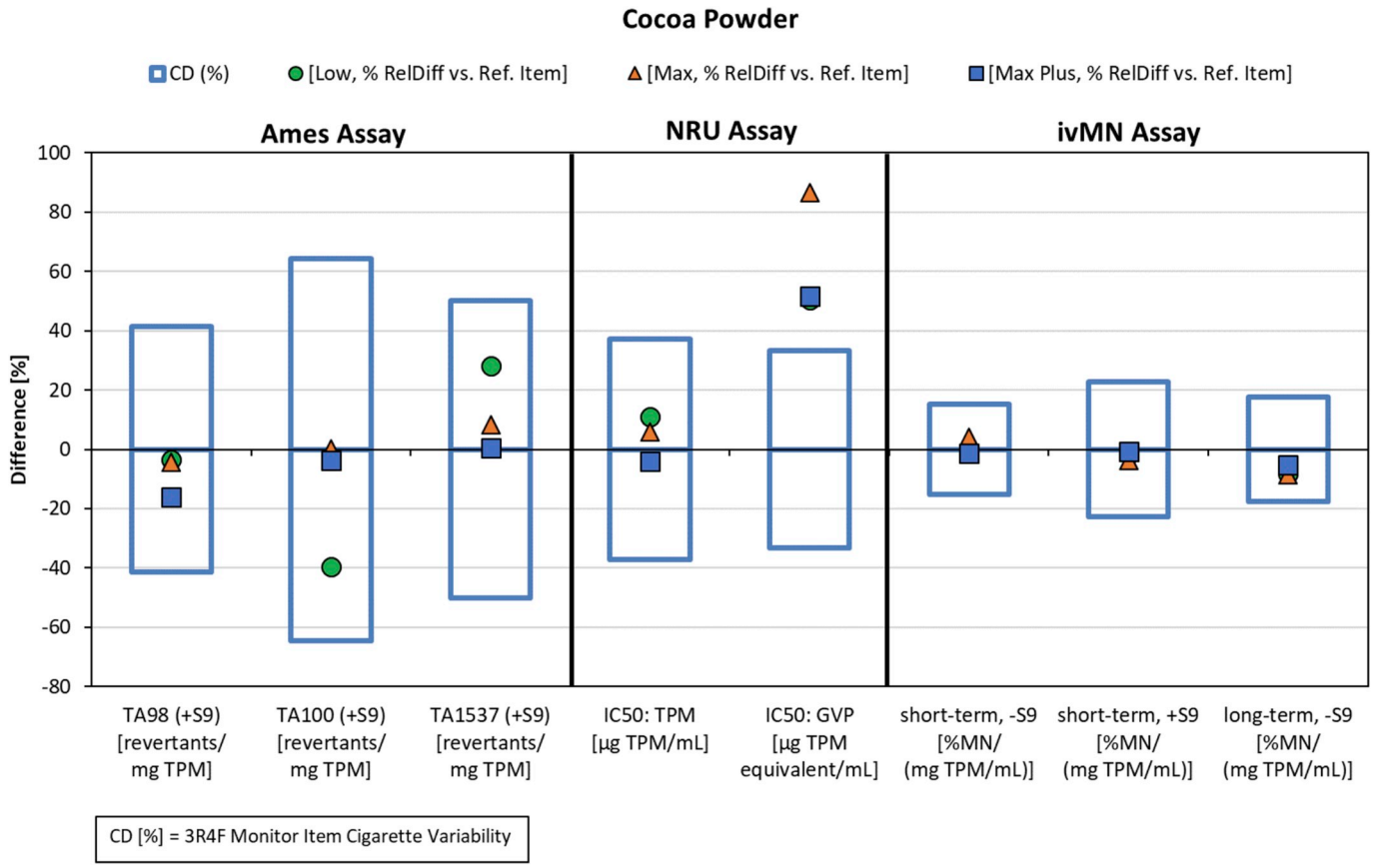

Fig. 23. Relative percentage difference (% RelDiff) between ivMN and Ames assay linear regression slopes and IC_{50} of the NRU assay for TPM/GVP from MS of cigarettes containing Low (0.5%), Max (1.0%) and Max Plus (1.5%) levels of cocoa powder and the corresponding reference cigarette containing no additive compared to the 3R4F monitor cigarette variability.

(Max and Max Plus), and CO (Max) (see Fig. 15). However, these differences were not statistically significant and did not show consistent additive-level increases.

The results are shown in Figs. 15–16**,** and further details are provided in Tables S24–S25 in the Supplementary Material on the journal's website.

3.2.1.5. Sorbitol. The smoke chemistry study showed statistically significant and meaningful increases for acrolein (Max Plus +81%) and formaldehyde (Max Plus +102%) for the test cigarettes containing sorbitol as single additive as compared to the additive-free reference cigarette, which were exceeding the 3R4F monitor cigarette variability.

In addition, the differences in emissions between test and additive-free reference cigarettes exceeded the inherent variability for acetaldehyde, acetone, butyraldehyde, crotonaldehyde, propionaldehyde, NAB, NNK, cadmium (Max Plus addition level), and water (Max level) (see Fig. 17). However, these differences were not statistically significant and did not show consistent additive-level increases.

The results are shown in Figs. 17–18**,** and further details are provided in Tables S26–S27 in the Supplementary Material on the journal's website.

3.2.2. Additive mixtures

The additive Mix 1 contained as target values 1% propylene glycol, 1% glycerol, plus the following additional top flavors: 0.02% fenugreek extract, 0.03% geraniol, 0.001% guaiacol, 0.01% maltol.

The additive Mix 2 contained as target values 2% propylene glycol, 1.5% glycerol, 2% sorbitol, plus the following additional top flavors: 0.02% fenugreek extract, 0.03% geraniol, 0.001% guaiacol, and 0.01% maltol.

The additive Mix 3 contained as target values 1% propylene glycol, 1.5% glycerol, 0.8% liquorice, 0.4% cocoa powder, 0.4% carob bean extract, 0.025% fig juice concentrate, 1% guar gum plus the following additional top flavors: 0.02% fenugreek extract, 0.03% geraniol, 0.001% guaiacol, 0.01% maltol.

The smoke chemistry study showed no statistically significant increases or decreases in any analytes for the test cigarettes containing Mix 1, Mix 2, and Mix 3 additives compared to the additive-free reference cigarette, which were exceeding the 3R4F monitor cigarette variability with the exception of a statistically significant increase in the yield of water for the test cigarette containing the Mix 2 additives. In addition, there were sporadic cases (e.g., acetaldehyde, acetone, and acrolein, for Mix 1 (see Fig. 19)) in which the relative percentage difference between the test cigarettes with additives and the additive-free reference cigarette did exceed the inherent variability of the analytical method. However, these differences were not statistically significant. The results are shown in Figs. 19–21**,** and further details are provided in Tables S28–S33 in the Supplementary Material on the journal's website.

3.3. In vitro toxicology

The 3R4F monitor cigarette, an additive-free reference cigarette and test cigarettes containing three levels of each single additive, or a mixture of additives were assayed for mutagenicity, cytotoxicity and genotoxicity in the Ames test, NRU assay and *iv*MN, respectively.

TPM was generated using the ISO smoking regime and extracted to a stock concentration of 10 mg/mL in anhydrous DMSO. Furthermore, the GVP was generated and tested in the Neutral Red Uptake assay only.

The *in vitro* assays were conducted to the relevant OECD and Health Canada guidelines (except for the NRU assay (T-102), where the

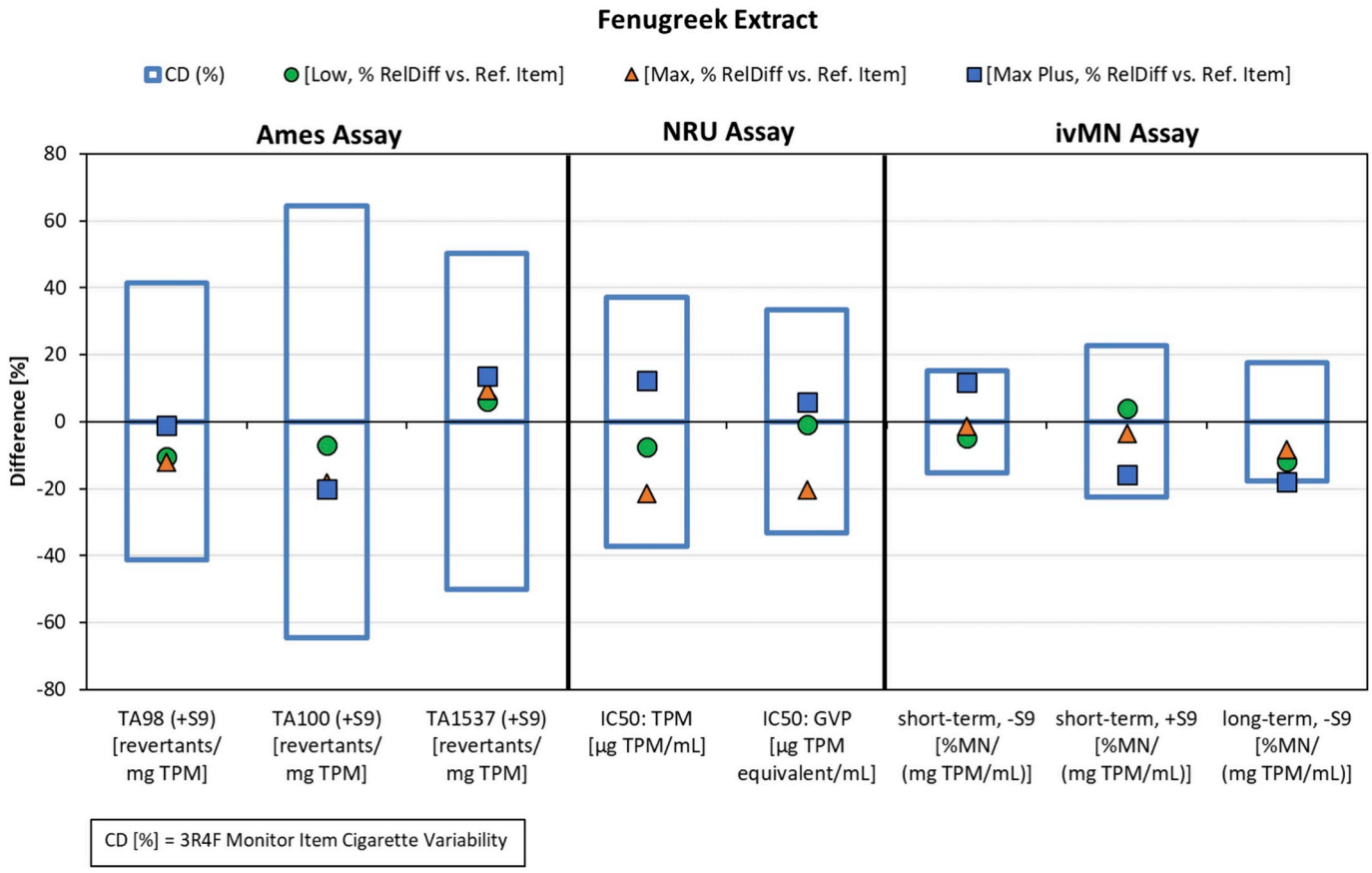

Fig. 24. Relative percentage difference (% RelDiff) between ivMN and Ames assay linear regression slopes and IC_{50} of the NRU assay for TPM/GVP from MS of cigarettes containing Low (0.01%), Max (0.02%) and Max Plus (0.03%) levels of fenugreek extract and the corresponding reference cigarette containing no additive compared to the 3R4F monitor cigarette variability.

PP + GVP was not conducted) and to GLP requirements.

The vehicle and positive controls in each assay and on each day of testing were within the historical control ranges used in this laboratory; the assays were therefore considered valid.

Regarding the results of the reference cigarette and the test cigarettes with the single additive (Low, Max, Max Plus levels) and the mixtures added, concentration related and reproducible increases in the Ames assay in revertants were generally observed in tester strains TA98, TA100, and TA1537 in the presence of S9 following treatment with the TPM samples. For all single additives and mixtures, strains were included in the figures (Figs. 22–37) and the statistical analysis when the specific activity slope was significantly different from zero. In the remaining strains and treatment condition, no increases in revertants, which were reproducible and significantly different from zero, were observed. Furthermore, when the TPM samples were tested in the *iv*MN assay, positive responses were observed in each treatment condition. In the NRU, the TPM and GVP samples induced concentration-related decreases in cell viability and an IC_{50} value could be derived in each instance. Statistical analysis was used to compare the test and reference cigarettes to each other and to the variability of 3R4F monitor cigarette responses.

3.3.1. Single additives

In the NRU Assay, there were no statistically significant and consistent additive-level related increases or decreases in the concentrations that reduced the number cells to 50% of that in the untreated control (IC_{50}) for mainstream TPM and GVP for the test cigarettes containing the 13 single additives compared to the additive-free reference cigarette, which were exceeding the 3R4F monitor cigarette variability. The results are shown in Figs. 22–34 and further details are provided in Tables $S34_{c-d}$ – $S46_{c-d}$ in the Supplementary Material on the journal's website.

Beyond the formal assessment that did not identify any statistically significant and consistent additive-level related increases and decreases, there were sporadic cases (e.g., cocoa powder, guaiacol) in which the relative percentage difference between the test cigarettes with additive and the additive-free reference cigarette did exceed the inherent method variability for the GVP of test cigarettes. However, these differences were not statistically significant.

In the Ames assay, there were no statistically significant and consistent additive-level related increases or decreases in the linear slopes of the dose-response curves of the TPM extracts for any of the five strains, in the presence and absence of S9 metabolic activation (+S9, -S9), for the test cigarettes containing the 13 single additives (carob bean, cocoa, fenugreek, fig juice, geraniol, glycerol, guaiacol, guar gum, liquorice, maltol, menthol, propylene glycol, and sorbitol) compared to the additive-free reference cigarette, which were exceeding the 3R4F monitor cigarette variability. The results are shown in Figs. 22–34 and further details are provided in Tables $S34_{a-b}$ – $S46_{a-b}$ in the Supplementary Material on the journal's website.

In the *iv*MN assay, there were no statistically significant and consistent additive-level related increases or decreases in the slopes of the dose/response curves of the mainstream TPM for the test cigarettes containing the 13 single additives compared to the additive-free reference cigarette, which were exceeding the 3R4F monitor cigarette variability. The results are shown in Figs. 22–34, and further details are provided in Tables $S34_{e-f}$ – $S46_{e-f}$ in the Supplementary Material on the journal's website.

Beyond the formal assessment that did not identify any statistically significant and consistent additive-level related increases and

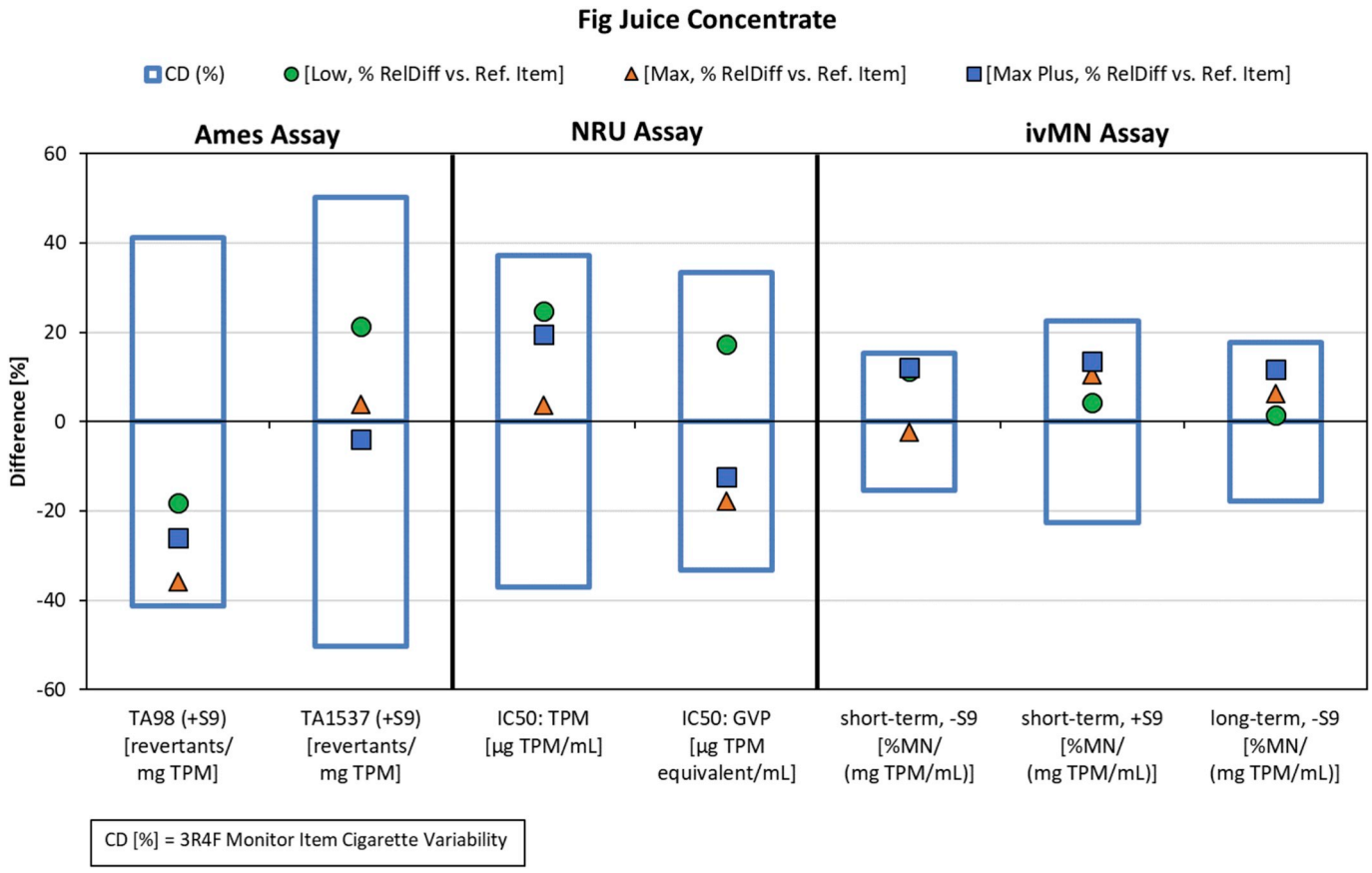

Fig. 25. Relative percentage difference (% RelDiff) between ivMN and Ames assay linear regression slopes and IC_{50} of the NRU assay for TPM/GVP from MS of cigarettes containing Low (0.01%), Max (0.02%) and Max Plus (0.03%) levels of fig juice extract and the corresponding reference cigarette containing no additive compared to the 3R4F monitor cigarette variability.

decreases, the relative percentage difference between the test cigarettes with the *l*-menthol Max Plus level and the additive-free reference cigarette exceed the inherent method variability for the short-term + S9 assay condition. However, the difference at the Max Plus level was not statistically significant.

3.3.2. Additive mixture

In the NRU assay, there was no statistically significant increase or decrease in the concentration that reduced the number cells to 50% of that in the untreated control (IC_{50}) for mainstream TPM and GVP for the test cigarettes containing the three additive mixtures compared to the additive-free reference cigarette, which were exceeding the 3R4F monitor cigarette variability. The results are shown in Fig. 37– and further details are provided in Tables $S47_{c-d}$ – $S49_{c-d}$ in the Supplementary Material on the journal's website.

In the Ames assay, there were no statistically significant increases or decreases in the linear slopes of the dose/response curves of the TPM for any of the five strains, in the presence and absence of S9 metabolic activation (+S9, -S9), for the test cigarettes containing the three additive mixtures compared to the additive-free reference cigarette, which were exceeding the 3R4F monitor cigarette variability. The results are shown in Figs. 35–37 and further details are provided in Tables $S47_{a-b}$ – $S49_{a-b}$ in the Supplementary Material on the journal's website.

In Fig. 36, the regression slope variability for strain TA102 (+S9) for the 3R4F monitor cigarette was not determined due to the historical slopes not being significantly different from zero. As such, there is no box plot for strain TA102 (+S9). The test cigarette containing Mix 2 additives and the reference cigarette both had slopes for strain TA102 (+S9) which were statistically significant from zero and the following mean values were calculated: 677.6 ± 638.0 revertants/mg TPM for the reference cigarette and 363.5 ± 157.0 revertants/mg TPM for the test cigarette containing Mix 2. Although the reference cigarette was tested multiple times in the Ames assay with TA102 (+S9), in only this case was the slope above zero. In our view, the results for the TA102 (+S9) obtained with the reference cigarette and the test cigarette containing Mix 2 were borderline and a chance finding. Above all, the difference in mean slopes was not statistically significant.

In the *iv*MN assay, there were no statistically increases or decreases in the slopes of the dose/response curves of the mainstream TPM for the test cigarettes containing the three additive mixtures compared to the additive-free reference cigarette, which were exceeding the 3R4F monitor cigarette variability. The results are shown in Figs. 35–37 and further details are provided in Tables $S47_{e-f}$ – $S49_{e-f}$ in the Supplementary Material on the journal's website.

4. Discussion

4.1. Carob bean

Carob bean is widely used in many consumer goods, such as foods and cosmetics and in pharmaceuticals, so there is a long history of consumer exposure to this additive (Burdock, 2010). The scientific literature demonstrates that carob bean has no carcinogenic (NTP, 1982a; Melnick et al., 1983; JECFA, 1981; EFSA ANSI, 2017), mutagenic/genotoxic (EFSA ANSI, 2017), or reprotoxic properties (Domanski et al., 1980 in JECFA, 1981; FDRL, 1972; Morgareidge, 1972; EFSA ANSI, 2017).

In several pyrolysis experiments, propylene glycol and acetic acid were determined as main degradation products when carob bean was pyrolyzed (Baker and Bishop, 2005). Based on the complex chemical

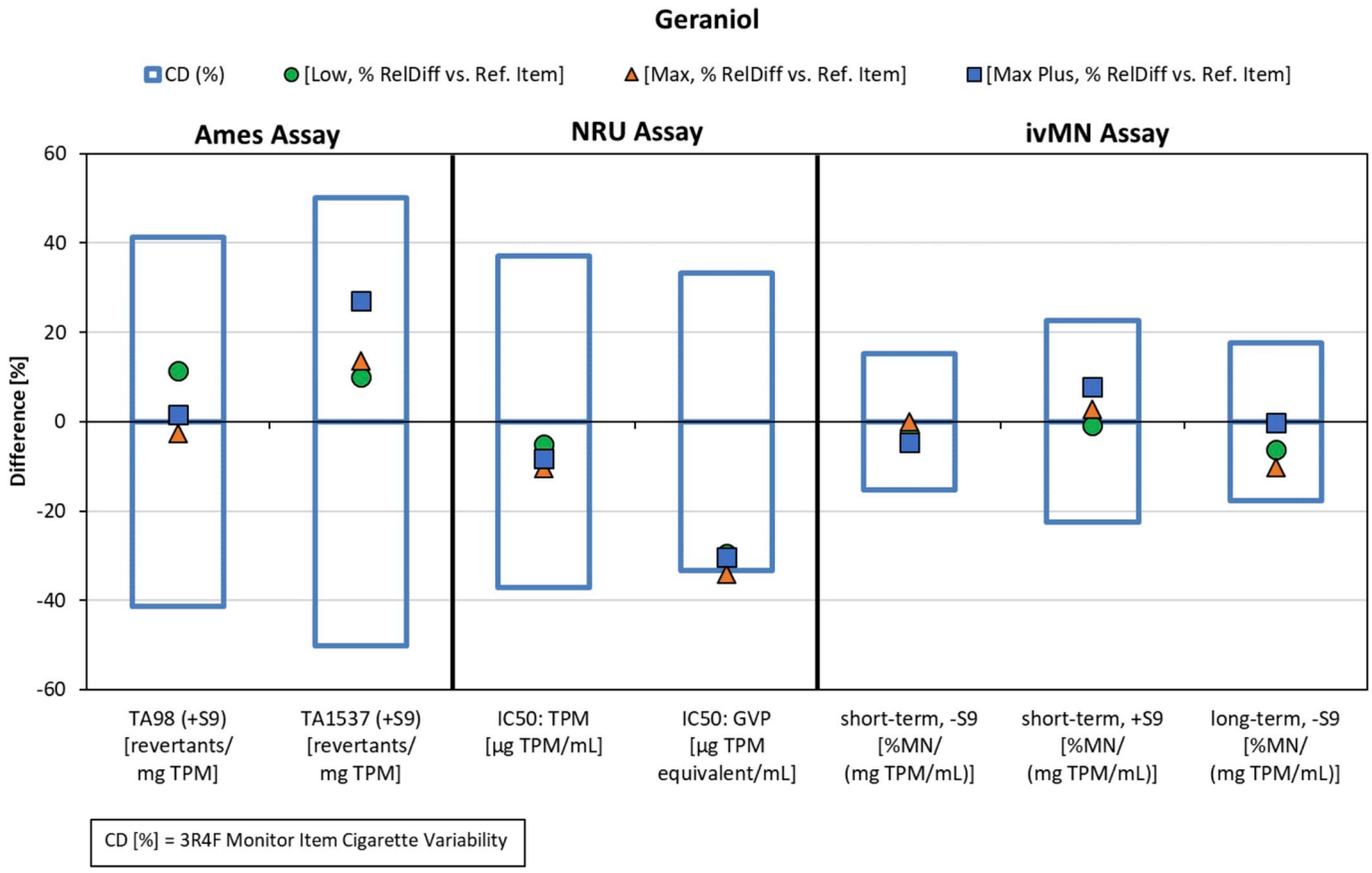

Fig. 26. Relative percentage difference (% RelDiff) between ivMN and Ames assay linear regression slopes and IC_{50} of the NRU assay for TPM/GVP from MS of cigarettes containing Low (0.015%), Max (0.030%) and Max Plus (0.045%) levels of geraniol and the corresponding reference cigarette containing no additive compared to the 3R4F monitor cigarette variability.

composition and non-volatile nature of the natural constituents, as well as preliminary indication of degradation during pyrolysis, the components of carob bean extract are unlikely to transfer intact and no transfer rates were found in literature. Furthermore, the scientific literature demonstrates that carob bean, when used as a tobacco additive, does not increase either the *in vitro* or the *in vivo* toxicity of mainstream cigarette smoke. Inclusion levels between 0.0001% and 4% carob bean in test cigarettes resulted in isolated, inconsistent instances of significant increases (e.g., *o*-toluidine) and decreases (e.g., CO) in emissions when compared to control cigarettes (Baker et al., 2004b; Baker et al., 2004c; Coggins et al., 2011a; Roemer et al., 2014; Rustemeier et al., 2002), with no impact on the biological activity of cigarette smoke *in vitro* (Baker et al., 2004a; Coggins et al., 2011a; Roemer et al., 2002, 2014) and *in vivo* (Baker et al., 2004c; Coggins et al., 2011a; Gaworski et al., 1998, 1999; Schramke et al., 2014; Vanscheeuwijck et al., 2002).

In summary, the results of our chemistry and *in vitro* studies were in line with published data showing, no statistically significant and meaningful increases in smoke constituents, cytotoxicity, mutagenicity, and genotoxicity when carob bean was tested as a single additive up to a maximum inclusion level of 0.60%.

4.2. Cocoa

Cocoa is the key raw material in chocolate manufacturing, so there is a long history of consumer exposure to this additive, mainly through confectionary and dairy products and beverages (Aprotosoaie, 2016; Craig and Nguyen, 1984). The scientific literature demonstrates that cocoa has no carcinogenic (IARC, 1991; IARC, 2016), mutagenic/genotoxic (EFSA CEF Panel, 2017), or reprotoxic properties (Tarka et al., 1986; Tarka, 2010; EFSA CONTAM Panel, 2008).

In pyrolysis experiments, acetic acid, acetol, and furfuryl alcohol were determined as main degradation products when cocoa was pyrolyzed (Baker and Bishop, 2005). The transfer of theobromine, a constituent of relevance found in cocoa, into MS was determined to be approximately 13% by Zaidi (1974) without further information of the construction of the cigarette used. Furthermore, the scientific literature demonstrates that cocoa when used as a tobacco additive does not increase either the *in vitro* or the *in vivo* toxicity of mainstream cigarette smoke. Inclusion levels between 0.0002% and 4.84% cocoa in test cigarettes resulted in isolated, inconsistent instances of significant increases (e.g., catechol) and decreases (e.g., acetaldehyde) in emissions when compared to control cigarettes (Baker et al., 2004b, 2004c; Coggins et al., 2011b; Roemer et al., 2010; Rustemeier et al., 2002), with no impact on the biological activity of cigarette smoke *in vitro* (Baker et al., 2004a; Coggins et al., 2011b; Roemer et al., 2002, 2010, 2014) and *in vivo* (Baker et al., 2004a; Carmines, 2002; Coggins et al., 2011b; Gaworski et al., 1998, 1999; Roemer and Hackenberg, 1990; Schramke et al., 2014; Vanscheeuwijck et al., 2002).

In summary, the results of our chemistry and *in vitro* studies were in line with published data showing no statistically significant and meaningful increases in smoke constituents, cytotoxicity, mutagenicity, and genotoxicity when cocoa powder was tested as a single additive up to a maximum inclusion level of 1.5%.The theobromine transfer rate of approximately 4.5%, which we calculated in our study, was lower than the published rate of 14% (Zaidi, 1974) which may be due to differences in cigarette construction and analytical methods.

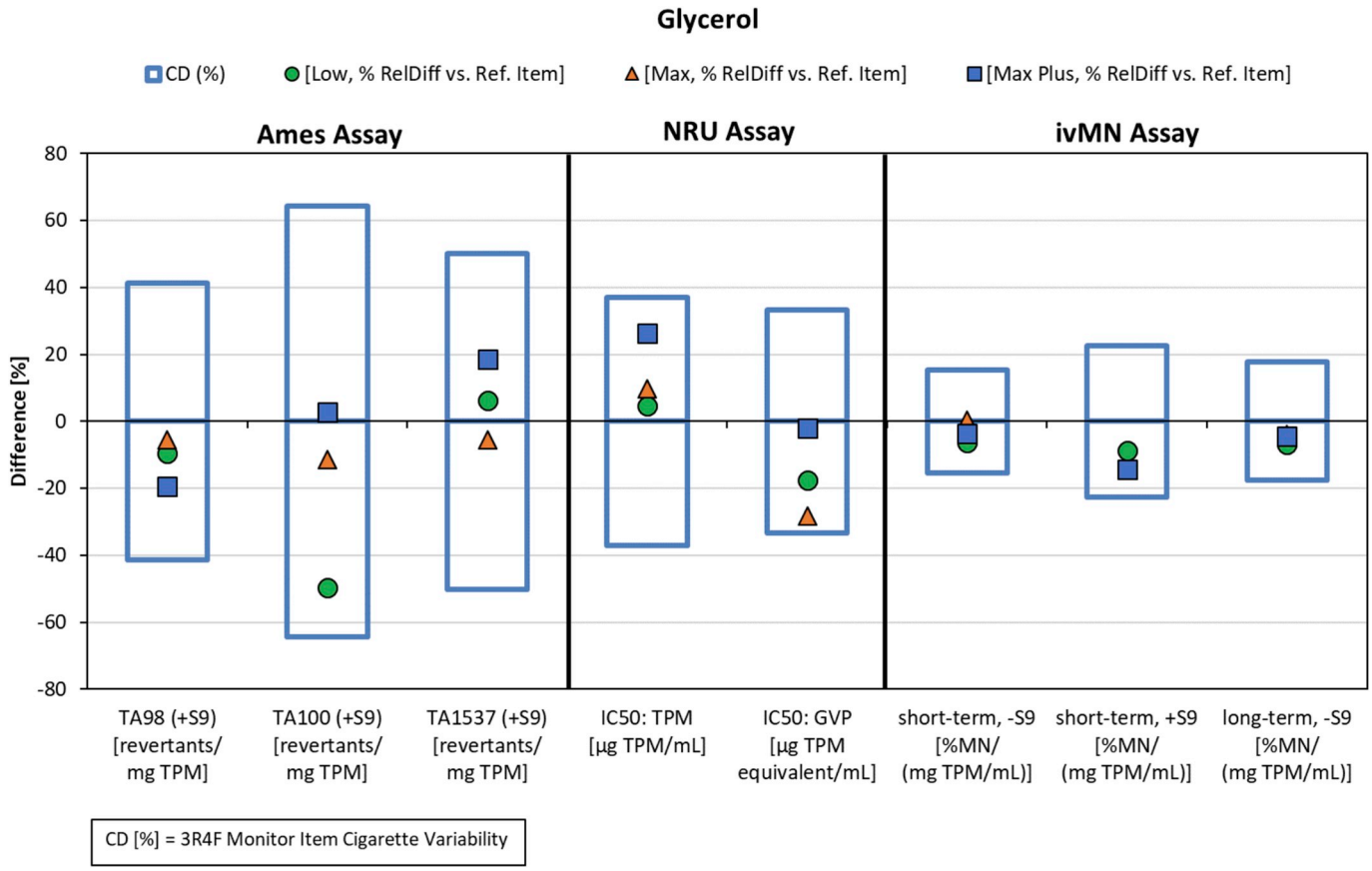

Fig. 27. Relative percentage difference (% RelDiff) between ivMN and Ames assay linear **regression** slopes and IC_{50} of the NRU assay for TPM/GVP from MS of cigarettes containing Low (2.5%), Max (5.0%) and Max Plus (6+%) levels of glycerol and the corresponding reference cigarette containing no additive compared to the 3R4F monitor cigarette variability.

4.3. Fenugreek

Fenugreek extract is widely used in many consumer goods (Waqas et al., 2010), such as foods (Ahmad et al., 2016; Burdock, 2010; Wankhede et al., 2016), and medicinal products (EMA, 2010), so there is a long history of consumer exposure to this additive (Burdock, 2010). The scientific literature demonstrates that fenugreek has no carcinogenic, mutagenic/genotoxic (Deshpande et al., 2016a; Flammang et al., 2004), or reprotoxic properties (Deshpande et al., 2016b; Deshpande et al., 2017; EMA, 2010).

In pyrolysis experiments, ethyl linoleate, ethyl palmitate, stearate, and palmitic acid were determined as main degradation products when fenugreek was pyrolyzed (Baker and Bishop, 2005). Based on the complex chemical composition and non-volatile nature of the natural constituents, as well as preliminary indication of degradation during pyrolysis, the components of fenugreek extract are unlikely to transfer intact and no transfer rates were found in literature. Furthermore, the scientific literature demonstrates that fenugreek extract, when used as a tobacco additive, does not increase either the *in vitro* or the *in vivo* toxicity of mainstream cigarette smoke. Inclusion levels between 0.0004% and 1% fenugreek in test cigarettes resulted in isolated, inconsistent instances of significant increases (e.g., acrolein) and decreases (e.g., formaldehyde) in emissions when compared to control cigarettes (Baker et al., 2004b, 2004c; Coggins et al., 2011a; Roemer et al., 2014; Rustemeier et al., 2002), with no impact on the biological activity of cigarette smoke *in vitro* (Baker et al., 2004a; Coggins et al., 2011a; Roemer et al., 2014; Roemer et al., 2002) and *in vivo* (Baker et al., 2004a; Gaworski et al., 1998, 1999; Schramke et al., 2014; Vanscheeuwijck et al., 2002).

In summary, the results of our chemistry and *in vitro* studies were in line with published data showing no consistent, and statistically significant increases in smoke constituents, cytotoxicity, mutagenicity, and genotoxicity when fenugreek was tested as a single additive up to a maximum inclusion level of 0.03%.

4.4. Fig

As one of the oldest known human foods (Barolo and Mostacero, 2014), figs as a fruit have a well-established safety profile. The chemical components of figs are food constituents that form part of the normal diet of humans (Burdock, 2010), and as such, figs are very well tolerated. In toxicity experiments carried out with fig fruit, no sign of toxicity was observed (Bhanushali et al., 2014; Kannur and Khandelwal, 2014; Alamgeer et al., 2017). Fig juice is not classified as carcinogenic or mutagenic/genotoxic, and it is not toxic to reproduction.

In pyrolysis experiments, acetic acid, furfural, and sorbic acid were determined as main degradation products when fig juice was pyrolyzed (Baker and Bishop, 2005). Based on the complex chemical composition and non-volatile nature of the natural constituents, as well as preliminary indication of degradation during pyrolysis, the components of fig juice are unlikely to transfer intact and no transfer rates were found in literature. Furthermore, the scientific literature demonstrates that fig juice, when used as a tobacco additive, does not increase either the *in vitro* or the *in vivo* toxicity of mainstream cigarette smoke. Inclusion levels between 0.0005% and 1.17% fig juice in test cigarettes resulted in isolated, inconsistent instances of significant increases (e.g., formaldehyde) and decreases (e.g., NNN and NNK) in emissions when compared to control cigarettes (Baker et al., 2004b, 2004c; Rustemeier et al., 2002), with no impact on the biological activity of cigarette smoke *in vitro* (Baker et al., 2004a; Renne et al., 2006; Roemer et al.,

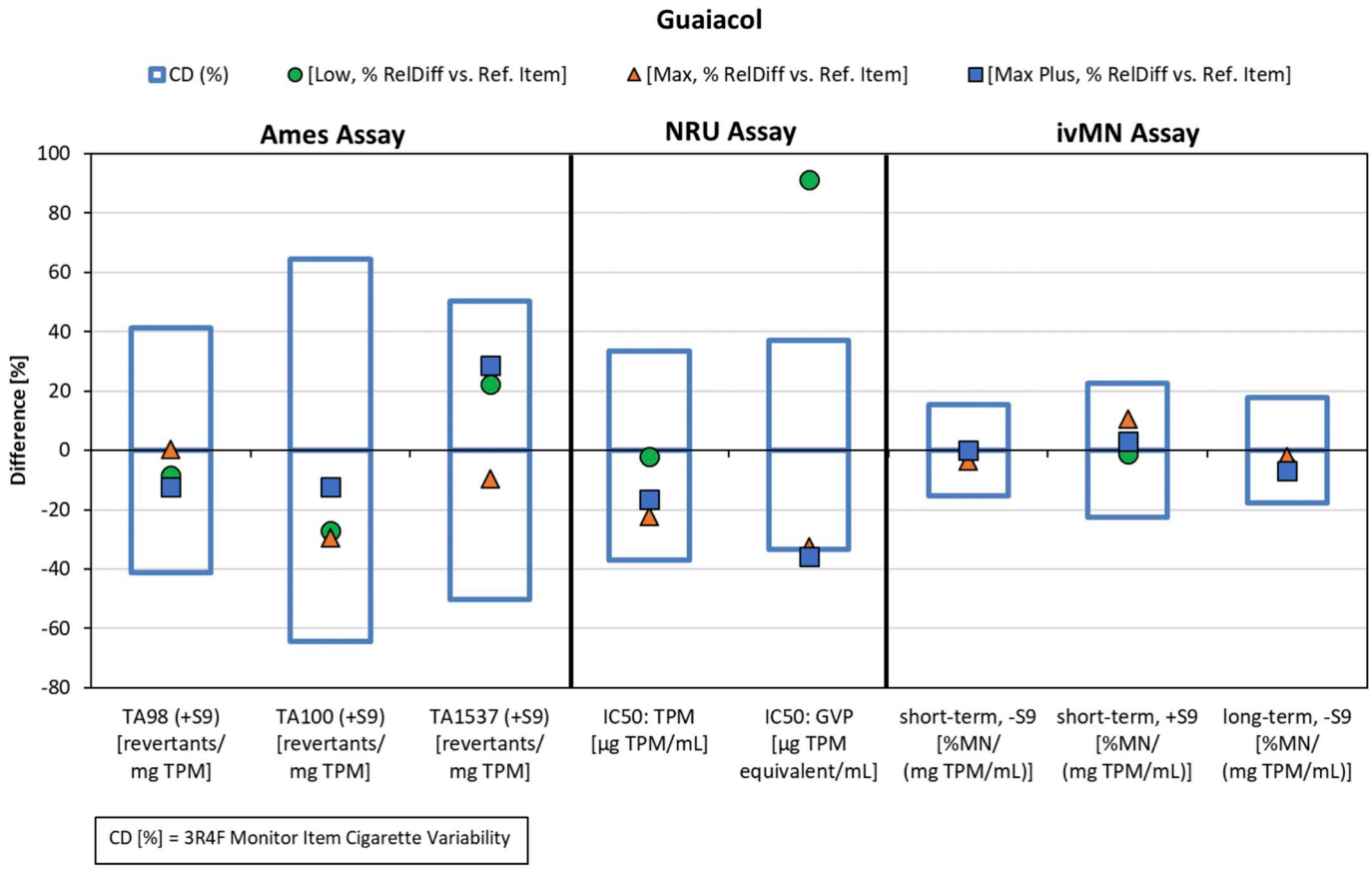

Fig. 28. Relative percentage difference (% RelDiff) between ivMN and Ames assay linear regression slopes and IC_{50} of the NRU assay for TPM/GVP from MS of cigarettes containing Low (0.0005%), Max (0.0010%) and Max Plus (0.0015%) levels of guaiacol and the corresponding reference cigarette containing no additive compared to the 3R4F monitor cigarette variability.

2002) and *in vivo* (Baker et al., 2004a; Gaworski et al., 1998, 1999; Vanscheeuwijck et al., 2002).

In summary, the results of our chemistry and *in vitro* studies were in line with published data showing no perseverative, consistent, and statistically significant increases in smoke constituents, cytotoxicity, mutagenicity, and genotoxicity when fig juice was tested as a single additive up to a maximum inclusion level of 0.3%.

4.5. Geraniol

Geraniol is widely used in foods (Lapczynski et al., 2008; EFSA CEF Panel, 2013) and many consumer goods, such as household cleaners (Lapczynski et al., 2008), cosmetics and perfumes (Chen and Viljoen, 2010), so there is a long history of consumer exposure to this additive. The scientific literature demonstrates that geraniol is not considered mutagenic/genotoxic (JECFA, 2004a; b), carcinogenic (JECFA, 2004a,b based on NTP, 1987), or a reproductive toxicant (JECFA, 2004a,b).

In pyrolysis experiments, it was reported that 85.6%–90.9% of the geraniol was transferred intact, along with some minor degradation products (Baker and Bishop, 2004; Purkis et al., 2011), but no transfer rates for geraniol from cigarettes into MS were reported. Furthermore, the scientific literature demonstrates that geraniol, when used as a tobacco additive, does not increase either the *in vitro* or the *in vivo* toxicity of mainstream cigarette smoke. Inclusion levels between 0.0001% and 0.0023% geraniol in test cigarettes resulted in isolated, inconsistent instances of significant increases (e.g., TPM) and decreases (e.g., cadmium) in emissions when compared to control cigarettes (Baker et al., 2004b, 2004c; Rustemeier et al., 2002; Roemer et al., 2014), with no impact on the biological activity of cigarette smoke *in vitro* (Baker et al., 2004b; Renne et al., 2006; Roemer et al., 2002, 2014) and *in vivo* (Baker and Bishop, 2004; Renne et al., 2006; Schramke et al., 2014; Vanscheeuwijck et al., 2002).

In summary, the results of our chemistry and *in vitro* studies were in line with published data showing no statistically significant and meaningful increases in smoke constituents, cytotoxicity, mutagenicity, and genotoxicity when geraniol was tested as a single additive up to a maximum inclusion level of 0.045%. A transfer rate of approximately 7.5% from cigarette tobacco to MS was determined for geraniol.

4.6. Glycerol

Glycerol is widely used in many consumer goods, such as foods (CIR, 2015; EFSA, 2017a) and cosmetics (CIR, 2015), and in pharmaceutical products (EFSA, 2017a), so there is a long history of consumer exposure to this additive. The scientific literature demonstrates that glycerol has no carcinogenic (EFSA, 2017a based on Hine et al., 1953), mutagenic/genotoxic (EFSA, 2017a; OECD SIDS, 2002), or reprotoxic properties (Wegener, 1953; OECD SIDS, 2002).

In pyrolysis experiments, it was reported that 99.8%–100% of the glycerol was transferred intact, along with some minor degradation products (Baker and Bishop, 2004; Purkis et al., 2011). In the present study, the glycerol transfer from cigarette tobacco into smoke was approximately 4.5%, which is in line with published transfer rates of 0.4%–8.1% for filtered cigarettes (Laurene et al., 1965, as cited by Carmines and Gaworski, 2005). Furthermore, our chemistry study showed an increase in water and decreases in the emissions of several toxicants (i.e., phenols, B[*a*]P, and NAB) that exceeded the inherent variability of the analytical method and were statistically significant. These results are consistent with results obtained in other studies, where glycerol was applied as a single additive up to 15% (Carmines

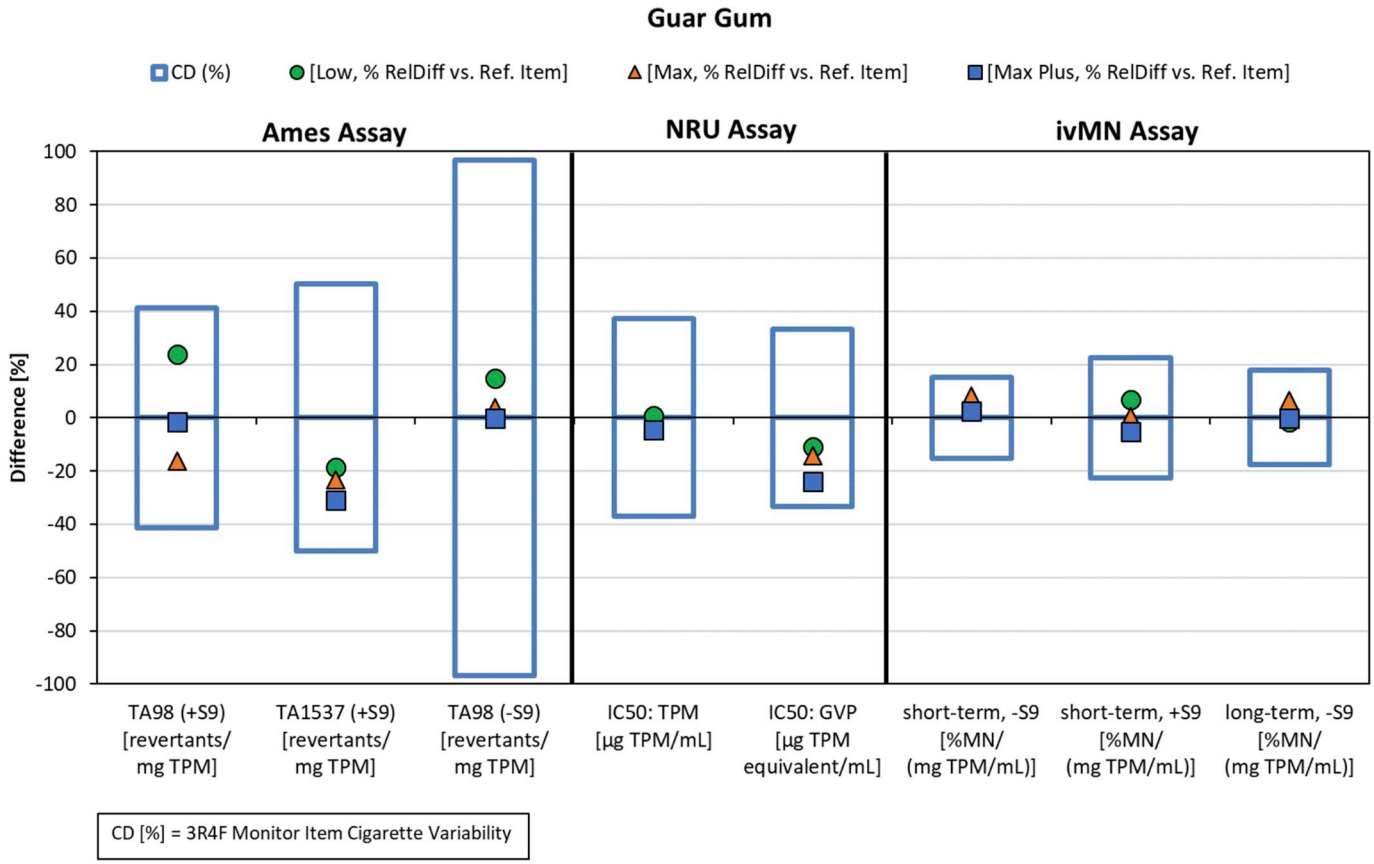

Fig. 29. Relative percentage difference (% RelDiff) between ivMN and Ames assay linear regression slopes and IC_{50} of the NRU assay for TPM/GVP from MS of cigarettes containing Low (0.5%), Max (1.0%) and Max Plus (1.5%) levels of guar gum and the corresponding reference cigarette containing no additive compared to the 3R4F monitor cigarette variability.

and Gaworski, 2005; Roemer et al., 2010) or as part of a mixture up to 11.4% (Baker et al., 2004b, 2004c; Rustemeier et al., 2002). However, Carmines and Gaworski (2005) found an increase in acrolein when glycerol levels of 10% or 15% were added to cigarette tobacco and ISO smoking conditions were used, whereas Roemer et al. (2002) reported an increase in acrolein for 5.5% glycerol under Health Canada Intense smoking conditions. No increase of acrolein was reported for levels of 5% or 5.5% under ISO smoking conditions, which is in line with the results of the present study. Overall, smoke chemistry data suggest that the addition of glycerol does not increase the toxicity of cigarette smoke. The results of our *in vitro* studies confirmed this conclusion showing no statistically significant and meaningful increases in cytotoxicity, mutagenicity, and genotoxicity when glycerol was tested as a single additive up to a maximum inclusion level of 6.0+%. The scientific literature confirmed our results; inclusion levels between 0.006% and 15% glycerol in test cigarettes had no impact on the biological activity of cigarette smoke *in vitro* (Baker et al., 2004b, 2004c; Carmines and Gaworski, 2005; Combes et al., 2013; Roemer et al., 2002, 2010) and *in vivo* (Baker et al., 2004a; Carmines and Gaworski, 2005; Gaworski et al., 1998, 1999; Heck et al., 2002; Vanscheeuwijck et al., 2002).

4.7. Guaiacol

Guaiacol is widely used in many consumer goods, such as foods (Dorfner et al., 2003), cosmetics, and personal care products (ECHA, 2018), and in pharmaceuticals (Chang et al., 2000), so there is a long history of consumer exposure to this additive. For Guaiacol, SCHEER (2016) mentioned a negative AMES assay, a positive genotoxicity (SCE) test in human lymphocytes and the need for further studies. However, guaiacol has been documented to give a negative result in the *in vivo* micronucleus assay (ECHA, 2018). Therefore, the scientific literature demonstrates that guaiacol has no carcinogenic (Hirose et al., 1989), mutagenic/genotoxic (EFSA, 2006, 2008; ECHA, 2018), or reprotoxic properties (no CLP classification).

In pyrolysis experiments, it was reported that 92.5%–99.8% of the guaiacol was transferred intact, along with some minor degradation products (Baker and Bishop, 2004; Purkis et al., 2011; Czegny et al., 2016). No transfer rates of guaiacol from cigarette tobacco into smoke were reported in literature and no reliable transfer rates could be determined in the present study, because the guaiacol yields for the additive-free reference cigarette and the test cigarettes were similar, taking into consideration the analytical method variability. The transfer study demonstrates that the guaiacol transfer resulting from the tobacco additive guaiacol is negligible compared to the guaiacol released from tobacco lignin pyrolysis. Furthermore, the scientific literature demonstrates that guaiacol, when used as a tobacco additive, does not increase the *in vitro* or the *in vivo* toxicity of mainstream cigarette smoke. Inclusion levels between 0.00001% and 0.003% guaiacol in test cigarettes resulted in isolated, inconsistent instances of significant increases (e.g., 4-aminobiphenyl) and decreases (e.g., catechol) in emissions when compared to control cigarettes (Baker et al., 2004a, 2004b; Roemer et al., 2014), with no impact on the biological activity of cigarette smoke *in vitro* (Baker et al., 2004a, 2004b; Jansson et al., 1986; Roemer et al., 2014) and *in vivo* (Baker et al., 2004a; Gaworski et al., 1998; Gaworski et al., 1999; Schramke et al., 2014).

In summary, the results of our chemistry and *in vitro* studies were in line with published data, showing no statistically significant and meaningful increases in smoke constituents, cytotoxicity, mutagenicity, and genotoxicity when guaiacol was tested as a single additive up to a

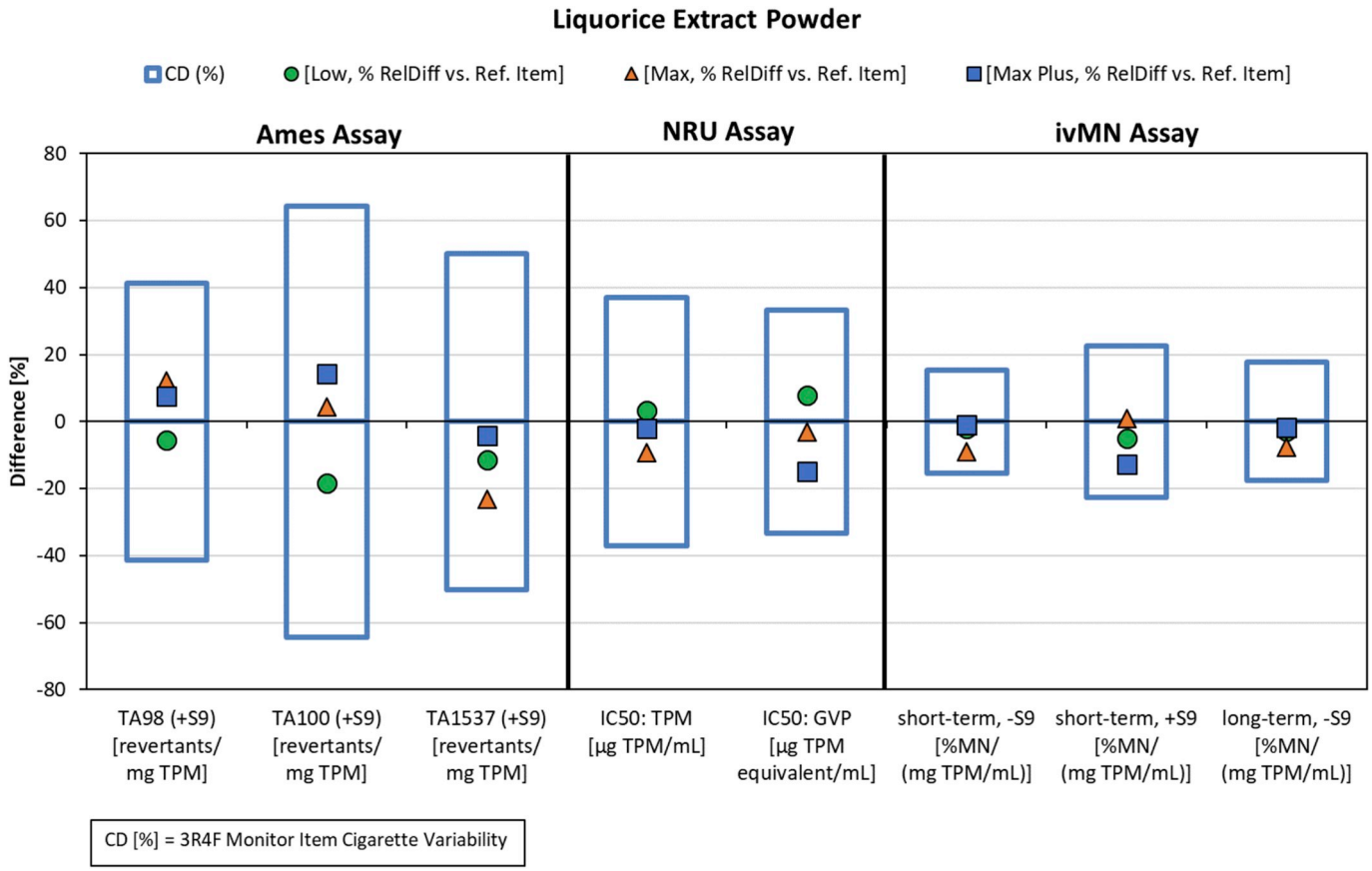

Fig. 30. Relative percentage difference (% RelDiff) between ivMN and Ames assay linear regression slopes and IC_{50} of the NRU assay for TPM/GVP from MS of cigarettes containing Low (0.6%), Max (1.2%) and Max Plus (1.8%) levels of liquorice and the corresponding reference cigarette containing no additive compared to the 3R4F monitor cigarette variability.

maximum inclusion level of 0.0015%.

4.8. Guar gum

Guar gum is widely used in many consumer goods, such as foods (Mudgil et al., 2014) and cosmetics (Windholz et al., 1976) and in pharmaceuticals (Martindale, 2014), so there is a long history of consumer exposure to this additive. The scientific literature demonstrates that guar gum has no carcinogenic (NTP, 1982b; EFSA, 2017b), mutagenic/genotoxic (Stanford Research Inst., 1972a,b; Zeiger et al., 1992; EFSA, 2017b), or reprotoxic properties (Collins et al., 1987; EFSA, 2017b).

In pyrolysis experiments, hydroxymethylfurfural, acetol, acetic acid, and methyl pyruvate were determined as main degradation products when guar gum was pyrolyzed (Baker and Bishop, 2005). Based on the complex chemical composition and non-volatile nature of the natural constituents, as well as preliminary indication of degradation during pyrolysis, the components of guar gum are unlikely to transfer intact, and no transfer rates were found in literature. Furthermore, the scientific literature demonstrates that guar gum, when used as a tobacco additive, does not increase either the *in vitro* or the *in vivo* toxicity of mainstream cigarette smoke. Inclusion levels between 0.01% and 9% guar gum in test cigarettes resulted in isolated, inconsistent instances of significant increases and decreases in emissions when compared to control cigarettes (Baker et al., 2004c; Coggins et al., 2011a, 2013), with no impact on the biological activity of cigarette smoke *in vitro* (Baker et al., 2004a; Coggins et al., 2011a, 2013) and *in vivo* (Baker et al., 2004a; Coggins et al., 2011a).

Summarizing our study results, our smoke chemistry study showed statistically significant and meaningful increases for formaldehyde (68%, 100%) and cadmium (46%, 47%) at the Max (1.0%) and Max Plus (1.5%) levels for the test cigarettes containing guar gum as single additive. When the cadmium level was analyzed in the present guar gum sample, a level of 0.005 mg/kg (5 ppb) cadmium was determined. With a transfer rate of 10% (3%–10% cadmium transfer for a filter cigarette were previously reported by Piade et al., 2015), less than 0.1% of the cadmium increase determined between the reference and the test cigarettes could be explained by the cadmium impurity of the food-grade guar gum sample. Other mechanisms changing cadmium emissions were discussed in literature. Piade et al. (2015) showed that part of the cadmium compounds are part of the GVP and can be filtered by activated charcoal filters. However, our experimental cigarettes have the same cigarette construction. Therefore, difference in filtration and in transfer rates are not expected.

In particular for formaldehyde and cadmium emissions, literature data showed inconsistent changes in smoke chemistry for test cigarettes with guar gum compared to reference cigarettes. When guar gum was added as single additive up to 2.2%, Coggins et al. (2011a) found a statistically significant 26% increase for formaldehyde at the medium level of 1.1%, but no increase in formaldehyde at the highest level and no increase for cadmium for all levels. In addition, the various pyrolysis methods published in literature did not report an increase in formaldehyde and cadmium for guar gum, because none of these methods are able to detect trace elements or low molecular weight aldehydes. However, our present *in vitro* data should show an increase in cytotoxicity. At least the increase in formaldehyde is expected to increase the cytotoxicity responses (Stabbert et al., 2017), whereas cadmium compounds were negative in the bacterial mutagenicity assay and tested positive mainly in *in vivo* genotoxicity tests (IARC, 2012). Taking into consideration that formaldehyde is known to be part in TPM as

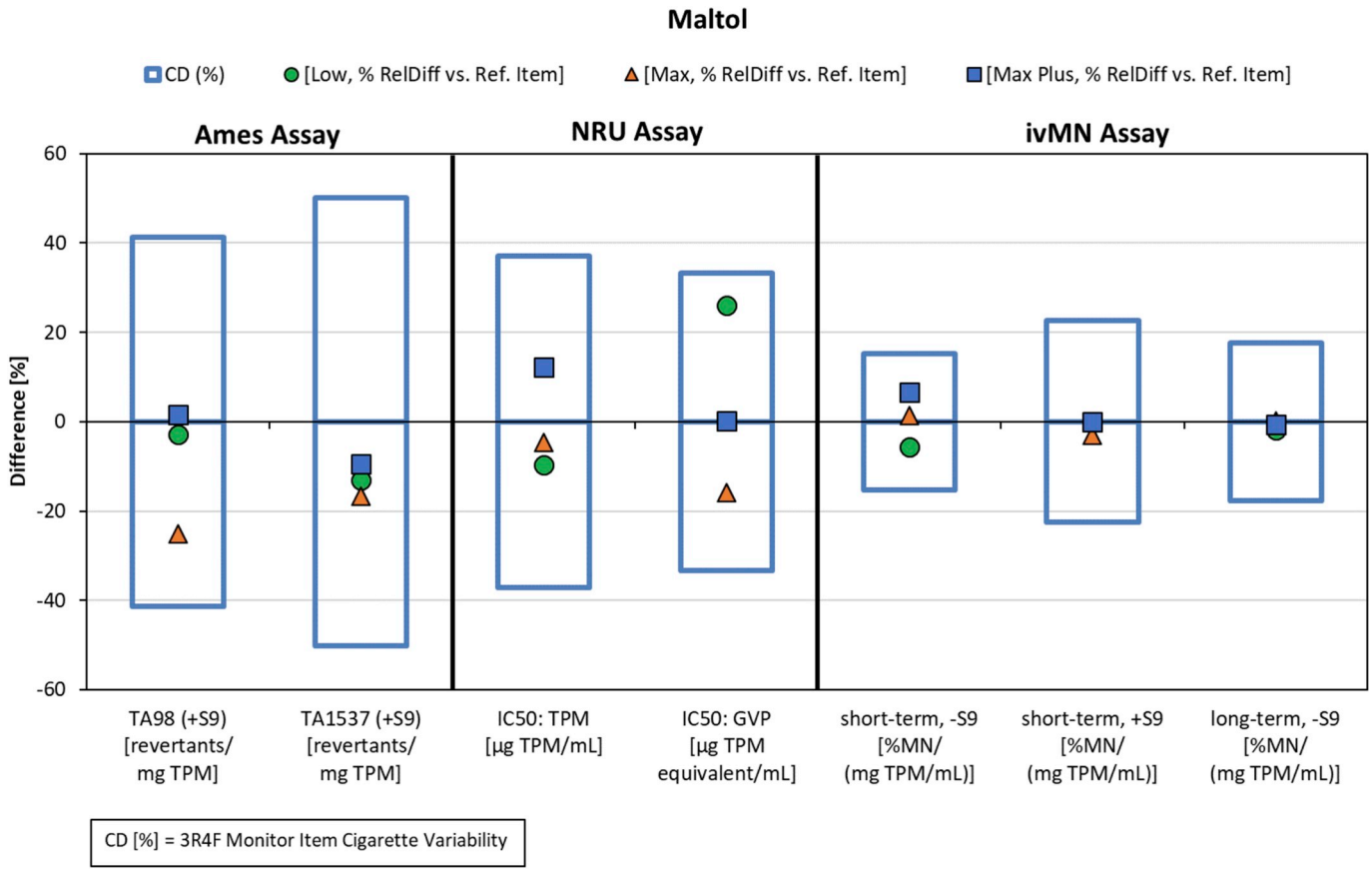

Fig. 31. Relative percentage difference (% RelDiff) between ivMN and Ames assay linear regression slopes and IC_{50} of the NRU assay for TPM/GVP from MS of cigarettes containing Low (0.005%), Max (0.010%) and Max Plus (0.015%) levels of maltol and the corresponding reference cigarette containing no additive compared to the 3R4F monitor cigarette variability.

well as in GVP and is known to increase the cytotoxicity, with a two-fold increase of formaldehyde from 17.3 to 34.6 µg/cig at the Max Plus level, we would expect to see a slight numerical increase in the NRU for both MS fractions. However, both MS fractions showed no statistically significant changes for all levels and the GVP a slight numerical decreasing trend. Taking all the information together, the sources for the increases in cadmium and formaldehyde are unknown and the results are contradictory to literature data and our *in vitro* results.

4.9. Liquorice

Liquorice and its derivatives are widely used in many consumer goods, such as foods (SCF, 2003), flavorants, cosmetics (CIR EP, 2007), and medicines, so there is a long history of consumer exposure to this additive. The scientific literature demonstrates that liquorice has no carcinogenic (JECFA, 2005; SCF, 2003, 2005), mutagenic/genotoxic (JECFA, 2005; SCF, 2003, 2005), or reprotoxic properties (Food and Drug Research Laboratories, 1972; Mantovani et al., 1988; JECFA, 2005; SCF, 2003, 2005).

In pyrolysis experiments, acetic acid, acetol, and furfuryl alcohol were determined as main degradation products when liquorice was pyrolyzed (Baker and Bishop, 2005). In a further study by Carmines and colleagues (Carmines et al., 2005) the major component of liquorice extract, glycyrrhizic acid, was not observed in the pyrolysis studies, suggesting that glycyrrhizic acid would not be present in mainstream cigarette smoke. Two other studies (Sakagami, 1973; Purkis et al., 2011) showed that when liquorice extract or glycyrrhizic acid were added to tobacco, the compounds underwent full degradation and no glycyrrhizic acid was detected in MS. In the present study, the measured glycyrrhizic acid concentrations in smoke were all below the detection limit. As such, there was no measurable transfer into the smoke. Furthermore, the scientific literature demonstrates that liquorice extract powder, when used as a tobacco additive, does not increase either the *in vitro* or the *in vivo* toxicity of mainstream cigarette smoke. Inclusion levels between 0.0001% and 8.0% liquorice extract powder in test cigarettes resulted in isolated, inconsistent instances of significant increases (e.g., benzo[*a*]anthracene) and decreases (e.g., NNN, NNK) in emissions when compared to control cigarettes (Carmines et al., 2005; Baker et al., 2004a; Roemer et al., 2014; Rustemeier et al., 2002*et a*), with no impact on the biological activity of cigarette smoke *in vitro* (Carmines et al., 2005; Baker et al., 2004a; Roemer et al., 2014; Roemer et al., 2002, 2014, 2014) and *in vivo* (Baker et al., 2004a; Carmines et al., 2005; Gaworski et al., 1998, 1999; Schramke et al., 2014; Vanscheeuwijck et al., 2002).

In summary, the results of our chemistry and *in vitro* studies were in line with published data showing no statistically significant and meaningful increases in smoke constituents, cytotoxicity, mutagenicity, and genotoxicity when liquorice extract powder was tested as a single additive up to a maximum inclusion level of 1.8%.

4.10. Maltol

Maltol is reported to occur naturally in a wide variety of foods (Burdock, 2010; EFSA FEEDAP, 2016) including wheat and rye bread, milk, and butter, and it is used as food flavoring, so there is a long history of consumer exposure to this additive. The scientific literature demonstrates that maltol has no carcinogenic (Gralla et al., 1969; EFSA CEF Panel, 2015), mutagenic/genotoxic (EFSA CEF Panel, 2015; Beevers, 2013, 2015), or reprotoxic properties (King, 1978).

In pyrolysis experiments, 99.8%–100% of the maltol remained

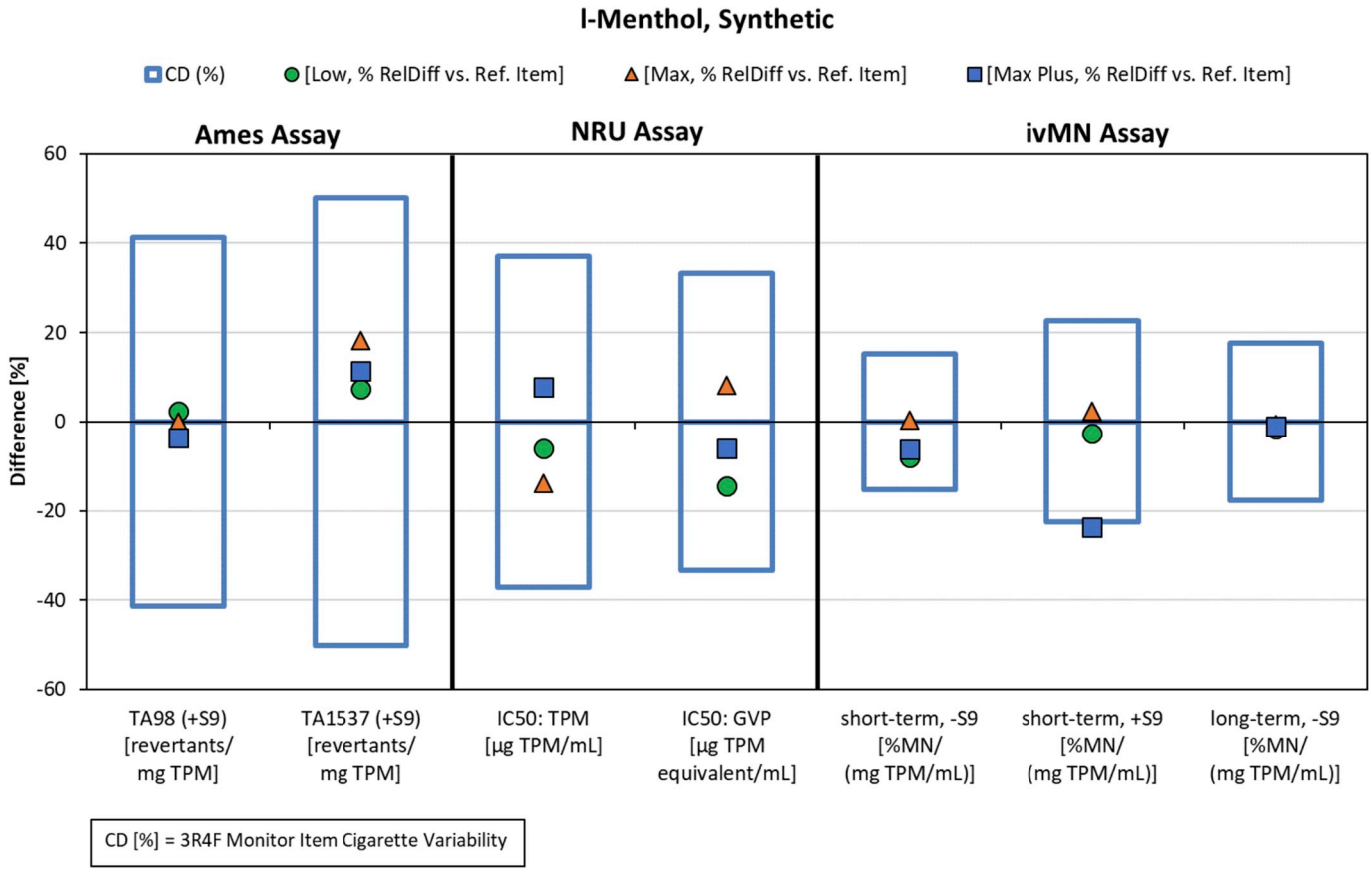

Fig. 32. Relative percentage difference (% RelDiff) between ivMN and Ames assay linear regression slopes and IC_{50} of the NRU assay for TPM/GVP from MS of cigarettes containing Low (0.6%), Max (1.2%) and Max Plus (1.8%) levels of *l*-menthol, synthetic and the corresponding reference cigarette containing no additive compared to the 3R4F monitor cigarette variability.

intact when the sample was heated (Baker and Bishop, 2004; Purkis et al., 2011). So far, no transfer rates of maltol into cigarette smoke have been published in literature. In the present study, the maltol transfer from cigarette tobacco into smoke was approximately 4.9%.

The scientific literature demonstrates that maltol, when used as a tobacco additive, does not increase either the *in vitro* or the *in vivo* toxicity of mainstream cigarette smoke. Inclusion levels between 0.00002% and 1.0% maltol in test cigarettes resulted in isolated, inconsistent instances of significant increases (e.g., NNK) and decreases (e.g., *N*-nitrosodimethyl-amine) in emissions when compared to control cigarettes (Baker et al., 2004a, 2004b; Coggins et al., 2011c; Roemer et al., 2014; Rustemeier et al., 2002), with no impact on the biological activity of cigarette smoke *in vitro* (Baker et al., 2004a, 2004b; Coggins et al., 2011c; Renne et al., 2006; Roemer et al., 2002, 2014) and *in vivo* (Baker et al., 2004a, 2004b; Gaworski et al., 1998, 1999; Renne et al., 2006; Schramke et al., 2014; Vanscheeuwijck et al., 2002).

In summary, the results of our chemistry and *in vitro* studies were in line with published data showing no statistically significant and meaningful increases in smoke constituents, cytotoxicity, mutagenicity, and genotoxicity when maltol was tested as a single additive up to a maximum inclusion level of 0.015%.

4.11. Menthol

Menthol is widely used in many consumer goods, such as foods and oral hygiene products, and in topical therapeutic preparations (Heck, 2010), so there is a long history of consumer exposure to this additive. The scientific literature demonstrates that menthol has no carcinogenic (OECD, 2003), mutagenic/genotoxic (JECFA, 1999; OECD, 2003), or reprotoxic properties (FDA, 1973; OECD, 2003).

In pyrolysis experiments when a sample was heated, 99.0%–97.4% of the menthol remained intact, along with some minor degradation products (Baker and Bishop, 2004; Purkis et al., 2011). Menthol transfer rates between 20.5% and 28.3% were determined for unventilated cigarettes (Jenkins et al., 1970; Best, 1972; and Purkis et al., 2011), whereas transfer rates of around 10% were reported for contemporary cigarettes (Best, 1993). The transfer rate determined in the present study was about 10%, which is in line with the published transfer rates of contemporary cigarettes.

The scientific literature demonstrates that menthol, when used as a tobacco additive, does not increase the *in vitro* or the *in vivo* toxicity of mainstream cigarette smoke. Inclusion levels between 0.0002% and 10% menthol in test cigarettes resulted in isolated, inconsistent instances of significant increases (e.g., tar) and decreases (e.g., catechol) in emissions when compared to control cigarettes (Baker et al., 2004a; Rustemeier et al., 2002), with no impact on the biological activity of cigarette smoke *in vitro* (Baker et al., 2004a; Noriyasu et al., 2013; Rakieten et al., 1952; Renne et al., 2006; Roemer et al., 2002) and *in vivo* (Baker et al., 2004a; Gaworski et al., 1998, 1999; Ha et al., 2015; Renne et al., 2006; Vanscheeuwijck et al., 2002).

In summary, the results of our chemistry and *in vitro* studies were in line with published data, showing no statistically significant and

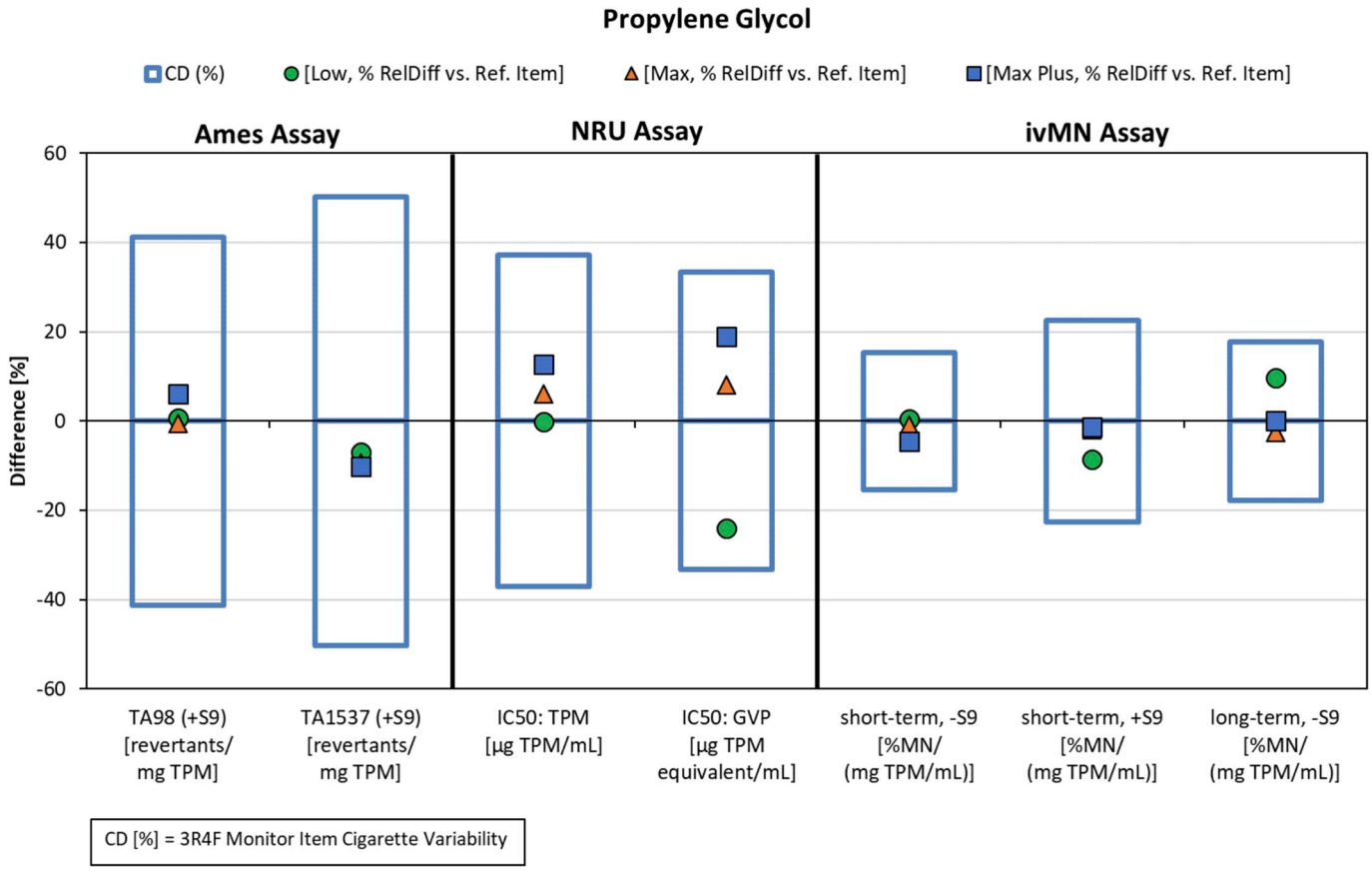

Fig. 33. Relative percentage difference (% RelDiff) between ivMN and Ames assay linear regression slopes and IC_{50} of the NRU assay for TPM/GVP from MS of cigarettes containing Low (2.5%), Max (5.0%) and Max Plus (6 + %) levels of propylene glycol and the corresponding reference cigarette containing no additive compared to the 3R4F monitor cigarette variability.

meaningful increases in smoke constituents, cytotoxicity, mutagenicity, and genotoxicity when menthol was tested as a single additive up to a maximum inclusion level of 1.8%.

4.12. Propylene glycol

Propylene glycol is widely used in many consumer goods, such as foods and cosmetics, and in pharmaceuticals (Werley et al., 2011; Fowles and Pottenger, 2013), so there is a long history of consumer exposure to this additive. The scientific literature demonstrates that propylene glycol has no carcinogenic (NTP, 2004a; NTP, 2004b), mutagenic/genotoxic (ECHA 2010a, 2010b, 2010c, 2010d; Hayashi et al., 1988; Ishidate et al., 1984; Fowles and Pottenger, 2013), or reprotoxic properties (ATSDR, 1997; NTP, 2004b; Fowles and Pottenger, 2013).

In pyrolysis experiments, it was reported that 86.3%–99.4% of the propylene glycol was transferred intact, along with some minor degradation products (Baker and Bishop, 2004; Purkis et al., 2011). Propylene glycol transfer rates between 7.3% and 8.8% for an unventilated cigarette containing ^{13}C labelled propylene glycol were reported (Purkis et al., 2011). The transfer rate determined in the present study with ventilated cigarettes was about 1%. The test cigarettes used in our study had a ventilation rate of 36%; therefore, a lower transfer rate is expected for our test cigarettes compared to those used by Purkis and colleagues.

The scientific literature demonstrates that propylene glycol, when used as a tobacco additive, does not increase either the *in vitro* or the *in vivo* toxicity of mainstream cigarette smoke. Inclusion levels between 0.57% and 10% propylene glycol in test cigarettes resulted in isolated, inconsistent instances of significant increases and decreases in emissions (e.g., acrolein, nicotine) when compared to control cigarettes (Baker et al., 2004b, 2004c; Rustemeier et al., 2002; Gaworski et al., 2010; Coggins et al., 2013), with no impact on the biological activity of cigarette smoke *in vitro* (Baker et al., 2004b, 2004c; Coggins et al., 2013; Gaworski et al., 2010; Roemer et al., 2002) and *in vivo* (Baker et al., 2004a, 2004c; Gaworski et al., 1999, 2010; Heck et al., 2002; Vanscheeuwijck et al., 2002).

In summary, our chemistry study showed decreases in the emissions of phenol and *m* + *p*-cresols that exceeded the inherent variability of the analytical method and were statistically significant. Therefore, the results of our chemistry and *in vitro* studies were in line with published data, showing no statistically significant and meaningful increases in smoke constituents, cytotoxicity, mutagenicity, and genotoxicity when propylene glycol was tested as a single additive up to a maximum inclusion level of 6.0 + %.

4.13. Sorbitol

Sorbitol is widely used in many consumer goods, such as foods and cosmetics and in pharmaceuticals (JECFA, 1982; Kearsley and Deis, 2006), so there is a long history of consumer exposure to this additive.

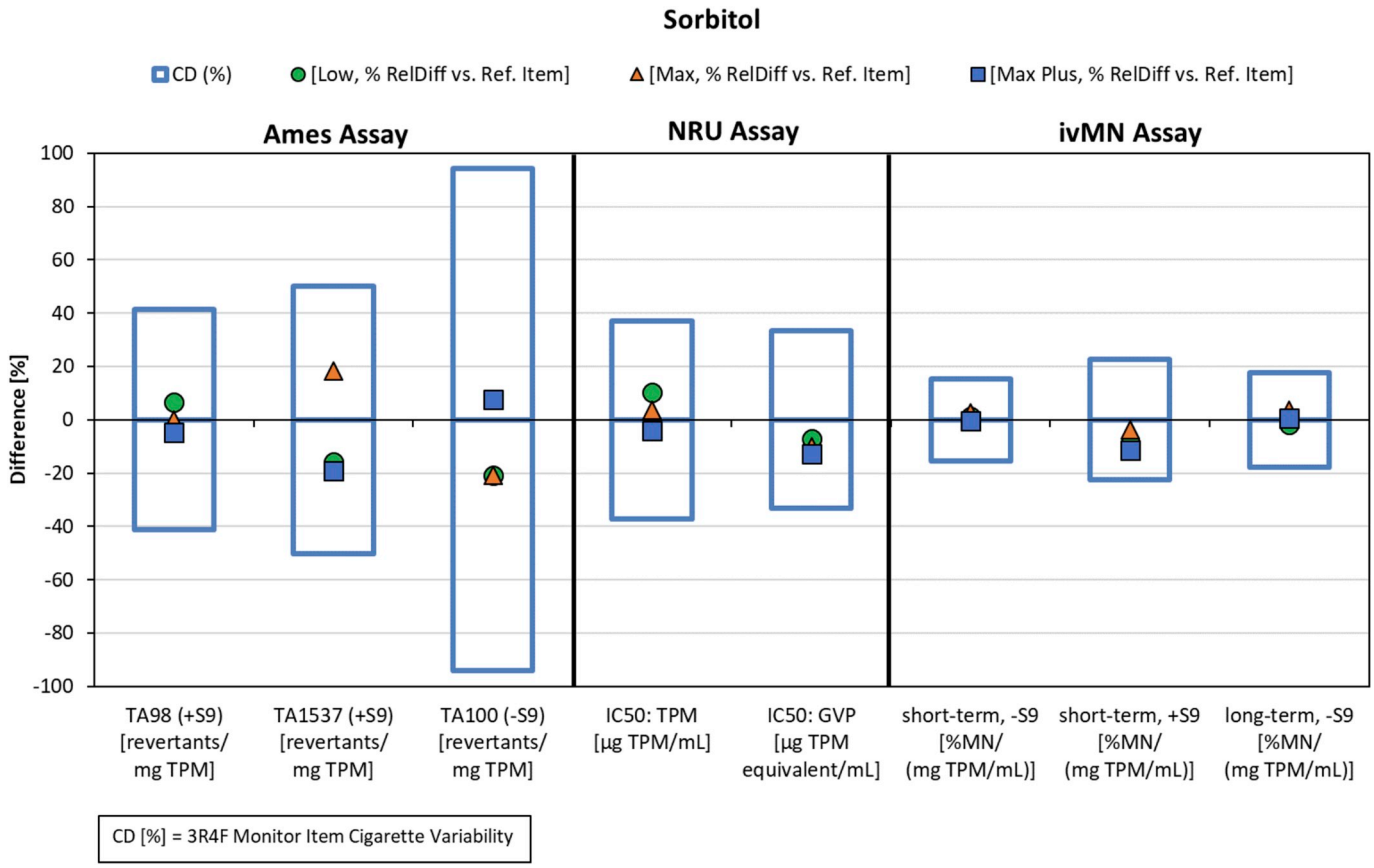

Fig. 34. Relative percentage difference (% RelDiff) between ivMN and Ames assay linear regression slopes and IC_{50} of the NRU assay for TPM/GVP from MS of cigarettes containing Low (0.6%), Max (1.2%) and Max Plus (1.8%) levels of sorbitol and the corresponding reference cigarette containing no additive compared to the 3R4F monitor cigarette variability.

The scientific literature demonstrates that sorbitol has no carcinogenic (Hunter et al., 1978), mutagenic/genotoxic (Felzenszwalb et al., 2013; Fujita and Sasaki, 1986; Chételát, 1980; Gallandre, 1980; Stanford Research Inst., 1972a,b), or reprotoxic properties (MacKenzie et al., 1986; Palmer et al., 1978).

In pyrolysis experiments, furfural, propylfuran, acetylfuran, and furanone were determined as main degradation products when sorbitol was pyrolyzed (Baker and Bishop, 2005). Sorbitol is a solid material and breaks down completely during pyrolysis, forming mainly furfural. When cigarettes were smoked with and without sorbitol, no statistically significant difference in the furfural yields was detected between test and reference cigarettes. Presumably, during cigarette smoking the sorbitol does not completely decompose, as in the pyrolysis study. Consequently, in this case the pyrolysis result are not predictive for the smoke composition of cigarettes with sorbitol. Based on the non-volatile nature of sorbitol and indications of its degradation during pyrolysis, sorbitol is unlikely to transfer intact and no transfer rates were found in literature.

The scientific literature demonstrates that sorbitol, when used as a tobacco additive, does not increase either the *in vitro* or the *in vivo* toxicity of mainstream cigarette smoke. Inclusion levels between 1.5% and 10% sorbitol in test cigarettes resulted in isolated, inconsistent instances of significant increases (e.g., water) and decreases (e.g., nicotine, NNK) in emissions when compared to control cigarettes (Coggins et al., 2011d; Baker et al., 2004a, 2004c), with no impact on the biological activity of cigarette smoke *in vitro* (Coggins et al., 2011d; Baker et al., 2004a; Baker et al., 2004c) and *in vivo* (Baker et al., 2004a, 2004c; Coggins et al., 2011d).

Summarizing our study results, our smoke chemistry study showed increases for acrolein (81%) and formaldehyde (102%) at the Max Plus (1.8%) level for the test cigarettes containing sorbitol as single additive. In particular for aldehyde emissons, literature data showed a statistically significant 9% decrease in acetaldehyde and a statistically significant 23% increase in acrolein at the highest level of 10% sorbitol, but no increase in formaldehyde for all levels (Coggins et al., 2011d). No statistically significant changes were reported for all aldehydes at the lower levels of 4.5% and 1.5% sorbitol. In addition, the various pyrolysis methods published in literature did not report an increase in formaldehyde and acrolein for sorbitol, because none of these methods are able to detect low molecular weight aldehydes. However, our *in vitro* data should show an increase. Taking into consideration that formaldehyde is known to be part in TPM and in GVP, acrolein occurs mainly in the GVP, and both are known to increase the cytotoxicity, we would expect to see an increase in cytotoxicity for both MS fractions. Stabbert et al. (2017) showed that 50% of GVP cytotoxicity of the Kentucky Reference cigarette 1R4F was explained by acrolein. Based on this result, an 80% increase for acrolein at the Max Plus level should result in an increase of about 40% in the GVP cytotoxicity response. However, both MS fractions showed no statistically significant changes for all levels.

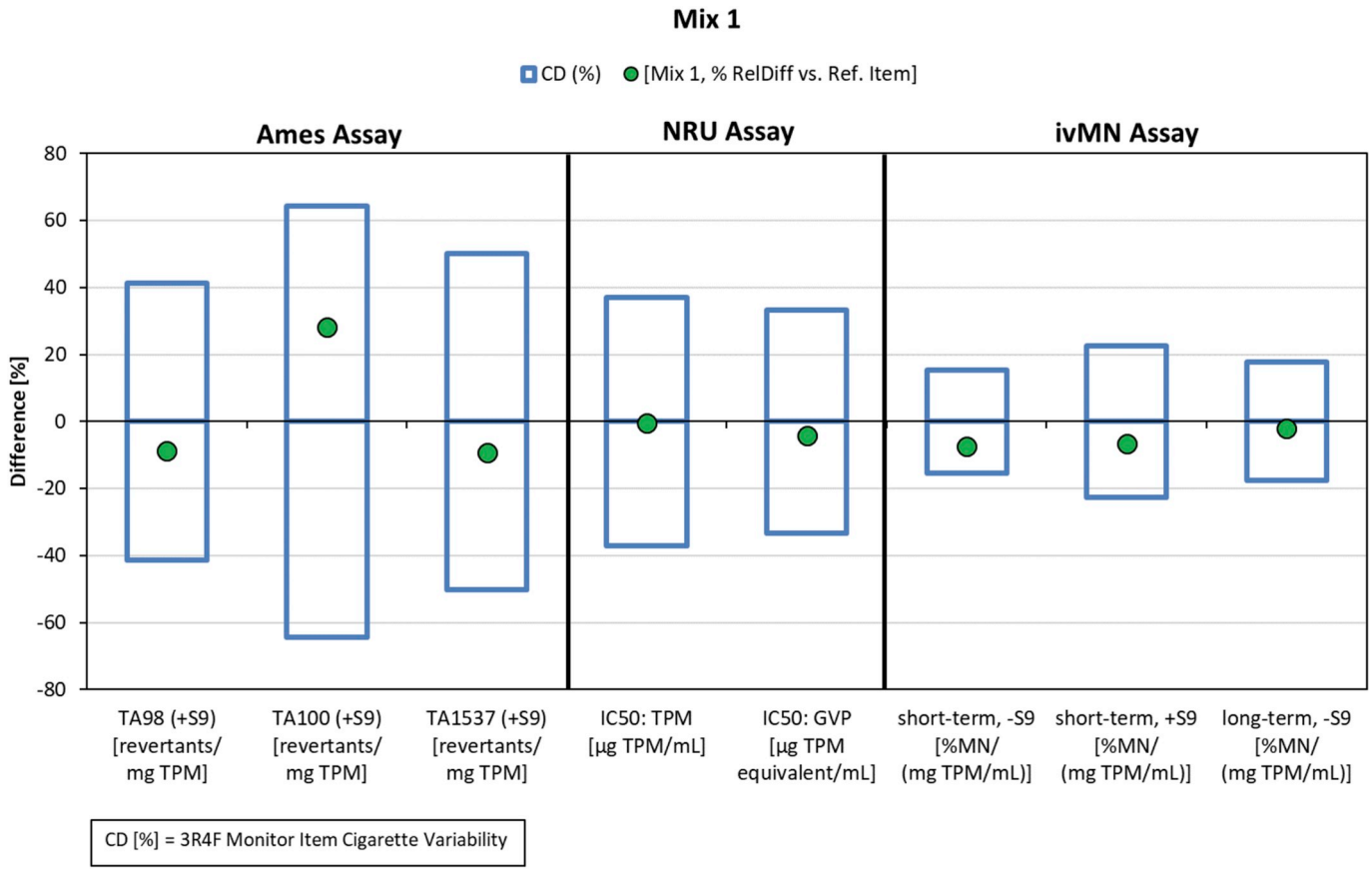

Fig. 35. Relative percentage difference (% RelDiff) between ivMN and Ames assay linear regression slopes and IC_{50} of the NRU assay for TPM/GVP from MS of cigarettes the additive Mix 1 and the corresponding reference cigarette containing no additive compared to the 3R4F monitor cigarette variability.

The sorbitol used in this study is the food-grade, liquid sorbitol E420 (ii) which contained 77.7% sorbitol, 2.4% mannitol, 0.11% reducing sugars, and 10.8% total sugars according to the supplier's specification and compliant to the regulation COMMISSION REGULATION (EU) No 231/2012. To what extend the impurities of the E420 (ii) grade sorbitol are the sources of the increase in formaldehyde and acrolein is unknown. Coggins et al. (2011d) did not provide further information on the purity of their test substances. However, most of the other carbohydrates investigated by Coggins et al. (2011d) did not show a statistically significant increase in acrolein or small increases at very high inclusion levels. Taking all the information together, the sources for the increases in acrolein and formaldehyde are unknown and the results are contradictory to our *in vitro* results.

4.14. Additive mixtures

When the additives were applied as mixtures to the tobacco of test cigarettes, chemical analyses showed no statistically significant differences in smoke emissions between cigarettes with and without the additive mixtures, which exceeded the inherent variability of the analytical method. This result is consistent with those obtained in other additive mixture studies. Overall, smoke chemistry data give no indication that the additions of additive mixtures increase the toxicity of cigarette smoke (Baker et al., 2004a; Carmines, 2002; Renne et al., 2006).

There was no statistically significant increase in biological activity, which exceeded the inherent variability of the methods when the test cigarettes containing the additive mixtures 1, 2 and 3 were tested in *in vitro* assays (Ames test, NRU, and *iv*MN) and the results were compared to those of the reference cigarette. Other studies have resulted in the same outcome for additive mixtures. The *in vitro* toxicology data affirm that the additive mixtures 1, 2, and 3 do not increase the toxicity of tobacco smoke.

5. Limitations

There is a general concern about the sensitivity of the applied methods/assays in particular for the *in vitro* assays when used in a comparative testing approach. While SCHEER (2016) questions that the methods have sufficient discriminatory power, Oldham et al. (2012) concluded that, “the discriminatory power of these studies is suitable for the detection of differences in the toxicity of mainstream cigarettes smoke that may potentially be introduced by the use of ingredients.”

Our studies were performed to fulfill the regulatory requirement of the EU Tobacco Products Directive 2014/40/EU for cigarettes and Roll Your Own tobacco containing an additive that is included in the priority list established by Commission Implementing Decision (EU) 2016/787. Article 6(2) in the Tobacco Products Directive provides that the studies have to examine whether additives have the effect of increasing the toxicity, or CMR properties of any of the products concerned “*to a significant or measurable degree.*” The legislature considers only significant or measureable increases to be relevant. It is the intrinsic toxicity of cigarette smoke that establishes the baseline for determining what constitutes an increase of “significant or measurable degree” in Article

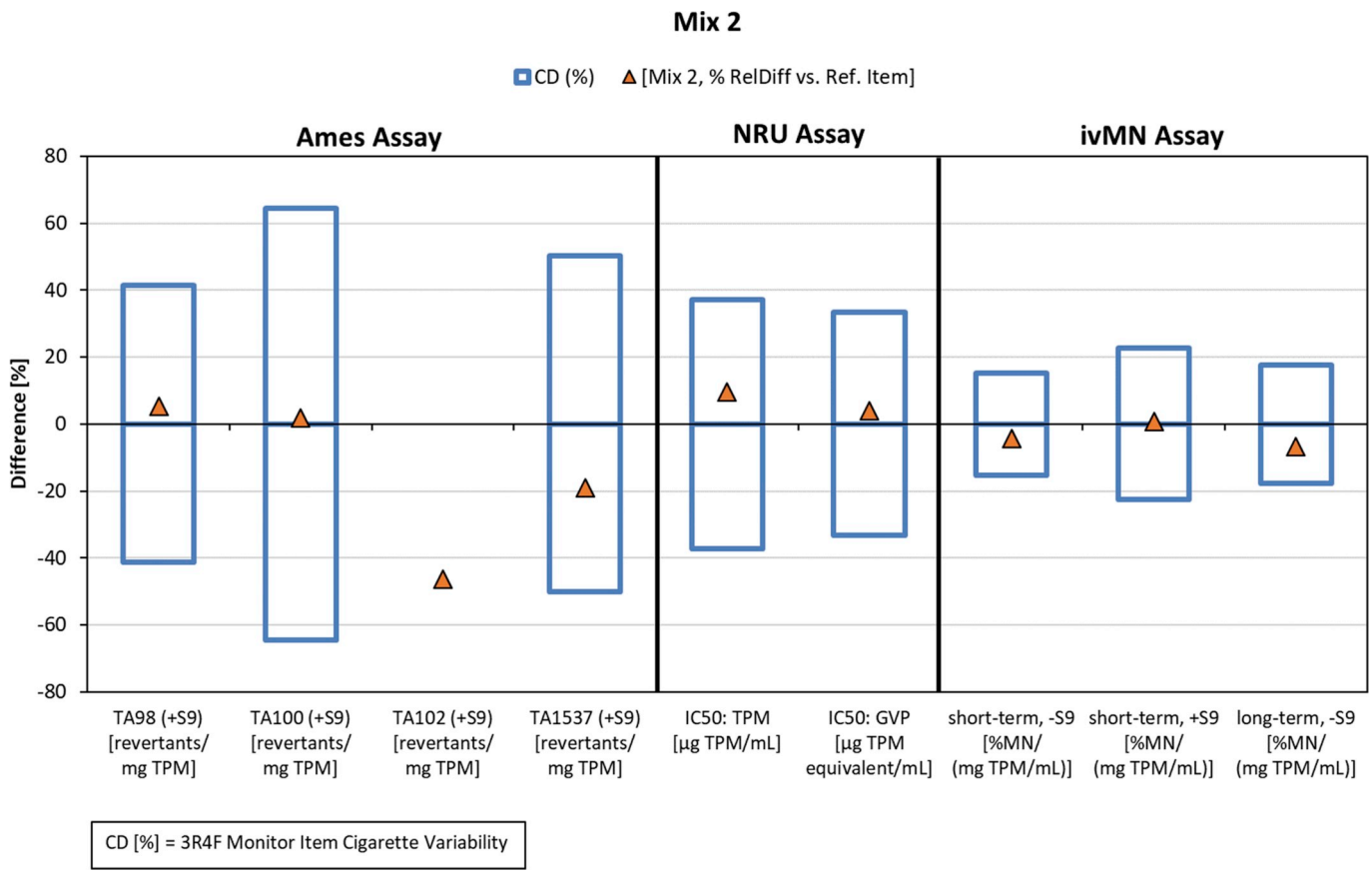

Fig. 36. Relative percentage difference (% RelDiff) between ivMN and Ames assay linear regression slopes and IC_{50} of the NRU assay for TPM/GVP from MS of cigarettes containing the additive Mix 2 and the corresponding reference cigarette containing no additive compared to the 3R4F monitor cigarette variability.
Note: For the 3R4F monitor cigarette, the regression slope variability for strain TA102 (+S9) was not determined due to historical slopes not being significantly different from zero. Test cigarettes containing Mix 2 additives and the reference cigarette both had significant slopes (i.e. greater than zero) in strain TA102 (+S9), however the difference in mean slopes was not statistically significant.

6(2). The legislature was aware of potential insignificant or non measureable increases and did not consider them relevant. Therefore, the discriminative power of Labstat's state-of-the-art analytical methods and *in vitro* assays is appropriate to meet the legislature's objectives.

6. Conclusions

The data presented in this publication shows the results of comprehensive smoke chemistry and *in vitro* studies (i.e., Ames assay, NRU assay, ivMN assay) required by EU TPD (2014/40/EU) Article 6(2) that were commissioned by a consortium of 12 tobacco companies to independent CROs.

Transfer rates for geraniol, glycerol, liquorice extract powder, maltol, *l*-menthol (synthetic), and propylene glycol were determined which were in the range between 1% and 11%. Guaiacol yields for the additive-free reference cigarette and the test cigarettes were similar taking into consideration the analytical method variability. Therefore, no reliable transfer rates for guaiacol could be calculated. For liquorice extract powder, glycyrrhizin was below the detection limit in smoke. Therefore, no transfer rates glycyrrhizin were calculated.

Comparisons of the 39 WHO smoke emissions in smoke from cigarettes with and without priority additives resulted in differences that, with only a few exceptions, were minor and mostly well within the inherent variability of the analytical method, not statistically significant, and did not show consistent additive-related increases or decreases. However, test cigarettes with guar gum showed an increase in formaldehyde and cadmium; test cigarettes with sorbitol showed an increase in formaldehyde and acrolein; test cigarettes with glycerol showed a decrease in phenols, B[*a*]P and NAB; and test cigarettes with propylene glycol showed a decrease in phenol and *m* + *p*-cresols. When the additives were tested as mixtures these changes were not observed. None of the increases or decreases in smoke chemistry translated into measured changes of *in vitro* toxicity using the assays selected for the study. Comparisons of the *in vitro* toxicity of smoke from cigarettes with and without priority additives resulted in differences that were minor and well within the inherent variability of the assays, not statistically significant, and did not show consistent additive-related increases or decreases. Thus, it was concluded that the addition of priority additives had no effect on the *in vitro* toxicity of the cigarette smoke under the conditions applied in the study. The results obtained in the present studies are consistent with those of similar studies reported in the scientific literature (Gaworski et al., 2011).

Conflict of interest statement

All authors with the exception of ER are employees of the tobacco companies that formed the “Priority Additives Tobacco Consortium”. ER contributed as a consultant and as such was compensated for his work.

Funding statement

This work was supported equally by each of the 12 Tobacco companies that together formed the Priority Additives Tobacco Consortium.

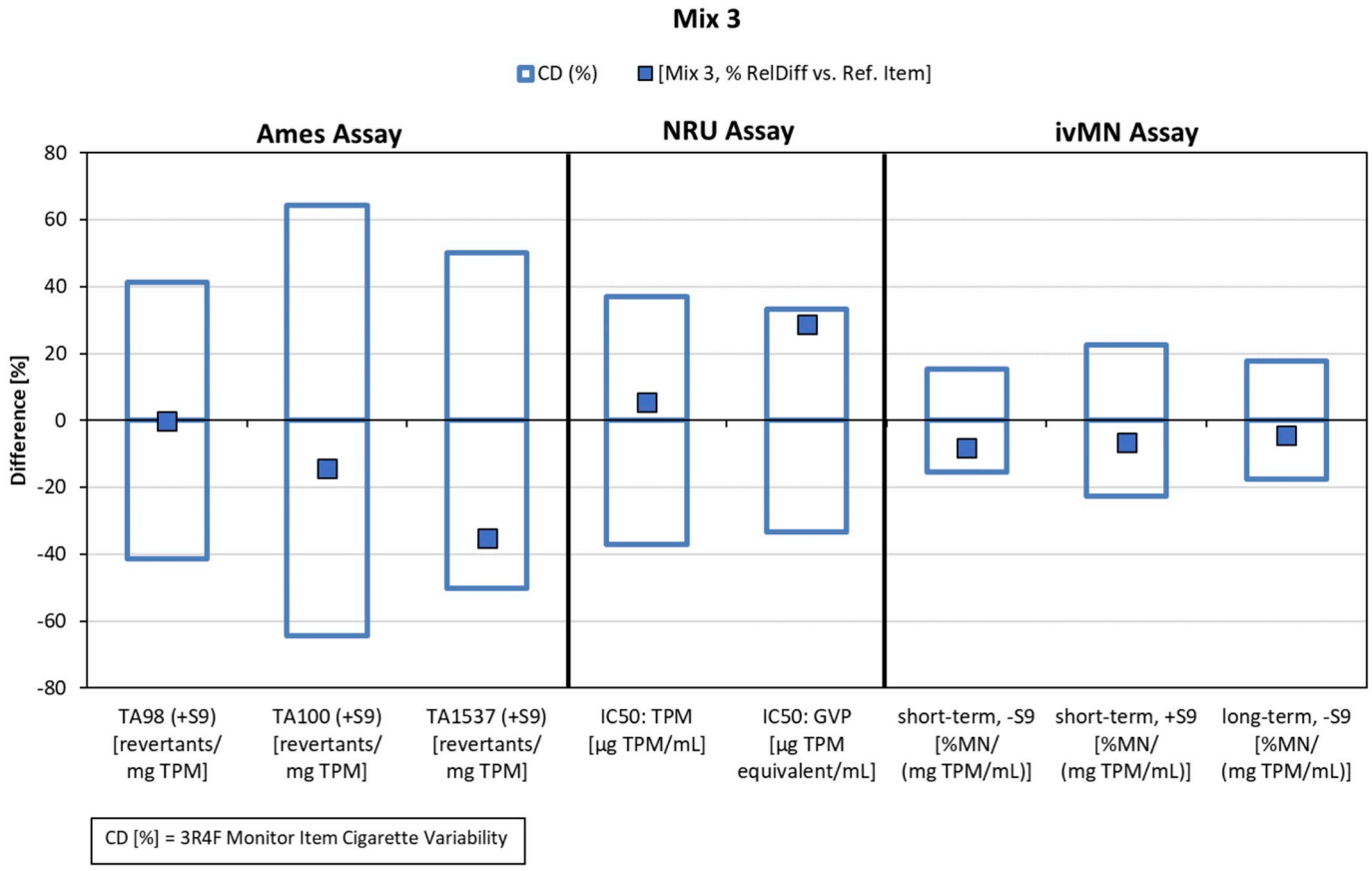

Fig. 37. Relative percentage difference (% RelDiff) between ivMN and Ames assay linear regression slopes and IC_{50} of the NRU assay for TPM/GVP from MS of cigarettes containing the additive Mix 3 and the corresponding reference cigarette containing no additive compared to the 3R4F monitor variability.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yrtph.2019.03.002.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.yrtph.2019.03.002

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