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The Addition of Cocoa, Glycerol, and Saccharose to the Tobacco of Cigarettes: Implications for Smoke Chemistry, *In Vitro* Cytotoxicity, Mutagenicity and Further Endpoints*

by

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SUMMARY

The cigarette ingredients cocoa powder, glycerol, and saccharose were investigated regarding their potential effect on the resulting mainstream smoke, i.e., smoke chemistry (Hoffmann analytes), mammalian cell cytotoxicity (Neutral Red Uptake assay), and bacterial mutagenicity (Ames assay). Each ingredient was added at three concentrations to the tobacco of a 6 mg and 10 mg 'tar' yield experimental American blend filter cigarette (obtained under ISO/FTC smoking regime). The lowest application concentration was equivalent to the normal approximate use level of the ingredients; the highest application level was up to 5-fold higher. The resulting data were compared with the respective control cigarettes without addition of the ingredients.

The addition of cocoa powder did not lead to any consistent effects on the measured mainstream smoke analytes. Neither the *in vitro* cytotoxicity nor the *in vitro* mutagenicity was affected by cocoa addition. The addition of glycerol resulted in a decrease in the delivery of several smoke constituents (generally around 20%), e.g. aldehydes, phenolics, and *N*-nitrosamines. Water in the particulate phase (TPM) was distinctly increased (up to +150%). The cytotoxicity of the TPM was decreased (approx. −15%). Mutagenicity was not affected. Saccharose addition consistently increased formaldehyde delivery in smoke by up to 40% and decreased tobacco-specific *N*-nitrosamines by up to approximately 20%. The increase in formaldehyde is discussed in the context of the human smoker. The cytotoxicity was not affected by the addition of saccharose, while the mutagenicity of the TPM was decreased in tester strain TA98 with metabolic activation (−15%).

The results are in agreement with currently available literature. Some investigations summarized in this publication are novel and have not yet been reported in the literature. Based on the total evidence, it can be concluded that the three ingredients added at their current use levels do not increase the inherent toxicity of the cigarette smoke. [Beitr. Tabakforsch. Int. 24 (2010) 117–138]

ZUSAMMENFASSUNG

Die Zigarettenadditive Kakaopulver, Glycerin und Saccharose wurden bezüglich eines potentiellen Effekts auf den Rauch, d.h., Rauchchemie (Hoffmann-Analyte), Toxizität in Säugetierzellen (Neutralrot-Aufnahme-Test) und Mutagenität in Bakterien (Ames-Test) untersucht. Jedes Additiv wurde jeweils in drei Konzentrationen zu zwei Zigaretten gegeben, die unter ISO/FTC-Abrauchbedingungen 6 mg oder 10 mg 'Teer' lieferten. Die Zigaretten waren Filterzigaretten vom Typ American Blend. Die niedrigste Zugabekonzentration entsprach der allgemeinen Gebrauchskonzentration der Additive; die höchste Zugabekonzentration war bis zu 5-fach höher. Die Ergebnisse wurden verglichen mit denen von den entsprechenden Kontrollzigaretten ohne die Zugabe von Additiven.

Die Zugabe von Kakaopulver hatte keinen konsistenten Effekt auf die gemessenen Hauptstromrauch-Analyten.

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Weder die Zytotoxizität *in vitro*, noch die Mutagenität *in vitro* wurden durch die Kakaozugabe beeinflusst. Die Zugabe von Glyzerin resultierte in der Abnahme von mehreren Rauchbestandteilen (im Allgemeinen ca. 20%) wie z.B. Aldehyde, phenolische Bestandteile und *N*-Nitrosamine. Wasser in der Partikelphase (TPM) war deutlich erhöht (bis zu +150%). Die Zytotoxizität des TPMs war erniedrigt (ca. −15%). Die Mutagenität war unverändert. Die Zugabe von Saccharose führte zu einer konsistenten Zunahme von Formaldehyd im Rauch bis zu 40% und verringerte die tabakspezifischen *N*-Nitrosamine bis zu 20%. Die Zunahme an Formaldehyd wird im Kontext des Rauchers diskutiert. Die Zytotoxizität wurde durch die Zugabe von Saccharose nicht beeinflusst, während die Mutagenität des TPMs im Teststamm TA98 mit metabolischer Aktivierung verringert war (−15%).
Die Ergebnisse stimmen mit denen aus der verfügbaren Literatur überein. Einige der hier zusammengefasst vorgestellten Untersuchungen sind neu, d.h. sie waren bisher noch nicht Gegenstand von Publikationen in der wissenschaftlichen Literatur. In Anbetracht aller Daten ergibt sich übereinstimmend, dass die drei Additive die inherente Toxizität von Zigarettenrauch nicht erhöhen, wenn sie den Zigaretten in den gegenwärtigen Gebrauchskonzentrationen zugegeben werden. [Beitr. Tabakforsch. Int. 24 (2010) 117–138]

RESUME

L'effet potentiel de l'addition à une cigarette de différents ingrédients - poudre de cacao, glycérine et saccharose -a été évalué par l'analyse chimique des rendements en composés de la «liste de Hoffmann» de sa fumée principale, ainsi que par la détermination de la cytotoxicité (essai de fixation du rouge neutre) et de la mutagénicité (essai d'Ames) de celle-ci. Chaque ingrédient a été ajouté au tabac (mélange Américain sans addition d'ingrédient) à trois différents niveaux. Deux types de cigarette-filtre expérimentales ont été produits à partir de chacun de ces mélanges pour obtenir des articles ayant des rendements ISO en goudron de 6 et de 10 mg. Le taux d'addition le plus bas a été choisi pour être représentatif du niveau d'addition usuel de chacun de ces ingredients et le plus élevé correspond à jusqu'à 5 fois ce taux. Tous les résultats analytiques ont été comparés à ceux obtenus sur la fumée des cigarettes de contrôle respectives, produites avec un mélange n'ayant reçu aucune addition d'ingrédient.
L'addition de poudre de cacao n'a permis d'observer aucun effet systématique sur les rendements des composés de la fumée principale analysés. Observées *in-vitro*, ni la cytotoxicité ni la mutagénicité de la fumée ne sont affectées par l'addition de poudre de cacao. L'addition de glycérine a conduit à une réduction (le plus souvent de l'ordre de 20%) des rendements de certains composés de la fumée comme les aldéhydes, les phénols et les *N*-nitrosamines. La quantité d'eau présente dans la phase particulaire de la fumée (TPM) a augmenté substantiellement (jusqu'à 150%). La cytotoxicité du TPM a baissé (de 15% approximativement) tandis que sa mutagénicité n'était pas affectée. L'addition de saccharose a systématiquement augmenté (jusqu'à 40%) le rendement en formaldéhyde dans la fumée, tandis que celui des nitrosamines spécifiques au tabac chutait d'environ 20%. L'augmentation des rendements en formaldéhyde est discutée en termes d'exposition pour le fumeur. La cytotoxicité n'a pas été affectée par l'addition de saccharose, alors que ceci provoquait une décroissance de la mutagénicité du TPM pour les tests pratiqués sur les souches TA98 après activation métabolique (−15%).
Ces résultats sont en accord avec ceux de la littérature. Certaines des déterminations résumées dans cette étude n'avaient encore jamais fait l'objet de publication. Sur la base des résultats pris dans leur globalité, on peut conclure que l'addition des 3 ingrédients aux niveaux pratiqués actuellement n'augmente pas la toxicité inhérente de la fumée des cigarettes. [Beitr. Tabakforsch. Int. 24 (2010) 117–138]

INTRODUCTION

Background

In American blend cigarettes, some ingredients, often called casings, are added to the tobacco blend in quantities around or above 1%. They are non volatile ingredients added early on in the manufacturing process and consist primarily of sugars, humectants and plant extracts such as licorice, cocoa and carob bean. The use of casings in the manufacture of cigarettes goes back to the early part of the 20th century (1). Indeed, the first so-called American Blend cigarette containing sugar, licorice and cocoa as major casings was introduced in 1913 (2). The major ingredients used in today's production, including sugar, licorice, cocoa, and the process for applying them, are very similar to the practices of that time (1). Then, as now, casings are used to improve the ability to process the tobacco (moisture retention and pliability), to re-balance the sugar/nitrogen ratio of air cured Burley tobacco and to enhance the taste and smoke characteristics of the blend (3). Further information about the actual quantities can be found on the websites of the major cigarette manufacturers. Because of the relatively high ingredient concentrations, there is a concern that ingredients may affect smoke composition, and thus, potentially impact the inherent toxicity of cigarette smoke. In 2007, the German Federal Ministry of Food, Agriculture, and Consumer Protection (Bundesministerium für Ernährung, Landwirtschaft und Verbraucherschutz, BMELV) initiated a project to test the hypothesis that the three ingredients cocoa, glycerol and saccharose applied to cigarette tobacco change the delivery of selected toxic mainstream smoke constituents. The Chemical and Veterinary Surveillance Agency Sigmaringen, Department 3, Section 31: Tobacco (Chemisches und Veterinär-Untersuchungsamt Sigmaringen, CVUA) was assigned to provide a study plan and to perform the experimental part of the project. The German cigarette industry was invited by the BMELV - via the Association of the German Cigarette Manufacturers (Verband der Cigarettenindustrie, VdC) - to manufacture and provide the experimental cigarettes for this study in accordance with the specifications obtained from the CVUA. The manufacturing and distribution of the experimental cigarettes was handled by a single international cigarette company based in Germany

and funded by the VdC. All parties considered the CVUA study plan to be scientifically valid and to present an in-depth approach to test the study hypothesis. The study plan included the use of two cigarette types, differing in their ISO 'tar' yield, two machine smoking regimens and three ingredient levels for both cigarette types for each ingredient type. In a spot check at the manufacturing factory, the CVUA checked whether their specifications were met. The cigarettes were made available to the CVUA and interested German cigarette manufacturers. Thus, in parallel to the chemical analytical work at the CVUA on these cigarettes, the authors of this publication initiated smoke chemistry analyses of these experimental cigarettes in an independent contract laboratory (Labstat International ULC, Study NS37B) and performed in addition *in vitro* cytotoxicity and mutagenicity testing in their affiliated research laboratories. There are several studies and reviews published in the peer reviewed literature, dealing with the effects of the tobacco ingredients cocoa, glycerol, and saccharose on the chemistry (e.g., 4–15) and/or toxicity of smoke (e.g., 16–22). Most studies have focused on the effects of sugars. Nearly all of these studies have been initiated and sponsored by the cigarette industry and most of them performed in the laboratories of the cigarette industry. Thus, the studies presented here on the delivery of selected mainstream smoke constituents allow, for the first time, a direct comparison of data obtained by the cigarette industry with those produced by an official governmental laboratory (CVUA data will be published as a separate publication in the same journal).

METHODS

Cigarettes

The experimental cellulose acetate filter cigarettes resemble marketed American blend cigarettes with 6 and 10 mg ISO 'tar' yields. The filler contained 50% Virginia (flue cured), 20% Burley (air cured) and 10% Oriental tobacco together with 20% stems. The tobacco weight was 611 and 674 mg/cigarette and the length of the cellulose acetate filter was 27 and 22 mm for the 6 and 10 mg 'tar' cigarette, respectively. The filter ventilation was approximately 47% for the low and 27% for the high 'tar' cigarette. For the cigarettes of both 'tar' classes, the ingredients cocoa powder (CAS No. 657-27-2), glycerol (CAS No. 56-81-5), and saccharose (CAS No. 57-50-1) were added separately at three application levels (Table 1). The cocoa powder had a fat content of 10 to 12%. The purity of glycerol and saccharose was higher than 99%.

Smoke Chemistry

Mainstream smoke generation and chemical analyses, at the request of Philip Morris International, were performed at Labstat International ULC, a laboratory accredited by the Standard Council of Canada according to the international ISO/IEC quality standard 17025 (23). The results of all analyses with their accompanying standard errors are presented on a per cigarette basis in the Appendix, allowing the reader to perform recalculation of the data according to preferred calculation basis (Tables A to D).

The cigarettes were smoked according to two standard smoking regimens, to the International Organization for Standardization (ISO) regimen 3308 (24), i.e., a puff duration of 2 s, a puff interval of 60 s, and a puff volume of 35 mL with no blocking of the filter ventilation holes and according to the intense regimen specified by Health Canada specifications (HCI), referred to as Method T-115 (25), i.e., a puff duration of 2 s, a puff interval of 30 s, and a puff volume of 55 mL with complete blocking of the ventilation holes. Smoking, generally, took place on Borgwaldt 20-port rotary smoking machines.

The smoke was analyzed using the Health Canada Official Test Methods, i.e., those methods required by the Canadian authorities for yearly submission of emission data for Canadian market cigarettes. These methods are in accordance with the relevant ISO methods. The specific details of these methods have been described in detail as the methods T-101 to T-104, T-107 to T-112 and T-114 to T-116 (25). These analytes have been specifically suggested to be of major importance for assessment of cigarette product changes. They are commonly referred to as the "Hoffmann Analytes" and consist of a list of mutagens, carcinogens, and potent irritants present in cigarette smoke (26).

Total Particulate Matter (TPM) was determined gravimetrically from the smoke trapped on glass fiber filters which were also used for sample collection of individual particle phase analytes (see below). Nicotine was determined by capillary gas chromatography with nitrogen-sensitive detection from a 2-propanol extract of the TPM filter. Water was determined from the same 2-propanol extract by Karl Fischer titration. 'Tar' yield was calculated as the TPM yield minus the nicotine and water yields. Ammonia was trapped in a dilute sulfuric acid solution and determined by ion chromatography using a suppressed-conductivity detector. Aromatic amines were determined by extracting TPM-filters with dilute hydrochloric acid, filtration and dichloromethane washing, followed by back extraction into hexane after basification. The hexane extracts were dried, derivatized with pentafluoropropionic acid anhydride and trimethylamine, cleaned-up by solid

Table 1. Specifications of the experimental cigarettes. Ingredients added in % of tobacco weight.

Ingredient	Cigarette 'tar' level	
	6 mg/cig	10 mg/cig
None (control)	—	—
Cocoa powder	0.40	0.40
	1.10	1.10
	2.20	2.20
Glycerol	1.50	1.50
	3.00	3.00
	5.50	5.50
Saccharose	1.55	1.55
	2.10	2.10
	4.80	4.80

phase extraction and analyzed by gas chromatography with a mass-selective detector. Benzo(*a*)pyrene was extracted from TPM-filters with hexane and determined by GC-MS. The extract was cleaned-up using a silica and a NH2-plus solid phase cartridge in series. The unfiltered mainstream tobacco smoke was scrubbed of its volatile carbonyls using an acidified solution of 2,4-dinitrophenylhydrazine in acetonitrile. Carbonyls, derivatized as hydrazones, were determined by liquid chromatography with UV detection. Hydrogen cyanide was extracted from TPM-filters with dilute sodium hydroxide and pooled with a sodium hydroxide solution from the impinger trap, where the gas/vapor phase was trapped. For the measurement, an automated continuous flow colorimetric analyzer was used. Carbon monoxide was determined by non-dispersive infrared photometry. Nitrogen oxides were determined by chemiluminescence. Tobacco-specific *N*-nitrosamines were trapped using both, a citrate/phosphate buffer solution and a glass fiber filter. The buffer solution and the glass fiber filter extract were combined and concentrated extracts were purified by adsorption chromatography on alumina. The concentrated eluate was analyzed by gas chromatography with a thermal energy analyzer. Phenols were extracted from a TPM-filter with dilute acetic acid. An aliquot of the TPM extract was syringe filtered, diluted and subjected to reversed-phase liquid chromatography with a fluorescence detector. Isoprene, 1,3-butadiene, benzene, toluene, and acrylonitrile in mainstream tobacco smoke were trapped in cold traps containing methanol and analyzed by gas chromatography with a mass selective gas detector.

In vitro *cytotoxicity*

Mainstream smoke generation, TPM sampling, chemical characterization, and biological assays on the samples were performed by Philip Morris Research Laboratories certified as being compliant to the "Good Laboratories Practice Regulations" (27). The results of all assays are presented in the Appendix (Table E).
The cigarettes were smoked according to the ISO 3308 (24) using a 20-port Borgwaldt smoking machine. TPM was collected on a glass fiber filter and extracted with dimethyl sulfoxide. The water-soluble fraction of the gas/vapor phase (GVP) was trapped in ice-cold phosphate-buffered saline. For each of the cigarette types, three TPM and GVP samples were produced.
The Neutral Red Uptake Cytotoxicity Assay on the TPM was performed as published by BABICH and BORENFREUND (28) and BORENFREUND and PUERNER (29) according to Protocol 3a (29). In short, mouse embryo BALB/c 3T3 cells (American Type Culture Collection ATCC #163, Manassas, Virginia, USA) were exposed for 24 h to culture medium supplemented with 5% fetal bovine serum to which TPM or GVP solutions/suspensions were added. For each sample, four replicate 96-well micro titer plates were used, each with eight smoke concentrations. In most cases up to 160 µg TPM/mL medium was used. Each smoke concentration was replicated six times per micro titer plate. After exposure, the medium was replaced by medium containing the dye neutral red (25 µg/mL). After a 3-h incubation period, neutral red was determined photometrically after washing and adding 100 µL/well of an extraction solution (1% acetic acid in 50/50 ethanol/water). The amount of neutral red taken up is directly proportional to the number of viable cells. Acrolein was used as positive control. The cytotoxic response was characterized as the EC_{50} value, i.e., the concentration that decreased the number of viable cells by 50% relative to the solvent control. The EC_{50} values (mg TPM/mL exposure medium) 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, $b = EC_{50}$, and a,c = form factors. For each of the three TPM samples, one EC_{50} was calculated. The mean EC_{50} value was used to characterize each TPM and GVP fraction.

In vitro *mutagenicity*

The assay was performed by Philip Morris Research Laboratories. The results of all assays are presented in the Appendix (Table E).
The cigarettes were smoked according to the ISO 3308 (24) using a 20-port Borgwaldt smoking machine. TPM was collected on a glass fiber filter and extracted with dimethyl sulfoxide. TPM samples were prepared in duplicate.
The bacterial cell mutagenicity assay, commonly referred to as the Ames assay was applied as the plate incorporation version of the *Salmonella* Reverse Mutation Assay and performed as published (30) in general accordance to OECD guideline No. 471 (31). Mutagenicity towards *Salmonella typhimurium* strains TA98, TA100, TA102, TA1537, and TA1535 (all obtained from B.N. Ames, Berkley, CA, except for TA1535 which was obtained from Trinova Biochem GmbH, Giessen, Germany) was determined in the presence and in the absence of a metabolic activation system consisting of the postmitochondrial fraction of the livers from rats treated with Aroclor 1254 (S9, Cytotest Cell Research, Rossdorf, Germany). For each sample, three doses which were expected to cover 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 36 ± 1 °C for 44–48 h. The number of His+ revertant colonies was determined with an automatic colony counter. 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 two samples.

Data analysis

To separate, in a transparent and reproducible way, spurious chance derived data, from those due to real effects, we followed a three-step approach. This approach seems

to be especially useful when dealing with a high number of false positive statistical significances due to multiple statistical testing. This would be expected in a study of the type presented here, due to the large number of statistical comparisons performed. The approach combined conventional statistical requirements and practices with the application of predefined fixed decision rules. As such, this approach follows the principles of evidence based toxicology (32, 33). The three steps were:

- A generally accepted statistical test to identify statistically significant differences: For smoke chemistry and mutagenicity, data were tested using the DUNNETT test. For cytotoxicity, data were tested using Student's *t*-test. All statistical comparisons were made at $p < 0.05$, without correction for multiple comparisons.
- A check, if the normal variability of the chemical analytical/biological method (due to the given laboratory procedures at the time period of interest) together with the variability of cigarette production (e.g., variations in the percentages of each of the filler components, filler density, filter ventilation, and paper permeability), is lower than the magnitude of the difference that caused a statistical significance. This check prevents the reliance on statistical significances due to chance results caused by abnormal, chance derived, low variations in the data sets of the samples that are compared.
  This check, in the following text, is referred to as the Minimum Detectable Difference (MDD): The MDD is the smallest difference in the means between two samples that would show up as statistically significant when using Student's *t*-test, the mean standard deviation (s_{pooled}) for the respective method derived from historical data from standard reference cigarettes in the given laboratory over the respective time period, a type I error (α) of 0.05, a type II error (β) of 0.20, and the number of replicates (n) used for this method.
  $MDD = (SQR(2s_{pooled}^2/n))*(t_{1-\alpha/2,\ 2(n-1)}+t_{1-\beta,\ 2(n-1)})$.
  For the mutagenicity data an equivalent approach based on Monte Carlo simulation was applied.
  Typical relative MDDs for the smoke chemistry data presented here are less than 10%, e.g., for 'tar', nicotine, and carbon monoxide, 15% to 20%, e.g., phenolics and aromatic amines, and up to 25% (e.g., for formaldehyde). MDDs for the cytotoxicity of the particulate matter and the gas/vapor phase are 10% and 30%, respectively. MDDs for the mutagenic response in the most responsive bacterial tester strains TA98 and TA100 with metabolic S9-activation are about 15% and 25%, respectively.
- A check on data consistency, i.e., whether a statistical significant difference that is larger than the respective MDD can be confirmed as a true effect by at least two of the following three criteria: Dose dependency under the conditions showing the statistical difference ('tar' class and smoking regimen), similar differences in the groups of the cigarette of the other 'tar' category, and similar differences in the group of the other smoking regimen.

Vice versa differences that were not statistically significant were considered as real if they met the above criteria.

RESULTS

Cocoa

The addition of cocoa to the experimental cigarettes did not result in any consistent effects on the measured mainstream smoke analytes expressed on a per cigarette basis or after normalization of the data and expressed on an equal TPM or equal nicotine weight basis.

There were 32 statistical significant differences in the delivery of smoke components, 13 of which could be excluded as irrelevant since the numerical difference between the samples derived from cigarettes with and without the addition of cocoa were too small compared to the inherent variation of the analytical method (MDD approach). None of the 19 remaining statistical significances could be confirmed, either by the results obtained with the cigarettes of the second 'tar' category or using the second smoking regimen (Figure 1, Tables A to D).

The *in vitro* cytotoxicity of the TPM, as measured in the NRU assay, was not affected by the addition of cocoa. The cytotoxicity of the GVP was decreased by 10% to 15% for all cigarettes with added cocoa. As this decrease is lower than the discriminatory power of this assay, it can only be taken as an indication for a reduction in cytotoxicity (Figure 2, Tables E and F).

The *in vitro* mutagenicity of the TPM, as measured in the Ames assay, was not affected by the addition of cocoa in any of the tester strains with and without metabolic activation. Increases and decreases in mutagenic activity for the smoke of the cocoa containing cigarettes were within the normal statistical variation (Figure 3, Tables E and F).

Glycerol

The addition of glycerol to the experimental cigarettes resulted in several consistent decreases in the measured mainstream smoke analytes. These decreases were more pronounced when the cigarettes were smoked under the ISO/FTC than when smoked under the HCI smoking regimen. Some of the observed decreases when cigarettes were smoked according to the ISO/FTC smoking regimen were not observed using the HCI smoking regimen. Observed decreases were still apparent when the data were normalized on equal TPM and on equal nicotine weight basis.

There were 75 statistical significant differences in the delivery of smoke components, 6 of which could be excluded as irrelevant as the numerical difference between the samples derived from cigarettes with and without the addition of glycerol were too small compared to the inherent variation of the analytical method (MDD approach). 61 of the 69 remaining statistical significances could be confirmed, either by the results obtained with the cigarettes of the second 'tar' category or using the second smoking regimen.

Distinct decreases (normalized on equal TPM) were observed for most aldehydes, e.g., for crotonaldehyde which was reduced up to 25% under ISO/FTC smoking conditions. Most of the decreases were not apparent using the HCI smoking regimen. Even more distinct were the reductions for the phenolic substances where reductions,

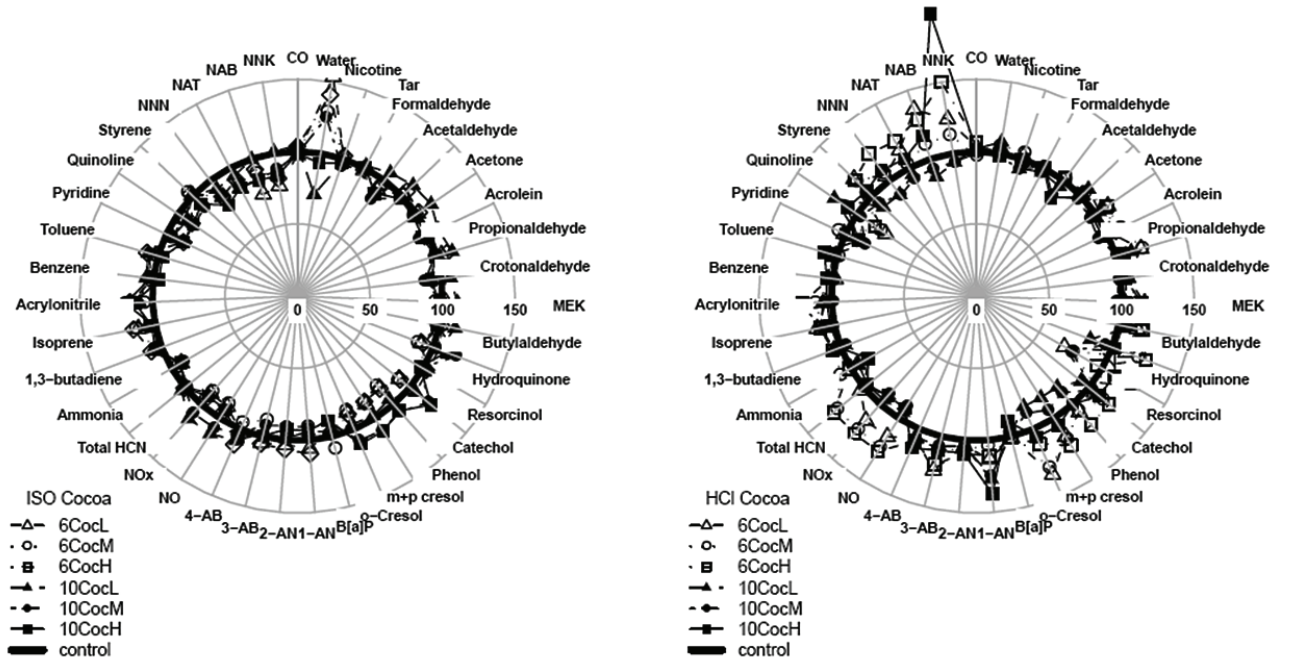

Figure 1. Smoke constituent concentrations in TPM of cigarettes with the addition of cocoa as an ingredient to the filler relative (%) to control cigarettes without cocoa addition, smoked under ISO/FTC or HCI smoking regimen. Group code: 6 or 10 indicates 'tar' target yield (mg) under ISO/FTC conditions. L, M, or H indicates low, medium, or high ingredient addition level. Abbreviations: MEK = methyl ethyl ketone, B[*a*]P = benzo(*a*)pyrene, 1-AN = 1-aminonaphthalene, 2-AN = 2-aminonaphthalene, 3-AB = 3-aminobiphenyl, 4-AB = 4-aminobiphenyl, NNN = *N'*-nitrosonornicotine, NAT = *N'*-nitrosoanatabine, NAB = *N'*-nitrosoanabasine, NNK = 4-(*N'*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone.

e.g., for phenol and cresol, were around 50%. HCN was decreased up to 25% under ISO/FTC but not under HCI conditions. Pyridine and quinoline as well as tobacco-specific *N*-nitrosamines were decreased by around 20% to 25% under both smoking conditions. Increases due to the addition of glycerol were seen for water (up to 150% under ISO/FTC and 25% under HCI conditions) and ammonia (up to 25% under ISO/FTC and 50% under HCI conditions) as shown in Figure 4 and Tables A to D.

The term TPM includes the amount of water in the particulate matter. As glycerol increased the amount of water in TPM, it seems of special importance to relate the yields of the smoke constituents also to a basis that is not affected by the increased yield of water, e.g., on a per mg nicotine basis. Using this calculation basis, the effects on the aldehydes and HCN were not evident but those on the phenolics, pyridine, and quinoline remained. The tobacco-specific *N*-nitrosamines showed increases as well as decreases on an equal nicotine weight basis (data not shown).

The *in vitro* cytotoxicity of the TPM was decreased for all glycerol containing experimental cigarettes by approximately 15% compared to the glycerol-free control cigarette. The cytotoxicity of the GVP was not affected. Considering the discriminatory power of this assay, the decreased cytotoxic activity for the TPM has to be considered as a real effect due to the addition of glycerol (Figure 2, Tables E and F).

The *in vitro* mutagenicity of the TPM was not affected in all tester strains either with or without metabolic activation by the addition of glycerol to the tobacco. Increases and decreases in mutagenic activity for the smoke of the glycerol containing cigarettes were within the normal statistical variation (Figure 3, Tables E and F).

Saccharose

Saccharose added to the tobacco of the experimental cigarettes did lead to two consistent effects on the measured mainstream smoke analytes, i.e., an increase in formaldehyde and a decrease in tobacco-specific *N*-nitrosamines. These two effects were the same when normalized on equal TPM or equal nicotine basis.

There were 51 statistical significant differences in the delivery of smoke components, 21 of which could be excluded as irrelevant since the numerical difference between the samples derived from cigarettes with and without the addition of saccharose were too small compared to the inherent variation of the analytical method (MDD approach). Nine of the 30 remaining statistical significances could be confirmed, either by the results obtained with the cigarettes of the second 'tar' category or the second smoking regimen. Formaldehyde was consistently increased up to 40% by the addition of saccharose. The tobacco-specific *N*-nitrosamines on the other hand were decreased by up to approximately 20% (Figure 5, Tables A to D).

The *in vitro* cytotoxicity of the TPM and the GVP was not affected by the addition of saccharose to the tobacco (Figure 2, Tables E and F).

The *in vitro* mutagenicity of the TPM was decreased in the most responsive tester strain/metabolic condition,

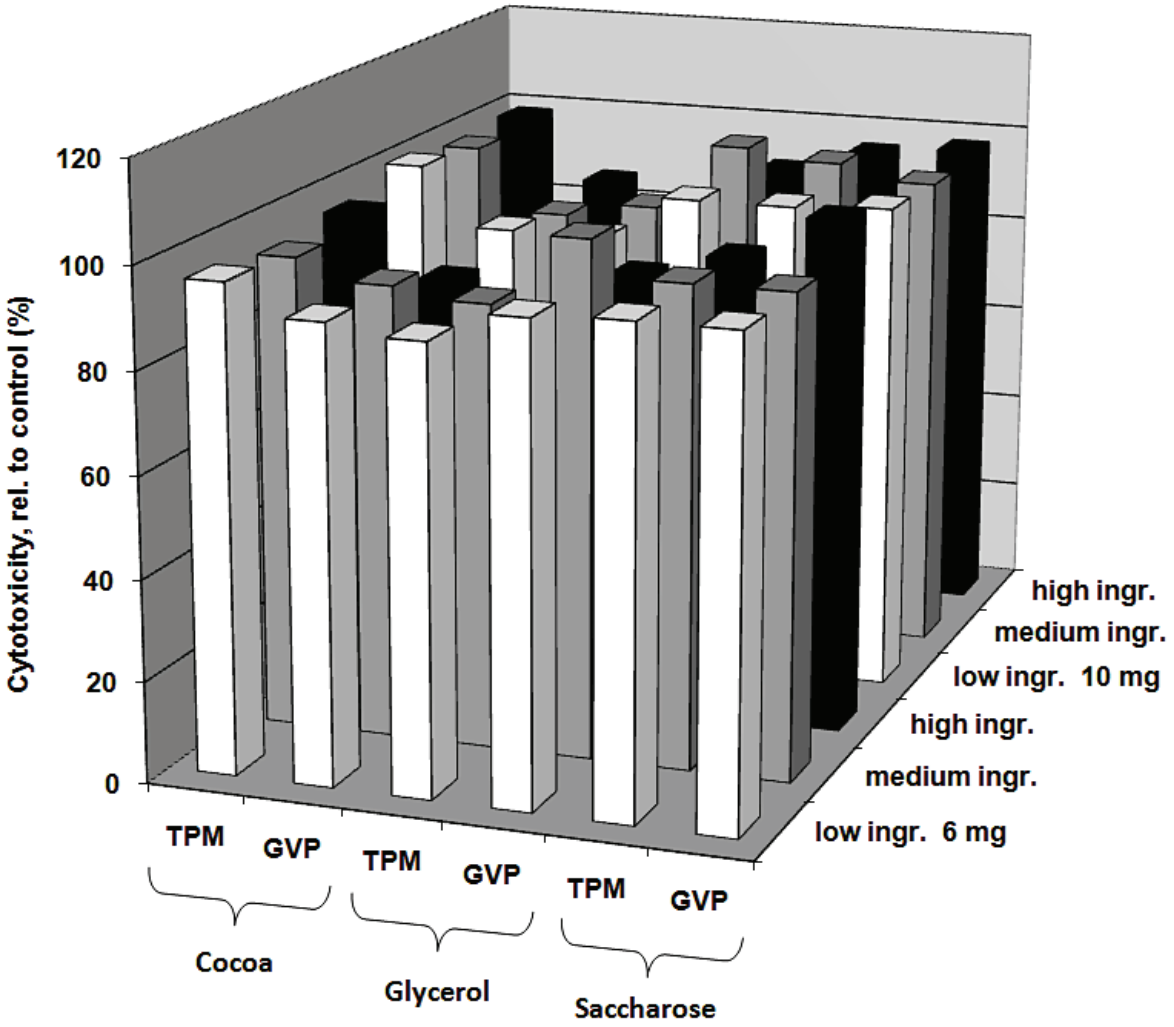

Figure 2. Cytotoxicity of TPM and GVP of cigarettes with the addition of cocoa, glycerol, or saccharose as ingredients to the filler relative (%) to control cigarettes without ingredient addition, smoked under ISO/FTC smoking regimen. The designation "6 mg" and "10 mg" refers to the tar yield target of the cigarettes used.

TA98+S9, by the addition of saccharose to the tobacco by approximately 15%. The tester strain TA100+S9, which is the second responsive tester strain for tobacco smoke mutagens, did not show an effect. The other tester strains/metabolic conditions showed increases and decreases in mutagenic activity within the normal statistical variation (Figure 3, Tables E and F).

DISCUSSION

General

Due to the study plan, there were several possibilities to assess the consistency of the data, i.e., consistency between experimental cigarettes with different ingredient concentrations, different 'tar' levels, and different smoking regimens. Using the MDD approach permitted discrimination between the expected large number of spurious statistical artifacts and real effects and assured that no true effect due to the addition of ingredients was overlooked because of missing statistical significance. As such, there is a high degree of confidence that the effects considered as reliable reflect the actual impact of the use of cocoa, glycerol, or saccharose as ingredients added to cigarette tobacco.

Using the Evidence-Based Toxicology approach (32, 33), it seems to be reasonable to introduce guiding rules for the interpretation of the unknown outcomes into the study protocol. In particular for the interpretation of smoke chemistry data that are inherently subject to a considerable number of false positive statistical test results due to multiple statistical testing.

In addition to the mainstream smoke constituent analyses required by Health Canada, we assessed the *in vitro* toxicity of the mainstream smoke of all test and control cigarettes. Smoke chemistry data constitute an important part of each toxicological hazard assessment of cigarette ingredients (and other product changes). However, assessing the impact of certain cigarette design factors on the toxicity of cigarette smoke requires both chemical analyses and biological assays. Although smoke chemistry analyses are able to determine increases and decreases in defined substances,

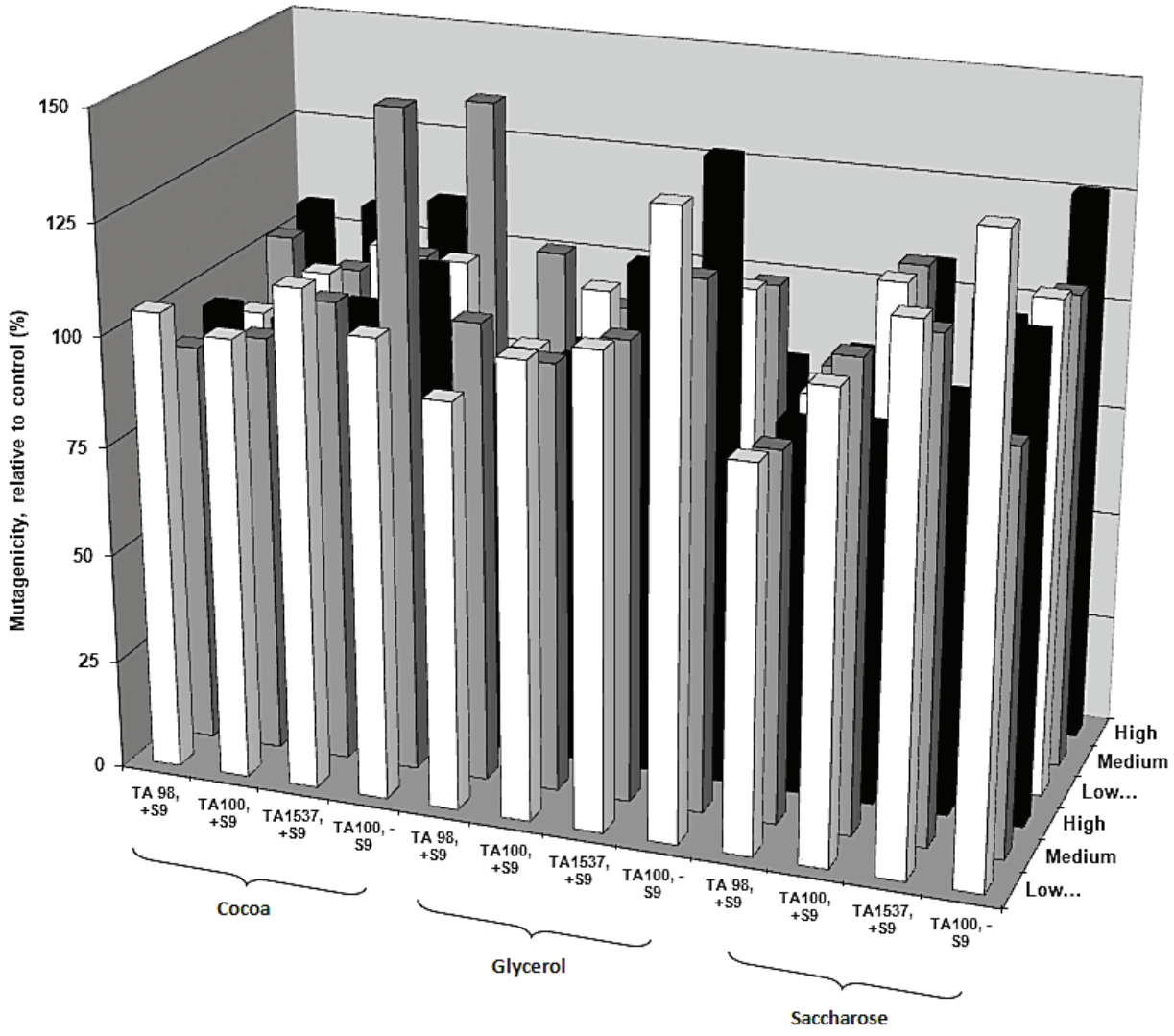

Figure 3. Mutagenicity of TPM of cigarettes with the addition of cocoa, glycerol, or saccharose as ingredients to the filler relative (%) to control cigarettes without ingredient addition, smoked under ISO/FTC smoking regimen. The designation "6 mg" and "10 mg" refers to the tar yield target of the cigarettes used.

they cannot predict the impact of these changes on the overall toxicity of cigarette smoke. This is especially true when one considers that the number of smoke constituents that are actually determined is rather limited compared to the more than five thousand constituents identified up to now. Biological assays are rather unspecific regarding substances that cause a response. The response towards cigarette smoke is thought to be the overall result caused by smoke constituents with the same mechanisms and includes the interactions with others in an antagonistic or synergistic way. Accordingly, the two types of detection systems - chemical and biological - complement one another. As stated in the DIN Technical Report (34) *in vitro* test systems are regarded as a central part of the test strategy (bacterial mutagenicity assay and cytotoxicity assay), which may be complemented by a set of screening tests for biochemical reactivities (SH-index, radical index, oxidative potential) and "[T]he determination of special analytes in tobacco smoke (e.g., "Hoffmann Analytes") can round off the test program".

In vivo data are generally considered to add more weight to the evidence of an effect in toxicological studies. The project presented here does not include *in vivo* studies. However, there are rat inhalation studies available in the literature supporting the assessment.

Human data, if existent, would be the most conclusive data, but human studies dealing directly with the use of the three ingredients in cigarettes are not available. Nevertheless, countries with and without the predominant use of cigarette ingredients allow an analysis of epidemiological data under this aspect.

Taken together, all sources of information are important and only the consideration of all available data allows a valid conclusion regarding the use of these cigarette ingredients.

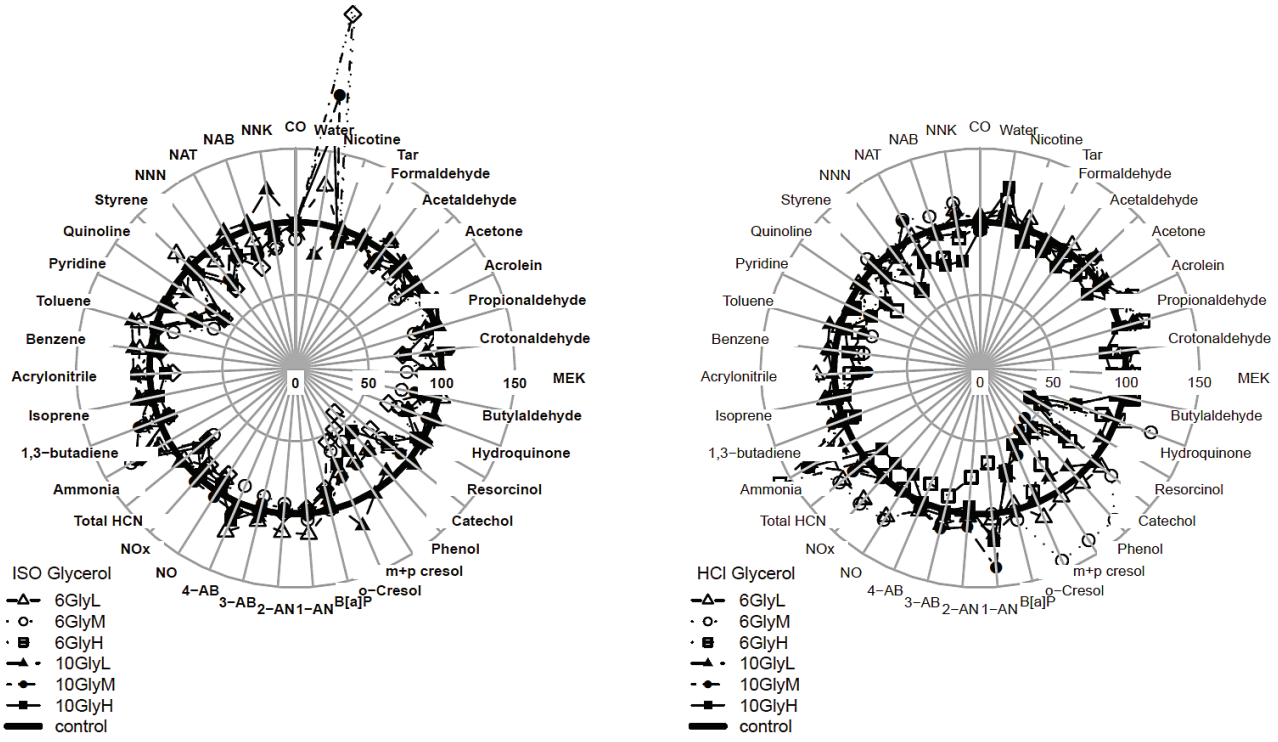

Figure 4. Smoke constituent concentrations in TPM of cigarettes with the addition of glycerol as an ingredient to the filler relative (%) to control cigarettes without glycerol addition, smoked under ISO/FTC or HCI smoking regimen. Group code and abbreviations: see Figure 1.

The discussion in this publication does not pretend to give a complete review of the studies available in literature on the use of the three ingredients in the context of cigarette smoke, nor does it provide detailed data on the outcomes of these studies. Especially, comments on differences in study design, sample size, and laboratory variability are not discussed. It is rather a short comparison of our data for consistency with those that are most readily available to the experts in this field. The cited references are complemented with the results of some unpublished data in cases where there are no literature data available. These unpublished data, however, have already been made available by PMI to the respective ministries in the EU as part of the annual ingredient reporting requirements in accordance with Directive 2001/37/EC (35).

Cocoa

Cocoa and cocoa products are foodstuff items and classified as "Generally Recognized As Safe (GRAS)" under the US food additive review program (21 CFR 182.20, Part 130). Beside quality standards, there are no restrictions on their use as food under any jurisdiction. Cocoa is approved for use in tobacco products as an additive in several countries. There is only one country (Canada) that affirmatively prohibits the use of cocoa on tobacco products.

Concerns have been raised that cocoa added to tobacco would exert a bronchodilating effect thereby enhancing the uptake of nicotine and increasing the addictive properties of cigarette smoke. It has also been suggested that theobromine and other constituents in cocoa would have direct psychoactive effects (36).

Approximately 2% of cocoa is theobromine (37, 38, 39), the compound associated with the speculated bronchodilating effect of cocoa (40). Using conservative assumptions (0.4% cocoa in 800 mg cigarette filler, mainstream/sidestream ratio: 20/80, 40 cigarettes per day, 100% unchanged transfer to mainstream smoke, 100% absorption), this translates to an uptake of approximately 0.1 mg theobromine per cigarette or 4 mg theobromine per day. This daily uptake from cigarette smoking was considered marginal compared to that taken up from food (100 to 1,000 mg per day (41)). In other words, 1,000 to 10,000 cigarettes would have to be smoked per day to reach such levels (assuming 100% transfer and absorption). The daily uptake from cigarette smoking is far below what is required to produce a pharmacological, i.e., bronchodilating, effect (38, 42, 43, 44). A comprehensive review by the Netherlands National Institute for Public Health and Environment (Dutch Rijksinstituut voor Volksgezondheid en Milieu, RIVM) also concluded "that the level of the psychoactive compounds of cocoa in cigarettes is probably too low to exert any local bronchoactive effects" (41).

Finally, the RIVM also concluded that the individual level of the psychoactive compounds in cigarette smoke from added cocoa are too low to increase the addiction of cigarette smoke (41).

The addition of cocoa powder to the tobacco filler of experimental cigarettes did not lead to any consistent effects on the measured mainstream smoke analytes. There is no smoke chemistry study available in the current literature on the effects of cocoa added as a single ingredient to the tobacco of cigarettes. However, there is a not

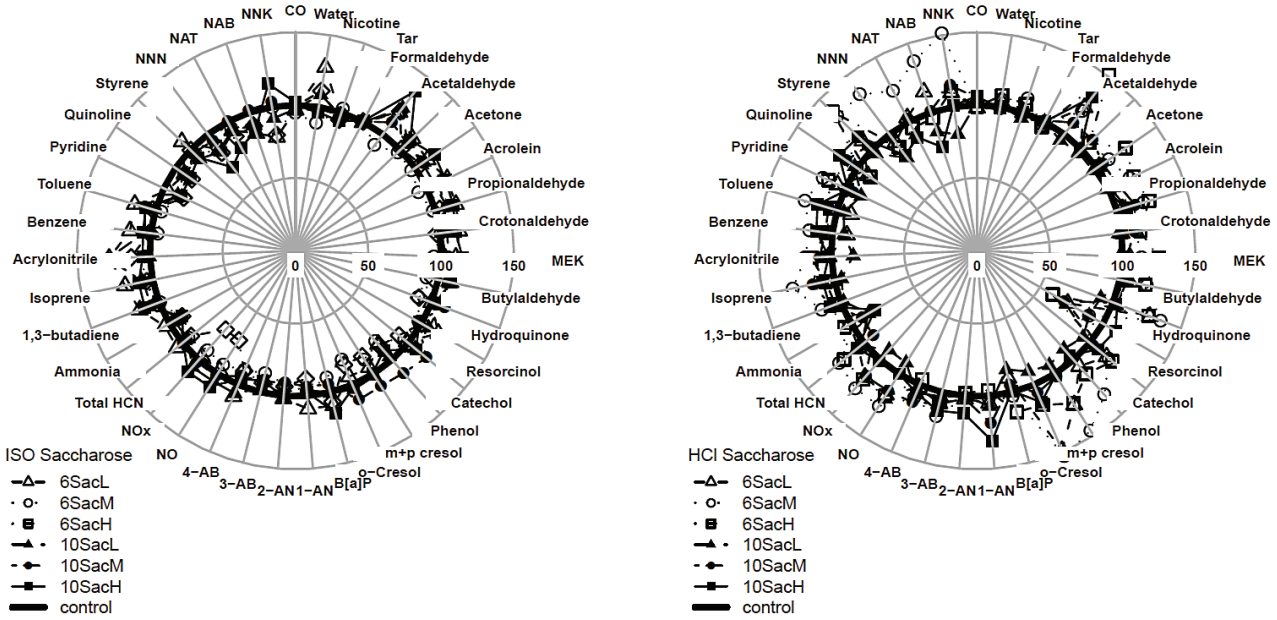

Figure 5. Smoke constituent concentrations in TPM of cigarettes with the addition of saccharose as an ingredient to the filler relative (%) to control cigarettes without saccharose addition, smoked under ISO/FTC or HCI smoking regimen. Group code and abbreviations: see Figure 1.

yet published report from a study sponsored by Philip Morris (PM) that corroborates the outcome of the present study (45). Smoke chemistry studies in which cocoa was added as a component of an ingredient mixture to the cigarette filler did not give rise to any toxicological concern (8, 20).

In the present study, addition of cocoa to the tobacco filler suggested reduced *in vitro* cytotoxicity of mainstream smoke GVP of the resulting smoke. This result requires further clarification as there are no cytotoxicity studies available in the current literature on the effects of cocoa added as a single ingredient to the tobacco of cigarettes. However, a not yet published report from a study sponsored by PM indicates that the addition of cocoa up to 4% in the tobacco filler does not affect the cytotoxicity of the GVP (46). Cytotoxicity studies where cocoa was added as a component of an ingredient mixture to the cigarette filler did not give rise to any toxicological concern (18, 20).

The addition of cocoa to the tobacco filler of experimental cigarettes did not alter the *in vitro* mutagenicity of the resulting mainstream smoke. There is no mutagenicity study available in the current literature on the effects of cocoa added as a single ingredient to the tobacco of cigarettes. However, in a not yet published report from a PM-sponsored study addition of cocoa up to an application level of 4% also resulted in no increase in the mutagenicity of TPM (47). Similarly, mutagenicity studies in which cocoa was added as a component of an ingredient mixture presented no toxicological concern (18, 20).

In vivo studies were not performed in this project and inhalation studies with cigarettes that differ only by the addition of cocoa have not been reported. However, there is an unpublished internal report from a nose-only inhalation study with male and female rats sponsored by PM (48). In this study, the authors concluded: *"The only biologically significant histopathological changes seen at the end of the exposure period which occurred at an increased incidence and/or severity compared to the Control Cigarette group were squamous cell hyperplasia-metaplasia and keratinization in the lower medial region of the arytenoid larynx, and increased goblet cell activity in the left lung of the male rats in the High Cigarette group [40,000 ppm cocoa]. The number of goblet cells in the bronchial epithelium in the lung (left and right) was statistically significantly increased in the High Cigarette group males compared to the Control Cigarette group males at the end of the exposure period."* A further analysis of this finding which was only found in male rats, but not in the female rats, revealed that the statistical significance was due to an abnormal low response in the male control group. When the data were compared to the female control group or to the results obtained from the exposure of male rats to a standard reference cigarette, which was similar in construction to the control cigarette, no abnormality in the group with the addition of the high cocoa concentration to the cigarette filler was observable.

Dermal carcinogenicity (skin painting) studies on the particulate phase of mainstream smoke from cigarettes with and without the addition of cocoa powder have been reported by the US National Cancer Institute and concluded that cigarettes with the addition of cocoa *"appeared to increase the tumorigenicity"* of the corresponding cigarette smoke (49). ROEMER and HACKENBERG (50), using a more extensive study protocol, however, were not able to corroborate this finding. They concluded that their data *"do not provide evidence that the biological activity of the condensates, as indicated by the occurrence of non-tumorous and tumorous lesions, is enhanced by the addition of cocoa"*.

Further studies in which cocoa was added as a component of an ingredient mixture at up to 9.7% did not give rise to

any toxicological concern in both, smoke inhalation studies (19, 20, 69) or a dermal carcinogenicity study (51).
Publications comparing morbidity and mortality of smokers of cigarettes with and without the addition of cocoa as the only determinant are not available. However, data are available that can be used to compare smokers in markets where there are essentially no ingredients added to the cigarettes with those markets where ingredients as cocoa, glycerol and saccharose are normally added to the tobacco of cigarettes. An analysis of these data concluded that there is no difference observable regarding the morbidity and mortality (52).

Glycerol

Glycerol has been characterized as a food additive with GRAS status (21 CFR 182.1320). Beside the obligations to exercise good manufacturing practices, i.e., to add only those levels to food that are necessary to impart the desired effect (such as maintain moisture and soften texture) no restrictions on the use of glycerol are known. Glycerol is approved for use in tobacco products as an additive in several countries and there is no country that affirmatively prohibits the use of glycerol on tobacco products.
The oral toxicity of glycerol is rather low with an LD_{50} in rats of greater than 25 mg/kg body weight and with a no-observed-effect-level (NOEL) of 10 mg/kg body weight (53). Glycerol is not genotoxic in an *in vitro* battery of assays (54) or tumorigenic in rats when given up to 20% to the diet (53). The inhalation toxicity in rats is also rather low. Nose-only exposure for 2 weeks, 5 days/week, 6 h/day at 1.0, 1.9, and 3.9 mg glycerol/L and 0.662 mg/L for 13 weeks produced minimal to mild squamous metaplasia of the epithelial lining at the base of the epiglottis of the larynx but not at 0.033 and 0.167 mg glycerol/L for 13 weeks (55). Assuming a glycerol transfer rate of 3.5% into the mainstream smoke (see below), a glycerol addition of 5% (*w/w*) to the tobacco (typical glycerol concentrations in cigarettes are around 2%), a tobacco weight of 800 mg/cigarette, and 8 puffs/cigarette of 50 mL each, the calculated glycerol concentration in smoke would be 3.5 mg/L. However, this concentration would represent the peak glycerol concentration during the short time during puff inhalation and not a concentration in the breathing air over six hours. The deposited glycerol in the respiratory tract can be expected to be immediately diluted by the epithelial lining fluid and metabolized shortly thereafter. The daily inhaled dose for the rats at the NOEL-concentration of 0.167 mg glycerol/L can be calculated using a minute volume of 0.7 mL breathing air/g body weight (56) as 40 mg/kg body weight. The inhaled glycerol dose for a 75 kg smoker with a consumption of 40 cigarettes/day would be 0.8 mg/kg body weight, i.e., by a factor of 50 lower than the NOEL-dose.
Glycerol in the smoke was not measured in the present study, but glycerol has been reported to be distilled unchanged into the smoke to a significant amount. Older studies report a transfer rate of approximately 10% into mainstream smoke (57). A more recent publication reports on a transfer rate of 3.5% (58). The latter transfer rate does not seem to support the previous assertion. However, considering that the absolute mass of glycerol at an application level of, e.g. 5%, will require the addition of approximately 40 mg glycerol to the filler; the transfer rate of 3.5% would then translate into a glycerol concentration of approximately 20% in the mainstream TPM of a cigarette with a TPM yield of 8 mg.
In the present study, addition of glycerol to the tobacco resulted in a large increase (up to +150%) in the concentration of water in the TPM under ISO/FTC smoking conditions. Under HCI smoking conditions this increase was still observable, but far lower (up to +25%). As glycerol is used as a humectant in cigarette production, due to its hydroscopic property, this increase was expected and has previously been reported as an effect of glycerol added as a single ingredient at 5, 10, and 15% to cigarette tobacco (59). A similar increased water concentration in TPM has been reported in a study in which the cigarette filler contained 4% glycerol together with 7% of other ingredients, including 5% propylene glycol, a humectant which has similar hygroscopic characteristics as glycerol (8). However, in another study where glycerol was added at 7% with propylene glycol at 1%, the increase in water in TPM was only around 12% (13).
The smoke chemistry data obtained in the present study showed an 18% increase in acrolein at the 5.5% glycerol addition level under the HCI, but not under the ISO/FTC smoking regimen. In another study in which the cigarette filler contained 5% glycerol, no effect on acrolein delivery was observed; however, at addition levels of 10% and 15% the increase of acrolein in mainstream smoke was 19% and 23%, respectively, using ISO/FTC smoking conditions (59).
Similarly, in another study using the ISO/FTC smoking regimen in which glycerol was added at 4% together with another 7% of other ingredients, no increase in acrolein was observed (8). At 7% glycerol and 1% propylene glycol addition levels using the ISO/FTC regimen an increase in acrolein of 15% was found (13). These data suggest, that an effect on acrolein delivery only occurs above an addition level of 5% glycerol.
The decreases in several smoke components, e.g., aldehydes, phenolics, pyridine, quinoline, and tobacco-specific *N*-nitrosamines due to the addition of glycerol to the tobacco filler was also observed in other studies (8, 59).
The increase in ammonia observed in the present study could not be confirmed in another study where glycerol was added at 7% (13). Ammonia was not measured in the other studies cited above. As such this finding remains inconclusive.
The *in vitro* cytotoxicity of the TPM in the present study was decreased by approximately 15% in experimental cigarettes with glycerol addition compared to the respective control cigarettes. This reduction was confirmed in another study at an application level of 15% glycerol. In addition, a reduction in the cytotoxicity of the GVP was also found at an addition level of 5% glycerol (59).
The *in vitro* mutagenicity of the TPM was not affected in the present study by the addition of glycerol to the tobacco filler. This was confirmed at an application level of 5% glycerol in another study (59). At an application level of 15% glycerol, the authors found a 10% decrease in mutagenicity using the most responsive tester strain TA98 with metabolic activation.

In vivo studies were not performed in the current study. However, in an inhalation study assessing glycerol addition to cigarettes, there was no increase in toxicity, especially no increased irritation in the respiratory tract of rats exposed to the smoke of cigarettes containing 5.1% glycerol (17). This result is in agreement with the results of a not yet published, PM-sponsored nose-only inhalation study with male and female rats in which the irritative changes in the respiratory tract were decreased at the highest level, of glycerol addition i.e., 15% (60).

The US National Cancer Institute has evaluated the dermal tumorigenicity of two condensate doses from cigarettes with and without the addition of 2.8% glycerol (49). While the tumor rates were practically identical at the low condensate dose, the tumor rate at the high condensate dose was higher in the glycerol cigarettes (57% compared to 40%). As this difference was within the historical response variation of the laboratory, the NCI concluded that glycerol *"may contribute to tumorigenicity"*. In three comparative dermal carcinogenicity studies in which glycerol was added at 2.4% with a mixture of other cigarette ingredients to different cigarettes, no increase in tumorigenicity due to the addition of glycerol was evident (51).

Publications comparing morbidity and mortality of smokers of cigarettes with and without the addition of glycerol are not available.

Saccharose

Saccharose is a foodstuff with GRAS status according to the US food additive review program (21 CFR 184.1854). Saccharose is approved for use in tobacco products as an additive in several countries. There is only one country (Canada) that affirmatively prohibits the use of saccharose on tobacco products.

Smoke chemistry analysis in the present study showed consistently an increase in formaldehyde and a decrease in tobacco-specific *N*-nitrosamines in experimental cigarettes containing addition of saccharose to the tobacco filler and smoked using either ISO/FTC or HCI smoking regimen. Both effects are pronounced and in agreement with other publications although the observed increase in formaldehyde observed in our study is clearly at the upper limit of those reported (14, 15). As formaldehyde has been classified as a suspected carcinogen (61, 62), non-genotoxic human carcinogen (63), or definite human carcinogen (64), this increase in formaldehyde calls for further analysis.

According to information provided by the major cigarette manufacturers on their internet websites, typical application levels of saccharose to the tobacco of American blended cigarettes are between 1% and 3%.

The sugar addition to the tobacco of American blended market cigarettes only partially replenishes the sugar that is lost during the air curing process of Burley tobacco. Burley tobacco, which accounts for approximately 30% of the tobacco in American blend cigarettes, is virtually completely depleted of sugars during curing due to the action of catabolizing enzymes. When comparing experimental cigarettes without the addition of saccharose to experimental cigarettes with up to 5% saccharose, the addition results in an increase in formaldehyde yield by approximately 30%–40% on equal TPM or nicotine basis. However, as there is only a partial sugar replenishment in American blend market cigarettes, the sugar concentration in American blend market cigarettes is actually lower than in Virginia-type market cigarettes. Virginia-type cigarettes are composed of so-called flue-cured tobaccos and do not include cured Burley tobacco (typical Virginia cigarette markets are the United Kingdom, Canada, and Australia). Our analysis of 81 marketed Virginia-type cigarettes compared to 121 marketed American blended cigarettes, confirmed an approximately 20% lower formaldehyde delivery for the American blended cigarettes with sugar addition, i.e., 37 µg formaldehyde/mg nicotine compared 45 µg formaldehyde/mg nicotine in Virginia type cigarettes without the addition of sugars (65).

As laid out above, experimental cigarettes with the addition of saccharose do not reflect that market cigarettes with the addition of saccharose are generally lower in sugar than those without. Nevertheless, one might be interested in the effect of saccharose addition in these experimental cigarettes on formaldehyde delivery and examine the effect in the context of human exposure and cancer risk.

In the present study, the average increase in formaldehyde delivery of all test cigarette with the highest amount of sucrose (4.8%) added compared to control is 14.1 µg formaldehyde/mg nicotine. A German population-based biomonitoring study on smokers reported a nicotine dose of approx. 1 mg/cigarette and an average cigarette consumption of 13.5 cigarettes per day (66). If one assumes that formaldehyde is as retained as nicotine (95–98%), then the total incremental exposure due to adding 4.8% sucrose is 190 µg formaldehyde/day. This uptake is equivalent to a 24 h continuous formaldehyde exposure to 7 ppb, at a breathing rate of 20 m^3/day (67).

A risk assessment provided by the Chemical Industry Institute of Toxicology (68) with input by the US EPA predicted excess respiratory tract cancer risk of additional exposure to formaldehyde in both smokers and non-smokers. The underlying CIIT model has been accepted and widely used by several national and international standards-setting bodies. According to the CIIT's risk assessment (69, 70), which predicts additional cancer risk for 80-years environmental continuous formaldehyde exposure, the additional cancer risk at 1 ppb and 10 ppb formaldehyde for non-smokers and for smokers are 2.94×10^{-9} and 4.72×10^{-8}, and 2.97×10^{-8} and 4.77×10^{-7}, respectively. The cancer risk estimates for smokers and non-smokers associated with increased exposure to formaldehyde at both 1 ppb and 10 ppb are below the de minimis risk of 1×10^{-6}.

A comprehensive review article by the German Federal Agency for Risk Assessment (71) might be of help for the interested reader to further bring formaldehyde exposure into the general context of cigarette smoke-related exposure.

The *in vitro* cytotoxicity of the TPM was not affected in the present study by the addition of saccharose to the tobacco filler. In a not yet published PM-sponsored study no effect on *in vitro* cytotoxicity was observed on addition of up to 10% saccharose (72). Studies where up to a concentration of 10.5% saccharose in a mixture with other ingredients was applied to cigarette filler do not suggest an increase in *in vitro* cytotoxicity of the mainstream smoke (18, 20).

The *in vitro* mutagenicity of the TPM was not increased by the addition of saccharose in the present study. Similar results have been reported using application levels up to 5% saccharose. In the presence of a metabolic activation system, even a reduction of the mutagenic activity due to the addition of saccharose was observed (16).
Studies in which up to 10.5% saccharose in a mixture with other ingredients was applied to the cigarette filler do not suggest an increased *in vitro* mutagenicity of the mainstream smoke (18, 20).
In vivo studies were not included in the current assessment. However, a not yet published PM-sponsored nose-only inhalation study with male and female rats concluded that, *"An increased number of goblet cells in the bronchial epithelium of the lung were seen in the 100,000 ppm males; however, neither the histopathological changes in the nose nor an increased number of goblet cells was seen in the 36,000 or 72,000 ppm Test Cigarette groups. ... The no-observed-effect level (NOEL) in this study was 72,000 ppm of Sucrose"*. A similar observation was not found in female rats (73).
Studies where saccharose was applied up to a concentration of 10.5% in a mixture with other ingredients to cigarettes filler do not indicate increased inhalation toxicity of smoke from experimental cigarettes with the addition of saccharose (16, 20, 74).

CONCLUSIONS

The data obtained in the present study are in agreement with the wealth of reported literature data. The chemistry data provided by Labstat International ULC may be compared with data obtained in an official governmental laboratory[1]. In addition, the current study presents new data on the effects of the addition of the three ingredients as single substances to experimental cigarettes for some test systems.
The addition of cocoa to cigarette filler did not result in any consistent changes in the yields of a standard panel of smoke components nor in any change in the *in vitro* cytotoxicity and mutagenicity of the smoke.
The addition of glycerol to cigarette filler resulted in several decreases of smoke components and an increase in water and possibly ammonia. The *in vitro* cytotoxicity of the TPM was slightly decreased, while the cytotoxicity of the GVP was not affected. The *in vitro* mutagenicity was also not affected.
The addition of saccharose to the tobacco filler of experimental cigarettes increased the delivery of formaldehyde and decreased the delivery of tobacco-specific *N*-nitrosamines compared to control cigarettes. The observed increase in formaldehyde was not reflected in an increase in the *in vitro* cytotoxicity and mutagenicity of the smoke. Under non-experimental, i.e., consumer use conditions, the addition of saccharose is actually related to decreased exposure to formaldehyde compared to a smoker of Virginia type cigarettes that traditionally contain no addition of sugar. Accordingly, the observed effect on formaldehyde delivery is considered as a real experimental result but does not appear to impact the human situation.
In summary, the data confirm that cigarette smoke is toxic both with and without addition of ingredients to the tobacco filler. However, there are no indications that cocoa, glycerol, or saccharose as tobacco ingredients at their current use levels increase the biological activity of tobacco smoke.

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Table A: Smoke chemistry, 6 mg ISO 'tar' yield cigarette, ISO smoking. Yield data in bold and italic are statistically significant relative to control; those that are underlined in addition are considered as real effects (see chapter data analysis). M = mean, SE = standard error of mean.

Parameter	Unit of measure	Control		Cocoa						Glycerol						Saccharose					
				0.4%		1.1%		2.2%		1.5%		3.0%		5.5%		1.6%		2.1%		4.8%	
		M	SE	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE
TPM	(mg/cig)	7.19	0.04	6.93	0.18	7.04	0.12	7.01	0.15	7.1	0.3	7.49	0.24	7.09	0.14	6.34	0.14	7.35	0.23	7.18	0.15
CO	(mg/cig)	6.45	0.11	6.06	0.30	6.45	0.05	6.52	0.11	6.2	0.2	5.88	0.37	6.05	0.28	***5.50***	0.17	6.24	0.13	6.39	0.03
Water	(mg/cig)	0.169	0.074	0.251	0.066	0.214	0.022	0.233	0.029	0.212	0.016	***0.457***	0.014	0.409	0.052	0.191	0.009	0.154	0.055	0.188	0.054
Nicotine	(mg/cig)	0.539	0.011	0.525	0.019	0.536	0.006	0.538	0.003	0.517	0.012	0.536	0.022	0.498	0.009	***0.465***	0.009	0.568	0.017	0.511	0.006
'Tar'	(mg/cig)	6.48	0.09	6.15	0.09	6.29	0.11	6.24	0.12	6.4	0.2	6.49	0.20	6.19	0.08	5.68	0.14	6.63	0.18	6.48	0.17
Puff count	(per cig)	7.09	0.08	7.09	0.08	7.32	0.14	7.31	0.05	6.8	0.1	7.18	0.06	7.07	0.07	6.90	0.13	7.40	0.19	7.13	0.06
Formaldehyde	(µg/cig)	15.5	0.4	13.7	0.9	14.6	0.3	14.1	1.3	16.5	0.7	14.2	0.7	13.5	1.2	16.3	0.3	14.3	1.0	18.5	0.7
Acetaldehyde	(µg/cig)	315	9	307	13	311	9	321	13	321	18	311	13	280	10	309	15	314	12	318	9
Acetone	(µg/cig)	175	6	170	10	168	6	174	6	173	8	151	6	148	4	170	8	174	7	183	4
Acrolein	(µg/cig)	30.6	1.4	28.9	2.0	29.0	0.5	30.0	1.9	32.3	1.9	31.9	1.3	30.3	1.1	31.1	2.5	29.2	1.2	30.9	1.4
Propionaldehyde	(µg/cig)	28.0	1.2	27.9	1.7	27.5	0.8	29.2	1.4	27.9	1.8	24.6	0.7	25.7	0.9	28.3	1.8	28.1	1.0	28.8	0.91
Crotonaldehyde	(µg/cig)	7.82	0.45	7.34	1.13	7.10	0.18	7.18	0.40	7.9	0.6	7.80	0.86	5.70	0.68	7.81	0.83	8.03	0.56	7.63	0.30
MEK	(µg/cig)	39.6	1.3	39.1	3.1	37.1	1.7	38.4	1.5	39.2	2.4	31.4	2.8	34.0	1.8	38.5	1.4	38.5	1.7	40.0	0.8
Butylaldehyde	(µg/cig)	23.8	0.7	23.0	1.9	22.8	0.6	23.5	0.5	24.1	1.4	18.4	0.9	21.0	1.6	22.8	1.5	22.3	1.0	22.9	0.4
Hydroquinone	(µg/cig)	40.4	0.3	***35.5***	0.4	***35.1***	0.8	***36.5***	0.7	***33.0***	1.4	***31.5***	0.8	***27.1***	0.3	***35.6***	0.8	***37.2***	0.3	***36.7***	0.3
Resorcinol	(µg/cig)	< 1.74	—	< 1.74	—	< 1.74	—	< 1.74	—	< 1.74	—	< 1.74	—	< 1.74	—	< 1.74	—	< 1.74	—	< 1.74	—
Catechol	(µg/cig)	40.4	1.4	35.2	2.2	35.8	0.8	35.9	1.5	***33.4***	0.6	***32.1***	0.8	***26.3***	0.5	35.2	0.6	39.8	0.2	37.0	1.1
Phenol	(µg/cig)	12.6	0.3	***10.3***	0.2	***10.2***	0.5	***10.5***	0.3	***9.3***	0.3	***7.7***	0.2	***5.0***	0.3	***9.5***	0.2	***10.7***	0.4	11.0	0.3
m+p-Cresol	(µg/cig)	8.74	0.15	***7.45***	0.15	***7.46***	0.15	***7.36***	0.20	***6.6***	0.2	***5.46***	0.19	***4.27***	0.13	***7.30***	0.21	7.91	0.21	***7.48***	0.17
o-Cresol	(µg/cig)	2.99	0.07	2.52	0.11	2.50	0.13	2.58	0.09	***2.3***	0.1	***1.92***	0.10	***1.62***	0.07	2.48	0.16	2.48	0.05	2.54	0.05
Benzo[*a*]pyrene	(ng/cig)	6.16	0.14	5.69	0.17	6.56	0.13	5.82	0.05	6.0	0.0	6.42	0.36	5.17	0.15	5.94	0.05	5.65	0.18	6.57	0.14
1-Aminonaphthalene	(ng/cig)	11.1	0.7	11.3	0.6	9.9	0.6	11.9	0.6	12.6	0.6	12.1	0.9	10.6	0.4	10.7	0.5	11.0	0.2	9.8	0.12
2-Aminonaphthalene	(ng/cig)	8.34	0.20	8.29	0.23	7.44	0.65	8.66	0.20	9.3	0.2	8.06	0.57	8.21	0.31	7.48	0.43	7.83	0.57	7.79	0.09
3-Aminobiphenyl	(ng/cig)	2.07	0.11	2.08	0.06	1.79	0.08	2.12	0.04	2.2	0.0	1.94	0.09	2.04	0.02	1.79	0.07	1.83	0.06	1.93	0.04
4-Aminobiphenyl	(ng/cig)	1.55	0.07	1.51	0.06	1.45	0.11	1.69	0.03	***1.9***	0.1	1.42	0.03	1.63	0.02	1.49	0.05	1.41	0.03	1.39	0.07
NO	(µg/cig)	111	5	97	6	96	3	102	2	99	3	107	3	94	3	95	2	106	5	***81***	1
NO_x	(µg/cig)	118	5	102	6	102	2	107	3	106	3	113	3	103	2	101	2	110	5	***85***	1
Total HCN	(µg/cig)	76.2	3.5	73.2	3.9	73.8	3.9	74.8	2.3	74.2	2.6	***57.2***	1.9	***58.1***	2.8	70.5	3.2	73.8	1.7	70.4	4.37
Ammonia	(µg/cig)	8.76	0.08	7.80	0.33	7.87	0.12	8.06	0.09	9.3	0.5	11.83	1.11	10.50	0.07	8.01	0.12	8.49	0.18	***7.86***	0.17
1,3-Butadiene	(µg/cig)	24.8	0.5	25.2	0.3	25.2	0.2	26.0	0.4	25.3	0.5	28.6	1.6	22.6	1.0	25.0	1.8	25.2	1.2	25.2	0.4
Isoprene	(µg/cig)	194	4	209	2	***215***	4	***218***	1	210	3	209	12	193	7	204	12	213	10	202	2
Acrylonitrile	(µg/cig)	5.11	0.37	5.68	0.36	5.29	0.19	5.79	0.31	5.5	0.1	5.37	0.45	4.23	0.20	5.77	0.01	5.41	0.35	5.77	0.06
Benzene	(µg/cig)	26.9	0.7	26.0	0.1	26.6	0.2	27.1	0.7	28.5	0.1	26.5	2.2	24.7	0.8	27.1	0.7	26.1	0.6	27.2	0.2
Toluene	(µg/cig)	42.6	1.7	41.8	0.5	42.0	1.0	44.9	2.5	47.0	0.5	38.5	4.1	41.2	1.4	43.1	0.9	41.7	1.3	43.5	0.3
Pyridine	(µg/cig)	9.51	0.78	8.78	0.60	8.95	0.58	8.07	0.52	9.4	0.1	***6.12***	0.66	7.15	0.27	8.15	0.34	8.48	0.48	8.07	0.30
Quinoline	(µg/cig)	0.288	0.008	0.261	0.011	0.269	0.002	0.249	0.007	0.270	0.011	***0.203***	0.001	***0.168***	0.008	0.235	0.006	0.273	0.006	***0.249***	0.004
Styrene	(µg/cig)	5.69	0.37	5.52	0.27	5.81	0.41	5.67	0.26	6.4	0.3	5.44	0.58	5.44	0.28	5.43	0.22	5.66	0.24	5.67	0.15
NNN	(ng/cig)	61.1	3.3	***50.4***	0.2	***50.2***	1.2	51.4	1.1	55.0	2.8	***42.9***	1.7	***40.9***	1.9	***46.8***	1.7	56.3	2.8	***51.4***	0.6
NAT	(ng/cig)	53.0	1.0	***43.1***	1.7	***45.5***	1.6	47.0	0.7	50.2	1.6	47.7	1.7	***44.3***	1.2	***43.6***	0.4	51.6	0.9	***47.0***	0.4
NAB	(ng/cig)	8.98	0.12	***6.43***	0.12	7.89	0.23	8.10	0.33	8.1	0.2	***7.64***	0.33	***6.41***	0.21	***6.74***	0.25	8.29	0.40	***8.10***	0.19
NNK	(ng/cig)	21.1	0.8	***15.7***	0.8	17.6	0.9	17.0	0.7	20.0	0.9	18.1	0.8	17.7	0.3	***14.7***	0.6	21.5	0.5	17.0	0.4

Table B: Smoke chemistry, 10 mg ISO 'tar' yield cigarette, ISO smoking. Yield data in bold and italic are statistically significant relative to control; those that are underlined in addition are considered as real effects (see chapter data analysis). M = mean, SE = standard error of mean.

Parameter	Unit of measure	Control		Cocoa						Glycerol						Saccharose					
				0.40%		1.1%		2.2%		1.5%		3.0%		5.0%		1.6%		2.1%		4.8%	
		M	SE	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE
TPM	(mg/cig)	12.6	0.3	12.2	0.1	12.8	0.6	12.9	0.3	12.2	0.5	12.8	0.2	13.3	0.3	12.0	0.2	13.0	0.2	12.9	0.2
CO	(mg/cig)	9.75	0.11	9.82	0.08	9.70	0.48	9.84	0.32	9.22	0.33	9.81	0.16	10.19	0.31	8.99	0.26	9.93	0.02	10.13	0.02
Water	(mg/cig)	0.655	0.086	0.450	0.007	0.830	0.117	0.625	0.084	0.494	0.061	***1.248***	0.043	***1.144***	0.085	0.673	0.051	0.651	0.064	0.645	0.134
Nicotine	(mg/cig)	0.873	0.020	0.823	0.006	0.840	0.031	0.864	0.017	0.846	0.031	0.789	0.015	0.836	0.017	0.811	0.020	0.899	0.013	0.836	0.011
'Tar'	(mg/cig)	11.1	0.3	10.9	0.1	11.1	0.5	11.4	0.2	10.8	0.4	10.7	0.2	11.4	0.2	10.5	0.2	11.5	0.2	11.4	0.1
Puff count	(per cig)	7.80	0.22	7.73	0.06	8.02	0.15	7.59	0.12	7.44	0.09	7.62	0.09	7.65	0.07	7.53	0.13	7.71	0.06	7.85	0.12
Formaldehyde	(µg/cig)	24.7	1.0	25.5	0.8	21.1	1.9	22.4	0.6	26.1	2.0	23.4	1.8	23.4	0.8	28.7	1.1	28.1	1.2	***34.6***	1.5
Acetaldehyde	(µg/cig)	457	8	487	21	452	12	456	22	449	21	464	31	459	6	460	7	***501***	3	***510***	7
Acetone	(µg/cig)	245	3	264	13	246	8	243	9	239	9	228	12	240	5	248	3	***267***	2	***290***	6
Acrolein	(µg/cig)	46.1	0.3	47.8	1.9	43.5	2.0	44.9	1.1	47.8	2.1	49.8	2.9	50.7	0.5	47.4	1.6	50.4	0.8	***53.6***	0.1
Propionaldehyde	(µg/cig)	40.5	0.9	43.4	1.7	40.4	1.5	41.0	1.8	39.9	2.0	35.8	2.0	41.2	1.0	41.2	0.7	***45.2***	0.5	***46.6***	0.5
Crotonaldehyde	(µg/cig)	14.6	0.5	15.0	1.1	13.1	0.8	13.9	0.9	12.7	0.7	15.6	1.4	11.7	0.3	14.0	0.2	15.8	0.3	16.5	0.7
MEK	(µg/cig)	57.8	1.7	61.6	3.4	58.0	2.1	57.8	2.3	55.4	2.0	51.3	2.8	57.0	1.1	56.6	0.4	62.7	0.3	***67.7***	1.7
Butylaldehyde	(µg/cig)	32.6	1.0	34.8	1.8	32.5	0.6	32.9	1.6	30.4	1.2	27.3	2.2	31.0	1.3	33.0	0.6	35.8	0.2	36.2	1.1
Hydroquinone	(µg/cig)	53.8	1.2	54.1	2.2	63.9	3.5	60.4	1.5	53.6	1.2	52.2	1.9	46.3	0.9	54.4	1.8	60.6	1.9	56.1	1.2
Resorcinol	(µg/cig)	< 1.74	—	< 1.74	—	< 1.74	—	< 1.74	—	< 1.74	—	< 1.74	—	< 1.74	—	< 1.74	—	< 1.75	—	< 1.75	—
Catechol	(µg/cig)	51.5	1.7	52.2	0.5	60.7	7.3	62.6	1.8	52.0	1.8	48.4	1.6	43.7	0.9	51.7	0.8	***61.5***	2.0	56.2	2.2
Phenol	(µg/cig)	20.5	1.0	18.9	0.2	22.2	2.7	22.2	1.0	18.5	1.0	***15.7***	0.5	***12.4***	0.5	18.9	0.1	23.7	1.4	19.1	0.4
m+*p*-Cresol	(µg/cig)	13.1	0.6	12.3	0.3	14.5	1.3	14.8	0.6	12.8	0.8	10.4	0.4	***9.2***	0.4	12.6	0.4	14.8	0.6	13.3	0.3
o-Cresol	(µg/cig)	4.35	0.40	4.05	0.21	4.79	0.46	4.95	0.22	4.91	0.30	3.57	0.08	3.29	0.25	4.26	0.16	4.98	0.17	4.52	0.05
Benzo[*a*]pyrene	(ng/cig)	9.86	0.35	9.20	0.24	8.92	0.05	8.93	0.20	8.82	0.22	9.60	0.45	9.06	0.45	9.40	0.16	9.58	0.12	11.51	0.55
1-Aminonaphthalene	(ng/cig)	17.2	0.2	15.5	0.6	15.8	0.7	16.4	0.7	16.4	0.7	17.6	0.7	17.7	0.7	15.6	0.8	16.5	1.0	17.0	0.6
2-Aminonaphthalene	(ng/cig)	11.7	0.2	11.1	0.2	10.7	0.5	11.1	0.9	11.1	0.6	11.3	0.1	12.2	0.6	10.8	0.5	10.9	1.1	12.0	0.3
3-Aminobiphenyl	(ng/cig)	2.78	0.18	2.62	0.10	2.59	0.11	2.83	0.23	2.81	0.04	2.69	0.04	2.96	0.13	2.56	0.13	2.65	0.22	2.61	0.16
4-Aminobiphenyl	(ng/cig)	2.08	0.11	2.17	0.04	2.17	0.07	2.24	0.14	2.09	0.06	2.24	0.07	2.47	0.03	2.09	0.12	2.00	0.18	2.02	0.02
NO	(µg/cig)	133	6	144	6	145	8	126	5	120	3	140	10	133	2	133	3	139	5	149	6
NO_x	(µg/cig)	141	7	154	6	155	9	134	5	126	3	148	11	145	1	143	3	148	5	160	6
Total HCN	(µg/cig)	134	4	133	5	135	3	137	5	132	5	105	7	121	2	121	0	137	4	136	5
Ammonia	(µg/cig)	14.4	0.3	12.9	0.4	13.3	0.3	13.7	0.4	15.2	0.3	***18.4***	0.5	***19.0***	0.9	13.1	0.3	13.5	0.3	13.2	0.2
1,3-Butadiene	(µg/cig)	34.2	1.2	35.2	2.1	35.6	0.6	36.5	1.1	36.1	0.5	***39.6***	0.9	33.4	0.5	33.5	0.4	37.3	0.7	38.6	1.7
Isoprene	(µg/cig)	281	8	288	18	288	0	295	7	296	12	289	7	283	6	271	5	309	5	314	15
Acrylonitrile	(µg/cig)	8.00	0.33	8.41	0.80	9.62	0.25	9.17	0.06	8.54	0.56	8.81	0.65	7.52	0.11	7.62	0.51	***10.59***	0.34	8.57	0.56
Benzene	(µg/cig)	36.2	1.2	36.6	1.1	37.3	0.2	35.6	0.7	39.0	1.8	36.5	1.0	36.2	0.4	33.9	0.9	38.8	1.0	38.9	2.4
Toluene	(µg/cig)	57.7	2.9	59.5	3.2	62.0	0.9	57.1	2.1	64.7	3.8	58.0	2.0	60.3	0.6	55.0	1.1	63.9	1.7	62.9	3.1
Pyridine	(µg/cig)	17.5	0.6	16.1	0.3	15.5	1.5	15.6	0.6	15.9	0.6	13.9	1.1	***13.8***	0.5	14.8	0.6	15.9	0.8	15.6	0.8
Quinoline	(µg/cig)	0.505	0.011	0.451	0.012	0.444	0.003	0.451	0.016	0.438	0.022	***0.359***	0.004	***0.318***	0.006	0.447	0.018	0.470	0.021	0.453	0.022
Styrene	(µg/cig)	9.46	0.15	9.20	0.16	8.49	0.72	8.90	0.33	9.22	0.13	9.31	0.89	9.21	0.28	8.16	0.34	9.45	0.20	8.91	0.44
NNN	(ng/cig)	87.0	4.9	77.8	4.0	76.6	0.9	69.9	3.0	82.2	3.4	66.6	3.3	***65.5***	2.5	70.0	2.2	84.5	4.0	***63.7***	2.6
NAT	(ng/cig)	75.7	1.8	68.5	1.5	***65.5***	0.3	***65.7***	1.8	77.1	1.5	74.5	3.0	69.5	1.7	66.4	1.1	78.0	3.3	***61.7***	1.1
NAB	(ng/cig)	11.6	0.1	11.1	0.2	9.9	0.2	10.0	0.5	11.7	0.4	12.0	0.4	10.1	0.6	***10.0***	0.3	12.1	0.3	10.3	0.1
NNK	(ng/cig)	28.1	1.5	27.5	1.4	25.2	0.5	24.3	0.7	33.5	0.5	27.9	1.0	27.1	0.3	24.6	0.4	29.9	0.7	33.3	0.9

Table C: Smoke chemistry, 6 mg ISO 'tar' yield, HCl smoking. Yield data in bold and italic are statistically significant relative to control; those that are underlined in addition are considered as real effects (see chapter data analysis). M = mean, SE = standard error of mean.

Parameter	Unit of measure	Control		Cocoa						Glycerol						Saccharose					
				0.4%		1.1%		2.2%		1.5%		3.0%		5.5%		1.6%		2.1%		4.8%	
		M	SE	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE
TPM	(mg/cig)	43.2	1.7	40.9	1.4	40.9	2.8	39.3	0.6	41.1	2.1	41.4	1.8	44.1	1.2	39.4	1.6	37.7	0.6	38.1	0.4
CO	(mg/cig)	26.5	1.1	25.9	1.3	24.5	1.3	25.7	0.7	25.0	1.0	23.9	1.0	26.2	0.7	23.6	0.5	22.7	1.0	23.1	0.2
Water	(mg/cig)	12.8	0.7	12.5	0.3	12.8	1.5	11.6	0.5	12.8	0.6	13.0	0.7	15.2	0.5	12.1	0.0	11.8	0.4	12.3	0.2
Nicotine	(mg/cig)	1.63	0.05	1.58	0.06	1.62	0.03	1.47	0.06	1.66	0.02	1.56	0.06	1.52	0.02	1.50	0.04	1.57	0.04	1.53	0.04
'Tar'	(mg/cig)	28.7	0.9	26.9	1.3	26.4	1.3	26.2	0.2	26.6	1.5	26.8	1.1	27.3	0.7	25.8	1.6	24.4	0.8	24.3	0.4
Puff count	(per cig)	8.44	0.14	8.41	0.13	8.74	0.07	8.27	0.16	8.56	0.17	8.28	0.19	8.37	0.10	8.01	0.30	8.46	0.13	8.52	0.10
Formaldehyde	(µg/cig)	73.2	8.0	69.5	2.4	70.8	1.2	67.7	5.7	72.9	3.8	66.5	0.9	72.5	2.7	82.1	1.3	77.0	3.0	97.8	3.3
Acetaldehyde	(µg/cig)	1131	29	1081	7	1117	10	1027	20	1087	40	1165	20	1095	30	1011	40	1057	16	1064	49
Acetone	(µg/cig)	539	16	560	7	557	5	537	13	525	8	534	9	550	22	503	12	518	5	588	34
Acrolein	(µg/cig)	119	1	***115***	0	***123***	1	***114***	1	126	1	128	4	***140***	5	112	3	120	0	124	5
Propionaldehyde	(µg/cig)	85.2	2.7	95.3	3.1	96.0	3.2	81.6	1.6	88.4	2.8	91.2	3.1	100.7	3.8	81.1	2.1	88.7	3.0	92.1	7.7
Crotonaldehyde	(µg/cig)	45.7	0.5	45.3	1.3	45.5	0.5	42.8	0.2	42.6	0.2	44.9	0.8	43.8	1.1	41.7	2.1	44.0	1.4	45.5	3.0
MEK	(µg/cig)	124	8	136	3	130	2	128	2	123	2	126	6	134	4	119	3	122	2	137	11
Butylaldehyde	(µg/cig)	67.3	2.7	72.4	1.5	73.9	3.9	71.8	2.0	66.3	0.5	67.4	0.6	73.7	2.6	65.8	1.5	69.8	2.1	70.2	5.7
Hydroquinone	(µg/cig)	109	6	113	6	123	5	123	2	108	5	130	0	99	5	126	6	128	5	120	1
Resorcinol	(µg/cig)	3.31	0.03	***2.21***	0.05	***2.40***	0.05	***2.90***	0.04	1.32	—	1.55	0.12	1.63	0.08	***2.17***	0.05	***2.59***	0.11	***1.76***	0.07
Catechol	(µg/cig)	97.1	3.7	96.5	1.3	103.8	4.1	104.3	4.3	98.9	2.6	108.0	3.7	78.6	3.0	101.8	2.6	99.1	3.9	102.6	3.7
Phenol	(µg/cig)	19.7	0.8	19.9	0.2	19.7	0.9	21.2	1.7	19.6	0.9	***26.2***	0.1	***11.7***	0.4	18.9	1.1	22.7	0.9	19.7	1.1
m+*p*-Cresol	(µg/cig)	13.7	0.6	15.0	0.3	15.3	0.6	15.3	0.5	13.6	0.6	***18.3***	0.2	***10.9***	0.4	15.7	0.9	17.6	0.9	15.0	0.2
o-Cresol	(µg/cig)	4.05	0.11	5.15	0.27	4.96	0.06	4.11	0.20	4.29	0.12	***5.56***	0.34	3.79	0.29	***5.50***	0.30	***5.40***	0.19	4.12	0.07
Benzo[*a*]pyrene	(ng/cig)	19.2	0.3	18.3	1.2	17.6	0.4	17.7	0.7	17.6	0.9	19.7	1.4	14.3	0.6	***15.8***	0.3	17.3	0.9	19.4	0.4
1-Aminonaphthalene	(ng/cig)	26.7	1.9	28.6	1.9	26.3	1.0	26.9	1.2	26.5	2.4	25.6	0.5	17.6	0.3	25.5	1.9	23.9	1.3	22.7	0.2
2-Aminonaphthalene	(ng/cig)	18.6	0.9	18.3	0.9	18.6	0.6	18.5	0.3	18.2	1.5	18.3	0.2	14.3	0.6	17.4	1.0	16.4	0.5	16.2	0.6
3-Aminobiphenyl	(ng/cig)	4.76	0.15	5.61	0.47	4.95	0.29	5.18	0.09	4.99	0.35	5.02	0.23	4.36	0.11	4.69	0.24	4.85	0.09	4.51	0.11
4-Aminobiphenyl	(ng/cig)	4.01	0.14	4.06	0.07	4.26	0.21	3.98	0.15	4.06	0.29	3.99	0.26	3.52	0.26	3.73	0.19	3.84	0.09	3.41	0.29
NO	(µg/cig)	281	8	305	18	329	18	326	16	320	15	331	12	281	5	300	19	309	13	293	7
NO_x	(µg/cig)	305	9	333	20	356	19	353	16	347	17	363	13	306	8	323	18	335	11	318	9
Total HCN	(µg/cig)	341	8	334	10	402	17	394	17	393	9	407	42	349	22	327	12	364	8	356	28
Ammonia	(µg/cig)	32.3	2.0	29.2	1.0	33.0	1.4	29.6	1.6	36.0	1.5	***42.2***	0.2	***52.2***	0.9	29.6	0.7	27.0	1.2	29.9	1.1
1,3-Butadiene	(µg/cig)	83.7	3.4	83.1	1.0	83.5	1.1	80.4	5.8	83.1	3.0	77.8	1.3	80.3	2.4	77.2	2.7	83.6	1.6	76.9	3.7
Isoprene	(µg/cig)	631	2	668	14	647	15	632	30	652	14	618	6	643	11	581	20	711	39	590	28
Acrylonitrile	(µg/cig)	23.0	0.4	22.6	0.6	24.2	0.9	25.2	2.1	24.5	0.9	***18.1***	0.1	20.9	1.2	21.2	1.2	23.6	0.8	23.1	1.9
Benzene	(µg/cig)	83.3	2.2	77.1	4.8	77.7	3.4	77.2	2.5	81.4	2.7	***63.6***	1.2	***71.3***	1.5	68.5	4.9	84.4	4.8	76.5	5.3
Toluene	(µg/cig)	147	4	141	12	138	5	138	3	140	5	***109***	1	130	4	122	9	159	7	137	8
Pyridine	(µg/cig)	44.2	1.8	42.4	2.7	43.7	2.4	39.8	1.2	41.0	3.0	40.6	2.5	42.8	2.3	42.5	1.6	45.1	2.6	44.0	1.3
Quinoline	(µg/cig)	0.663	0.009	***0.480***	0.024	***0.499***	0.029	***0.515***	0.029	0.635	0.032	***0.496***	0.008	***0.469***	0.035	***0.549***	0.012	0.577	0.031	***0.520***	0.018
Styrene	(µg/cig)	23.3	0.9	25.9	0.3	25.0	1.8	24.6	0.6	22.5	1.3	23.9	1.8	23.8	2.4	23.1	1.3	27.2	1.9	28.4	1.1

Table D: Smoke chemistry, 10 mg ISO 'tar' yjf rø-ÍI DJsmpl joh.!Yield data in bold and italic are statistically significant relative to control; those that are underlined in addition are considered as real effects (see chapter data analysis). M = mean, SE = standard error of mean.

Parameter	Unit of measure	Control		Cocoa						Glycerol						Saccharose					
				0.4%		1.1%		2.2%		1.5%		3.0%		5.5%		1.6%		2.1%		4.8%	
		M	SE	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE
TPM	(mg/cig)	48.5	1.5	47.7	0.8	50.3	1.3	48.5	3.2	46.7	2.1	47.1	0.4	51.8	1.7	49.7	3.2	47.1	1.3	45.6	0.6
CO	(mg/cig)	26.4	1.8	26.5	2.0	27.1	0.4	26.5	0.5	26.7	1.0	26.3	0.2	26.6	1.7	27.6	0.5	27.0	0.5	26.4	0.7
Water	(mg/cig)	13.9	0.6	14.8	0.5	14.1	0.6	14.0	0.7	14.7	0.9	14.2	0.4	18.5	1.2	14.4	0.9	13.2	0.0	13.0	0.5
Nicotine	(mg/cig)	1.96	0.03	1.80	0.06	1.90	0.02	1.91	0.07	1.93	0.02	1.86	0.02	1.90	0.07	1.94	0.09	1.93	0.06	1.85	0.07
'Tar'	(mg/cig)	32.6	1.0	31.2	0.7	34.3	0.7	32.5	2.4	30.1	1.4	31.0	0.2	31.4	1.0	33.3	2.2	32.0	1.3	30.8	0.3
Puff count	(percig)	9.46	0.07	8.83	0.25	9.52	0.10	9.62	0.20	9.73	0.16	9.37	0.31	9.28	0.09	9.16	0.37	9.41	0.18	9.78	0.22
Formaldehyde	(µg/cig)	86.9	4.3	81.4	2.4	78.5	3.1	73.7	1.5	82.0	3.0	75.7	4.0	79.5	1.4	84.8	3.2	93.3	1.7	***107.9***	4.6
Acetaldehyde	(µg/cig)	1169	31	1228	49	1181	68	1157	15	1239	18	1247	14	1133	19	1167	33	1246	49	1075	22
Acetone	(µg/cig)	604	14	612	17	597	28	586	17	610	12	576	14	576	15	599	12	620	18	586	13
Acrolein	(µg/cig)	130	1	129	4	126	6	125	3	140	3	142	3	***144***	2	131	4	135	2	123	5
Propionaldehyde	(µg/cig)	96	4	105	4	101	2	100	4	103	4	96	1	100	3	104	2	105	3	96	6
Crotonaldehyde	(µg/cig)	51.2	1.7	52.7	2.0	52.9	3.8	51.5	0.5	52.1	0.9	51.1	1.7	47.3	2.2	51.4	3.0	54.1	3.1	48.1	0.3
MEK	(µg/cig)	146	2	148	4	147	8	150	1	142	6	135	3	140	3	147	6	145	3	139	4.3
Butylaldehyde	(µg/cig)	77.0	2.5	82.1	2.1	81.2	1.7	79.3	5.4	79.3	3.2	71.8	1.7	77.7	1.7	82.7	2.1	77.5	0.9	76.1	4.0
Hydroquinone	(µg/cig)	155	9	128	7	152	7	158	1	140	0	***103***	3	***116***	3	144	10	152	7	150	7
Resorcinol	(µg/cig)	3.19	0.14	***3.89***	0.05	***2.53***	0.01	3.50	0.12	2.92	0.07	1.63	0.11	1.32	—	2.91	0.06	***2.14***	0.13	3.31	0.13
Catechol	(µg/cig)	130	8	119	7	139	5	142	2	129	6	***85***	2	103	6	126	4	130	0	131	2
Phenol	(µg/cig)	37.0	1.9	30.6	1.8	34.2	1.3	37.0	1.8	31.9	1.7	***16.7***	0.4	***21.3***	0.6	34.1	1.3	35.4	1.8	33.3	1.4
m+*p* Cresol	(µg/cig)	25.2	0.8	***20.6***	0.6	24.4	0.5	26.8	1.1	21.5	0.4	***12.3***	1.1	***15.4***	0.1	24.0	1.8	25.0	0.9	24.0	0.1
o-Cresol	(µg/cig)	7.27	0.30	5.94	0.08	6.60	0.18	7.70	0.39	6.74	0.19	***4.58***	0.32	***4.39***	0.05	6.69	0.41	7.50	0.30	7.71	0.10
Benzo[*a*]pyrene	(ng/cig)	22.5	0.7	21.7	1.1	23.0	1.1	20.2	0.4	22.2	0.7	***15.5***	0.7	18.4	1.4	19.3	0.6	20.9	1.1	21.0	1.0
1-Aminonaphthalene	(ng/cig)	25.0	3.0	31.4	2.5	32.5	0.9	34.2	1.2	27.5	2.3	33.2	1.6	31.6	2.5	27.5	2.0	29.1	0.3	30.9	2.5
2-Aminonaphthalene	(ng/cig)	20.3	0.8	20.8	1.2	21.9	1.1	22.2	0.7	19.7	0.9	21.5	0.8	20.6	1.2	20.9	1.8	20.2	0.9	21.5	1.4
3-Aminobiphenyl	(ng/cig)	5.48	0.07	5.77	0.23	6.10	0.49	6.03	0.35	5.48	0.14	5.99	0.26	5.91	0.06	5.77	0.31	5.77	0.30	5.85	0.04
4-Aminobiphenyl	(ng/cig)	4.26	0.26	4.45	0.06	4.88	0.37	4.84	0.27	4.29	0.11	4.33	0.08	4.55	0.13	4.61	0.26	4.68	0.29	4.23	0.21
NO	(µg/cig)	354	9	330	13	330	29	326	15	343	18	342	15	326	13	329	12	392	9	332	12
NO_x	(µg/cig)	383	10	361	13	363	29	354	15	374	18	375	15	357	15	357	14	428	10	362	13
Total HCN	(µg/cig)	411	20	393	26	427	11	399	25	427	12	446	18	384	30	433	18	370	2	410	6
Ammonia	(µg/cig)	41.9	2.3	38.6	2.4	37.5	1.9	36.0	1.0	44.0	0.8	***54.5***	2.4	***60.4***	2.2	36.7	1.1	37.1	0.5	32.0	1.0
1,3-Butadiene	(µg/cig)	88.8	2.3	91.6	2.0	88.9	2.3	89.7	1.2	89.4	2.8	92.7	3.2	86.4	2.4	88.7	0.9	92.2	2.2	90.5	3.0
Isoprene	(µg/cig)	688	22	737	4	721	13	706	20	701	43	714	46	694	14	670	15	772	7	701	13
Acrylonitrile	(µg/cig)	26.3	1.6	27.4	0.9	26.9	1.1	26.9	1.1	25.6	1.5	19.7	0.5	23.5	2.2	22.9	1.4	27.5	1.4	25.3	0.8
Benzene	(µg/cig)	87.8	4.2	89.0	1.9	89.9	5.4	91.7	1.2	89.2	3.7	76.2	2.4	85.8	2.1	82.8	3.6	90.0	3.9	91.5	2.2
Toluene	(µg/cig)	156	6	161	4	167	8	170	3	162	5	***125***	3	160	5	152	6	162	7	168	7
Pyridine	(µg/cig)	57.9	1.9	49.8	2.9	51.4	2.0	51.9	1.5	53.1	0.6	***47.0***	2.1	51.0	2.1	52.1	3.6	53.1	1.3	53.6	1.7
Quinoline	(µg/cig)	0.836	0.020	0.869	0.025	0.751	0.059	0.804	0.048	0.675	0.015	0.723	0.025	0.624	0.073	0.779	0.033	0.789	0.033	0.807	0.080
Styrene	(µg/cig)	29.7	1.4	25.9	2.5	28.7	2.3	31.2	0.9	28.5	1.6	26.1	1.2	28.1	1.6	29.3	2.7	30.1	2.2	31.6	1.4
NNN	(ng/cig)	181	2	156	4	165	5	197	8	145	7	168	6	***132***	9	***148***	8	173	3	***140***	5
NAT	(ng/cig)	161	7	151	7	153	4	172	7	140	5	176	3	141	5	148	6	159	3	133	3
NAB	(ng/cig)	25.8	0.8	22.3	1.1	24.9	1.4	30.1	1.8	23.7	1.0	24.3	0.8	***20.4***	0.6	22.8	0.3	25.3	1.1	***18.2***	0.7
NNK	(ng/cig)	61.0	1.8	56.4	1.9	59.8	1.3	121.0	10.4	54.4	2.3	62.5	2.4	***48.3***	2.1	***50.8***	1.8	68.1	1.8	58.2	1.3

Table E: Cyototoxicity and mutagenicity, 6mg ISO 'tar' yield cigarette, ISO smoking. Asterics mark statistical significant differences relative to respective control ($p < 0.05$).

Ingredient type	Unit of measure	Cocoa				Glycerol				Saccharose			
Ingredient level		Control	0.4%	1.1%	2.2%	Control	1.5%	3.0%	5.5%	Control	1.6%	2.1%	4.8%
Neutral Red Uptake Assay													
PP, 1/EC50	(mL/cig)	96.8 ± 1.3	84.9 ± 1.6*	84.0 ± 1.4*	88.0 ± 2.8*	95.5 ± 4.1	87.6 ± 3.5	76.8 ± 2.4*	74.7 ± 1.9	85.3 ± 2.0	74.7 ± 0.6*	82.8 ± 2.0	78.4 ± 1.4*
GVP, 1/EC50	(mL/cig)	48.1 ± 1.4	38.8 ± 1.2*	39.6 ± 1.3*	37.8 ± 0.5*	50.4 ± 2.9	48.9 ± 2.6	46.6 ± 2.9	47.1 ± 2.0	46,4 ± 0.5	40.7 ± 1.6	45.3 ± 1.4	46.3 ± 1.8
Ames Assay, PP													
TA98, +S9	(rev/cig)	19,399 ± 1,018	19,036 ± 584	16,654 ± 880	17,701 ± 688	19,399 ± 1,018	18,402 ± 903	21,218 ± 837	18,106 ± 925	19,399 ± 1,018	15,531 ± 760*	16,140 ± 865	16,118 ± 835
TA100, +S9	(rev/cig)	9,188 ± 367	8,704 ± 543	8,194 ± 566	8,301 ± 640	9,188 ± 367	9,794 ± 668	9,426 ± 729	9,105 ± 478	9,188 ± 367	8,867 ± 564	9,593 ± 782	7,680 ± 539
TA102, +S9	(rev/cig)	−296 ± 99	−128 ± 155	−106 ± 70	−134 ± 76	−296 ± 99	−71 ± 157	−277 ± 79	74 ± 163	−296 ± 99	−98 ± 109	−213 ± 97	−58 ± 90
TA1535, +S9	(rev/cig)	15 ± 14	1 ± 11	1 ± 13	6 ± 11	15 ± 14	27 ± 10	31 ± 9	14 ± 9	15 ± 14	15 ± 12	8 ± 10	−4 ± 8
TA1537, +S9	(rev/cig)	2,978 ± 248	3,175 ± 235	2,937 ± 248	2,843 ± 192	2,978 ± 248	3,311 ± 206	3,272 ± 259	3,655 ± 167	2,978 ± 248	3,301 ± 181	3,321 ± 196	2,754 ± 279
TA98, -S9	(rev/cig)	33 ± 21	35 ± 19	22 ± 23	43 ± 19	33 ± 21	45 ± 25	61 ± 18	114 ± 25	33 ± 21	111 ± 28	97 ± 20	106 ± 17
TA100, -S9	(rev/cig)	654 ± 155	648 ± 144	1,002 ± 87	685 ± 101	654 ± 155	942 ± 141	825 ± 230	974 ± 151	654 ± 155	843 ± 147	593 ± 124	701 ± 131
TA102, -S9	(rev/cig)	−26 ± 69	20 ± 70	111 ± 73	−6 ± 99	−26 ± 69	47 ± 129	27 ± 100	169 ± 102	−26 ± 69	−56 ± 88	33 ± 101	139 ± 130
TA1535, -S9	(rev/cig)	31 ± 10	8 ± 11	6 ± 9	22 ± 14	31 ± 10	12 ± 11	20 ± 13	0 ± 11	31 ± 10	6 ± 13	−8 ± 10	−1 ± 13
TA1537, -S9	(rev/cig)	103 ± 17	104 ± 16	102 ± 18	108 ± 12	103 ± 17	111 ± 18	72 ± 21	104 ± 15	103 ± 17	92 ± 16	84 ± 15	117 ± 16

Table F: Cyototoxicity and mutagenicity, 10 mg ISO 'tar' yield cigarette, ISO smoking. Asterics mark statistical significant differences relative to respective control ($p < 0.05$).

Ingredient type	Unit of measure	Cocoa				Glycerol				Saccharose			
Ingredient level		Control	0.4%	1.1%	2.2%	Control	1.5%	3.0%	5.5%	Control	1.6%	2.1%	4.8%
Neutral Red Uptake Assay													
PP , 1/EC50	(mL/cig)	147 ± 1	140 ± 5.7	140 ± 6	146 ± 5	145 ± 6	133 ± 3	124 ± 1*	125 ± 6*	1345 ± 3	133 ± 5	140 ± 9	137 ± 1
GVP, 1/EC50	(mL/cig)	73.6 ± 2.0	60.7 ± 1.5*	60.0 ± 1.0*	62.7 ± 1.1*	69.5 ± 4	70.1 ± 5	68.8 ± 4	74.4 ± 4.3	67.3 ± 1.1	67.4 ± 1.5	67.7 ± 1.3	70.3 ± 1.8
Ames Assay, PP													
TA98, +S9	(rev/cig)	29,581 ± 1,316	25,919 ± 1,362	30,787 ± 1,645	31,968 ± 1,988	29,581 ± 1,316	26,075 ± 948	33,422 ± 1,555	25,519 ± 1,185	29,581 ± 1,316	25,038 ± 1,113	27,399 ± 1,314	25,520 ± 824
TA100, +S9	(rev/cig)	13,817 ± 903	13,521 ± 770	13,410 ± 1,113	15,026 ± 1,158	13,817 ± 903	14,244 ± 995	14,032 ± 1,395	14,607 ± 1,170	13,817 ± 903	15,322 ± 1078	16,505 ± 1,378	15,121 ± 1,041
TA102, +S9	(rev/cig)	−452 ± 178	−262 ± 116	−460 ± 128	−296 ± 108	−452 ± 178	−148 ± 258	−320 ± 124	101 ± 388	−452 ± 178	−178 ± 242	−62 ± 239	−178 ± 264
TA1535, +S9	(rev/cig)	19 ± 13	9 ± 19	25 ± 22	20 ± 20	19 ± 13	−2 ± 15	46 ± 21	14 ± 21	19 ± 13	14 ± 18	21 ± 15	−16 ± 18
TA1537, +S9	(rev/cig)	4,794 ± 422	5,057 ± 504	4,936 ± 313	5,393 ± 391	4,794 ± 422	3,886 ± 339	5,177 ± 450	4,234 ± 417	4,794 ± 422	4,207 ± 216	5,450 ± 478	4,717 ± 356
TA98, -S9	(rev/cig)	−3 ± 31	39 ± 24	115 ± 30*	70 ± 32	−3 ± 31	97 ± 38*	14 ± 30	54 ± 40	−3 ± 31	133 ± 32*	42 ± 29	111 ± 37*
TA100, -S9	(rev/cig)	1,229 ± 185	1,275 ± 145	1,702 ± 225	880 ± 198	1,229 ± 185	1,309 ± 242	1,342 ± 416	1,095 ± 275	1,229 ± 185	1,374 ± 224	1,442 ± 207	1,581 ± 243
TA102, -S9	(rev/cig)	59 ± 159	64 ± 128	185 ± 108	−112 ± 162	59 ± 159	257 ± 155	−148 ± 196	−8 ± 184	59 ± 159	243 ± 162	99 ± 140	50 ± 175
TA1535, -S9	(rev/cig)	−13 ± 17	20 ± 20	30 ± 22	10 ± 18	−13 ± 17	−12 ± 18	44 ± 29	−16 ± 13	−13 ± 17	−29 ± 13	20 ± 27	−3 ± 21
TA1537, -S9	(rev/cig)	122 ± 40	191 ± 27	116 ± 25	125 ± 21	122 ± 40	189v23	113 ± 29	170 ± 34	122 ± 40	214 ± 28	184 ± 28	205 ± 26