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Fractionation, Chemical and Toxicological Characterization of Tobacco Smoke Components

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Résumé

La fumée du tabac est un aérosol extrêmement complexe constitué de milliers de composés répartis entre la phase particulaire et la phase vapeur. Il a été démontré que les effets toxicologiques de cette fumée sont associés aux composés appartenant aux deux phases. Plusieurs composés biologiquement actifs ont été identifiés dans la fumée du tabac; cependant, il n'y a pas d'études démontrant la relation entre les réponses biologiques obtenues via les tests in vitro ou in vivo et les composés présents dans la fumée entière du tabac. Le but de la présente recherche est de développer des méthodes fiables et robustes de fractionnement de la fumée à l'aide de techniques de séparation analytique et de techniques de détection combinés à des essais *in vitro* toxicologiques.

Une étude antérieure réalisée par nos collaborateurs a démontré que, suite à l'étude des produits de combustion de douze principaux composés du tabac, l'acide chlorogénique s'est avéré être le composé le plus cytotoxique selon les test *in vitro* du micronoyau. Ainsi, dans cette étude, une méthode par chromatographie préparative en phase liquide a été développée dans le but de fractionner les produits de combustion de l'acide chlorogénique. Les fractions des produits de combustion de l'acide chlorogénique ont ensuite été testées et les composés responsables de la toxicité de l'acide chlorogénique ont été identifiés. Le composé de la sous-fraction responsable en majeure partie de la cytoxicité a été identifié comme étant le catéchol, lequel fut confirmé par chromatographie en phase liquide/ spectrométrie de masse à temps de vol.

Des études récentes ont démontré les effets toxicologiques de la fumée entière du tabac et l'implication spécifique de la phase vapeur. C'est pourquoi notre travail a ensuite été focalisé principalement à l'analyse de la fumée entière. La machine à fumer Borgwaldt RM20S® utilisée avec les chambres d'exposition cellulaire de *British American Tobacco* permettent l'étude *in vitro* de l'exposition de cellules à différentes concentrations de fumée entière du tabac. Les essais biologiques *in vitro* ont un degré élevé de variabilité, ainsi, il faut prendre en compte toutes les autres sources de variabilité pour évaluer avec précision la finalité toxicologique de ces essais; toutefois, la fiabilité de la génération de la fumée de la machine n'a jamais été évaluée jusqu'à maintenant. Nous avons donc déterminé la fiabilité de la génération et de la dilution (RSD entre 0,7 et 12 %) de la fumée en quantifiant la présence de deux gaz de référence (le CH_4 par détection à ionisation de flamme et le CO par absorption infrarouge) et d'un composé de la phase particulaire, le solanesol (par chromatographie en phase liquide à haute performance).

Ensuite, la relation entre la dose et la dilution des composés de la phase vapeur retrouvée dans la chambre d'exposition cellulaire a été caractérisée en utilisant une nouvelle technique d'extraction dite par HSSE (Headspace Stir Bar Sorptive Extraction) couplée à la chromatographie en phase liquide/ spectrométrie de masse. La répétabilité de la méthode a donné une valeur de RSD se situant entre 10 et 13 % pour cinq des composés de référence identifiés dans la phase vapeur de la fumée de cigarette. La réponse offrant la surface maximale d'aire sous la courbe a été obtenue en utilisant les conditions expérimentales suivantes : intervalle de temps d'exposition/ désorption de 10 ±0.5 min, température de désorption de 200°C pour 2 min et température de concentration

cryogénique (cryofocussing) de -75°C. La précision de la dilution de la fumée est linéaire et est fonction de l'abondance des analytes ainsi que de la concentration (RSD de 6,2 à 17,2 %) avec des quantités de 6 à 450 ng pour les composés de référence. Ces résultats démontrent que la machine à fumer Borgwaldt RM20S® est un outil fiable pour générer et acheminer de façon répétitive et linéaire la fumée de cigarette aux cultures cellulaires *in vitro*.

Notre approche consiste en l'élaboration d'une méthodologie permettant de travailler avec un composé unique du tabac, pouvant être appliqué à des échantillons plus complexes par la suite ; ex : la phase vapeur de la fumée de cigarette. La méthodologie ainsi développée peut potentiellement servir de méthode de standardisation pour l'évaluation d'instruments ou de l'identification de produits dans l'industrie de tabac.

Mots-clés: Machine à fumer Borgwaldt RM20S®, acide chlorogénique, chambre d'exposition cellulaire, système d'exposition, fractionnement, chromatographie en phase gazeuse, HSSE, test in vitro du micronoyau, chromatographie en phase liquide, spectrométrie de masse, désorption thermale, phase vapeur, fumée entière de cigarette.

Abstract

Tobacco smoke is an extremely complex aerosol composed of thousands of constituents distributed amongst the particulate and vapor phases. Toxicological effects have been linked to compounds present in both of these phases. Many biologically active compounds have been identified within tobacco smoke; however, there is a lack of studies correlating specific *in vitro* or *in vivo* biological responses to components within whole tobacco smoke. The goal of this research was to develop reliable and robust smoke fractionation methods using analytical separation and detection techniques in combination with *in vitro* toxicological assays.

In a previous study by our collaborators, toxicological assessment of the particulate phase combustion products of twelve individual tobacco components revealed that the combustion products of chlorogenic acid were the most cytotoxic using the *in vitro* micronucleus test. Therefore, a preparative liquid chromatography method was developed in this work to fractionate the combustion products of chlorogenic acid to assess the bioactivity of these fractions and to identify the compounds responsible for the toxicity observed. The sub-fraction responsible for the most cytotoxic response comprised catechol, which was identified by liquid chromatography/time-of-flight mass spectrometry.

Emerging studies have highlighted the toxicological significance of whole tobacco smoke and specifically the vapor phase, which shifted our focus to whole smoke analyses. The Borgwaldt RM20S® smoking machine in combination with British American

Tobacco's *in vitro* cell exposure chamber allow for the generation of fresh cigarette smoke in various doses and delivery to cell cultures. *In vitro* biological assays have a high degree of variability, thus, all other sources of variability must be accounted for to accurately assess toxicological endpoints; however, the reliability of dose delivery of the instrument had not been assessed until now. We have determined the reliability (RSD from 0.7-12%) of smoke generation and dilution by quantifying two reference standard gases (CH_4 by flame ionization detection and CO by infrared absorption) and the tobacco particulate phase marker, solanesol (by high performance liquid chromatography-ultraviolet absorption detection).

The relationship between dose and diluted vapor phase components found within the exposure chamber was then characterized by developing a headspace stir-bar sorptive extraction-gas chromatography/mass spectrometry method. The method repeatability gave an RSD from 10-13% for five reference compounds identified in the vapor phase of cigarette smoke. The maximal peak area response was obtained using the following experimental conditions: exposure-to-desorption time interval of 10 ± 0.5 min, desorption temperature of 200 °C for 2 min, and a cryofocussing temperature of -75 °C. The dilution precision was found to yield a linear response of analyte abundance and was observed to be a function of concentration (RSD from 6.2-17.2 %) with quantities of 6-450 ng for the reference compounds. The findings obtained suggest the Borgwaldt RM20S® is a reliable tool to generate and deliver repeatable and linear doses of cigarette smoke to *in vitro* cell cultures.

Our approach began with designing the methodology to work with an individual tobacco component, which could then be applied to a more complex sample, *e.g.*, the vapor phase of cigarette smoke. The methodology developed can potentially serve as standardized methods for the assessment of instrumentation or screening of products for the Tobacco Industry.

Keywords : Borgwaldt RM-20S® smoking machine, chlorogenic acid, exposure chamber, exposure system, fractionation, gas chromatography, headspace stir-bar sorptive extraction, *in vitro* micronucleus test, liquid chromatography, mass spectrometry, thermal desorption, vapor phase, whole cigarette smoke.

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Figure 8-1: Experimental flow chart of the chlorogenic acid combustion product study. [1]Corresponds to LC/UV reversed-phase experimental conditions described in section 3.3.9 [2]Corresponds to LC/UV reversed-phase experimental conditions described in section 3.3.11 i

Figure 9-1: Representation of the nine types of mobile phase gradient elutions used. The solvent consisted of MeOH in 0.1% aqueous formic acid (pH 3-4), with concentrations of MeOH of 10-50 to 75% increasing over 24 min. ii

Figure 9-2: Total Ion Chromatograms (m/z 50-1000) of the comparison of the nine types of mobile phase gradient elutions used MeOH of 10-50 to 75% in 0.1 % aqueous formic acid, increasing over 24 min. iii

Figure 9-3: Total Ion Chromatograms (m/z 50-1000) representing the separations of the standard mixture using a gradient of 20-75% MeOH in 1% aqueous formic acid. Mixture contained: 2,5-dihydroxybenzoic Acid (1), *p*-hydroxybenzoic acid (2), caffeic acid (3), *p*-coumaric acid (4), 4-hydroxy-3-methoxycinnamic acid (5), benzoic acid (6) and trans-cinnamic acid (7).. iv

List of Abbreviations, Initials and Acronyms

1R4F	Kentucky reference cigarette blend with low nicotine, batch from 1983
2R4F	Kentucky reference cigarette blend with low nicotine, batch from 2002
3R4F	Kentucky reference cigarette blend with low nicotine, batch from 2006
ACN	Acetonitrile
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
BAT	British American Tobacco
BN	Binucleated
CBPI	Cytokinesis-block proliferation index
CE	Capillary electrophoresis
CEL	Cellulose
CFP	Cambridge filter pad
CGA	Chlorogenic acid
CHO	Chinese Hamster Ovary Cells
CIS	Cooled injection system
CK36	Canadian reference cigarette blend
COPD	Chronic obstructive pulmonary disease
CORESTA	Cooperation Centre for Scientific Research Relative to Tobacco
CSC	Cigarette Smoke Condensate
DCM	Dichloromethane
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EA	Ethyl acetate
EI	Electron ionization
ESI	Electrospray ionization
FD	Fluorescence detection
FID	Flame ionization detection

FTC	US Federal Trade Commission
GC	Gas chromatography
GLU	Glucose
GLY	Glycine
HPLC	High performance liquid chromatography
HSSE	Headspace stir-bar sorptive extraction
IR	Infrared
IS	Internal standard
ISO	International Standard Organization
IVMNT	*In vitro* micronucleus test
JPTP	John Payne Tar Predictor
LIG	Lignin
LC	Liquid chromatography
MAL	Malic acid
MeOH	Methanol
MS	Mass Spectrometry
MSD	Mass Selective Detector
NAB	N-nitrosoanabasine
NAT	N-nitrosoanatabine
NSERC	Natural Sciences and Engineering Council of Canada
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.
NNN	N-Nitrosonornicotine
NRU	Neutral red uptake
PDMS	Polydimethylsiloxane
PEC	Pectin
PRO	Proline
RSD	Relative standard deviation
RUB	RUBISCO
SBSE	Stir-bar sorptive extraction

SPME	Solid phase micro-extraction
STA	Starch
TCD	Thermal conductivity detection
TD	Thermal desorption
TDS	Thermal desorption system
TIC	Total ion chromatogram
TIOJ	Tobacco Institute of Japan
TOF	Time-of-flight
TPM	Total particulate matter
TRY	Tryptophan
TSNA	Tobacco-specific nitrosamines
TYR	Tyrosine
UV	Ultraviolet
VIS	Visible
XIC	Extracted ion chromatogram

To my family & friends,
especially Sandra, Lisa and Martine
and to my husband, Ranjit

Life is a laboratory. Experiment.
- Pawliszyn

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1 General Introduction and Research Objectives

1.1 Overview

Tobacco smoke is a complex mixture composed of thousands of components, distributed between the particulate and vapor phases. Extensive work has been carried out on the identification of compounds present in tobacco and its smoke due to its biological activity. Many biologically active compounds have been identified within tobacco smoke; however, there is a lack of studies correlating specific *in vitro* or *in vivo* biological responses to components within whole tobacco smoke due to its complex nature *i.e.,* the number and diverse range of compounds simultaneously distributed in the two phases. The general hypothesis that guided this work was as follows: the development of reliable and robust smoke fractionation methods makes it possible to assign toxicological effects to specific cigarette smoke components by using sophisticated analytical separation and detection techniques in combination with reliable toxicology assays. The novelty in this approach lies in the union of chemical characterization and toxicological studies in parallel. This thesis describes the development of methods for chemical characterization of tobacco smoke components, specifically the combustion products of chlorogenic acid, which is the most abundant tobacco leaf polyphenol (Chapter 3), and the vapor phase of cigarette smoke (Chapters 4 and 5). To prepare those readers who may be unfamiliar with this field of study, an introduction of some aspects to tobacco science is made in Chapter 1, which is concluded with the objectives of the research. The specific techniques used during fractionation and chemical analysis are described in detail in Chapter 2. *In vitro* toxicological assays have a high degree of variability, thus, all other sources of variability must be reduced or accounted to accurately assess toxicological endpoints. The

methodology developed (Chapters 4-5) can potentially serve as standardized methods for the assessment of instrumentation (Chapter 4) or screening of tobacco products (Chapter 3, 5) for the Tobacco Industry.

1.2 Tobacco

Tobacco used in cigarettes is a product processed from the leaves of the *Nicotiana* genus, namely *Nicotiana tabacum L.* (typically used in North America) and *Nicotiana rustica* subgenera plant species (Tso, 1999). Similar to other types of plants, the chemical constituents (*i.e.* sugars, alkaloids, N_2 and cellulose) vary based on leaf positioning and environmental factors, such as soil type and nutrient levels (Tso, 1999). Many plants in the *Solanaceae* family contain nicotine (Fig. 1-1), a powerful neurotoxin; however, *Nicotiana tabacum L.* contains higher levels of nicotine than most plants. Tobacco plants are grown and cultivated in a similar procedure to other agricultural products. In addition to seeding and cultivation, curing, aging, fermentation, blending and manufacturing processes take place prior to cigarette production.

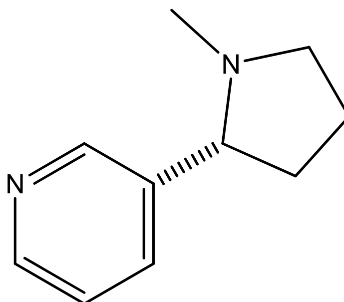

Figure 1-1: Structure of nicotine, a naturally occurring tobacco alkaloid.

1.2.1 Curing Process

Following cultivation, the leaves undergo a "curing" process (air, flue, sun or fire) that involves biochemical and chemical changes. Curing involves three stages which results in hydrolysis of starch and other components. The first is a biochemical process applied to tobacco leaf by controlling temperature and relative humidity, referred to as "yellowing". The second is a "fixing color" stage, which is followed by the third "drying process" involving dehydration to preserve leaves. Two of the most common types of curing processes are air- and flue-curing. Air-cured tobacco is hung in ambient, well ventilated areas and allowed to dry for a period of four to eight weeks (Palmer *et al.*, 1999). Air-cured tobacco is lower in sugar and higher in nicotine compared to using other types of curing and is typically used for Burley, Maryland and cigar tobaccos (Table 1-1). Flue-curing is a heat-driven process involving the slow ramping of temperature for a period of one week and is used for Virginia tobacco (Peedin, 1999). Flue-cured tobacco is higher in sugar and contains moderate levels of nicotine (Table 1-1).

Table 1-1: Typical composition of cigarette tobaccos: representative analyses of cigarette tobaccos (leaf web after aging, moisture-free basis) (Leffingwell, 1999).[1]

Component (%)[1]	Flue-cured type 13	Burley type 31	Maryland type 32	Oriental[2]
Total volatile bases as ammonia	0.282	0.621	0.366	0.289
Nicotine	1.93	2.91	1.27	1.05
Ammonia	0.019	0.159	0.13	0.105
Glutamine as ammonia	0.033	0.035	0.041	0.02
Asparagine as ammonia	0.025	0.111	0.016	0.058
α-Amino nitrogen as ammonia	0.065	0.203	0.075	0.118
Protein nitrogen as ammonia	0.91	1.77	1.61	1.19
Nitrate nitrogen as NO_3	trace	1.7	0.087	trace
Total nitrogen as ammonia	1.97	3.96	2.8	2.65
pH	5.45	5.8	6.6	4.9
Total volatile acids as acetic acid	0.153	0.103	0.09	0.194
Formic acid	0.059	0.027	0.022	0.079
Malic acid	2.83	6.75	2.43	3.87
Citric acid	0.78	8.22	2.98	1.03
Oxalic acid	0.81	3.04	2.79	3.16
Volatile oils	0.148	0.141	0.14	0.248
Alcohol-soluble resins	9.08	9.27	8.94	11.28
Reducing sugars as dextrose	22.09	0.21	0.21	12.39
Pectin as calcium pectate	6.91	9.91	12.41	6.77
Crude fiber	7.88	9.29	21.79	6.63
Ash	10.81	24.53	21.98	14.78
Calcium as CaO	2.22	8.01	4.79	4.22
Potassium as K_2O	2.47	5.22	4.4	2.33
Magnesium as MgO	0.36	1.29	1.03	0.69
Chlorine as Cl	0.84	0.71	0.26	0.69
Phosphorus as P_2O_5	0.51	0.57	0.53	0.47
Sulfur as SO_4	1.23	1.98	3.34	1.4
Alkalinity of water-soluble ash[3]	15.9	36.2	36.9	22.5

[1]In % except for pH and alkalinity.
[2]Blend of Macedonia, Smyrna and Samsun types.
[3]Milliliters of 1 N acid per 100 g tobacco.

[1] Quantification of an amino acid is commonly carried out by measuring the ammonia concentration of the α-amino group of that particular amino acid.

1.2.2 Aging Process

Freshly cured tobacco is not immediately processed since its smoke is pungent and irritating (Leffingwell, 1999). During aging, leaf tobacco is stored for days or even months on a farm to improve the aroma and texture of the leaves. In addition, leaves are separated based on physical imperfections, color, thickness, length, stalk position etc. Depending on the specific recipe, the tobacco is blended and cigarettes or other products can be manufactured.

1.2.3 Types of Tobacco

The most common types of tobacco are Burley, Virginia and Oriental. These main types are divided into grades based on where the tobacco is grown, which part of the plant it is taken from and other plant characteristics. Virginia, normally a flue-cured tobacco, is a brighter tobacco because it turns to a yellow/orange color during the curing process. This process causes degradation of chlorophyll and most carbohydrates are converted into simple sugars (Leffingwell, 1999). Virginia tobacco contains between 1-3.5 % nicotine and 5-25 % sugars (Leffingwell, 1999). In Canada, Virginia blends do not contain flavors or additives. Burley tobaccos typically undergo an air-curing process under ambient conditions in which the leaves turn brown with very low sugar levels. This type of tobacco is treated with sugars to replace those lost during the curing process and is combined with other types of tobacco or flavors prior to consumption. Oriental tobacco, also known as Turkish tobacco, is sun-dried, and contains small leaved plants, because it is grown in soil with limited supplies of nitrogen and water.

1.2.4 Consumption

Tobacco is generally consumed in smokable and smokeless forms, such as cigarettes, cigars, chewing tobacco, pipe smoking, snuff (dry smokeless form of tobacco, snorted through the nose) and snus (smokeless form of tobacco consumed by placing sachet under the upper lip). In this study, only smokable forms were analyzed.

1.3 Composition of Tobacco and Tobacco Smoke

Leaf tobacco consists of over 2000 components and upon partial combustion generates a complex aerosol containing more than 5000 components distributed between particulate and vapor phases (Rodgman *et al.*, 2009a, Rodgman *et al.*, 2009b).

1.3.1 Leaf Chemistry

The physical and chemical properties of leaf tobacco vary with genetics, agricultural practices, soil type and nutrients, weather conditions, plant disease, stalk positioning, harvesting and curing procedures (Leffingwell, 1999). The tobacco leaf is comprised of carbohydrates (starch, sugars, sugar esters, glucosides, cellulose, hemicelluloses and pectin), nitrogen containing compounds (proteins, amino acids, ammonia, nitrates and alkaloids including nicotine, cotinine, nornicotine, myosmine, nicotyrine, anabasine and anatabine), plastid pigments (chlorophylls and carotenoid pigments such as β-carotene, violaxanthin, lutein and neoxanthin), isoprenoids (degraded caratenoid products, acyclic isoprenoids and N-demethylated-derivatives such as solanesol and neophytadiene, carbocyclic diterpenoids, cembranoids, labdanoids and their degration

products), phenolics (polyphenols such as chlorogenic acid, rutin, scopoletin and scopolin, lignin and others), sterols (sterols, steryl esters and esterified steryl glucosides) and inorganics (Baker *et al.*, 2003, Leffingwell, 1999). The Krebs cycle is responsible for the metabolic carbon-nitrogen balance in plants, of which, carbon dioxide is provided from the photosynthesis process and inorganic nitrogen is absorbed from the soil. Therefore, where nitrogen supply is abundant, protein, amino acid and nicotine are produced in abundance (*i.e.* cigar and burley tobaccos). Conversely, where nitrogen supplies are limited (*i.e.* Oriental Tobacco), there is an accumulation of acetate in the Krebs cycle which results in the formation of terpenoids, carbohydrates, aromatic acids and resins. An intermediate nitrogen level is required for a flue-cured type tobacco (Table 1-1) (Leffingwell, 1999). Many types of compounds are common to leaf tobacco and tobacco smoke (Table 1-2).

Table 1-2: Number of compounds identified in tobacco and in smoke (Weeks, 1999) .

Classes of Chemicals	in Tobacco (#)	in Smoke (#)	common to both (#)
Carboxylic acids	450	69	140
Amino acids[1]	95	18	16
Lactones	129	135	39
Esters	529	456	314
Amides and imides	205	227	32
Anhydrides	10	10	4
Aldehydes	111	106	48
Carbohydrates	138	30	12
Nitriles	4	101	4
Ketones	348	461	122
Alcohols	334	157	69
Phenols	58	188	40
Amines	65	150	37
N-Heterocycles:			
Pyridines	63	324	46
Pyrroles and indoles	9	88	3
Pyrazines	21	55	18
Nonaromatic	13	43	7
Ethers	53	88	15
Hydrocarbons	184	429	114
Inorganics and metals	105	111	69

[1] Refers to natural and non-standard amino acids. The latter are constituents of proteins and biologically active peptides.

1.3.2 Distillation, Pyrolysis and Combustion

During a puff, air is drawn into a cigarette through the burning zone and mainstream smoke (produced during the puff) is formed (Fig. 1-2). In addition, sidestream smoke is formed (during smolder) by a natural convection flow of air at the burning zone. Many chemical and physical processes occur while a cigarette is burning since tobacco is in

the presence of varying amounts of oxygen and temperatures (ambient up to 950 °C) (Baker *et al.*, 2003). The burning process can be broken down into heat producing combustion and endothermic pyrolysis/distillation, yielding organic smoke products. Combustion is defined as the sequence of chemical reactions between a fuel and an oxidant and involves the production of heat and conversion of various compounds, whereas, pyrolysis is the chemical decomposition of organic materials by heat in the absence of oxygen. Distillation is the separation of components in mixtures based on differences in their volatilities in a boiling mixture. As a result of the burning process, other processes that may occur are: pyrosynthesis, sublimation and condensation. Pyrosynthesis occurs at high temperatures and is the fusion of simple compounds into complex ones. Sublimation is the phase transition from solid to gas, without becoming a liquid and condensation is the transition from gas to liquid or solid.

Temperature and heating rate of tobacco are extremely important during smoke generation. When air is drawn into the cigarette, oxygen is consumed by combustion with carbon-rich tobacco (at temperatures between 700-950°C), releasing carbon monoxide, carbon dioxide, water and heat. Downstream, pyrolysis occurs at between 200-600 °C with low oxygen levels. As the aerosol is drawn out of the pyrolysis zone, it rapidly cools as the diluted air enters (Baker *et al.*, 2003).

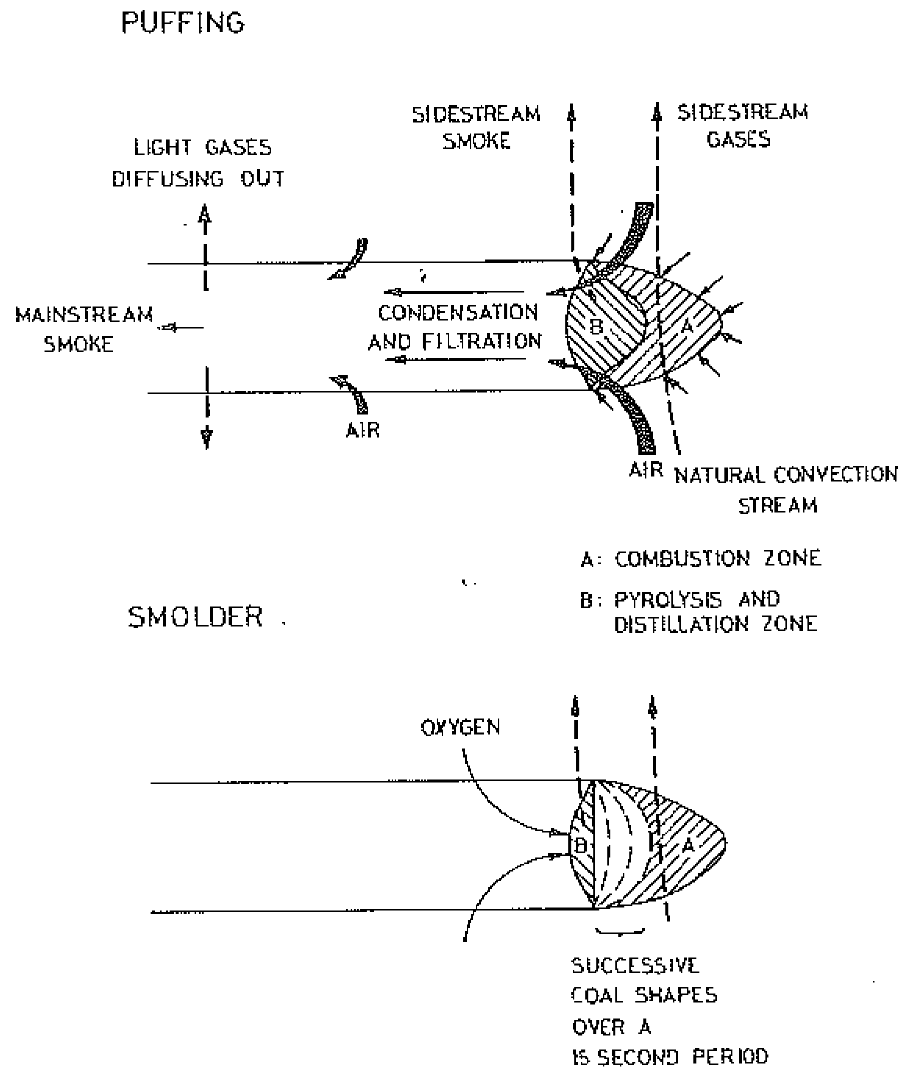

Figure 1-2: The burning cigarette and processes occurring during puffing (top) and during smoldering (bottom) (Baker, 1999).

1.3.3 Cigarette Smoke Chemistry

Fresh cigarette smoke is extremely complex, dynamic and reactive because of its physical properties and chemical composition. It's complexity has been compared to other widely studied mixtures, such as air (pollution) and diesel engine emissions (Borgerding *et*

al., 2005). As an aerosol, tobacco smoke components are distributed between the particulate and vapor phases. The chemical composition of whole mainstream smoke from an American blended cigarette (specific blend details not available, similar in composition to Ky3R4F, which is specified in section 1.4.2), smoked under standard conditions (35 mL puff for a 2 second duration, once every minute) is estimated in Table 1-3 (Dube *et al.*, 1982). Additionally, the yields of various compounds found in mainstream cigarette smoke are listed in Table 1-4.

Table 1-3: Approximate chemical composition of whole mainstream smoke (Baker, 1999).

Constituent	% by Weight	
	individual	by phase
Air		
N_2	62	75.9
O_2	13	
Ar	0.9	
Vapor Phase		
Water	1.3	19.6
CO_2	12.5	
CO	4	
H_2	0.1	
CH_4	0.3	
Hydrocarbons	0.6	
Aldehydes	0.3	
Ketones	0.2	
Nitriles	0.1	
Heterocyclics	0.03	
Methanol	0.03	
Organic acids	0.02	
Esters	0.01	
Other compounds	0.1	
Particulate Phase		
Water	0.8	4.5
Alkanes	0.2	
Terpenoids	0.2	
Phenols	0.2	
Esters	0.2	
Nicotine	0.3	
Other alkaloids	0.1	
Alcohols	0.3	
Carbonyls	0.5	
Organic acids	0.6	
Leaf pigments	0.2	
Other compounds	0.9	

Table 1-4: Some typical mainstream yield ratios for plain, unfiltered cigarettes of various types, smoked under standard smoking machine conditions (Baker, 1999).

Substance	Yield[1]	Substance	Yield[1]	Substance	Yield[1]
Small molecules		*Phenols*		*Aza-arenes*	
Carbonyl sulphide	18-42 µg	Phenol	60-140 µg	Quinoline	0.5-2.0 µg
HCN	160-500 µg	Cresols (*o*-, *m*-, *p*-)	11-37 µg	Isoquinoline	1.6-2.0 µg
CO	10-23 mg	Catechol	100-360 µg	Benzo[h]quinoline	10 ng
Hydrazine	20-40 µg	Hydroquinone	110-300 µg	Indole	16-38 µg
Methane	600-1000 µg				
Acetylene	20-40 µg	*Acids*		*Hydrocarbons*	
Nitrogen oxides	100-600 µg	Formic acid	210-490 µg	Isoprene	330-1100 µg
CO2	20-50 mg	Acetic acid	270-810 µg	Benzene	36-68 µg
H2O (gas phase)	3-14 mg	3-Methylvaleric acid	20-60 µg	Toluene	100-200 µg
NH3	50-130 µg	Lactic acid	60-170 µg	Limonene	15-50 µg
N2 (generated)	<10 µg	Benzoic acid	14-28 µg	Neophytadiene	66-230 µg
		Phenylacetic acid	11-38 µg		
Neutral heteroatom organics		Succinic acid	70-140 µg	*Polynuclear aromatic hydrocarbons*	
Acetonitrile	160-210 µg	Glycolic acid	40-130 µg	Naphthalene	2.6 µg
Benzonitrile	5-6 µg			Pyrene	45-140 ng
Acetamide	70-100 µg	*Amines, pyridines, alkaloids*		Benzo[a]pyrene	9-40 ng
Methyl chloride	150-600 µg	Methylamine	12-29 µg	Anthracene	24 ng
		n-Propylamine	1.6-3.4 µg	Phenanthrene	77 ng
Aldehydes, ketones and alcohols		n-Butylamine	0.5-1.5 µg	Fluoranthene	60-150 ng
Acetaldehyde	0.5-1.2 mg	Aniline	360 ng		
Propionaldehyde	175-250 µg	Pyridine	16-46 µg	*Nitrosamines*†	
Acetone	100-250 µg	3-Ethenylpyridine	11-30 µg	N-nitrosodimethylamine	10-40 ng
Acrolein	60-100 µg	Methylpyrazine	2-5 µg	N-nitrosodiethylamine	nd-25 ng
2-Butanone	~ 30 µg	Pyrrole	16-23 µg	N-nitrosopyrrolidine	6-30 ng
2-Furaldehyde	15-43 µg	Nicotine	0.8-2.3 mg	N-nitrosodiethanolamine	0-70 ng
Furfuryl alcohol	18-65 µg	Myosmine	13-33 µg	N'-nitrosonornicotine	0.2-3 µg
Cyclotene*	3-5 µg	Nicotyrine	4-40 µg	NNK‡	0.1-1 µg
Pyranone**	13-150 µg	Anatabine	2-20 µg	N'-nitrosoanatabine	0.3-5 µg
		2,3-'Bipyridyl	16-22 µg		
Phytosterols				*Inorganic constituents*	
β-Sitosterol	59 µg			Cadmium	100 ng
Campesterol	43 µg			Nickel	20-80 ng
Cholesterol	22 µg			Zinc	60 ng

*2-Hydroxy-3-methyl-2-cyclopentanone.
** 5,6-Dihydro-3,5-dihydroxy-2-methyl-4*H*-pryan-4-one.
† Data in the literature on levels of volatiles and nitrosamines may be in error, due to artifact formation during the extraction procedure.
‡ 4-(methylnitrosamino)-1-(30pyridyl)-1-butanone. Nd = not detected
[1] Yield refers to µg/cig mainstream smoke

There is no definitive way to separate and differentiate the particulate and vapor phases. Some components partition between the two phases, depending on time, temperature and smoke dilution (Baker, 1999). Based on the latter, it is generally accepted that the portion of smoke that passes through a Cambridge filter pad (CFP) at room temperature is defined as the vapor phase (volatile and semi-volatile compounds). The portion that is retained by the CFP is referred to as the particulate phase (semi-volatile and non-volatile compounds) or "total particulate matter" (TPM). Another common term used is "tar", which is defined as the weight of TPM minus the weight of nicotine and water. The CFP (glass fiber, 44 mm in diameter) retains cigarette smoke particles larger than 0.1 μm in diameter with an efficiency of approximately 99.9 % (Adam *et al.*, 2006, Baker, 1999). This efficiency is based on the nature and quantity of the sample, flow through the filter, temperature and moisture level (Baker, 1999).

1.4 Standard Reference Cigarette Samples

For research purposes, international standard reference cigarettes are manufactured by the Kentucky Tobacco Research & Development Center. The first blend was manufactured in 1974 and is referred to as Ky1R1. Blends vary based on composition of tobacco used, *i.e.*, percentage of Virginia flue-cured, Burley, Maryland, Oriental etc. Other blends include: Ky1R3F (blend used by the National Cancer Institute produced in 1974), Ky1R4F (low nicotine blend produced in 1983), Ky2R4F (similar to Ky1R4F blend but produced in 2002) and Ky1R5F (ultra-low nicotine blend produced in 1989) have been produced. Currently, the batch of cigarettes that is available is referred to as Ky3R4F

(similar to Ky1R4F blend but produced in 2006) and is a blend of Virginia flue-cured (35.41 %), Burley (21.62 %), Oriental (12.07 %), Maryland (1.35 %), Reconstituted (29.55 %), Glycerin (2.67 %) and an “Isosweet” sugar (6.41 %) (University of Kentucky, 2010a). The Ky3R4F was the only references cigarette used throughout this work.

1.5 Cigarette Smoke Sample Collection

1.5.1 Smoking Protocol (International Organization for Standardization)

The cigarette smoking process is highly variable between different smokers as well as between different smoking sessions for the same individual. Factors that influence the smoking behavior include human, social and environmental variables. The yield and composition of smoke compounds depends on the physical dimensions of the cigarette, puff volume (20-80 cm^3), the shape of the puff profile (*i.e.* the pressure/flow relationship), which dictates a puff duration (0.8-3 s), the number and frequency of puffs (typically 20-100 s) and residual butt length (19-28 mm) left at the end of smoking (Baker, 1999, Borgerding *et al.*, 2005). Paper type is an additional variable that may effect smoke composition.

A standard set of smoking procedures was implemented by government and industry and included employing a 35 cm^3 puff for a 2 second duration, every minute, while maintaining a butt length of 23 mm (International Organization for Standardization, 1999). These parameters were implemented to promote consistency in research (mainly

product comparisons) using automated smoking machines by the US Federal Trade Commission (FTC), International standard organization (ISO), Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) and Tobacco Institute of Japan (TIOJ). Due to the variation and higher intensity in smoking behavior between individual smokers, this smoking regime can not be used to predict consumer yields (Côté *et al.*, 2010).

1.5.2 Particulate Phase Collection

Extensive work has been carried out on phenols, terpenes and alkaloids among other particulate phase constituents, based on their biological/toxicological significance. As stated above, the most common technique for collecting the particulate phase of cigarette smoke is by using a CFP to trap total particulate matter (TPM) generated by a smoking machine. The CFP, a glass fiber filter, traps the particles ≥0.1 μm with >99 % efficiency (Baker, 1999). The particulate phase is subsequently extracted from the CFP using a solvent (Misra *et al.*, 2010). Other techniques that are used to trap the particulate phase include: electrostatic precipitation, jet impaction and the use of solid adsorbents (activated charcoal, silica gels, molecular sieves and tenax) (Baker, 1999, Dube *et al.*, 1982, Wynder *et al.*, 1967).

1.5.3 Vapor Phase Collection

Volatile organic compounds, hydrocarbons, carbon monoxide, methane, aldehydes and nitrosamines are among the compounds of biological interest, found in the vapor phase

of cigarette smoke. Collection of the vapor phase is quite challenging and has been achieved by employing sampling bag methods, cryogenic/cold traps, impingers, solvent traps, adsorbent traps, direct injection methods, and solid-phase microextraction (SPME) related techniques (Omori *et al.*, 1999, Ye, 2008, Dube *et al.*, 1982, Wynder *et al.*, 1967, Borgerding *et al.*, 2005). These techniques all have their own specific advantages and limitations. Gas bags allow for the exposure of whole smoke, without preconcentration and can average smoking from a number of cigarettes; however, smoke ageing may occur (which includes degradation of some compounds) and some compounds may deposit on the bag itself (Dong *et al.*, 2000, Omori *et al.*, 1999, Chen *et al.*, 2003). Impingers and traps under the use of cryogenic conditions may provide an alternative that is easy to use an internal standard and average smoking from a number of cigarettes; however, solvents are used and can cause interferences with some analytes and typically have a complex setting and time-consuming experimental work (Dong *et al.*, 2000, Darrall *et al.*, 1998, Byrd *et al.*, 1990). Direct injection methods provide un-aged smoke to be analyzed, where degradation of analytes are minimized; however, reproducibility and quantitation are challenging due to only sampling a fraction of a puff (Takanami *et al.*, 2003, Adam *et al.*, 2009). SPME and related techniques allow for solvent-free method, rapid pre-concentration and sorption in one step, homogeneous sampling and high reproducibility; however sensitivity can be low since these techniques are limited by the volume of the sorptive phase and quantitation may be challenging (Polzin *et al.*, 2007, Ye, 2008, Buszewski *et al.*, 2009).

1.6 Cigarette Smoke Sample Analysis

Tobacco smoke has been extensively characterized using many experimental approaches, including detailed chemical analyses of the smoke using chromatographic instrumentation coupled with a variety of detection techniques, analysis of smoke from precursor "doped" cigarettes, studying aerosols, investigating pyrolysis from fundamental perspectives, studies involving isotopically labeled compounds, fractionation studies, investigating mass transfer inside the cigarette and computer modeling of the formation processes and their interactions (Baker, 1999, Swain et al., 1969, Chen et al., 2003, Dong et al., 2000, Adam et al., 2009, Adam et al., 2006, Baggett et al., 1974, Wynder et al., 1967, Stedman, 1968, IARC, 1986, Borgerding et al., 2005, Polzin et al., 2007).

1.7 Toxicology of Tobacco Smoke

It is important to note that the "toxicology of tobacco smoke" is an extremely broad and complex subject and that the content of this chapter is limited to the literature provided.

1.7.1 Health Implications of Tobacco Use

The use of tobacco has been recorded across the world since the 10th century or earlier (Hoffmann *et al.*, 1998a). It was in the 1920's that adverse health effects began to be more prevalent and the first paper linking tobacco use and cancer was published in the late 1920s (Lickint, 1929). It was not until 1964 that the United States Surgeon General issued a report on Smoking and Health (US Surgeon General, 1964), suggesting a relationship between smoking and cancer, which was confirmed in the 1980s (US Surgeon General,

1982). At present, some of the known risks associated with tobacco use include: stroke, heart attack, chronic obstructive pulmonary disease (COPD), emphysema and various forms of lung, mouth and pancreatic cancers (Hoffmann *et al.*, 2001b, U.S. Department of Health and Human Services, 1989).

1.7.2 Toxicologically Relevant Constituents Present in Tobacco Smoke

During the smoking process, a complex mixture is inhaled into the respiratory tract and chemical, physical and physiological phenomena occur. There are a few important factors to consider when evaluating cigarette smoke toxicity. Some of these key factors include the complex nature of cigarette smoke, potential changes of the smoke material during collection and the analysis itself.

The toxicity of cigarette smoke is thought to be a function of the concentrations of individual toxicants present in smoke. A set of compounds referred to as the "Hoffmann analytes" were reported by Hoffmann in the 1990s (Hoffmann, 1993, Hoffmann *et al.*, 1998b, Hoffmann *et al.*, 1997). The Hoffmann analytes are summarized in Table 1-4 and the structures of some of these compounds are presented in Fig 1-3 and 1-4 (Hoffmann *et al.*, 2001b, Hoffmann *et al.*, 1997, Hoffmann, 1993, Hoffmann *et al.*, 2001a, Hoffmann *et al.*, 1990, Hoffmann *et al.*, 1998b, Rodgman *et al.*, 2003, Rodgman *et al.*, 2009b). A more recent list of 149 toxicants potentially found in cigarette smoke was published in 2003 (Rodgman *et al.*, 2003). Various tobacco smoke components have been established as tumor promoters, carcinogenic and/or co-carcinogenic (Borgerding *et al.*, 2005). Few toxicological studies have been carried out on specific Hoffmann analytes or other

biologically active smoke constituents that produce *in vitro* or *in vivo* biological responses from whole smoke (Borgerding *et al.*, 2005, Rodgman *et al.*, 2003). In fact, toxicological data is available for roughly 5% of the number of components present in tobacco smoke (Rodgman *et al.*, 2003, Rodgman *et al.*, 2009b).

Table 1-5: Summary of lists of toxicants by Hoffmann et *al*. from 1986 to 2001 (Hoffmann *et al.*, 2001b, Hoffmann *et al.*, 1997, Hoffmann, 1993, Hoffmann *et al.*, 2001a, Hoffmann *et al.*, 1990, Hoffmann *et al.*, 1998b, Rodgman *et al.*, 2003, Rodgman *et al.*, 2009b).

Group	Hoffmann Analyte	Group	Hoffmann Analyte	Group	Hoffmann Analyte
Polycyclic aromatic hydrocarbons	Benz[*a*]anthracene	N-Heterocyclic Amines	AαC	Phenols	Phenol
	Benzo[*b*]fluoranthene		MeAαC		*m*+*p*+*o*- Cresol
	Benzo[*j*]fluoranthene		Glu-P-1		Catechol
	Benzo[*k*]fluoranthene		Glu-P-2		Resorcinol
	Benzo[*a*]pyrene		PhIP		Hydroquinone
	Chrysene		IQ		Methyleugenol
	5-Methyl-chrysene		MeIQ		Caffeic acid
	Dibenz[*a,h*]anthracene		Trp-P-1	Chloroaromatic Compounds	DDT
	Dibenzo[*a,e*]pyrene		Trp-P-2		DDE
	Dibenzo[*a,h*]pyrene	Aldehydes and Ketones	Formaldehyde		Polychlorodibenzo-*p* -dioxins
	Dibenzo[*a,i*]pyrene		Acetaldehyde		Polychlorodibenzofurans
	Dibenzo[*a,l*]pyrene		Propionaledhyde	Inorganic Compounds	Hydrazine
	Indeno[1,2,3-*cd*]pyrene		Butyraldehyde		Hydrogen sulfide
Aza-arenes	Pyridine		Crotonaldehyde		Arsenic
	Quinoline		Acrolein		Beryllium
	Dibenz[*a,h*]acridine		Acetone		Cadmium
	Dibenz[*a,j*]acridine		2-Butanone		Chromium (VI)
	7*H*-Dibenzo[*c,g*]carbazole	Volatile Hydrocarbons	1,3-Butadiene		Cobalt
N-Nitrosamines	*N*-Nitrosodimethylamine		Isoprene		Nickel
	N-Nitrosoethylmethylamine		Benzene		Mercury
	N-Nitrosodiethylamine		Toluene		Lead
	N-Nitrosodi-*n* -propylamine		Styrene		Polonium-210
	N-Nitrosodi-*n* -butylamine	Miscellaneous Organic Compounds	Acetamide		Selenium
	N-Nitrosopyrrolidine		Acrylonitrile	Additional Compounds	Nicotine
	N-Nitrosopiperidine		Acrylamide		Carbon monoxide
	N-Nitrosodiethanolamine		1,1-Dimethyl-hydrazine		Ammonia
	N-Nitrososarcosine		Maleic hydrazide		Nitrogen oxides
	N′-Nitrosonornicotine		Methanol		Hydrogen cyanide
	4-(*N*-Methylnitrosamino)-1-(3-pyridyl)-1-butanone		Methyl isocyanate		
			Nitromethane		
	N'-Nitrosoanabasine		2-Nitropropane		
	N'-Nitrosoanatabine		Nitrobenzene		
	N-Nitrosomorpholine		Vinyl chloride		
Aromatic Amines	Aniline		Ethyl carbamate		
	2-Toluidine		Ethylene oxide		
	2,6-Dimethyl-aniline		Propylene oxide		
	1-Napthylamine		Di(2-ethylhexyl)phthalate		
	2-Napthylamine		Furan		
	3-Amino-biphenyl		Benzo[*b*]furan		
	4-Amino-biphenyl				

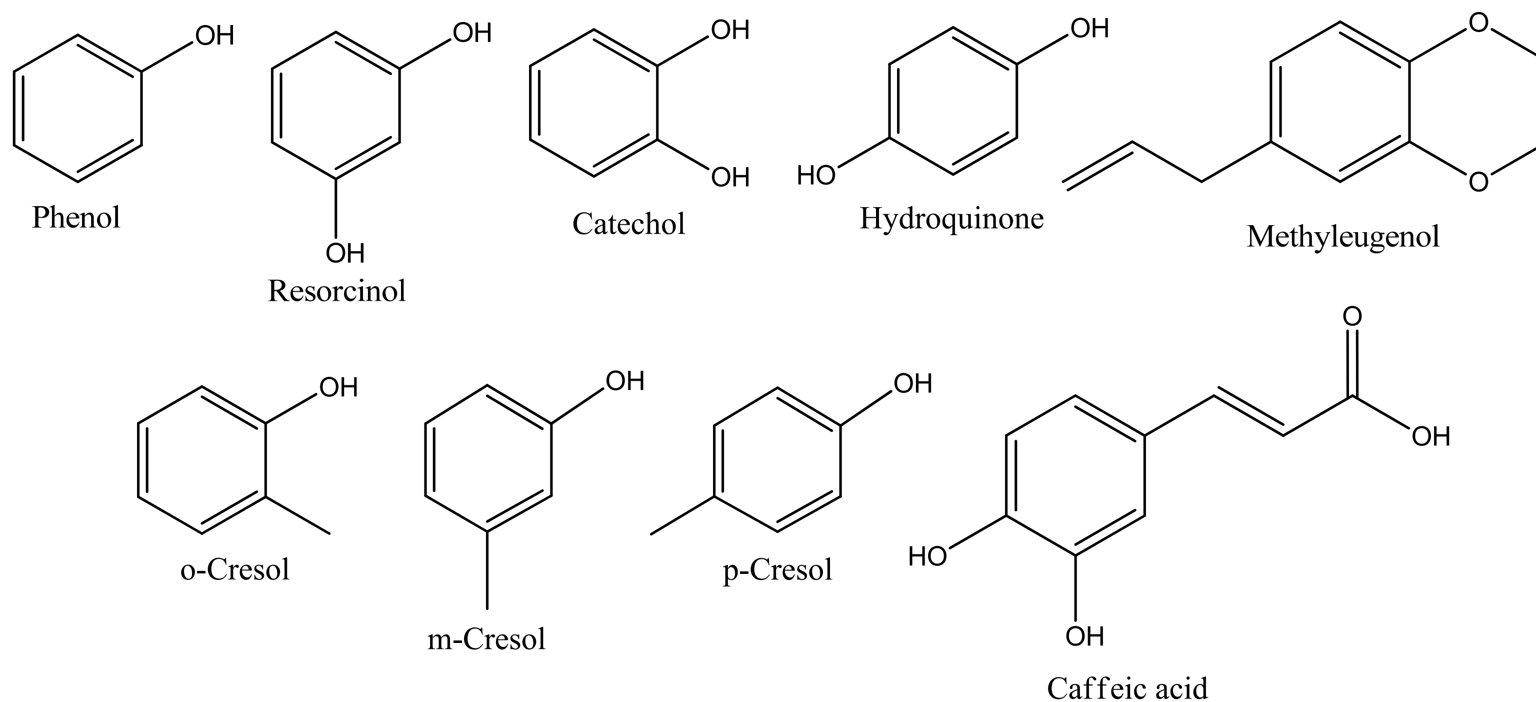

Figure 1-3: Hoffmann analytes, structures of phenolic compounds found primarily in the particulate phase of cigarette smoke (Rodgman *et al.*, 2003).

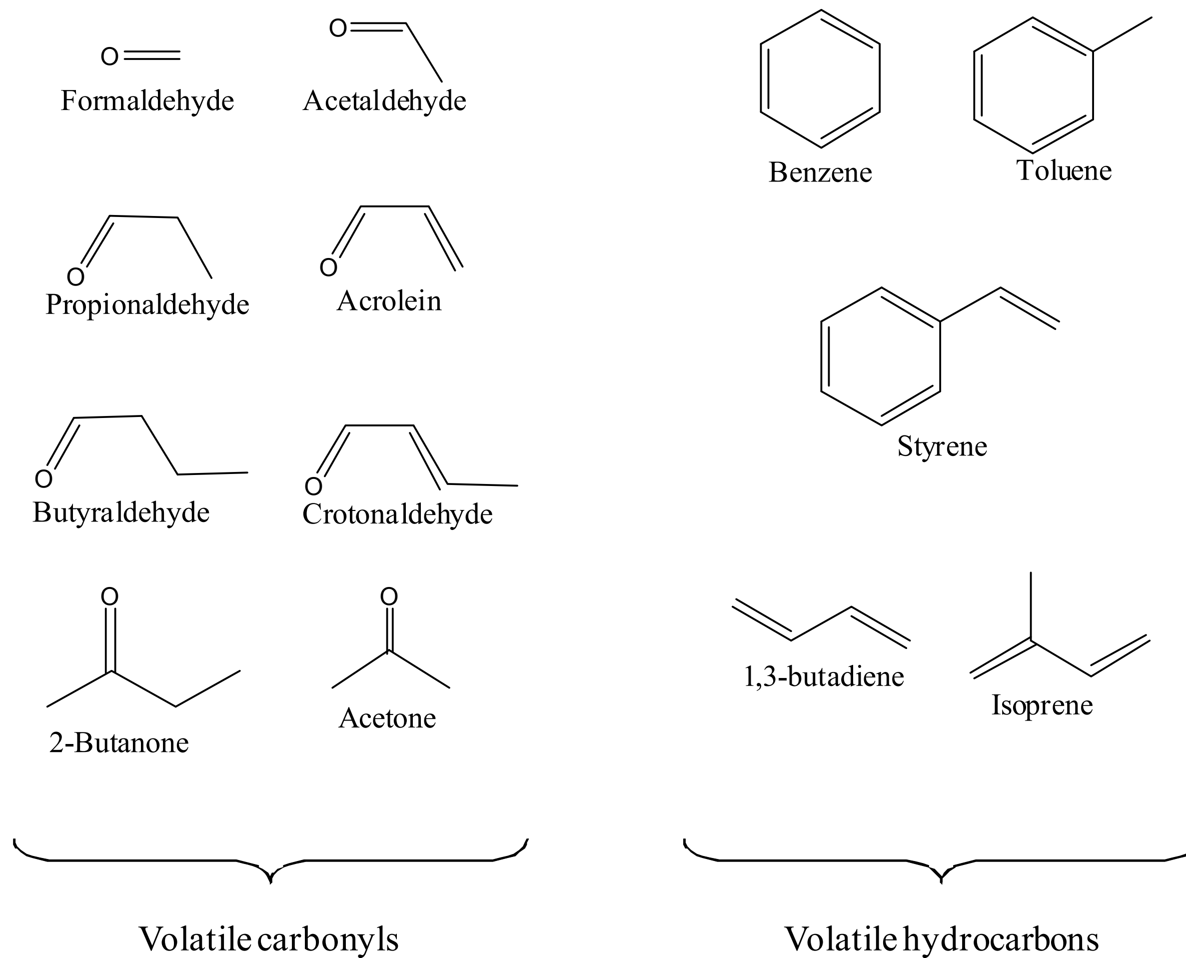

Figure 1-4: Hoffmann analytes, structures of volatile carbonyls (aldehydes and ketones) and hydrocarbons found primarily in the vapor phase of cigarette smoke (Rodgman *et al.*, 2003).

1.7.3 Traditional Approaches: *in Vitro* Assays

Historically, biological testing of cigarette smoke has been based on smoke generation procedures intended for product comparisons. Cigarette smoke *in vivo* studies involve the exposure of cigarette smoke to rodents via inhalation (Baumgartner *et al.*, 1980) or cigarette smoke condensates to rodents by using skin painting carcinogenesis testing (Walaszek *et al.*, 2007). This chapter has been limited to three particular *in vitro* bioassays used throughout the tobacco industry which have been developed by CORESTA and implemented by Health Canada (Health Canada, 2004a, Health Canada, 2004c, Health Canada, 2004b). These tests are the *in vitro* micronucleus test (IVMNT), neutral red uptake assay and Ames test and are all based on the exposure of cells to the particulate matter extracted by DMSO from a CFP.

The IVMNT is an *in vitro* genotoxicity and cytotoxicity assay used to detect compounds that induce the formation of micronuclei (*i.e.* small membrane bound DNA fragments) in the cytoplasm of mammalian cells at the interphase. Following exposure of cells to a genotoxic agent, the cells are stained with a fluorescent dye and the percentage of observed micronuclei is reported[2]. The frequency of micronuclei is visually determined and compared between samples and controls to determine the relative genotoxicities. Furthermore, the cell viability (cytotoxicity) can also be visually determined by comparing samples and controls for relative survivals. In this thesis work, only the IVMNT was used, therefore, this assay is further described in Chapter 2.

The neutral red uptake assay (NRU) is an *in vitro* technique used to assess the cytotoxic potential of chemical compounds. It is a cell viability chemo-sensitive assay based on the ability of viable cells to incorporate and bind neutral red, a supravital dye. Since the neutral red dye is taken up by the lysosomes (containing digestive enzymes), the assay examines the cellular membrane activity and energy status. In the presence of toxic agents, a decrease in the uptake of the dye occurs. The samples are assessed by measuring their optical densities at 540 nm (absorption maxima of neutral red dye) and are compared relative to the sample control. Therefore, viable and damaged cells can be differentiated.

The Ames test is an *in vitro* technique used to assess the mutagenic potential of chemical compounds. This test uses *Salmonella typhimuriun* bacterial strains that carry mutations in genes involved in histidine synthesis and require histidine for growth. The test is based on the mutant's ability to grow on a histidine-free medium. The bacteria are grown in the presence of limited histidine. When the histidine is depleted, only mutated bacteria that are able to produce histidine will survive. Following a 48 hour incubation period, the mutagenicity of a substance is proportional to the number of colonies observed. A positive test is indicative of a compound's mutagenicity and potential carcinogenicity.

1.7.4 Current Approaches: Whole Smoke Exposure Systems

A major drawback associated with traditional *in vivo* and *in vitro* exposure procedures is the inability to simultaneously expose cigarette smoke particulate and vapor phases to cells. Studies indicate that the vapor phase of cigarette smoke plays a major role

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[2] The determination of percent of % micronuclei is given in detail in Chapter 2 and 3.

in the toxicological responses (Bombick *et al.*, 1997c, Bombick *et al.*, 1997b, Phillips *et al.*, 2004, Wieczorek *et al.*, 2006, CORESTA, 2005). Thus, exposure to whole cigarette smoke (including both phases) should give a comprehensive description of cigarette smoke toxicity.

Developments in the last decade have introduced smoke generation systems, such as the Borgwaldt RM20S® smoking machine (Phillips *et al.*, 2005), Burghart Mimic Smoker-01® (Scian *et al.*, 2009a, Scian *et al.*, 2009b), the Vitrocell Smoking Robot VC 10® (Aufderheide *et al.*, 2000) and the development of novel *in vitro* exposure systems such as British American Tobacco's (BAT) exposure chamber (Phillips *et al.*, 2005) and the CULTEX system (Aufderheide *et al.*, 2000). These systems allow for the generation of fresh cigarette smoke in various dilutions (or doses) and direct delivery to cell cultures. The ability to introduce smoke diluted over a wide range is required for *in vitro* cell culture investigations because of the high sensitivity of these biological systems. Only the Borgwaldt RM20S® smoking machine was available to us and its operation and assessment are described in detail in Chapter 4.

1.8 Research Objectives

Tobacco smoke is a complex mixture composed of more than 5000 components, simultaneously distributed between the particulate and vapor phases (Rodgman *et al.*, 2009a, Rodgman *et al.*, 2009b). Based on the health implications of tobacco smoke, many studies have been conducted to identify the components present in tobacco and its smoke (Rodgman *et al.*, 2009a, Rodgman *et al.*, 2009b, Dong *et al.*, 2000, Omori *et al.*, 1999,

Chen *et al.*, 2003, Darrall *et al.*, 1998, Adam *et al.*, 2006, Polzin *et al.*, 2007, Baggett *et al.*, 1974, Baker, 1999). Furthermore, lists have been prepared containing biologically active components found within smoke (Hoffmann, 1993, Hoffmann *et al.*, 1998b, Hoffmann *et al.*, 1997, Rodgman *et al.*, 2003). In terms of toxicological testing, the majority of studies has been carried out on extracts of tobacco smoke particulate or vapor phases (Bombick *et al.*, 1997a, Wynder *et al.*, 1969). Nevertheless, there is a lack of studies correlating specific *in vitro* or *in vivo* biological responses to components within whole tobacco smoke (Borgerding *et al.*, 2005, Rodgman *et al.*, 2003) due to its complex nature *i.e.,* the number and diverse range of compounds simultaneously distributed in the two phases. Therefore, the general hypothesis that guided the current work was as follows: the development of reliable and robust smoke fractionation methods makes it possible to assign toxicological effects to specific cigarette smoke components by using sophisticated analytical separation and detection techniques in combination with reliable toxicology assays. The novelty in this approach lies in the union of chemical characterization and toxicological studies in parallel.

Given the complex nature of tobacco smoke, its simplification prior to analysis is required. One approach used to assess the composition and toxicity of cigarette smoke is to study the combustion of individual components found in leaf tobacco. This approach reduces the complexity of a whole smoke experiment and allows the development of a strategy to assess individual components or their respective combustion products, for application to the study of whole smoke. A previous study that applied this approach (Préfontaine *et al.*, 2006), and which inspired the research described in Chapter 3, was

based on the toxicological assessment of the particulate phase combustion products of twelve individual tobacco components using the IVMNT. The particulate phase components were targeted based on the available methodology, instrumentation and expertise required. Twelve major tobacco constituents were chosen for this study by Préfontaine *et al.*: lignin (LIG), chlorogenic acid (CLO in this figure only), malic acid (MAL), RUBISCO (RUB), tryptophan (TRY), tyrosine (TYR), glycine (GLY), proline (PRO), pectin (PEC), glucose (GLU), starch (STA) and cellulose (CEL). Additionally, two tobacco reference blends used in the tobacco science industry were assessed: CK36 and 1R4F. Based on the IVMNT results (Fig. 1-5), the combustion products of chlorogenic acid and lignin were found to be the most cytotoxic of the twelve sets of combustion products tested. Lignin is a complex biopolymer found in tobacco and chlorogenic acid is the most abundant polyphenol in tobacco. It is important to point out that chemical characterization (*i.e.*, identification) of the products generated by combustion of any one of the 12 starting compounds was not carried out. Chlorogenic acid was therefore chosen for further investigation based on its higher cytotoxic response and simpler structure than lignin.

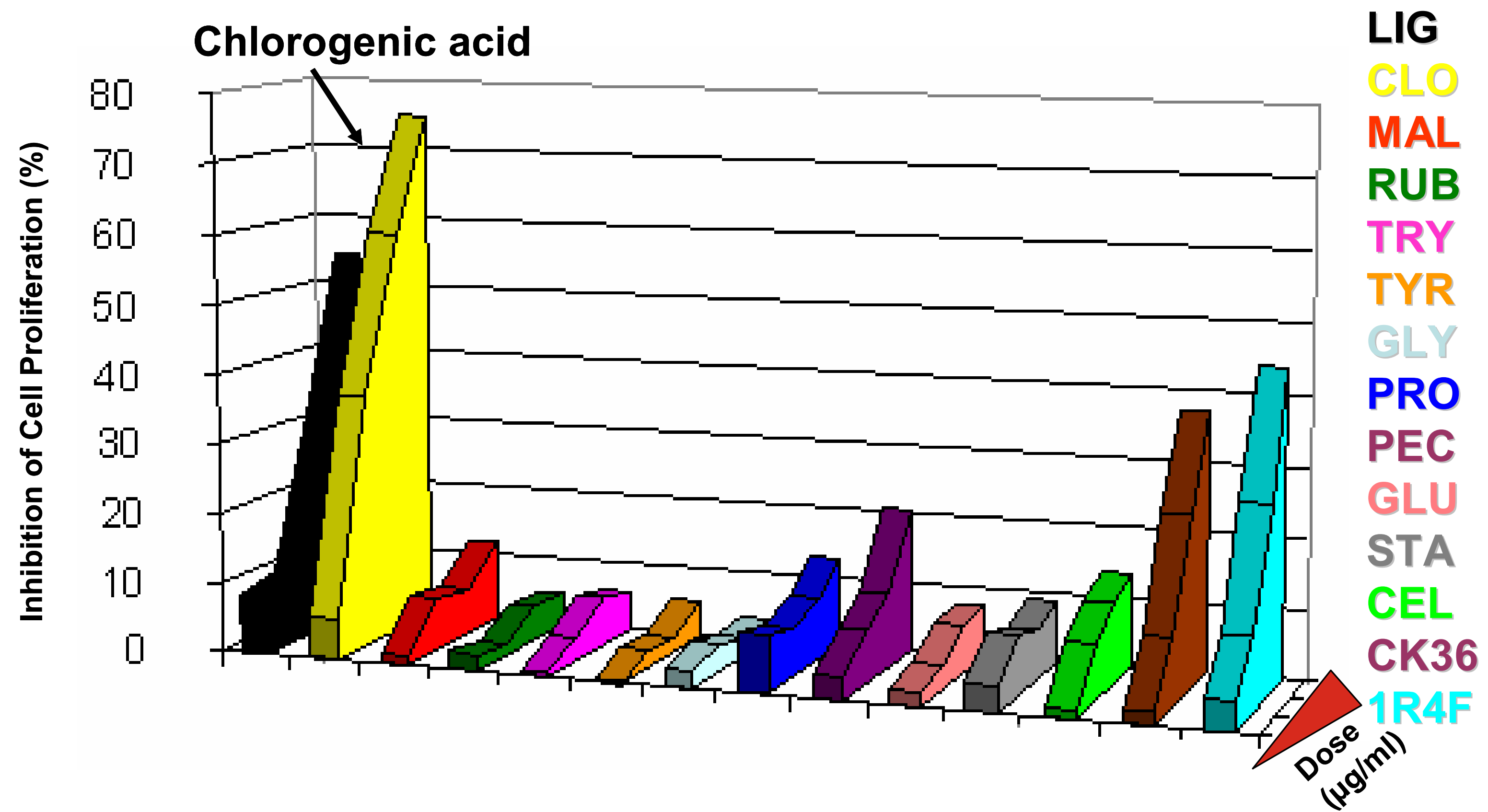

Figure 1-5: Cytotoxicity results (IVMNT) from the exposure of cells to the combustion products of twelve tobacco constituents (Préfontaine *et al.*, 2006).

Our first objective was to identify the components or groups of components resulting from the combustion of chlorogenic acid, responsible for the cytotoxicity results obtained from the IVMNT (Préfontaine *et al.*, 2006). Therefore, the challenge was to develop a method to further differentiate the combustion products of chlorogenic acid and test portions of them to identify the bioactive compounds by combining chromatography and toxicological assays. LC/MS was employed because separation and accurate mass identification were required, given the complexity of this type of unknown sample. Development of a method to characterize the combustion products of a single tobacco component like chlorogenic acid (less complex than whole tobacco smoke products), as presented in Chapter 3, provided us with a methodology that could potentially be applied to whole tobacco smoke products.

Emerging studies have highlighted the biological significance of whole tobacco smoke vapor phase volatiles and semi-volatiles (Bombick *et al.*, 1997b, Bombick *et al.*, 1997c, Phillips *et al.*, 2004, CORESTA, 2005, Wieczorek *et al.*, 2006). One of the main drawbacks for the assessment of the vapor phase and whole smoke has been a lack of techniques available to tackle the *in vitro* toxicological characterization. Recent advancements have led to the development of smoke generation systems, such as the Borgwaldt RM20S® smoking machine and novel *in vitro* exposure systems, such as British American Tobacco's (BAT) exposure chamber. These systems allow for the generation of fresh cigarette smoke in various doses and delivery to cell cultures. The instrumentation was introduced in our laboratory during the work on chlorogenic acid. Even if the study of combustion products from individual tobacco constituents can provide insight into their contribution to overall cigarette smoke toxicity, the study of whole cigarette smoke is more

representative of smoker exposure to the complex mixture generated during smoking. Thus, our objectives shifted to whole smoke analyses versus solely the particulate phase. *In vitro* cell culture studies had been carried out on the instrument mentioned above and a positive dose-response relationship was observed for cells exposed to cigarette smoke by the NRU assay (Phillips *et al.*, 2005). Doses of smoke were based on dilutions of smoke in air (v/v) that had been previously correlated to the quantity of TPM deposited on a CFP (Phillips *et al.*, 2005). However, the reliability of dose delivery from this type of instrumentation was not assessed and the model has been criticized as an unreliable model for toxicological testing. In general, *in vitro* and *in vivo* toxicological testing data have high variability, primarily based on the heterogeneity of the cell or tissue culture being exposed. Therefore, our second objective was to assess the reliability of the instrumentation in terms of repeatability, reproducibility and accuracy of the smoke generation and dilution for this type of system using standard reference gases and a cigarette particulate phase marker. This study is described in Chapter 4.

Once the smoke generation instrument was deemed reliable for cell exposure studies, our third objective was to develop a method to chemically characterize the vapor phase components generated by the Borgwaldt RM20S® within the exposure chamber itself, at a biologically significant dose. This characterization involved the collection of the vapor phase components after cigarette smoke dilution and transfer to the chamber to evaluate the dose linearity, as described in Chapter 5. Collection was achieved by stir-bar sorptive extraction and GC/MS was employed because separation and accurate mass identification were required, given the complexity of this type of unknown sample. By

assessing the reliability of smoke delivered to the exposure chamber by this instrument, the *in vitro* toxicological data generated by using this model can be more precisely correlated to the dose. Furthermore, developing a method to characterize the biologically active smoke vapor phase components can serve as a standard method for assessment of similar types of instrumentation and provide a screening tool to assess relative smoke bioactivity for potential harm reduced tobacco product comparisons.

2 Experimental Methodology

2.1 Smoke Generation Techniques

2.1.1 Generation of Partial Combustion Products from Individual Tobacco Components using the John Payne Tar Combustion Simulator

A John Payne Tar Predictor apparatus is commonly used to simulate partial (or complete) combustion of various samples, ranging from individual tobacco components to spiked tobacco mixtures. The samples generated can be analyzed for their chemical composition or tested for their respective biological activities. The sample is inserted in a quartz tube that is automatically driven into the instrument's furnace (Fig. 2-1). Quartz tubes are used because of their thermal resistance, which in turn allows for samples to be burned at temperatures mimicking the combustion of a cigarette (up to 950 °C). Experimental conditions detailed in Chapter 3 (*e.g.*, combustion temperature, sample size and flow rate) are carefully chosen to attempt to mimic the cigarette smoking process, since they are key factors contributing in the types and quantities of products formed (Chen, 2004).

During the partial combustion process (Fig, 2-2), atmospheric air is drawn through the quartz tube at constant air flow, forming smoke that passes through a CFP of diameter 55 mm, thereby trapping the TPM of the smoke. Silicone grease was used to prevent leaking of smoke from the tubing at specific locations. Following the partial combustion process, the CFP holder is blocked on both ends to reduce loss of the semi-volatile

components. The particulate phase on the CFP is then extracted with a solvent to produce concentrated extracts for further chemical or biological analysis.

The John Payne Tar Predictor was used in this study (Chapter 3) to partially combust pure chlorogenic acid in order to generate partial combustion products for further toxicological analyses. This type of instrumentation was chosen based on its ability to partially combust pure substances at a range of temperatures, in substantial quantities for further testing as well as its its accepted use in the Tobacco Industry.

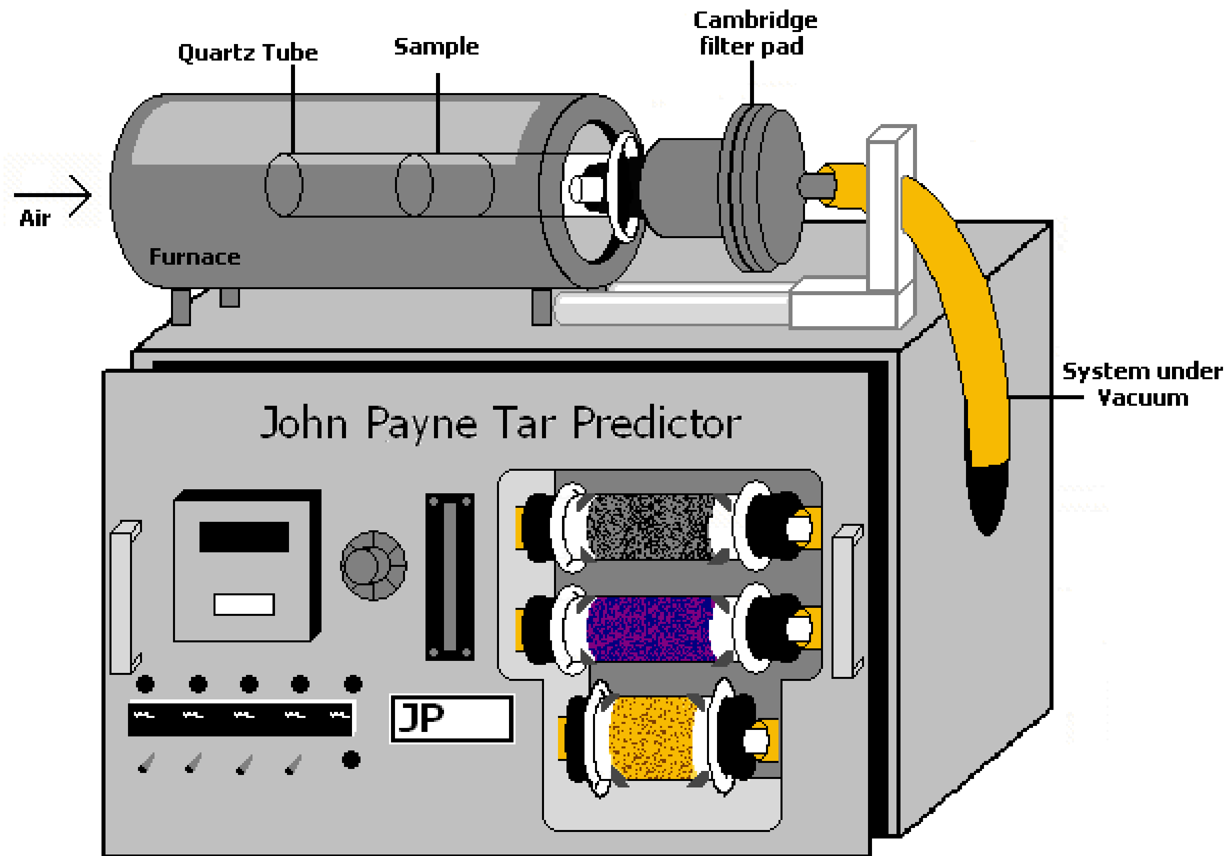

Figure 2-1: Schematic of the John Payne Tar Predictor combustion simulator used to generate partial combustion products of pure tobacco components or other samples. The quartz tube contains the sample to be combusted in the furnace for a fixed duration and temperature at a given air flow rate and the CFP collects the particulate phase as the smoke passes through.

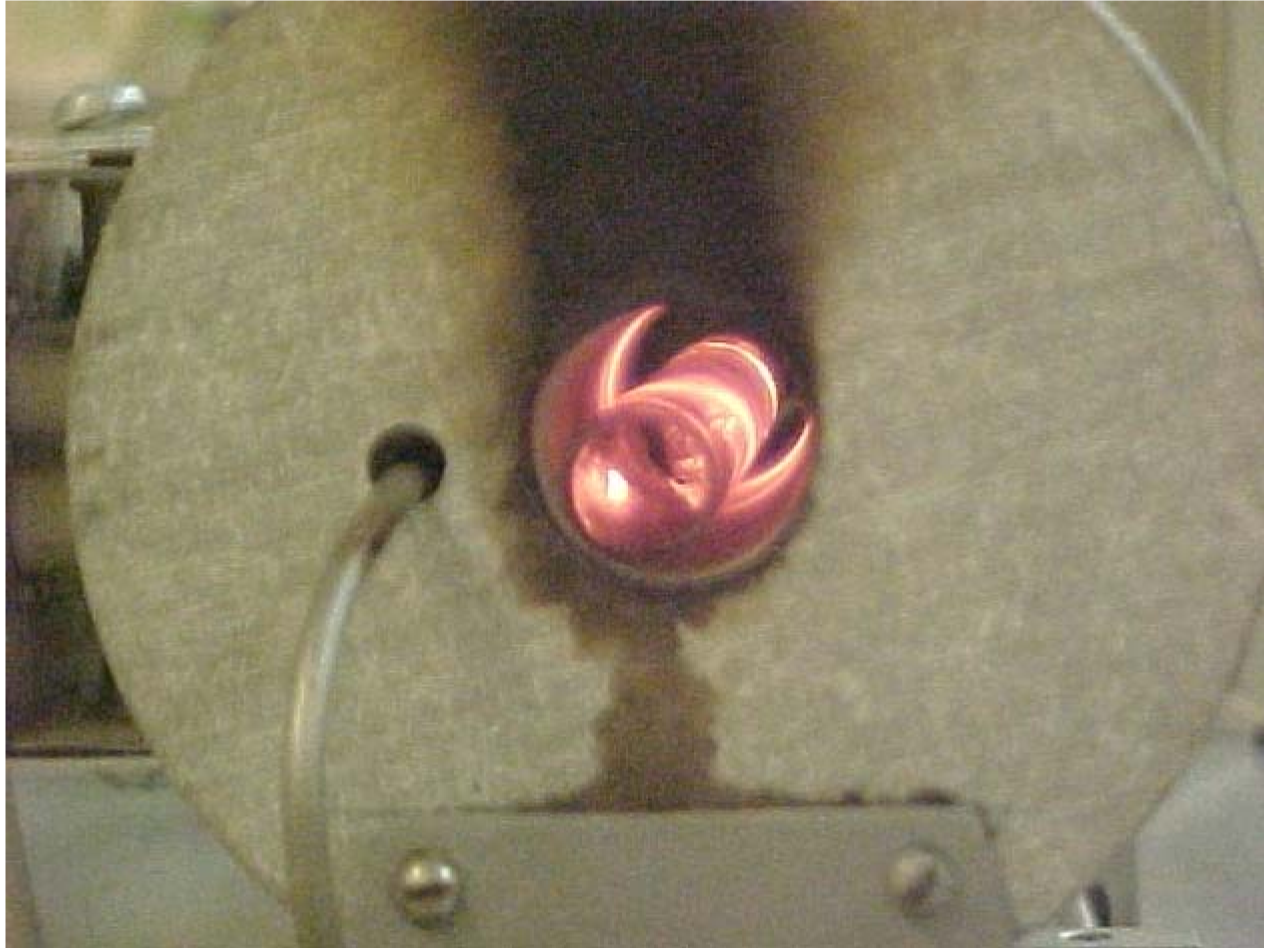

Figure 2-2: Photograph of the cross-section of the furnace during partial combustion of an individual tobacco constituent by the John Payne Tar Predictor.

2.1.1.1 Chlorogenic Acids and its Combustion Products: Application of the John Payne Tar Predictor

Phenolic components are important contributors to aroma, taste and toxicity. Phenolic compounds present in tobacco smoke are typically products of the thermal degradation of polyphenolic compounds. Some of the major polyphenolic compounds in tobacco are: chlorogenic acids (0.31- 4.3 %), rutin (0.15- 1.8 %) and scopoletin (0.003-0.012 %) (Wynder *et al.*, 1967). Chlorogenic acid isomers are bio-synthesized (esterification reaction) from quinic acid and caffeic acid (Fig. 2-3) and are classified as convertogenic[3] and/or clastogenic[4] (Stich *et al.*, 1981).

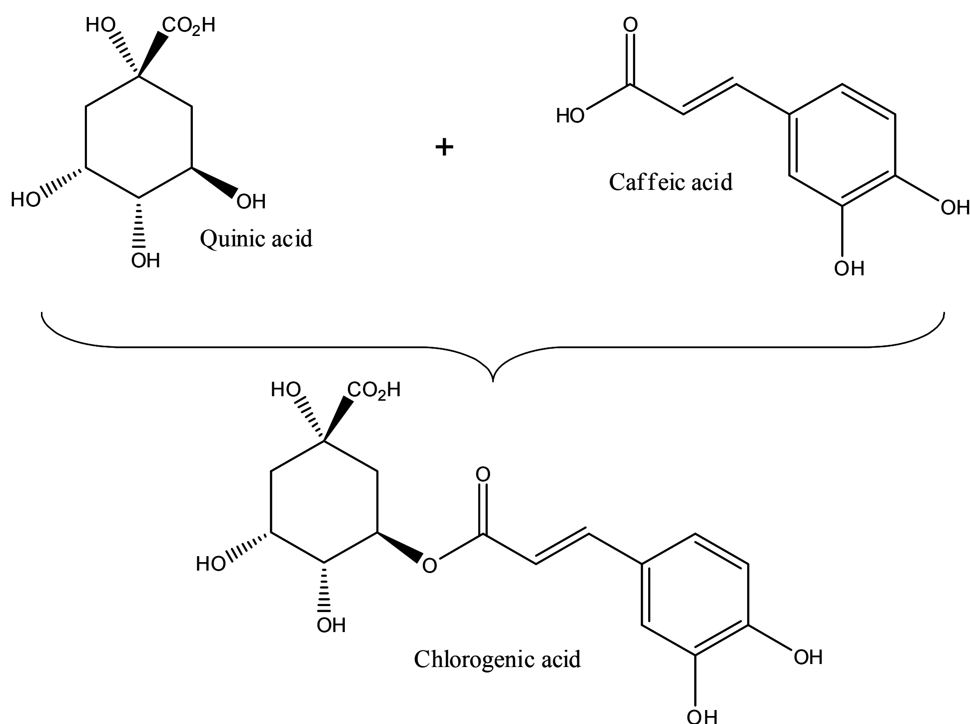

Figure 2-3: The product of quinic acid and caffeic acid, chlorogenic acids (class of isomers).

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[3] Convertogenic is defined as mitotic gene conversion at the gene locus trp 5 of a *Saccharomyces cerevisiae* D7 strain, resulting in the induction of tryptophan prototrophs.
[4] Clastogenic is defined as capable of causing chromosomal breakage.

The partial combustion of chlorogenic acid has been investigated under various temperatures (ambient to 800 °C) and has been found to generate benzene, phenol, benzoic acid and vinyl catechol among other bioactive products (Fig. 2-4) (Sharma *et al.*, 2002b, Baker, 1999, Schlotzhauer *et al.*, 1981). Vinyl catechol, a co-carcinogen[5], is one of the major compounds observed in the combustion products of chlorogenic acid and between 200-300 μg is present in the particulate phase of cigarette smoke (Schlotzhauer *et al.*, 1981, Hoffmann *et al.*, 1983). Other studies indicates that catechol hydroquinone and benzene (also combustion products of chlorogenic acid) inhibit human T cell lymphocyte proliferation (Li *et al.*, 1997, Snyder *et al.*, 1993). Moreover, the combustion products of chlorogenic acid have been shown to be cytotoxic[6], genotoxic[7], based on (IVMNT) results (Préfontaine *et al.*, 2006, Kaur *et al.*, 2009). In these studies, the John Payne Tar Predictor was used to generate the partial combustion products of the various tobacco constituents, including chlorogenic acid.

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[5] Co-carcinogenic is defined as a compound that promotes the effects of a carcinogen.
[6] Treatment of cells to a cytotoxic compound results in cell necrosis, losing membrane integrity resulting in cell lysis, or apoptosis, programmed cell death.
[7] Genotoxicity describes damage (deletion) to a cells genetic material affecting its integrity. Genotoxic substances may be mutagenic or carcinogenic, causing genetic mutations or tumor development.

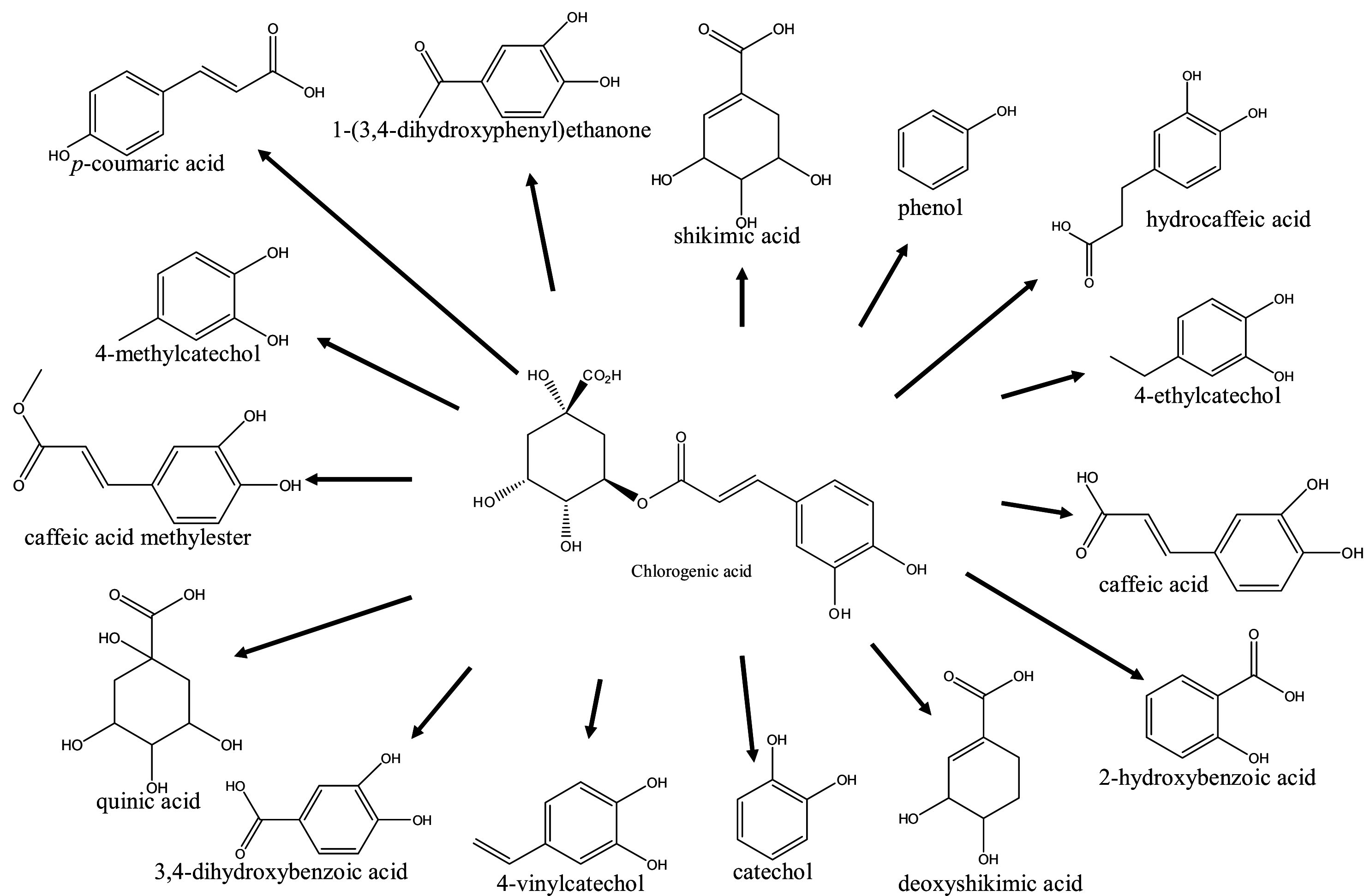

Figure 2-4: Some of the partial combustion products of chlorogenic acid.

2.1.2 Generation and Dilution of Cigarette Smoke by the Borgwaldt RM20S® Smoking Engine in Combination with BAT's Exposure Chamber for *in Vitro* Cell Culture Exposures

The Borgwaldt RM20S® (Fig. 2-5) is an automatic smoking machine that generates and dilutes fresh cigarette smoke for *in vitro* cell culture investigations (Borgwaldt KC, 2010). It has a rotary based engine that can simultaneously smoke four types of cigarettes for several hours, depending on the smoking regime used. Each cigarette corresponds to a specific port, syringe and exposure chamber (A-D); the machine smokes the cigarette, dilutes and delivers the smoke into the chambers. By incorporating a CFP before the exposure chamber, which is a modification we made specifically for the study, the particulate phase is trapped, resulting in the exposure of only the vapor phase to the cells. The instrument has an incorporated anemometer, allowing for controlled air flow, as well as an electrical lighter, butt detector and butt extractor. The latter allows several cigarettes to be smoked in sequence at a given port. The Borgwaldt RM20S® can be used with BAT's exposure chamber (Fig. 2-6) to enable cells or tissues to be exposed to the diluted smoke (*i.e.* doses) generated by the Borgwaldt RM20S®. Within the chamber, the cells or tissues lie on porous Transwell® inserts and are exposed to smoke at the air-liquid interface (Thorne *et al.*, 2009, Phillips *et al.*, 2005). The ability to introduce smoke diluted over a wide range is required for *in vitro* cell culture investigations because of the high sensitivity of these biological systems.

The Borwaldt RM20S® was used to generate and dilute smoke for reliability studies (Chapter 4) involving the measurement of solanesol in cigarette particulate phase and for

chemical characterization studies involving the measurement of various cigarette vapor phase components present within the BAT exposure chamber (Chapter 5). This type of instrumentation was chosen based on its ability to expose cells to whole smoke and on its increasing popularity and potential use in the Tobacco Industry. It is important to underline that in the studies presented in Chapter 4 and 5, no live cells were used.

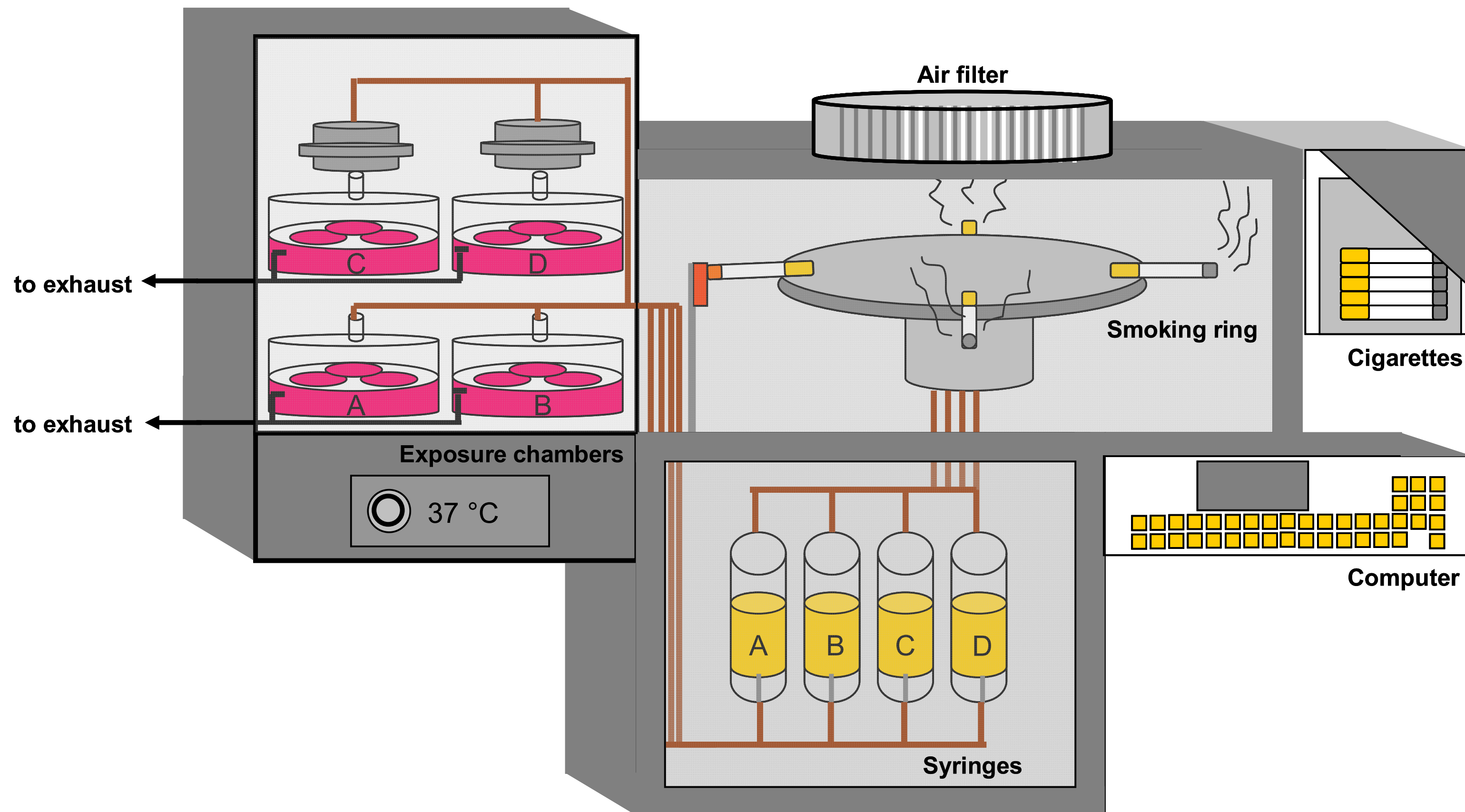

Figure 2-5: Schematic of the Borgwaldt RM20S® instrument in combination with BAT's exposure chamber (on left side of figure) used to generate and dilute cigarette smoke for *in vitro* cell or tissue culture exposures. *Exposure chambers A and B are exposed to whole cigarette smoke versus C and D which are exposed to vapor phase only in the figure.*

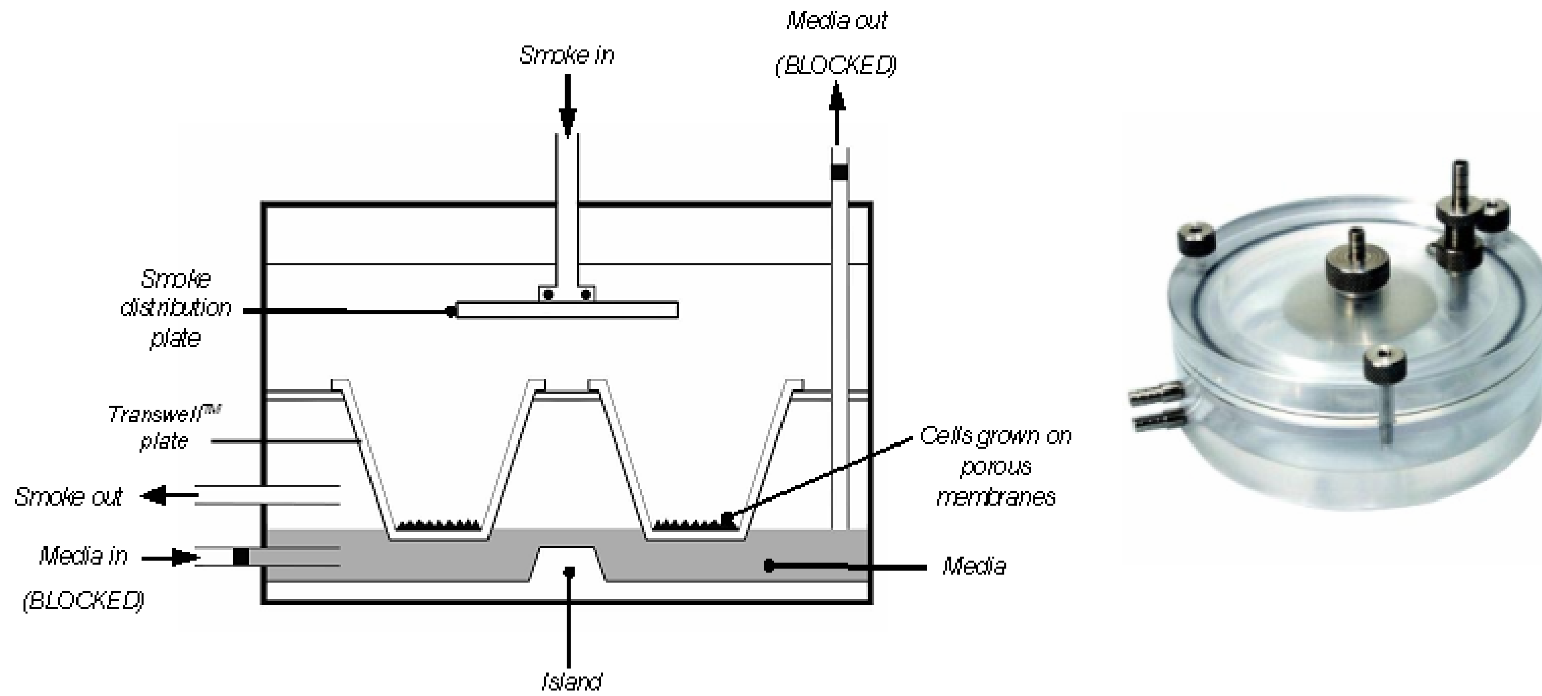

Figure 2-6: The BAT exposure chamber (Patent Publication Number WO 03/100417, (Thorne *et al.*, 2009).

2.2 Sample Collection Techniques

2.2.1 Extraction and Enrichment of Organic Compounds by using Stir-bar Sorptive Extraction (SBSE) or Headspace Stir-bar Sorptive Extraction (HSSE)

SBSE is a similar technique to SPME except the sorptive phase (*i.e.* PDMS) is on a glass-coated magnetic stirring bar for improved extraction/enrichment of analytes from aqueous samples. SBSE is based on the approximation that the partitioning coefficients between PDMS and water ($K_{PDMS/W}$) are proportional to the octanol/water coefficients ($K_{o/w}$). Thus, the only parameter governing recovery of the analyte from the sample is the ratio of partitioning constant and phase ratio between PDMS on the stir-bar and the water sample. The theoretical recovery (R_{TH}) can be determined by the following:

$$R_{TH} = \frac{M_{(PDMS)}}{M_{(O)}} = \frac{\alpha}{1+\alpha} \qquad \textbf{(Equation 2.1)}$$

$$\text{where: } \alpha = \frac{K_{O/W}}{\beta}$$

$$\beta = \frac{volume\ of\ water}{volume\ of\ PDMS}$$

$M_{(PDMS)}$ = mass of analyte present in the PDMS

$M_{(O)}$ = total amount of analyte originally present in the water

SBSE used to sample the vapor phase is referred to as headspace stir-bar sorptive extraction (HSSE). Basically, it is a static mode of operation carried out by suspending the

stir-bar in the vapor phase, which is in equilibrium (or not) with a solid or liquid matrix. For quantification, full equilibrium is not essential, *i.e.* as in SPME. For example, as long as the stirring period for a sample and standards are carried out for a similar fixed period, calibration may be accurate; depending on the sample being analyzed, some exceptions may apply. Approaching extraction equilibrium is preferred for maximum sensitivity (Baltussen *et al.*, 1999). As with SBSE and for SPME, analyte recovery is achieved through thermal desorption combined online with GC analysis of desorbed analytes. The preferred detection method is by MS, which offers a high sensitivity and specificity.

A commercially available product, the Twister™ stir-bar (Fig. 2-7), is coated with PDMS. This stir-bar can be placed in a sample (gaseous or liquid) and either magnetically agitated to provide stirring or left static. Upon contact with the sample, it absorbs and concentrates compounds onto its PDMS phase. Subsequently, it can be directly transferred to a thermal desorption system (TDS) then a cooled injection system (CIS, a programmable inline temperature concentration and injection technique), which is directly coupled to a GC/MS. Due to the amount (volume) of the PDMS coating, it is proven to be more sensitive (*i.e.* provides a higher enrichment factor) than conventional SPME. Typical SBSE devices, such as the Twister™, consist of a magnetic stirring bar encased in a glass sheath that is coated with a ≤ 1 mm film of PDMS and can contain between 55-219 μL of PDMS (compared to ≤ 0.5 μL in SPME) depending on the length of the stir-bar, which is between 1-2 cm.

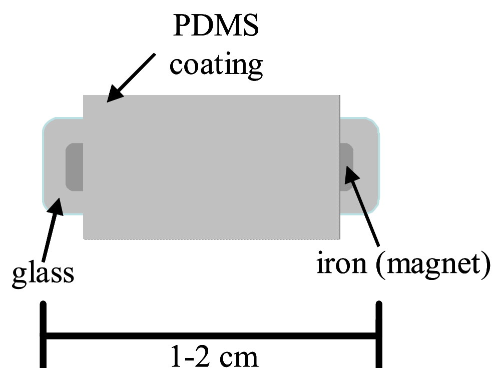

Figure 2-7: Schematic of a Twister™ stir-bar used for SBSE and HSSE applications.

HSSE was used for the chemical characterization studies involving the measurement of various cigarette vapor phase components present within the BAT exposure chamber. This technique was chosen based on the types of components present within the smoke sample (*i.e.* volatiles and semi-volatiles), the high sensitivity compared to SPME in order to analyze very dilute cigarette smoke and the physical size allowing it to be placed within the exposure chamber well.

2.3 Description of Techniques used for the Separation and Identification of Smoke Constituents

2.3.1 Chromatographic techniques: LC, HPLC and GC

HPLC/TOFMS and LC/MSD were employed for the chemical characterization studies of the partial combustion products of chlorogenic acid in the particulate phase (Chapter 3). Based on the nature of the analytes (phenolic compounds), reversed phase chromatography with a non polar stationary phase (C18 or ether linked phenyl stationary

phases used in Chapter 3 and Appendix B) and a mobile phase organic solvent such as, methanol was applied. Reversed phase separation involves using a stationary phase that is less polar than the mobile phase and is the most commonly used mode.

GC/MS was employed for the chemical characterization of cigarette smoke vapor phase components (Chapter 5). The components of interest were relatively non-polar semi-volatile and volatiles species. For this type of analysis by GC, elution is a function of volatility or boiling point. The stationary phase used was non polar and consisted of (5%-phenyl)-methylpolysiloxane (DB5-MS).

2.3.2 Fractionation by LC

Preparative scale LC is a non-destructive technique that can be used for fractionation of samples for further analysis or purification purposes. The sample (can exceed injections > 20 μL) is injected and migrates through a column (dimensions used in Chapter 3: 250 mm × 22 mm and 100 mm × 21.2 mm) composed of a stationary phase and mobile phase. Larger stationary phase particles are used and thus lower pressure for the mobile phase, allowing higher sample volumes to be injected. Fractions are collected at the column outlet. Separation of the sample occurs based on its partition between the mobile and stationary phases, in an identical manner to HPLC. Preparative LC uses the same types of detector as HPLC (except with MSD). The sample exits the detector and is collected as fractions into different tubes. These fractions can be further separated or analyzed for their chemical or toxicological properties. With MSD, which is a destructive technique, a small portion of the effluent exiting the column is split off the main stream for detection.

2.3.3 UV Absorbance Detection

Ultraviolet absorbance detection is the most common LC detection method because most solutes absorb light in the 180-350 nm wavelength range. Some of the compounds that absorb light in the UV range include those containing double bonds (π electrons) and unshared electrons. Simple detection systems use a mercury lamp, which has an intense line emission at 254 nm. More sophisticated instruments use a deuterium, or xenon lamp, which emit over ultraviolet and visible wavelengths, or a tungsten lamp for the visible region only, combined with a monochromator to select the specific wavelength for molecular absorption (Harris, 2006).

LC/UV was employed for the fractionation studies of the partial combustion products of chlorogenic acid (Chapter 3). This type of instrumentation was used because it allowed for a large-scale injection generating sufficient fractionation volumes for the subsequent toxicological testing. In addition, the types of solutes present in the samples generated (phenolic type compounds) absorb well at the 254 nm wavelength, so a mercury lamp detector could be used. LC/UV was used for the Borgwaldt RM20S® instrument reliability studies (Chapter 4), involving the quantification of solanesol (which absorbs well at 254 nm) in TPM.

2.3.4 Mass Spectrometry Detection (MSD)

Mass spectrometry is a technique that provides identification of the molecular mass and possibly information concerning the structure of a given analyte(s). For small organic molecules, the accuracy of the molecular mass can be measured within 5 ppm, depending

on the detector (*i.e.* to 0.001 amu for an analyte of mass 200 g/mol), which is often sufficient to confirm the molecular formula of a compound, depending on the complexity of the sample. Mass spectrometry is both qualitative and quantitative and is used for a large range of compounds. The technique is based on ionization of molecules to produce charged species that are separated according to their mass-to-charge (m/z) ratios and then detected with a high sensitivity electron multiplier or microchannel plate.

Given the complexity and unknown composition of the samples in this study, accurate mass measurement providing the empirical formula for given analytes was required in order to identify the compound(s) of interest without the use of standards (*i.e.,* ± 5 ppm provided by a TOFMS). In addition, MSD allows to monitor a wide range of compounds (m:z's). HPLC/TOFMS (equipped with an electrospray ionization source and referred to as simply LC/TOFMS in Chapter 3) was employed for the chemical characterization studies of the partial combustion products of chlorogenic acid in the particulate phase. LC/MSD (equipped with a quadrupole analyzer) was also used to identify the products that were responsible for the bioactivity assessed by the IVMNT (Chapter 3).

GC/MS (equipped with a electron impact ionization source and quadrupole analyzer) was employed for the chemical characterization studies of the cigarette smoke vapor phase generated, diluted and delivered by the Borgwaldt RM20S® and within the BAT exposure chamber (Chapter 5). This type of instrumentation was used because the target compounds which were present in cigarette smoke vapor phase were volatiles and semi-volatiles. Due to the complexity of cigarette smoke, in terms of types and number of

products, interest in volatile aromatic compounds and other volatiles/semi-volatiles species, GC/MS has been commonly used for the separation and identification of cigarette smoke products (Torikaiu *et al.*, 2005, Moldoveanu *et al.*, 2007, Nanni *et al.*, 1990).

2.3.5 Quantification by Flame Ionization Detection (FID)

FID which is usually coupled to GC, is used for the analysis of hydrocarbons and other flammable compounds. It is very sensitive and its response is linear with carbon content of a given analyte across a wide range of concentrations, thus, it is often coupled to GC.

The sample comprised of organic compounds enters the FID and components are pyrolyzed at the temperature of a hydrogen/air flame. Pyrolysis produces positively charged ions and electrons that conduct electricity through the flame. The ions are repelled towards the collector plates (tabular electrodes) that are connected to an ammeter. Upon hitting the plates, the ions induce a current that is detected by the ammeter. The signal is then amplified, integrated and displayed. Therefore, the current measured corresponds to the proportion of reduced carbon atoms in the flame. The pyrolysis products are vented through an exhaust port. The FID is mass sensitive rather than concentration sensitive.

A mini FID (not coupled to GC) was employed in the Borgwaldt RM20S® instrument reliability studies (Chapter 4), involving the quantification of a CH_4 standard gas. This method was used because it is routinely used for hydrocarbon analyses and it was readily available in our laboratory.

2.3.6 Quantification by Infrared Detection (IR)

IR spectroscopy is based on absorption of light in the infrared region of the electromagnetic spectrum and can be used to identify compounds or sample composition because of the narrow spectral bands achieved. Gases can be directly injected into IR spectrometers. The emission source is split into two beams (one passes through the sample, the other through the reference). The beams are both reflected back to the detector and two absorbance (or transmittance) signals are compared. IR spectroscopy is based on the fact that molecules have specific frequencies corresponding to discreet energy levels, at which its atoms vibrate and thus absorb light at exactly these energy levels.

An IR detector was employed in the Borgwaldt RM20S® instrument reliability studies, involving the quantification of a CO standard gas. This type of detector was used because CO is active in the IR region, but is not detected by FID, and it was readily available in our laboratory.

2.4 The *in Vitro* Micronucleus Test (IVMNT) used to Assess the Relative Bioactivity of Chlorogenic Acid Combustion Products

As mentioned in the previous section, the IVMNT in a short-term *in vitro* genotoxicity and cytotoxicity assay. Genotoxicity testing evaluates the effects of a particular test substance on DNA (DNA damage), which has been linked to carcinogenesis and arteriosclerosis (CORESTA, 2002). Chromosomal aberrations and gene mutations are

key results of DNA damage (Heddle *et al.*, 2010). Cytotoxicity is measured by cell viability (takes into account cell death by apoptosis and necrosis) and is a critical step in the disease process associated with carcinogenesis and emphysema (CORESTA, 2002). This assay has the advantages of avoiding the use of *in vivo* rodent toxicological testing, being applicable to various cell types, providing genotoxic and cytotoxic information simultaneously, being sensitive to tobacco smoke, fast and easy to perform compared to traditional tests (OECD, 2004). The major drawbacks related to this assay include: that it can not assess both vapor and particulate phases, simultaneously; solvents used to prepare extracts may cause interferences; and as with other in vitro biological assays, there can be a high variability between samples based on the heterogeneity of cell cultures (20 % RSD).

The IVMNT can detect compounds that induce the formation of micronuclei (*i.e.* small membrane bound DNA fragments) in the cytoplasm of mammalian cells at the interphase. The compounds that induce the formation of micronuclei are referred to as clastogenic (cause chromosomal loss) or aneugenic (interfere with normal chromosomal segregation) (CORESTA, 2002, OECD, 2004). The micronuclei may result from acentric fragments (chromosomes that do not contain a centromere-structural modification) or chromosomes that do not migrate with the rest of the chromosomes during anaphase of the cell cycle (CORESTA, 2002, OECD, 2004).

A fixed number of cells (5×10^5 cells) are harvested in 25 cm^3 flasks one day prior to exposure. The general procedure for the IVMNT begins with a 3 hour exposure of cells (mammalian cells such as: V79 Chinese hamster fibroblast cells; or human lymphocytes) to the test substance (CORESTA, 2002, OECD, 2004). Following exposure, cytochalasin B is added to block cell division, but not nuclear division. Cells are grown long enough to

undergo nuclear division and observe binucleated interphase cells (OECD, 2004). Those interphase cells that have undergone chromosomal damage, will contain additional micronuclei. The cells are harvested, fixed to glass slides, stained with a fluorescent dye (acridine orange) and microscopically analyzed for the presence of micronuclei. Positive (mitomycin C, a clastogen) and negative controls (1 % solvent, *i.e.,* DMSO) are included in every experiment. DMSO is typically used as the solvent for samples in *in vitro* assays and cigarette smoke total particulate matter, due to its excellent solvent properties for polar and non-polar compounds. At the 1 % concentration, DMSO does not interfere with the assay (Misra *et al.*, 2010). A positive test result is indicated by a dose-response relationship different than that of the negative control and is comprised of usually 3 test concentrations (OECD, 2004).

Slides are scored at 400× magnification according to Fenech's criteria (Fenech *et al.*, 2003).

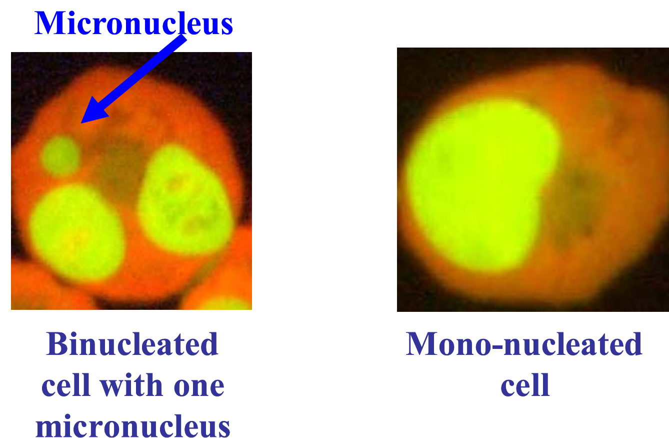

Figure 2-8: Photograph of fluorescently stained cells (binucleated cell with one micronucleus on left and a mono-nucleated cell on the right) obtained from the IVMNT assay.

The percentage of micronuclei, which is a measure of genotoxicity, is determined by first selecting 1000 binucleated cells and then counting the number of these having at least one micronucleus detected, as follows:

$$\%\,Micronuclei = \left(\frac{No.\,of\ binucleated\ cells\ with\ one\ or\ more\ micronuclei}{Total\ No.\,of\ binucleated\ cells} \right) \times 100$$ **(Equation 2-2)**

The percentage of inhibition of cell proliferation was calculated by first determining the Cytokinesis-Block Proliferation Index (CBPI) as follows:

$$CBPI = \left(\frac{No.\,binucleated\ cells + 2[No.\,of\ tri\text{-}, tetra\text{-}\ and\ multi\text{-}nucleated\ cells]}{Total\ No.\,cells\ -\ mitotic\ cells} \right)$$ **(Equation 2-3)**

$$\%\,Inhibition\ of\ cell\ proliferation = 100 - \left(\left[\frac{mean\ CBPI\ sample\ dose}{mean\ CBPI\ solvent\ control} \right] \times 100 \right)$$ **(Equation 2-4)**

The average and relative standard deviation (RSD) for the percentages of micronuclei and inhibition of cell proliferation are generally calculated from duplicate experiments.

3 Sequential Fractionation with Concurrent Chemical and Toxicological Characterization of the Combustion Products of Chlorogenic Acid

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Note on my contribution

My participation in research tasks: I carried out the majority of the experimental work and data analysis. Martine Lacasse was involved in a part of the method development for combustion was responsible for the experimental work involving the selective extraction of the extracts of chlorogenic acid combustion products, and Alexandra Fürtös was involved in training me on the LC/TOFMS instrument as well as providing advice on the separation method development[8].

Publication: I wrote the article which took into account comments/corrections provided by Alexandra Fürtös, André Morin (co-supervisor) and Karen C. Waldron (supervisor).

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[8] Mari Bratberg, a summer student I co-supervised with K.C. Waldron, also contributed to the method development, which is described in Appendix B: Maximization of the HPLC-MS Separation of a Standard Mixture of Seven Compounds found in the Combustion of Chlorogenic Acid

3.1 Abstract

Chlorogenic acid is the most abundant polyphenol found in the tobacco plant. The biological effects of its combustion products remain largely unknown. In this study, chlorogenic acid was burned at 640 °C for 2 min and the particulate matter of the smoke was collected onto Cambridge filter pads followed by selective extraction in five different solvents. Various fractions of the chlorogenic acid combustion products were tested for induction of micronuclei in V79 Chinese hamster fibroblast cells. Over 40 compounds were identified in the dimethyl sulfoxide (DMSO) extract by high performance liquid chromatography coupled to electrospray time-of-flight mass spectrometry (HPLC/TOF-MS). The DMSO extract was then fractionated into three major fractions by preparative LC. The fraction inducing the highest degree of toxicity was further separated into four sub-fractions. The sub-fraction responsible for the most toxic response was determined to contain catechol as its major component. The overall reproducibility of the combustion, the extraction procedure and the chemical characterization of the compounds responsible for the toxicity in the chlorogenic acid smoke were evaluated by LC/TOF-MS.

3.2 Introduction

Tobacco consists of over 2000 components and upon combustion generates more than 7000 compounds (Rodgman *et al.*, 2009b). Due to the highly complex nature of tobacco smoke, the exact mechanisms of toxicity are still unknown. For instance, a number of lists of cigarette smoke toxicants have been published in recent years, some of which have begun to estimate the relative toxicity of the compounds found in tobacco smoke

(Rodgman *et al.*, 2003). However, these approaches are unable to account for the complex chemical profile and potential interactions that may occur in cigarette smoke. Many studies have been carried out on whole tobacco smoke in efforts to determine the correlation between tobacco smoke components and their biological effects (Hoffmann *et al.*, 1997, Jansson *et al.*, 1986, Paschke *et al.*, 2002).

An alternative approach is to study the individual components found in leaf tobacco, which upon combustion generate a variety of bioactive species. Among the major groups of constituents found in tobacco, the polyphenol group accounts for about 10 % of the leaf dry weight (Stedman, 1968, Huber, 1989). Among the polyphenols, chlorogenic acid (CGA) (3-[[3-(3,4-dihydroxyphenyl)-1-oxo-2-propenyl]oxy]-1,4,5-trihydroxycyclohexanecarboxylic acid) is the most abundant single constituent. It represents about 1 – 3 % or higher of leaf dry weight of the tobacco plant (Runeckles, 1963, Wynder *et al.*, 1967). Several studies have identified components found in smoke from the combustion of CGA (Sharma *et al.*, 2002a, Schlotzhauer *et al.*, 1982, Sakuma *et al.*, 1982, Schlotzhauer *et al.*, 1992), and other studies have identified CGA as well as some of its combustion products as being genotoxic and carcinogenic (Stich *et al.*, 1981, Li *et al.*, 1997, Gopalakrishna *et al.*, 1994). Combustion of CGA principally generates pyrocatechol (more commonly known as catechol), phenol, hydroquinone, quinide, benzene and benzoic acid. Some of these phenolic compounds were reported to be toxic (Schlotzhauer *et al.*, 1992, McCue *et al.*, 2003, Robertson *et al.*, 1991) whereas catechol and phenol were reported to enhance carcinogenic processes induced by other compounds such as polynuclear aromatic hydrocarbons (Hoffmann *et al.*, 1983).

A few groups have reported toxicological data on individual components found in tobacco smoke (Li *et al.*, 1996, Li *et al.*, 1997, McCue *et al.*, 2003, Préfontaine *et al.*, 2006, Poirier *et al.*, 2001). A previous study (Préfontaine *et al.*, 2006) indicated that of twelve tobacco components tested, the combustion products of the two polyphenols, CGA and lignin contained the most bioactive components, evaluated by the *in vitro* micronucleus test (IVMNT). The IVMNT is an *in vitro* genotoxicity test used to identify chemicals that induce the formation of small, membrane-bound deoxyribonucleic acid fragments, called micronuclei, in the cytoplasm of interphase mammalian cells (Health Canada, 2004b, Health Canada, 1999, OECD, 2004, CORESTA, 2002). CGA is the least complex and most readily available of the two above polyphenolic compounds found in tobacco, therefore, it was chosen for further investigation. The objective of the current study was to identify the toxic compounds resulting from the combustion of CGA under atmospheric conditions. The so-called incomplete combustion conditions were adopted to mimic the process occurring during the burning of a cigarette. A strategy to partially combust, extract, fractionate and concurrently evaluate the chemical composition and relative toxicity of the combustion products of CGA by *in vitro* toxicological assays was designed. Our approach combines analytical chemistry and *in vitro* toxicology to expand knowledge on the toxicity of smoke constituents generated from the partial combustion (*i.e.*, burning) of one single tobacco component, chlorogenic acid.

3.3 Experimental

3.3.1 Chemicals and reagents

All standards and reagents used for the combustion reproducibility study were supplied by Sigma–Aldrich (St. Louis, MO, USA) and were of ≥ 99.0 % purity unless otherwise indicated: hydroquinone (123-31-9), phenol (108-95-2), *m*-cresol (108-39-4), *p*-cresol (106-44-5), *o*-cresol (95-48-7), pyrocatechol (120-80-9), resorcinol (180-46-3), 3,4-dihydroxybenzoic acid (90-50-3), caffeic acid (331-39-5), trans-cinnamic acid (140-10-3), ferulic acid (1135-24-6), 2,5-dihydroxybenzoic acid (490-79-9), *p*-hydroxybenzoic acid (99-96-7), 1,2-cyclohexanedione (765-87-7) at 97 %, *p*-coumaric acid (501-98-4) at 98 % and CGA (CAS 327-97-9) at ≥ 95 % purity. Glass wool (Pyrex brand wool filtering fiber) was purchased from Corning (Big Flats, NY, USA). The HPLC grade solvents used for the filter extraction and the CGA combustion reproducibility study were dimethyl sulfoxide (DMSO), methanol (MeOH), dichloromethane (DCM), ethyl acetate (EA), acetonitrile (ACN), acetic acid and formic acid, all purchased from Fisher Scientific (Whitby, Canada) and used without further purification. Water used for the filter extraction was either distilled water purified using a Milli-Q system (Millipore, Billerica, MA, USA), which consisted of a carbon cartridge, two high-capacity mixed ion exchange cartridges and a 0.45 μm filter (Chromatographic Specialties, Brockville, Canada) or HPLC grade water from Fisher Scientific. Formic acid for HPLC/MS studies was obtained from Fluka (Buchs, Switzerland). Benzoic acid (65-85-0) at 99.5 % purity was supplied by Laboratoire MAT (Beauport, Canada). Appropriate ventilation measures and protection of researchers were employed for all manipulations that involved the use of organic solvents and compounds

known or suspected to be toxic. The operation of all instruments used in this study was carried out according to the safety procedures recommended by the manufacturers.

3.3.2 Sample preparation

Aliquots composed of 0.5 g CGA dissolved in 5 mL of MeOH were mixed with the aid of a vortex then deposited onto a matrix of 0.5 g of glass wool in individual Petri dishes. To evaporate the MeOH, the sample was stored for at least 72 h in a conditioned room at 22.5 ^{o}C with 60 % relative humidity. Following the storage period, the corrected mass of CGA adsorbed on the matrix was determined to ±1.0 mg by subtracting the glass wool matrix and Petri dish mass (includes CGA adsorbed onto the Petri dish) from the total mass of the sample (mass of matrix, CGA aliquot and Petri dish).

3.3.3 Partial combustion of CGA and collection/extraction of the particulate phase

The CGA sample adsorbed onto the glass wool matrix was transferred from the Petri dish and packed (7.5 cm bed length) into a quartz combustion tube (outer dimensions: 26.5 cm × 1.2 cm, wall thickness: 1 mm). A John Payne Tar Predictor (JPTP) (John Payne Machinery Spares, Winchester, UK) apparatus was used to burn CGA and collect the particulate phase of its smoke. The quartz tube that contained the CGA sample was automatically driven into the furnace where the burning process was conducted at 640 ^{o}C ±10 ^{o}C for 2 min. During this time, atmospheric air was drawn through the quartz tube at 1.8 L/min, forming smoke that passed through the Cambridge filter of diameter 55 mm (Borgwaldt, Richmond, VA, USA) which trapped the particulate phase of the smoke, or

total particulate matter (TPM). Full combustion in air, by definition, should render all organic compounds to CO, CO_2 and H_2O. Therefore, to be accurate, the burning process employed here results in partial or incomplete combustion of CGA. Silicone grease was used to avoid leaking of smoke from the tubing at specific locations.

To allow for deposition of the particulate phase, each Cambridge filter was set aside for a period of 15 – 60 min. The Cambridge filter was weighed to ±0.1 mg before and after the burning process to determine the mass of collected TPM. The particulate matter collected on the Cambridge filter was extracted under vacuum, using a Büchner funnel, by adding drop-wise a specific volume of solvent as follows. For DMSO extraction, the volume of DMSO used was that needed to obtain a final concentration of 15 mg/mL of TPM, assuming 100 % extraction efficiency[9]. For the other solvents, the extraction volume was fixed at 10 mL per filter to obtain a suitable volume for the subsequent biological assay. The extraction solvent was then evaporated using a rotary evaporator (except when water was used) (Rotavapor-R, Büchi, Switzerland) followed by lyophilization (FreeZone 4.5 L Benchtop Freeze Dry System, Labconco, Kansas, MO, USA). The dry particulate matter (DPM), which refers to the residue remaining after the evaporation of the extraction solvent, was reconstituted in DMSO to give a final concentration of 15 mg/mL of DPM for the water, MeOH and EA extracts and 5 mg/mL for the DCM extract. A more dilute solution of the DCM extract was necessary to maintain a manageable volume since very little DPM was obtained. For each different solvent a new set of Cambridge filters with collected material from CGA burning was utilized. An “extract” resulted from pooling the

extraction solutions of three Cambridge filters unless otherwise stated. Extracts were then aliquoted into 1.5 mL vials and stored in the dark at -80 ^{o}C. All toxicity and chromatography experiments using the extracts were performed in duplicate, unless stated otherwise.

3.3.4 Mammalian cell cultures

The cellular lineage used for the IVMNT assay was an internationally registered V79 Chinese hamster cell line (lung fibroblast) obtained from the European Collection of Cell Cultures (V79 86041102 lot 04/C/016). Cells were cultured in complete culture medium (Dulbecco Modified Eagle Medium, DMEM; Gibco, Grand Island, NY, USA) supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (FBS) and 0.5 % (v/v) penicillin/streptomycin (50 units/mL, 50 μg/mL), both from Gibco. Cells were re-suspended by trypsinization (0.1 % trypsin, 1.06 mM EDTA; Gibco) at 37 ^{o}C. Subcultivation of cells was performed two times per week ((1.0–2.0) $\times$ 10^5 cells) into a 75 cm^3 Corning flask.

3.3.5 *In Vitro* Micronucleus Test (IVMNT)

The IVMNT was performed with V79 Chinese hamster fibroblast cells without metabolic activation (S9 fraction). Cells were grown in 25 cm^3 flasks at a concentration of 5.0×10^5 cells/mL in 10 mL of DMEM for 24 h. The culture medium was then replaced by the DMSO-dissolved extracts added to DMEM at the following concentrations to which the

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[9] DMSO is an aprotic polar solvent with a polarity index of 7.2 and is an excellent solvent for polar organic compounds, acids, alkalis and mineral salts. Thus, it is likely that DMSO has an extraction efficiency less than 100 % for some of the non-polar compounds found in the particulate phase of tobacco smoke.

cells were exposed for three hours: 5, 10, 15 and 20 µg of DPM (or TPM) per mL of DMEM. The positive control was mitomycin C (MMC, 0.8 µg/mL; Sigma–Aldrich) and the negative control was DMSO (1 % (v/v) in DMEM). After the 3 h exposure, cells were rinsed twice with Hanks' Balanced Salt Solution (HBSS, Gibco) and re-incubated for 17 h in DMEM containing 3 µg/mL cytochalasin B (which blocks cellular division, but does not block nuclear division). Cells were harvested by trypsinization, re-suspended in culture medium at 1.0×10^5 cells/mL and centrifuged onto microscopic slides at 1200 rpm for 8 min using a Cytospin 3 (Shandon, London, UK). Slides were then air dried, fixed in 90 % methanol (9 min at -20 ^{o}C) and stained with Acridine Orange solution for 30 s (12.5 mg/100mL of 1×-PBS; Sigma–Aldrich). Finally, slides were scored at 400× magnification according to Fenech's criteria (Fenech *et al.*, 2003). The percentage of micronuclei, which is a measure of genotoxicity, was determined by first selecting 1000 binucleated (BN) cells and then counting the number of these having at least one micronucleus detected, as follows:

$$\%Micronuclei = \left(\frac{No. of\ BN\ cells\ with\ one\ or\ more\ micronuclei}{Total\ no. of\ BN\ cells} \right) \times 100$$

where a micronucleus is defined as a particle surrounded by distinct borders, having a maximum of one third the size of the main nucleus and lying inside the cytoplasm (Frieauff *et al.*, 1998). The percentage of inhibition of cell proliferation was calculated by first

determining the Cytokinesis–block Proliferation Index (CBPI) (Fenech *et al.*, 2003) as follows:

$$\mathrm{CBPI} = \left(\frac{\mathrm{No.\,BN\,cells} + 2[\mathrm{No.\,of\,multi\text{-}nucleated\,cells}]}{\mathrm{Total\,no.\,of\,cells\ -\ mitotic\,cells}} \right)$$

where multi-nucleated cells are those having three or more nuclei

$$\%\mathit{Inhibition\ of\ cell\ proliferation} = 100 - \left(\left[\frac{\mathrm{mean\,CBPI\,sample\,dose}}{\mathrm{mean\,CBPI\,solvent\,control}} \right] \times 100 \right)$$

The average and relative standard deviation (RSD) for the percentages of micronuclei and inhibition of cell proliferation were calculated from duplicate experiments.

3.3.6 Reproducibility study of the CGA combustion

The precision of the combustion of CGA was evaluated by HPLC using a Waters 2695 Separation Module with a Waters 715 Ultra Wisp automatic injector (Milford, MA, USA). Detection was achieved with a Waters 2475 Multi wavelength fluorescence (FL) detector. The instrument was controlled by ChemStation Plus Family software version A.08.03 (Agilent Technologies). Separation was achieved on a Spherisorb, ODS2 analytical column (5 µm particles, 150 mm × 4.6mm) from Waters.

Reproducibility of the combustion method was determined by comparing the quantity of selected phenolic compounds obtained from four different combustions (Health Canada, 1999, Risner *et al.*, 1990), but using only 25% of the TPM from each. A quarter of each

Cambridge filter (one per combustion) was extracted with 10 mL of 1% (v/v) aqueous acetic acid for 30 min on an orbital shaker. The four extracts from the four combustions were each filtered through a 0.45 μm filter, of which, 2 mL was collected for analysis by HPLC/FL. The volume of each extract injected was 10 μL. Separation was achieved by gradient elution (0–100 % ACN in 1 % (v/v) aqueous acetic acid over 46 min) at a mobile phase flow rate of 1.2 mL/min. The total run time was 66 min. Quantification was achieved by external calibration as follows. A stock solution of 1.00 mg/mL of each standard compound was prepared in 1 % (v/v) aqueous acetic acid. From the stock solutions, six working solutions, ranging from 0 to 50 μg/mL, were prepared in 1 % (v/v) aqueous acetic acid, filtered through a 0.45 μm filter and transferred into 2 mL amber vials. A 20 μL volume of each working solution was injected in duplicate and a standard calibration curve was made by plotting the concentration of the working solutions versus their respective peak areas.

3.3.7 Reproducibility study of the extraction with DMSO and DCM

HPLC/MS was used to assess the precision of the DMSO and DCM extraction procedure. The instrument consisted of an 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) directly interfaced with an Agilent electrospray ionization single quadrupole mass spectrometer (LC/MSD). Injections of 5 μL (75 μg of product per injection) of DMSO or DCM extracts (the latter having been re-suspended in DMSO) were made onto an Eclipse XDB-C18 analytical column (5 μm particles, 150 mm × 4.6mm) from Agilent Technologies. Separation was achieved using a gradient elution of 0–80% MeOH in 0.1 % (v/v) aqueous formic acid over 24 min at a flow rate of 0.5 mL/min. The

total run time was 30 min. For mass spectrometric detection, ions were generated in negative electrospray mode with 4000 V applied on the capillary. The fragmentor was set at 70 V and the drying gas (N_2) was heated at 300°C and run at 10 L/min. Spectra were acquired from m/z 75 to m/z 575 at a rate of 0.94 s/cycle. The reproducibility of the method of extraction by DMSO and DCM was determined by comparing the peak areas (for duplicate injections) of the following 13 phenolic reference compounds consistently found in the 4 different DMSO extracts: catechol, hydroxyquinone, 4-methyl catechol, 4-vinyl catechol, 2-hydroxybenzoic acid, 4-hydroxybenzoic acid, 4-ethyl catechol, 1-(3,4-dihydroxyphenyl) ethanone, *p*-coumaric acid, coumaric acid, hydrocaffeic acid, quinic acid and caffeic acid methyl ester.

3.3.8 Analytical separation of the DMSO extract

Accurate mass-based identification of several products found in the DMSO extract was achieved using an Agilent 1100 HPLC system directly interfaced with a 6120 series electrospray ionization time-of-flight (TOF) mass spectrometer from Agilent Technologies. The LC/TOF-MS instrument was controlled by Agilent Mass Hunter software, and the data was processed by Analyst QS software (Agilent Technologies/Sciex). Samples were diluted 1:100 in HPLC grade water and 2 µL aliquots (0.3 µg of product per injection) were injected onto the Eclipse XDB-C18 analytical column. The chromatographic separation was performed in gradient mode (0–80 % MeOH in 0.1 % v/v, aqueous formic acid over 45 min) at a flow rate of 0.5 mL/min. The total run time was 60 min. For MS detection, ions were generated in negative electrospray mode with 4000 V applied on the capillary. The

fragmentor was set at 200 V and the heated drying gas (N_2 at 350°C) was run at 12 L/min. Spectra were acquired from m/z 50 to m/z 1000 at a rate of 0.94 s/cycle.

3.3.9 Preparative fractionation of the DMSO extract[10]

The LC system used for preparative fractionation of the DMSO extract consisted of a Gilson 215 LC Handler with 156 UV-VIS absorbance detector (Middletown, WI, USA) directly interfaced with an LCQ single quadrupole mass spectrometer from Thermo Fisher Scientific (Waltham, MA, USA). The instrument was controlled by XCalibur software, version 1.3 (Thermo Fisher) and Gilson Unipoint software. DMSO extracts (15 mg/mL) were injected (1.8 mL) and separations were performed on a Prevail C18 preparative column (5 μm particles, 250 mm × 22mm) from Alltech (Lexington, KY, USA) by gradient elution (0–80 % MeOH in 0.1 % (v/v) aqueous formic acid over 20 min) at a flow rate of 15 mL/min. The total run time was 30 min and the UV signal was recorded at 254 nm concomitant to monitoring the MS signal. Fractions of 8 mL each were collected every 39.1 s into borosilicate disposable culture tubes (10mm × 100mm; Fisher Scientific) and then pooled to give three major fractions spanning the following time intervals: 0-14.2 min, 14.2-23.5 min and 23.5-30 min. A second injection of 1.8 mL (27 mg) was treated identically and pooled with the corresponding major fractions from the first injection in round bottom flasks. The three (pooled) fractions were reduced in volume using a rotary evaporator for approximately 10 min at 30 ^{o}C under a moderate rotation speed. The flasks were then immersed and rotated in acetone/dry-ice to induce uniform sample freezing. Finally, the remaining liquids were lyophilized overnight and re-suspended in 50% MeOH

(aq), transferred into pre-weighed vials which were again rotavapped, lyophilized and weighed to obtain the correct mass for each fraction. The quantities of the products obtained were, 39.5, 22.5 and 7.9 mg respectively, for the first through third pooled fractions. The fractions were stored at -80 ^{o}C in clear glass vials. Approximately 29 % more material was collected than was injected (69.9 mg collected versus 54 mg injected, by calculation). This discrepancy is probably due[11] to residual DMSO in the first fraction that can not be entirely evaporated by lyophilization.

3.3.10 Chemical characterization and separation of fraction 2

The LC/TOF-MS described above, which is a high resolution system, was used for the chemical characterization of the most bioactive fraction of the DMSO extract. This was achieved by first using the lower resolution LC/MSD system (see section in Reproducibility studies) to optimize the separation of a test mixture representative of fraction 2, comprised of the following seven standards: caffeic acid, benzoic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, trans-cinnamic acid, 4-hydroxy-3-methoxycinnamic acid and 2,5-dihydroxybenzoic acid. This test mixture was injected onto four different stationary phases: Synergi Polar-RP (4 µm, 150mm × 4.6mm), Synergi Hydro-RP (4 µm, 150mm × 4.6mm), Gemini C18 (5 µm, 150 × 4.6mm) and Gemini C6-Phenyl (5 µm, 150mm × 4.6mm), all from Phenomenex (Torrance, CA, USA). Separations were carried out under nine different gradient elution conditions by varying the initial MeOH concentration as follows: 10, 15, 20, 25, 30, 35, 40, 45 and 50 %, in 0.1 % (v/v) aqueous

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[10] A detailed experimental flow chart is given in Appendix A.
[11] Assumption made.

formic acid, with the gradient applied up to 75 %, over the first 24 min in each case. The best gradient conditions were transferred to the higher resolution LC/TOF-MS instrument and applied to the separation of fraction 2 components. Samples were first diluted 100-fold in 50 % MeOH (aq) to make them compatible with the dynamic range of the LC/TOF-MS, then injections of 2 µL (corresponding to 0.3 µg of product) were made on the four columns listed above. The total run time was 30 min at a flow rate of 0.5 mL/min.

3.3.11 Preparative sub-fractionation of fraction 2

To sub-fractionate "fraction 2" of the DMSO extract by preparative LC, an injection of 2.0 mL was made on the instrument described for preparative fractionation of the DMSO extract. Samples (4.24 mg/mL in 75%, v/v, MeOH (aq)) were injected in duplicate and separations were performed on an AXIA packed Synergi Polar-RP preparative column (4 µm particles, 100 mm × 21.2mm) from Phenomenex. A Polar-RP security guard prep cartridge (15mm × 21.2 mm) from Phenomenex was installed upstream of the preparative column. The chromatographic separation was performed in gradient mode (15–75% MeOH in 0.1 %, v/v, aqueous formic acid over 20 min) at a flow rate of 6 mL/min. The total run time was 30 min and the UV signal was monitored at 254 nm concomitant with the MS signal. Fractions of 4 mL each were collected every 19.8 s into borosilicate disposable culture tubes (10mm × 100mm; Fisher Scientific) and then pooled to give four large sub-fractions spanning the following time intervals: 12.0-15.2, 15.2-16.4, 16.4-21.2 and 21.2-30 min. Each pooled sub-fraction was placed in a round-bottom flask and was treated as described above during the first fractionation step. The amounts of product obtained for the first through fourth pooled sub-fractions were 2.16, 1.49, 5.87 and 9.07 mg respectively. A

small portion of (major) fraction 2 of the DMSO extract was used for control studies. Samples were kept at -80°C in clear glass vials until utilization.

3.3.12 Chemical characterization of sub-fraction 1

The LC/TOF-MS system described above was used for the accurate mass identification of products present in the sub-fraction displaying the highest toxicity. Samples were diluted 1:100 in 50 % (v/v) MeOH (aq) and injections of 2 µL aliquots were performed on the Polar-RP column (4 µm, 150 mm × 4.6mm) followed by separation by gradient elution (0–80 % MeOH in 0.1%, v/v, aqueous formic acid over 24 min) at a flow rate of 0.5 mL/min. The total run time was 15 min.

3.3.13 Statistical analysis

The results for the combustion reproducibility study were tested for comparison of linearity between different groups of either extracts or fractions using the analysis of covariance (ANCOVA) method. For comparison between the DMSO extracts, the percentages of micronuclei (genotoxicity) and inhibition of cell proliferation were taken as the direct quantitative variable, the dose of exposure as the quantitative dependent covariable and the extraction solvent was taken as the qualitative covariable for two replicates. ANCOVA compares the dose–response linearity between each extract. Significant differences between extracts were determined by the Duncan's multiple comparison test and were considered significant when $p < 0.05$. Toxicological data obtained from the IVMNT for the different solvent extracts and fractionation studies were analyzed using XLSTAT software, version 7.5 (Addinsoft Brooklyn, NY, USA).

Analysis of variance (ANOVA) was used to evaluate the toxicity results where the dose, the CGA extracts, the fractions and the sub-fractions were all considered as factors. The dose by extract/fraction interaction was also included in the model. In order to assess differences between the CGA extracts/fractions for the different doses, the dose by extract interaction was investigated using multiple comparisons. More specifically, the extracts/fractions were analyzed by the Fisher least significant difference multiple comparison test with a Bonferroni correction to type 1 error to ensure that the overall risk was kept under $\alpha = 5\%$. In all cases, the background level of genotoxicity generated by the control solvent (1% DMSO) was subtracted from the micronuclei percentage values for all samples. As a result of the statistical analysis, the data were grouped as follows: A, B or C. Samples sharing the same letter, *i.e.*, lie within the same group, are not statistically different.

3.4 Results and discussion

The various toxicological studies carried out on tobacco smoke have been generally related to the combustion products of whole tobacco (Frenesius, 1985, Rodgman, 2003, DeMarini, 2004). The aim of our study was to characterize the toxicity of the combustion products of one individual tobacco component, CGA, which is the major polyphenolic component of tobacco. A few toxicological studies have reported on the genotoxicity of CGA (Stich *et al.*, 1981) and its incomplete combustion products (Schlotzhauer *et al.*, 1992, Hoffmann *et al.*, 1983, Préfontaine *et al.*, 2006). In addition, some chemical studies

have been published on the identification of CGA combustion products (Sharma *et al.*, 2002a, Sakuma *et al.*, 1982). However, no previous study on relating genotoxicity to the chemical composition of the combustion products of CGA has been made. The combustion conditions used in this study were chosen based on the range of temperatures found during the combustion of cigarettes, which occur between 300 °C and 900 °C and higher (Baker, 1981). The precision of our chemical analyses required a robust and reproducible means of simulating the partial combustion of CGA, which was why the JPTP apparatus was employed. Furthermore, this study was carried out at a single combustion temperature of 640 °C for simplicity.[12]

3.4.1 Reproducibility study of the CGA partial combustion process

In order to understand and quantify any variability in the toxicological and/or chemical analyses, it was deemed important to evaluate the precision of the CGA combustion method. This was assessed by comparing: (a) the phenolic content in four different extracts by HPLC/FL and (b) the genotoxicity and the degree of inhibition of cell proliferation between three of the four extracts using the IVMNT. The Cambridge filters were extracted with 1% (v/v) aqueous acetic acid for this study because this solution is known to extract phenolic compounds well (Risner *et al.*, 1990).

HPLC/FL showed that the concentration of hydroquinone, resorcinol, catechol and phenol (reported as a function of the quantity of TPM extracted per quarter filter) varied with an average RSD of 15.5% (and median RSD of 12.6%) (Table 3-1). *p*-Cresol was

often below the limit of quantification. The high polarity of the solvent may have impeded the extraction of *p*-cresol, thus explaining why the latter was barely detected[13]. To evaluate the relative proportion of each compound, their concentrations were normalized relative to hydroquinone (Table 3-2) for each experiment to eliminate the sampling error associated with extracting only ¼ of the filter pad. The relative (*i.e.* normalized) concentrations of the phenolic compounds resorcinol, catechol and phenol determined by HPLC/FL, showed an average of 11.1 % RSD (Table 3-2). This precision is in close agreement with the HPLC/FL determination of phenols in the particulate phase of mainstream cigarette smoke of the 1R5F reference cigarette reported recently by Moldoveanu and Kiser (Moldoveanu *et al.*, 2007). As seen in Table 3-1, the concentration of phenol varied the most among the four combustions; its RSD was more than twice that of the other phenolic compounds. Although phenol is the most volatile of the five species, ineffective trapping was ruled out as a source of its high variability because the temperature did not exceed 45 °C at the Cambridge filter pad position[14]. Although the experimental procedure was identical for each sample, it is possible that slight differences in the rotavaporation step may have contributed to losses of phenol in some samples. Calibration using an internal standard such as 4-chlorophenol deposited on the Cambridge filter pad prior to extraction (Moldoveanu *et al.*, 2007) would be necessary to confirm this.

The IVMNT method was chosen to measure the extracted TPM bioactivity because it is one of the *in vitro* toxicity tests recommended for tobacco smoke studies by

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[12] Inside a burning cigarette, temperatures range from ambient to 950 ºC (Baker, 1981) and many bioactive products are formed between 500-800 ºC (Torikai *et al.*, 2004). Below these mentioned temperatures, partial combustion would not be as efficient.
[13] Recovery tests not performed.

CORESTA and Health Canada (CORESTA, 2002, Health Canada, 2004b, OECD, 2004). Overall, although a variation of 11.1 % in normalized phenolic content was present between the extracts, this did not translate into a similar variation in bioactivity. The IVMNT data (Figure 3-1a and 3-1b) showed that the percentages of micronuclei and inhibition of cell proliferation among different extracts were not significantly different as per the Duncan's multiple comparison test. Therefore, we decided to continue with this method of combustion using the JPTP. However, to reduce the impact of the high variability between combustions, we pooled the extracts from three independent combustions to obtain one final pooled extract, which was then divided into equal aliquots and stored at -80°C for subsequent toxicological and chemical assays.

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[14] Phenol has a boiling point of 182 °C, thus, volatility was not the major source of variation at 45 °C.

Table 3-1: Reproducibility of the partial combustion process of CGA: phenolic compounds identified by HPLC/FD in 1% (v/v) aqueous acetic acid extracts of the TPM from ¼ of each of four Cambridge filters.

CGA combustion replicates	TPM qty on ¼ Cambridge filter (mg)	Phenolic content (µg/mg TPM)					Average
		Hydroquinone	Resorcinol	Catechol	Phenol	p-Cresol	
λexcitation (nm)	-	285	270	270	270	270	
λemission (nm)	-	325	310	310	298	305	
Combustion 1	27.8	16.1	0.8	32.9	10.2	0.1	
Combustion 2	33.2	19.5	1.0	40.4	16.1	0.1	
Combustion 3	32.9	19.2	1.0	34.1	10.4	< LOD	
Combustion 4	38.2	15.1	0.9	31.2	9.1	0.1	
Average	33.0	17.5	0.9	34.7	11.5	0.1	
RSD (%)	12.9	12.6	10.4	11.6	27.5	N/C[a]	15.5

[a] N/C: not calculated.

Table 3-2: Normalized phenolic content relative to hydroquinone (from Table 3-1).

CGA combustion replicates	Normalized quantity relative to hydroquinone					Average
	Hydroquinone	Resorcinol	Catechol	Phenol	p-Cresol	
Combustion 1	1.00	0.052	2.042	0.633	0.006	
Combustion 2	1.00	0.053	2.076	0.826	0.007	
Combustion 3	1.00	0.053	1.775	0.539	0.001	
Combustion 4	1.00	0.060	2.067	0.604	0.005	
Average	-	0.054	1.990	0.651	0.005	
SD	-	0.004	0.144	0.124	0.003	
RSD (%)	-	7.1	7.2	19.0	N/C[a]	11.1

[a] N/C: not calculated.

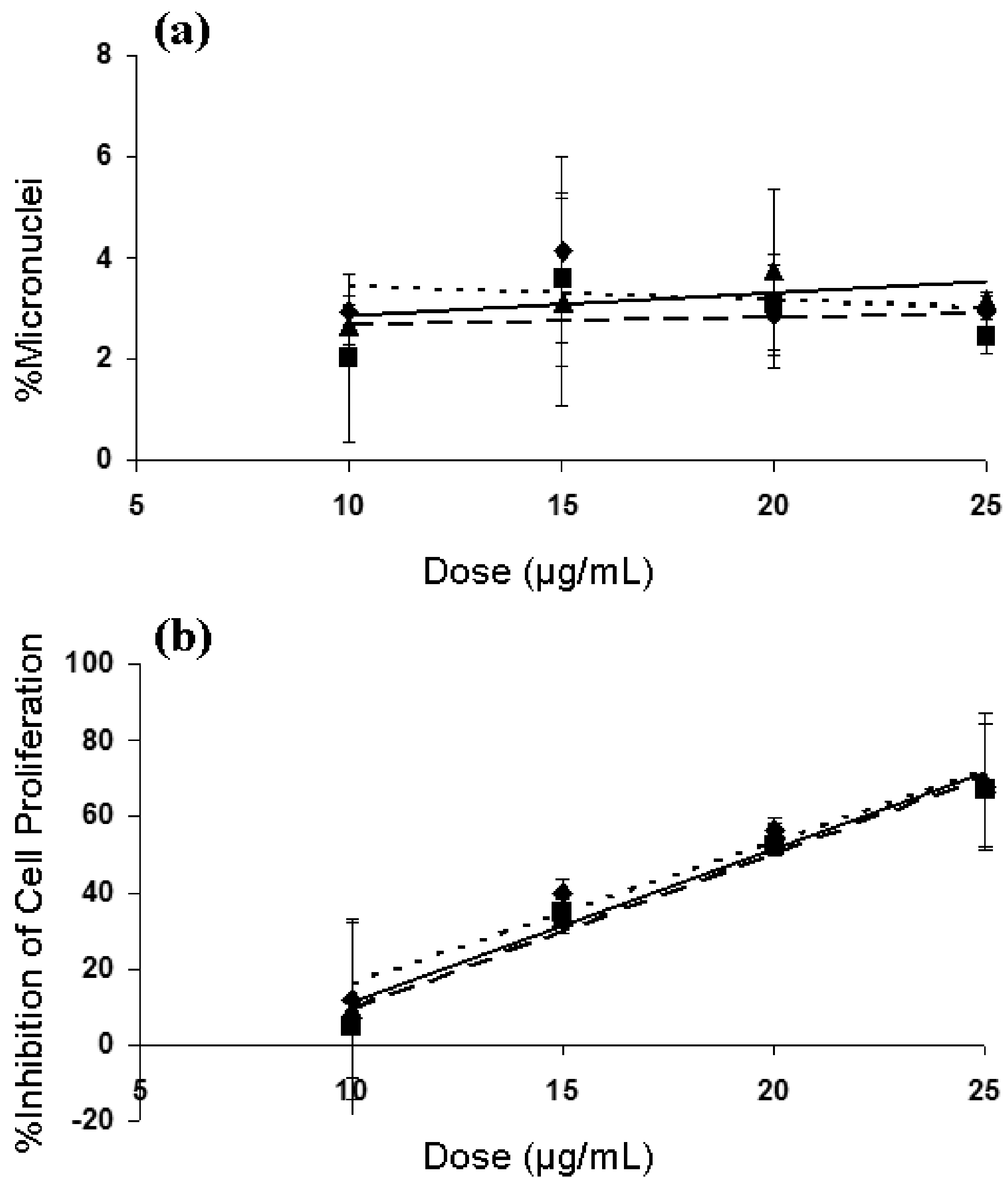

Figure 3-1: The genotoxic activity (% micronuclei), (a), and inhibition of cell proliferation (b), induced by DMSO extracts from three independent combustions of 0.5 g of CGA, on V79 cells exposed for 3 h without metabolic activation. Dose refers to the quantity of TPM or DPM (μg) per mL of medium. The three extracts were tested in the Duncan's test ($\alpha = 0.05$) and no statistically significant difference was found. (····◆····) combustion 1; (– ■ – –) combustion 2; —▲— combustion 3. Error bars indicate standard deviation.

3.4.2 Effect of extraction solvent

Selective solvent extraction was used to initiate the chemical characterization study of CGA combustion products. The five solvents, used in parallel, were DMSO (polarity index (P)=7.2, dipole moment[15] (DM)=3.96), water (P=9, DM=1.85), MeOH (P=5.1, DM=1.70), DCM (P=3.1, DM=1.60) and EA (P=4.4, DM=1.78), thus yielding five different extracts. These solvents were chosen due to their different polarity index values and because of the limited selectivity and high variability observed with 1% (v/v) aqueous acetic acid as an extraction solvent. Schlotzhauer and Chortyk showed that tobacco directly extracted with solvents of various polarity or "extraction strength" yielded extracts of different chemical composition (Schlotzhauer *et al.*, 1981). According to their miscibility and polarity index, the most hydrophilic products are preferentially extracted by DMSO, water and MeOH, whereas less hydrophilic products are found in the DCM and EA extracts. Generally, the phenolic compounds have amphiphilic properties, and thus should be found in every extract. In a previous work (Lacasse, 2007), GC/MS analysis of the five extracts of CGA combustion products showed the presence of phenolic compounds such as catechol, phenol, hydroquinone, ethyl catechol, benzoic acid and quinic acid in most of the extracts.

Based on their chemical composition and the relative amounts of each combustion product, certain extracts among the five tested were expected to induce a higher degree of genotoxicity and/or inhibit cell proliferation than others by the IVMNT. The percentage of micronuclei, or genotoxicity, is shown in Figure 3-2a for the five extracts. At a dose of 20

μg/mL, the genotoxicity induced by the DCM extract was significantly different from the water and MeOH extracts, but not from the DMSO and EA extracts. The inhibition of cell proliferation is shown in Figure 3-2b for the five extracts. At doses of 5 and 10 μg/mL there was no statistically significant difference in the genotoxicity induced, as evaluated by ANOVA. However, at doses of 15 and 20 μg/mL, the inhibition of cell proliferation induced by the DCM extract was significantly different from all other extracts except DMSO at 15 μg/mL, and except water and DMSO at 20 μg/mL. The negative values observed for the inhibition of cell proliferation reflect cell growth. Overall, the DMSO and DCM extracts induced higher biotoxicity compared to extracts obtained using water, EA and MeOH.

DCM is the least polar of the solvents tested and thus would be expected to extract phenolic compounds, which are known to be bioactive. DMSO on the other hand possesses excellent solvating powers; it dissolves both polar and non-polar compounds. Furthermore, a low concentration of DMSO (1 % (v/v) in DMEM) has low toxicity (Vignes, 2000), which was why the other extraction solvents were reconstituted in DMSO for the IVMNT assays[16].

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[15] Dipole moment is related to polarity and describes the distance of separation between charges (nature of bonds). Solvent-solute interactions are affected by their dipole moments.

[16] Following re-constitution in DMSO, extracts were analyzed to ensure that initial solvents were not present to contribute to toxic response.

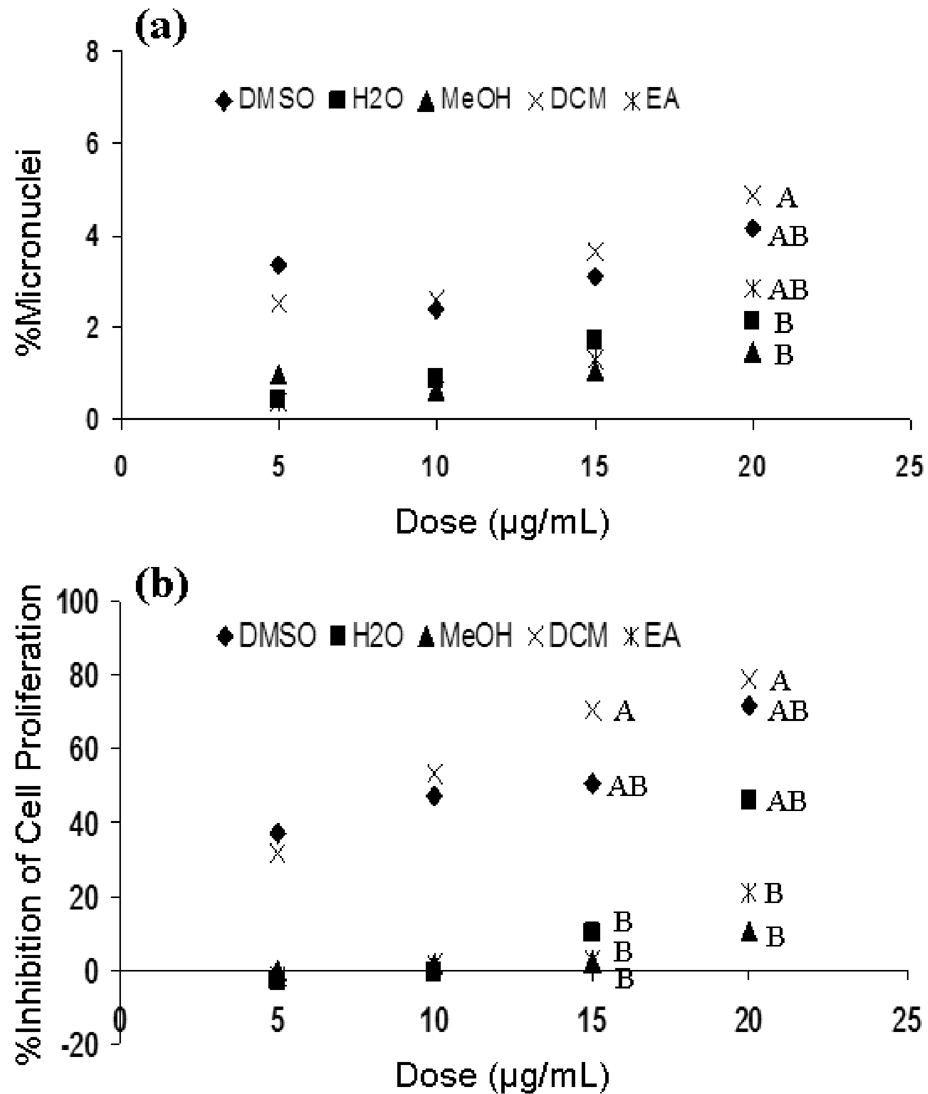

Figure 3-2: The genotoxic activity (% micronuclei), (a), and inhibition of cell proliferation, (b), induced by DCM, DMSO, water, EA and MeOH extracts generated from combustions of 0.5 g of CGA, on V79 cells exposed for 3 h without metabolic activation. Dose refers to the quantity of TPM (µg) per mL of medium. n = 2 for all the condensates except DCM and DMSO, where n = 4. The dose/extraction solvent interactions were analyzed by the Fisher least significant difference multiple comparison test with a Bonferroni correction to type 1 error to ensure that the overall risk was kept under $\alpha = 0.05$. Letters A, B and AB designate different statistical groups. Error bars have been removed for clarity.

3.4.3 Reproducibility study of the extraction with DMSO and DCM

Based on the results comparing extraction by five different solvents, the precision of the DMSO and DCM extraction procedures was evaluated by LC/MS to ensure a robust and reliable method. Four independent combustion experiments were carried out for both DMSO and DCM. Each extract obtained was injected in duplicate. The abundance (peak areas) of 13 reference compounds found in the extracts were monitored (Table 3-3). Retention times were highly reproducible (≤ 0.1% RSD) across the four extracts tested for both extraction solvents. The peak area precision of the DMSO extraction (< 10 % RSD) was nine times better than that of DCM (data not shown)[17]. This may have been due to the volatile nature of DCM; evaporation may have occurred during the extraction procedure leading to less reproducible results. Therefore, DCM extraction was not further investigated. In addition, DMSO was observed to extract a larger number of compounds, which is in keeping with its good solvating strength. With respect to biological activity, the DMSO extracts were not further tested by the IVMNT since the results above showed that variation in genotoxicity and inhibition of cell proliferation was minimal even though phenolic content varied greatly (15.5 % average RSD, Table 3-1).

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[17] For some compounds, the peak area precision was up to nine times better for DMSO extracts, *i.e.*, for quinic acid RSD from DCM extracts was 49 %, compared to 5.6 % for DMSO. The precision may have been due to the volatility of DCM, thus, leading to an inefficient extraction compared to DMSO.

Table 3-3: Reproducibility of the extraction by DMSO (n=4), with respect to peak area for 13 phenolic compounds identified by LC/MSD (negative mode).

Reference product name	Average peak area (10^3)	RSD (%)
Catechol	19 ± 1	5.3
Hydroxyquinone	400 ± 30	7.5
4-Methyl catechol	29 ± 2	6.9
4-Vinyl catechol	710 ± 40	5.6
2-Hydroxybenzoic acid	160 ± 10	6.3
4-Hydroxybenzoic acid	310 ± 20	6.5
4-Ethyl catechol	210 ± 20	9.5
1-(3,4-Dihydroxyphenyl)ethanone	25 ± 2	8.0
p-Coumaric acid (isomer 1)	170 ± 10	5.9
Coumaric acid (isomer 2)	34 ± 3	8.8
Hydrocaffeic acid	120 ± 6	5.0
Quinic acid	54 ± 3	5.6
Caffeic acid methyl ester	30 ± 3	10.0

3.4.4 Analytical separation of the DMSO extract

Accurate mass determination by LC/TOF-MS was used to identify the main components, and class of components, in the whole DMSO extract (Figure 3-3). Over 40 compounds were identified by negative ionization mode, which was used because the majority of the combustion products possessed alcohol and/or acidic functional groups. These results guided the choice of which fractions to pool for preparative LC.

Several phenolic compounds were present in the DMSO extract, which is consistent with previous studies of CGA (Sharma *et al.*, 2002a, Schlotzhauer *et al.*, 1992, Sakuma *et al.*, 1982). Based on the complexity of the combustion products of a single tobacco component like CGA, we can only begin to imagine the complexity of whole tobacco smoke. Only techniques like LC/MS and gas chromatography–MS (Moldoveanu *et al.*, 2007, Vaughan *et al.*, 2008) have the selectivity and resolution needed to provide reliable identification and quantification of such a large range of components. Although the DMSO extract was bioactive according to the IVMNT, it was difficult to identify the specific compounds responsible for bioactivity. Therefore, it was necessary to further simplify the extract. Some potential techniques to achieve this include: filtration, centrifugation, liquid–liquid extraction, solid-phase extraction and sample fractionation, among others. Fractionation by preparative scale LC was chosen based on its ability to divide the sample into precise portions having sufficient quantity for further analysis by the IVMNT.

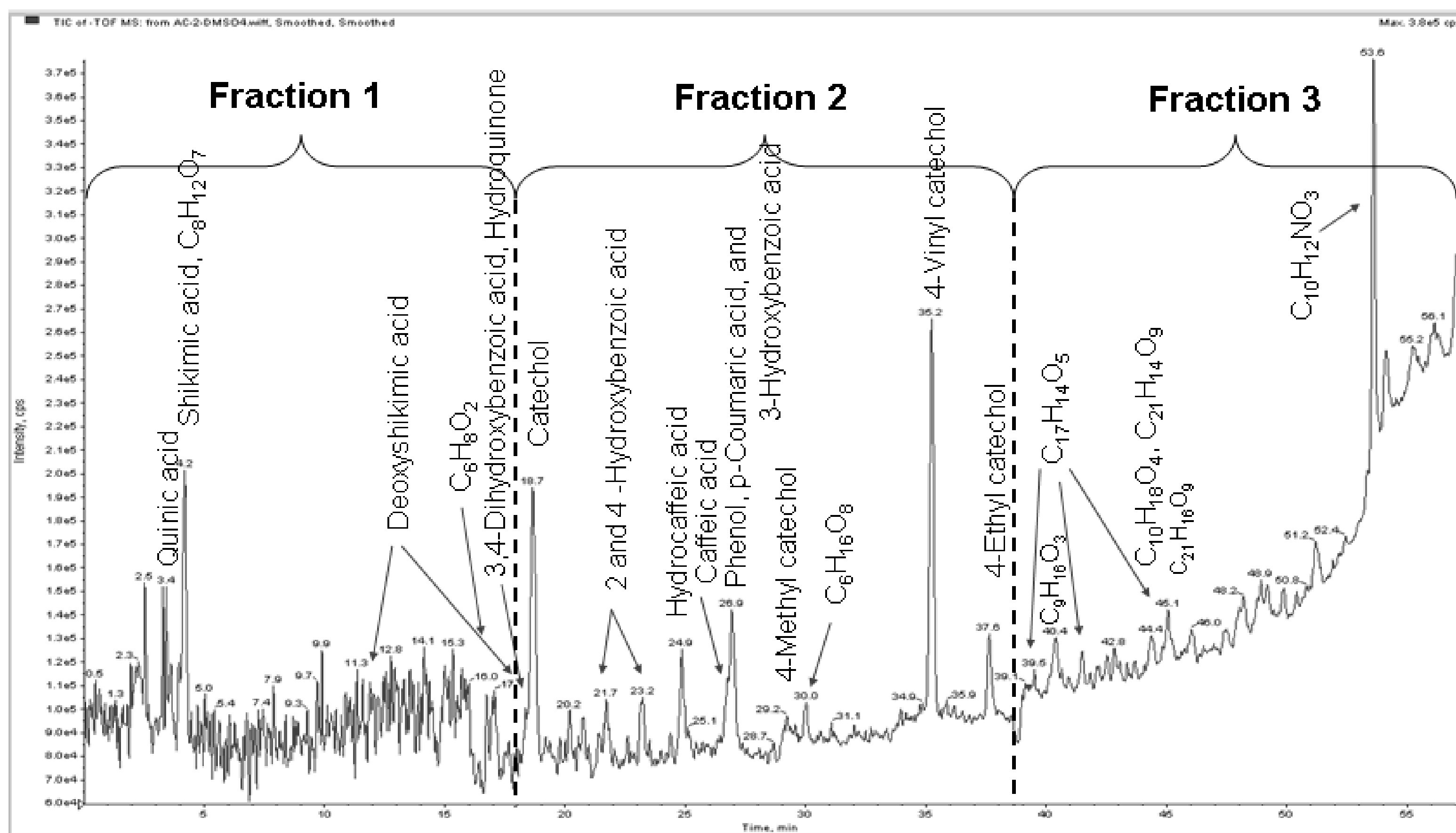

Figure 3-3: Total ion chromatogram of the DMSO extract showing the CGA combustion products, which were analyzed by LC/TOF-MS in negative mode. The dashed lines have been overlaid to represent the compounds isolated in the three main fractions collected.

3.4.5 Preparative fractionation of the DMSO extract

The DMSO extract was fractionated by preparative LC/UV (detection at 254 nm) into three major fractions, as indicated by the dotted lines in Figure 3-3. This allowed for determination of the difference in toxicity between fractions and presumably a convergence on the compounds responsible for the observed toxicity. The first fraction, which was selected to include quinic acid-related compounds and other non UV-absorbing species, contained 39.5 mg of product. The second fraction (22.5 mg) included catechol and its derivatives while the third (7.9 mg) comprised more hydrophobic compounds. Biotoxicity was assessed by the IVMNT. As illustrated (Figures 3-4a and 3-4b), among the three major fractions tested, fraction 2 induced the highest percentages of micronuclei and inhibition of cell proliferation compared to fractions 1 and 3. The increased level of toxicity generated by fraction 2 was likely due to the presence of phenolic compounds found in that fraction. Figure 3-4a shows that the whole DMSO extract as well as the second fraction induced the highest percentage of micronuclei. However, only the (whole) DMSO extract induced a significantly higher percentage of micronuclei at a dose of 35 μg/mL. Figure 3-4b shows that fraction 2 and the (whole) DMSO extract induced a significantly higher inhibition of cell proliferation compared to fractions 1 and 3 at doses of 15–35 μg/mL.

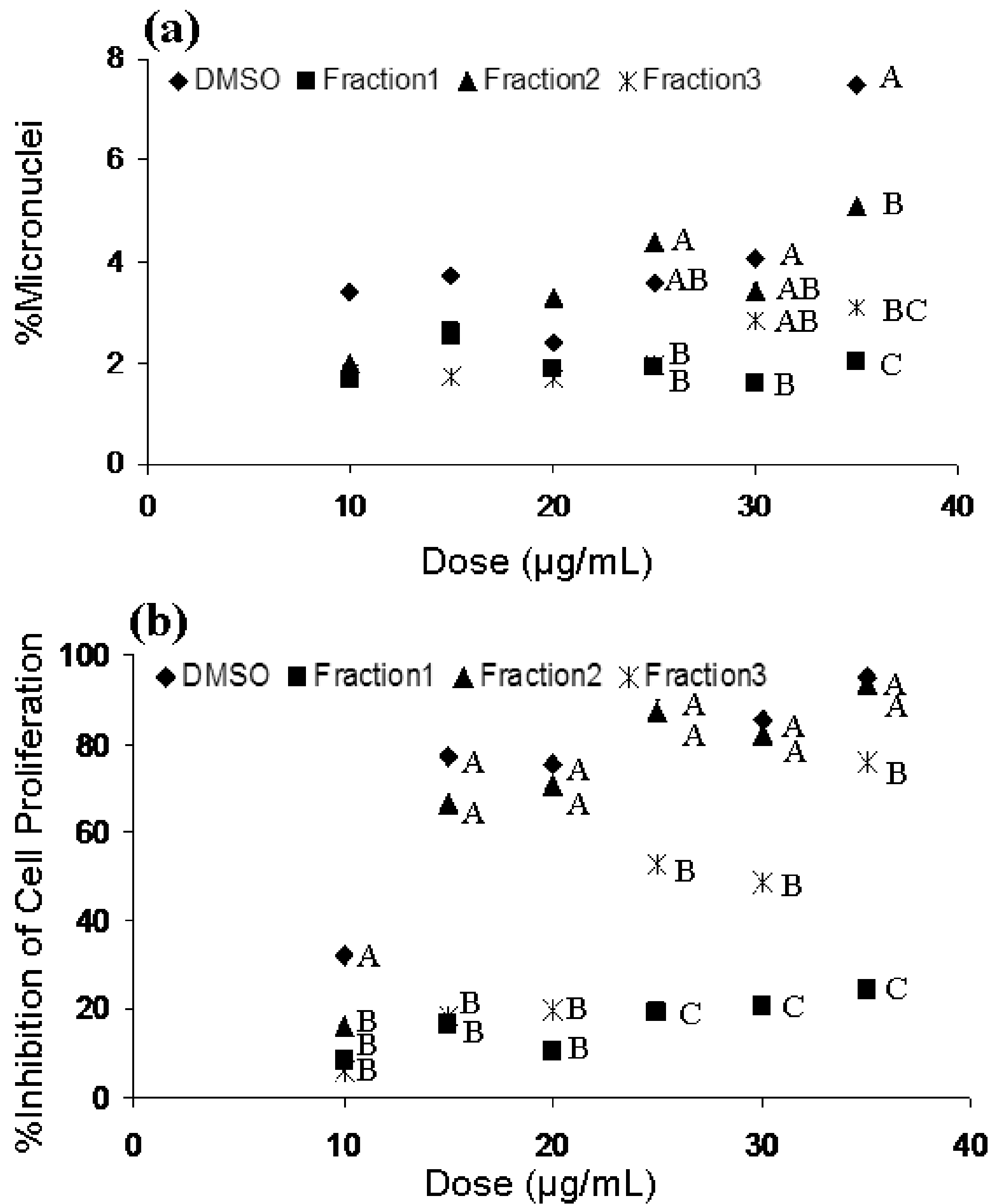

Figure 3-4: The genotoxic activity (% micronuclei), (a), and inhibition of cell proliferation (b), induced by (whole) DMSO extract, fractions 1–3. All other conditions as in Fig. 3-2. Error bars have been removed for clarity.

3.4.6 Chemical characterization and separation of fraction 2

Due to its overall higher bioactivity, the second fraction was re-analyzed by LC/TOF-MS with accurate mass measurement to assess its chemical composition. Fraction 2 was found to contain catechol and its derivatives (methyl catechol, ethyl catechol and vinyl catechol), phenol, hydrocaffeic acid, 1-(3,4-dihydroxyphenol)ethanone, 3,4-dihydroxybenzoic acid, *p*-hydroxycinnamic acid, *p*-coumaric acid, caffeic acid methyl ester, caffeic acid and hydroxybenzoic acid (Figure 3-3). Among these, the last 8 compounds (hydrocaffeic acid to hydroxybenzoic acid) have not been previously reported as carcinogens, mutagens or teratogens as opposed to catechol, phenol and caffeic acid (Gold *et al.*, 1997). As previously discussed, catechol and its derivatives are known to be responsible for induction of micronuclei and toxicity in the micronuclei assay (Robertson *et al.*, 1991) and thus could be responsible for the increased level of bioactivity of fraction 2.

The analytical separation of fraction 2 was optimized with respect to peak resolution with the objective of sub-fractionating it for further analysis to identify the compounds responsible for its bioactivity. Based on the compounds identified in fraction 2, a test mixture of seven standard compounds was prepared and a series of different stationary phases and eluant compositions were evaluated on the LC/MSD instrument as described in Materials and Methods. The best resolution for the test mixture was obtained with a 15 (or 20) to 75 % MeOH in 0.1 % (v/v) aqueous formic acid gradient over 23 min using the Polar-RP column (data not shown). This column, which is composed of an ether-linked phenyl stationary phase with polar end-capping, most likely enabled a more selective interaction with the aromatic compounds and improved their resolution. Subsequently, fraction 2 was analyzed under the optimized

conditions by LC/TOF-MS. This enabled separation of the quite abundant and bioactive catechol from three isomers of hydroxybenzoic acid (data not shown).

3.4.7 Preparative sub-fractionation of fraction 2 and chemical characterization of sub-fraction 1

The optimized analytical separation conditions used for fraction 2 of the DMSO extract were transferred to a Polar-RP preparative column for sub-fractionation. Figure 3-5 shows how we generated the four major sub-fractions of fraction 2 by preparative LC. The genotoxicity induced by these four sub-fractions, as well as by major fraction 2 and by the whole DMSO extract is shown in Figure 3-6a. No statistically significant difference (α=0.05) was measured by ANOVA between the six samples compared at the lower dose range (5 and 10 µg/mL). Whereas, at the dose ranges corresponding to 15 and 20 µg/mL there were statistically significant differences in terms of generation of micronuclei between sub-fraction 4, the DMSO extract and sub-fraction 2. The inhibition of cell proliferation induced by the four sub-fractions, by fraction 2 and by the whole DMSO extract is compared in Figure 3-6b. Sub-fraction 1 and the DMSO extract induced a higher percentage of inhibition of cell proliferation but were only significantly higher compared to sub-fractions 2 and 3 at doses of 15 and 20 µg/mL. Overall, the IVMNT showed that sub-fraction 1 induced the highest degree of genotoxicity and inhibition of cell proliferation compared to the other sub-fractions.

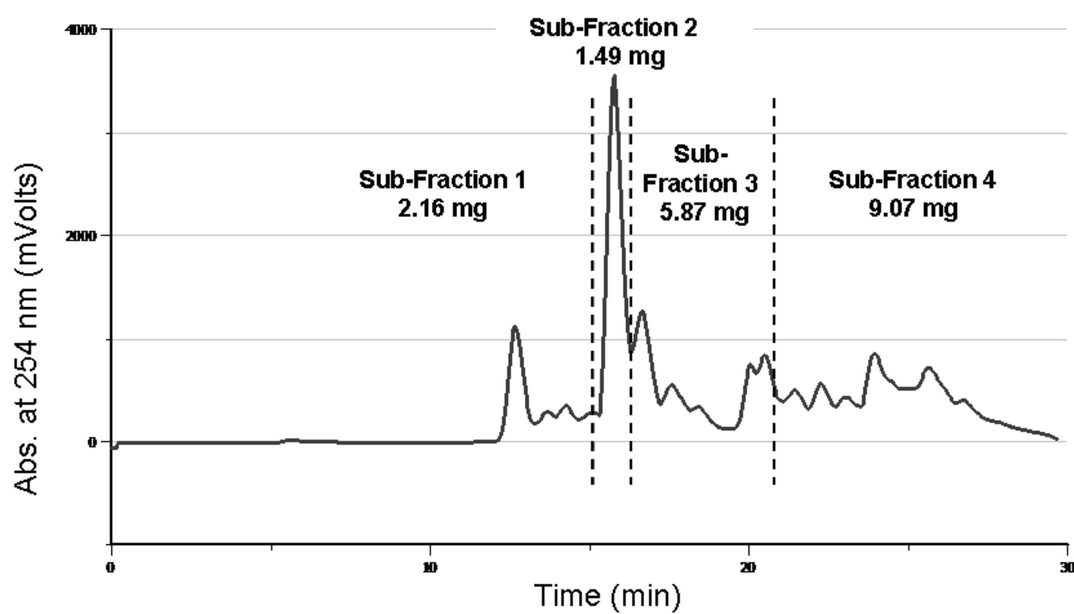

Figure 3-5: Preparative LC chromatogram (254 nm UV trace) of fraction 2 of the DMSO extract showing the sub-fractions collected. The dotted lines have been overlaid to represent the four main sub-fractions collected.

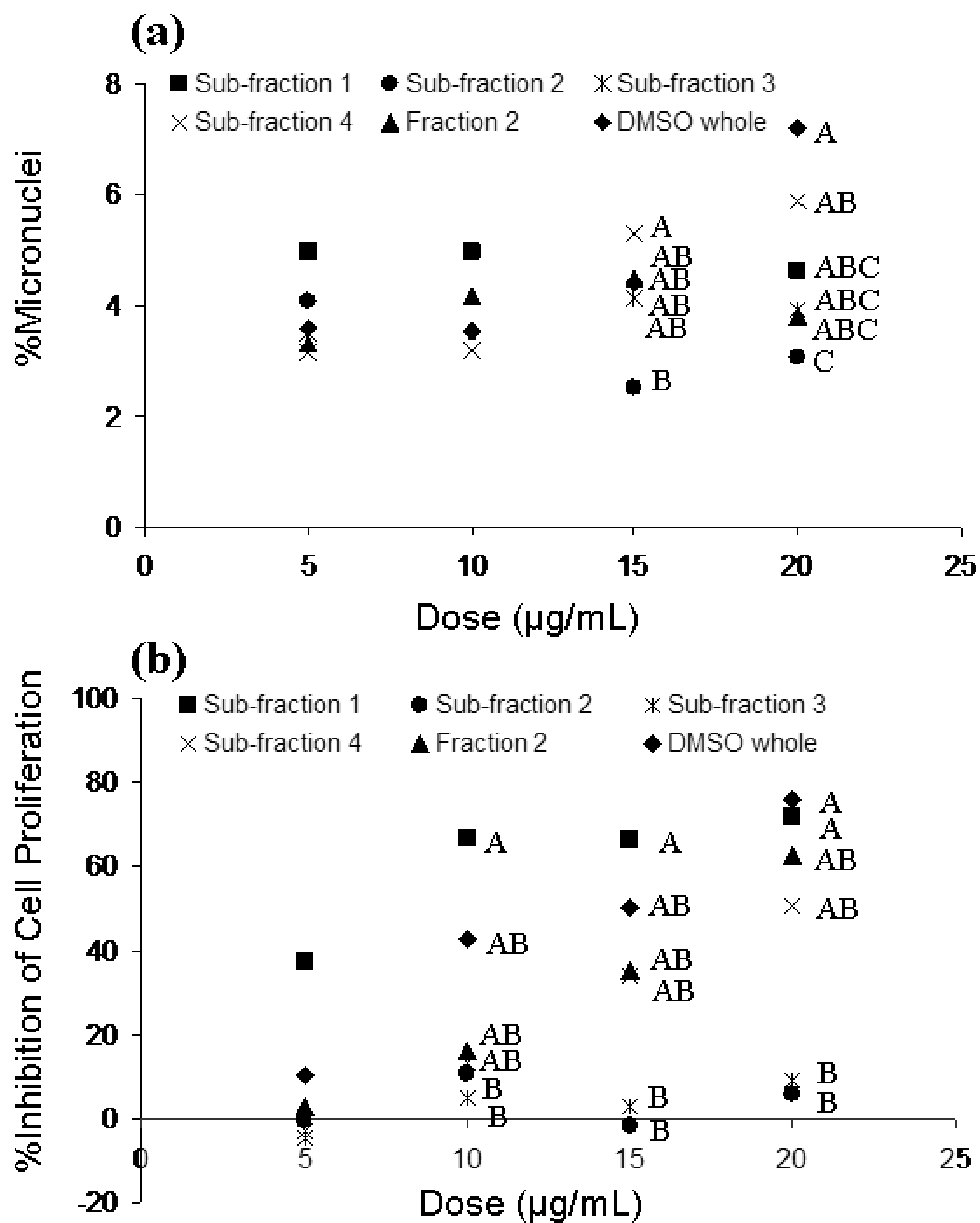

Figure 3-6: The genotoxic activity (% micronuclei), (a), and inhibition of cell proliferation (b), induced by DMSO extract, fraction 2 and sub-fractions 1–4. All other conditions as in Fig. 3-2. Error bars have been removed for clarity.

Subsequently, sub-fraction 1 was analyzed by LC/TOF-MS (Figure 3-7) and found to contain catechol as the major component, 3,4-dihydroxybenzoic acid and a third, less abundant compound with the empirical formula $C_6H_8O_2$. Based on this formula, some logical structures were deduced. One possible compound is 1,2-cyclohexanedione, for which no toxicology information was found in the literature A set of standards of 1,2-cyclohexanedione were prepared, but they were inactive in terms of toxicological response in the dose range of 5-20 μg/mL. A second possibility may be one of the isomers of dihydroxycyclohexadiene. Unfortunately, no standards were commercially available to test biotoxicity by the IVMNT. To the best of our knowledge, toxicological data is also not available for any of these isomers. Further structural analysis of the $C_6H_8O_2$ compound was beyond the scope of this study.

The second compound identified, 3,4-dihydroxybenzoic acid, is not known to be either genotoxic or an inhibitor of cell proliferation (Gold *et al.*, 1997). Catechol, on the other hand, which was *ca.* 10-fold more abundant than 3,4-dihydroxybenzoic acid (Figure 3-7), was confirmed to be genotoxic and inhibit cell proliferation as seen in Figures 3-8a and 3-8b for catechol standards (5-20 μg/mL dose range) assessed by the IVMNT. These results support previous findings in terms of the toxicological response (Chouchane *et al.*, 2006) and in terms of catechol being a product of the combustion of CGA (Gopalakrishna *et al.*, 1994, Schlotzhauer *et al.*, 1992, Robertson *et al.*, 1991, Hoffmann *et al.*, 1983, Schlotzhauer *et al.*, 1982). Vaughan *et al*. recently reported that mainstream smoke from the 1R4F reference cigarette contained essentially the same quantity of catechol as hydroquinone per cigarette, whereas flue-cured tobacco cigarettes delivered an amount of catechol twice that of hydroquinone (Vaughan *et al.*, 2008). We also obtained a 2-to-1 ratio of catechol to hydroquinone in the extracted

particulate matter after partial combustion of CGA (Table 3-2). This suggests that CGA is one of the major sources of phenolic components in flue-cured tobacco (Vaughan *et al.*, 2008), thus demonstrating that our choice to investigate the burning of the single tobacco component CGA is a simpler yet valid alternative to using whole tobacco smoke to study the relationship between toxicity and fractional chemical composition.

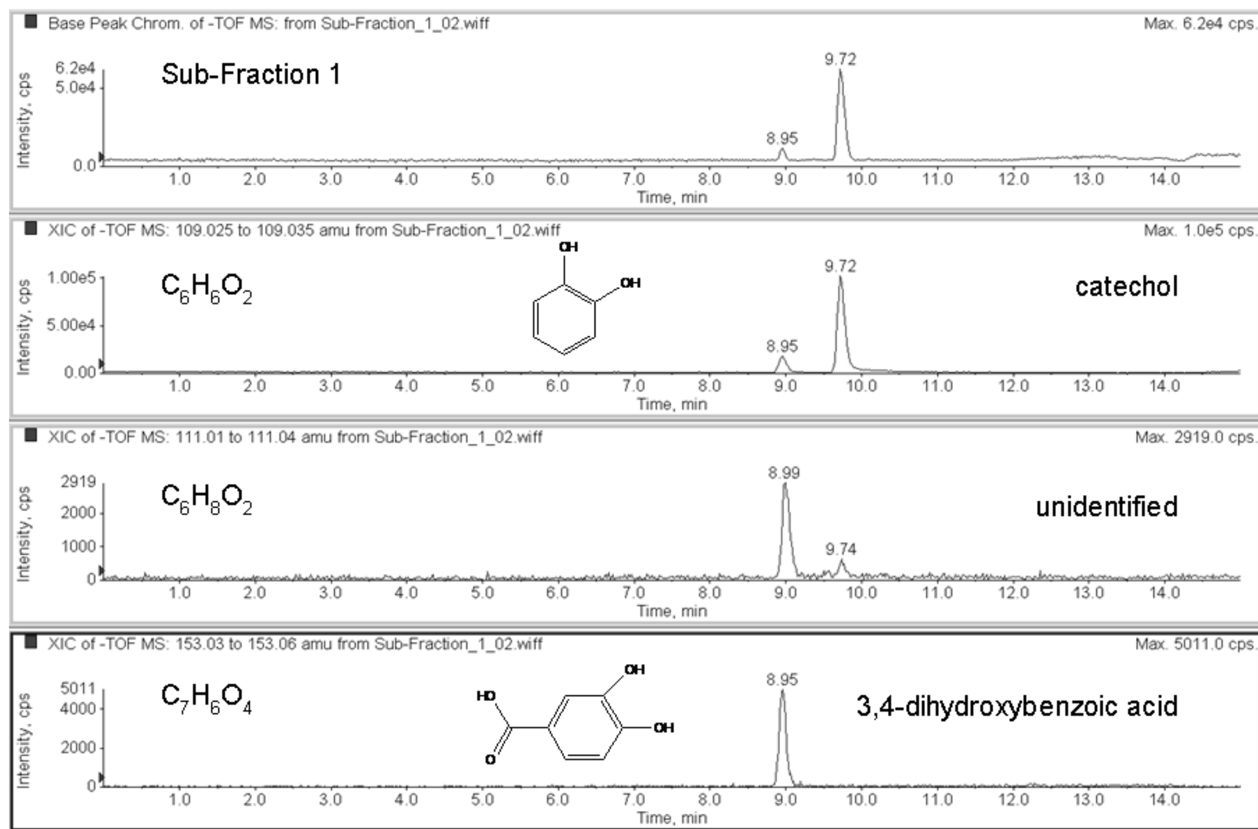

Figure 3-7: Base peak LC/TOF-MS chromatogram (upper most trace) and extracted ion chromatograms (lower traces) of sub-fraction 1 of fraction 2 of the DMSO extract of CGA combustion products. Separation conditions are given in Section 2.

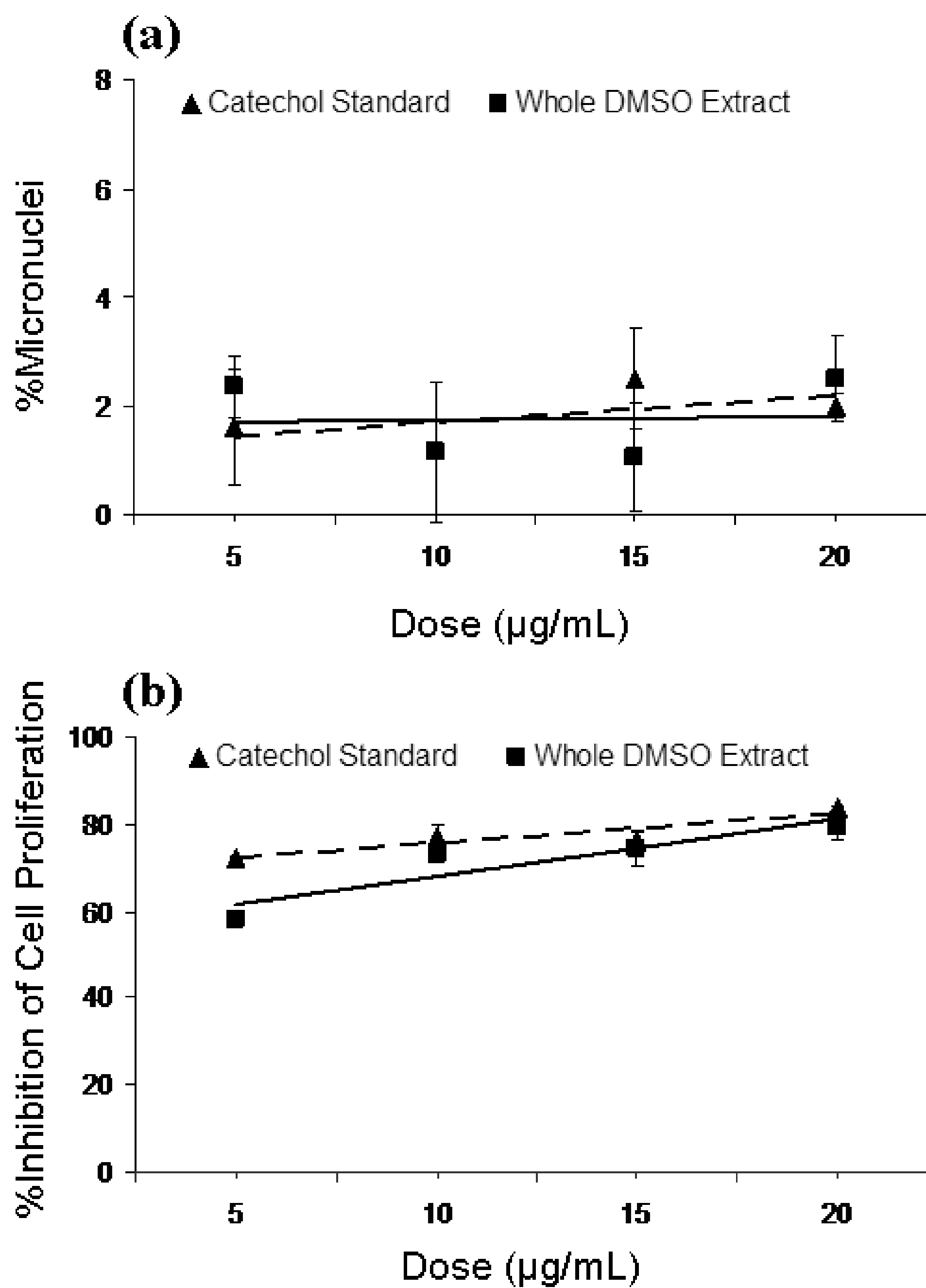

Figure 3-8: The genotoxic activity (% micronuclei), (a), and inhibition of cell proliferation (b), induced by DMSO extract and catechol standards. All other conditions as in Fig. 3-1. (—■—) DMSO; (- ▲ -) catechol standard**.** Error bars indicate standard deviation.

3.5 Conclusion

A multidisciplinary study comprising solvent extraction, fractionation, bioassay and state-of-the-art LC/MS allowed us to systematically narrow in on the biotoxic components in the particulate matter produced from the incomplete combustion (*i.e.* burning) of chlorogenic acid (CGA). Extraction with DMSO followed by successive chromatographic fractionation combined with accurate mass identification and use of the IVMNT for bioactivity identified catechol, 3,4-dihydroxybenzoic acid and a minor, unidentified constituent ($C_6H_8O_2$) as being components of the most bioactive sub-fraction of CGA combustion products. 3,4-Dihydroxybenzoic acid has not been reported to be genotoxic or an inhibitor of cell proliferation. Catechol, on the other hand, was the major component present in the most toxic sub-fraction and is known to be toxic. By testing catechol standards alone, we were able to confirm that catechol is indeed genotoxic and blocks cell proliferation in the dose working range. We suspect that catechol is therefore the major component responsible for the bioactivity resulting from the whole DMSO extract. Furthermore, a positive correlation was established between CGA (compared to other polyphenolic compounds) found in tobacco and catechol and ethyl-catechol found in smoke (Schlotzhauer *et al.*, 1992, Schlotzhauer *et al.*, 1982). This demonstrates that in terms of chemistry, our approach of studying a single component is not only valid but is also relevant. The relationship between CGA and catechol would support the reduction of CGA in tobacco in order to reduce catechol.

Our research carried out on the combustion products of CGA may not be directly correlated to the smoke from all cigarette types due to the fact that the combustion of a single tobacco component does not take into account possible interactions between multiple

components during combustion. Also, the conditions of tobacco combustion, such as heating rate and atmospheric gas concentration have been shown to influence the relative proportions of the products (Baker, 1981). However, our methodology allows for the analysis of a simpler product mixture. Also, we cannot directly relate the toxicological results from the *in vitro* assays to *in vivo* toxicity since there are detoxification pathways involved in the latter. Finally, only the compounds detected by LC/MS in negative mode were accounted for. Nonetheless, our approach combining toxicology with chemical identification has contributed to a better understanding of the toxicity of a single tobacco component, CGA.

3.6 Acknowledgements

Graduate bursaries for Navneet Kaur were provided by the Natural Science and Engineering Council of Canada (NSERC) and CORESTA. We thank N. Poirier for help with culture maintenance and the IVMNT, and J. Dumont for help with the HPLC/FL analyses. We also thank D. Sekhon and K. Venne for their assistance with the various LC/MS instruments at U. of Montréal and M. Bratberg for her contribution to LC/MSD method development.

4 Evaluation of Precision and Accuracy of the Borgwaldt RM20S® Smoking Machine Designed for *In Vitro* Exposure

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Note on my contribution

My participation in research tasks: I carried out the majority of the experimental work and data analysis. Martine Lacasse (technician) was involved in a part of the method development for methane analysis, Jean-Philippe Roy (technician) assisted in the carbon monoxide experimental work and Jason Adamson (technician) was responsible for all data obtained for methane analysis from the laboratory in the UK (reproducibility studies). Statistical analyses were carried out by Graham Errington (Statistician) under the supervision of Marianna Gaca.

Publication: I wrote the article, which took into account comments/corrections provided by Jean-Louis Cabral, Marianna Gaca, André Morin (co-supervisor) and Karen C. Waldron (supervisor). Jason Adamson was responsible for illustrating Fig. 4-1

4.1 Abstract

The Borgwaldt RM20S® smoking machine enables the generation, dilution and transfer of fresh cigarette smoke to cell exposure chambers, for *in vitro* analyses. We present a study determining [18]the precision (repeatability r, reproducibility R)[19] and accuracy of smoke dose generated by the Borgwaldt RM20S® system and delivery to exposure chambers. Due to the aerosol nature of cigarette smoke, the repeatability of the dilution of the vapor phase in air was assessed by quantifying two reference standard gases methane (CH_4, r between 29.0-37.0 and RSD between 2.2-4.5%) and carbon monoxide (CO, r between 166.8 -235.8 and RSD between 0.7-3.7%). The accuracy of dilution (percent error) for CH_4 and CO was between 6.4-19.5% and between 5.8-6.4%, respectively, over a 10-1000-fold dilution range. To corroborate our findings, a small inter-laboratory study was carried out for CH_4 measurements. The combined dilution repeatability had an r between 21.3-46.4, R between 52.9-88.4, RSD between 6.3-17.3% and error between 4.3-13.1%. Based on the particulate component of cigarette smoke (3R4F), the repeatability (RSD = 12%) of the undiluted smoke generated by the Borgwaldt RM20S® was assessed by quantifying solanesol using high performance liquid chromatography with ultraviolet detection (HPLC/UV). Finally, the repeatability (r between 0.98-4.53 and RSD between 8.8-12%) of the dilution of generated smoke particulate phase was assessed by quantifying solanesol following various dilutions of

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[18] Originally published as “confirming the precision” and amended as “determining the precision” in the thesis.

[19] Repeatability measurements correspond to the agreement of individual results obtained from testing the same sample, in the same laboratory, having a difference in one of the following: analyst, apparatus or the day (Centre d’expertise en analyse environnementale du Québec, 2007). Reproducibility measurements correspond to the agreement of individual results obtained from testing one sample, in different laboratories, by a different analyst, on a different apparatus, on a different day (Centre d’expertise en analyse environnementale du Québec, 2007).

cigarette smoke. The findings in this study suggest the Borgwaldt RM20S® smoking machine is a reliable tool to generate and deliver repeatable and reproducible doses of whole smoke to *in vitro* cultures.

4.2 Introduction

Cigarette smoke is a complex aerosol mixture composed of over 5000 compounds (Rodgman *et al.*, 2009b, Rodgman *et al.*, 2009a) distributed between the particulate and the vapor phases. There are a number of different methods that have been used to investigate and assess the biological effects of cigarette smoke. These range from the design of an inhalation machine for studies involving rodent *in vivo* whole smoke exposure (Baumgartner *et al.*, 1980) to the use of skin painting carcinogenesis experiments using cigarette smoke condensates (Walaszek *et al.*, 2007). A number of *in vitro* techniques have been reported that involve the exposure of submerged cellular cultures to either total particulate matter (TPM) or to aqueous cigarette smoke extracts (Cantral *et al.*, 1995, Lannan *et al.*, 1994, Nakayama *et al.*, 1985).

These above methods may not be representative of the whole smoke exposure and toxicity. In addition, many *in vivo* studies involve direct animal exposure to cigarette smoke, often resulting in "forced inhalation" which may give rise to artifacts, considering that the animal is under distress throughout the exposure process. With respect to skin painting carcinogenesis studies, the test material is not identical to smoke inhaled by a smoker and is thus not representative of the major route of potential human exposure (Walaszek *et al.*, 2007). For toxicological assessment of TPM, the use of dimethylsulfoxide (DMSO) as the TPM extraction solvent from a Cambridge filter pad (CFP) (Health Canada, 2004b), may not be desirable for certain biological assay endpoints because DMSO is

known to act as an antioxidant, is a potent scavenger of free radicals and may possibly interfere with assays measuring oxidative stress (Kishioka *et al.*, 2007, Misra *et al.*, 2010). Another limitation of most *in vitro* studies is the incompatibility for simultaneous assessment of the vapor and particulate phase of cigarette smoke. Recent studies indicate that the vapor phase of cigarette smoke plays a major role in the toxicological responses (Bombick *et al.*, 1997c, Bombick *et al.*, 1997b, Phillips *et al.*, 2004, Wieczorek *et al.*, 2006, CORESTA, 2005). Thus, exposure to whole cigarette smoke (including both phases) should give a comprehensive description of cigarette smoke toxicity.

To study the toxicity of whole cigarette smoke, accurate and precise generation, dilution and delivery of the smoke would be desirable. Recent advancements have led to the development of advanced smoke generation systems, such as the Borgwaldt RM20S® smoking machine (Phillips *et al.*, 2005), Burghart Mimic Smoker-01® (Scian *et al.*, 2009a) , the Vitrocell Smoking Robot VC 10® (Aufderheide *et al.*, 2000). Also this has led to the development of novel *in vitro* exposure systems such as British American Tobacco's (BAT) exposure chamber (Phillips *et al.*, 2005) and the CULTEX system (Aufderheide *et al.*, 2000). These exposure systems have been designed to 'minimize the loss of smoke components and to mimic the events occurring in the smoker's respiratory tract' according to Scian et al. (Scian *et al.*, 2009a). They are direct exposure methods and thus are more representative of human inhalation than indirect methods using impingers (Lestari *et al.*, 2006) . These systems also allow for the generation of fresh cigarette smoke in various dilutions (or doses) and direct delivery to cell cultures. The ability to introduce smoke diluted over a wide range is required for *in vitro* cell culture investigations because of the high sensitivity of these biological systems. The Borgwaldt RM20S® smoking machine in combination with BAT's exposure chamber using Transwell® inserts, enables direct

exposure of *in vitro* cellular cultures to whole cigarette smoke at the air-liquid interface (Fig. 4-1) (Massey *et al.*, 1998, Phillips *et al.*, 2005). This smoking machine, first commercialized in 2005, can smoke up to eight cigarettes simultaneously with the smoke collected into eight independent syringes. Each syringe can dilute the cigarette smoke with air in ratios ranging from 1:1.14 to 1:4000 (smoke volume: air volume), which corresponds to a range of 87- 0.025 % (v/v) cigarette smoke in air. For biological exposures, doses tend to be in the range of 0.4- 5% (v/v) cigarette smoke in air (Phillips *et al.*, 2005). The dose at this range of whole smoke dilution has been correlated to the quantity of TPM deposited on a CFP and on the Transwell® inserts within BAT's exposure chamber (Massey *et al.*, 1998, Phillips *et al.*, 2005). However, the accuracy, precision and linearity of the smoke dose delivered to the exposure chamber by the Borgwaldt RM20S® smoking machine were not reported and therefore are the subject of the current study.

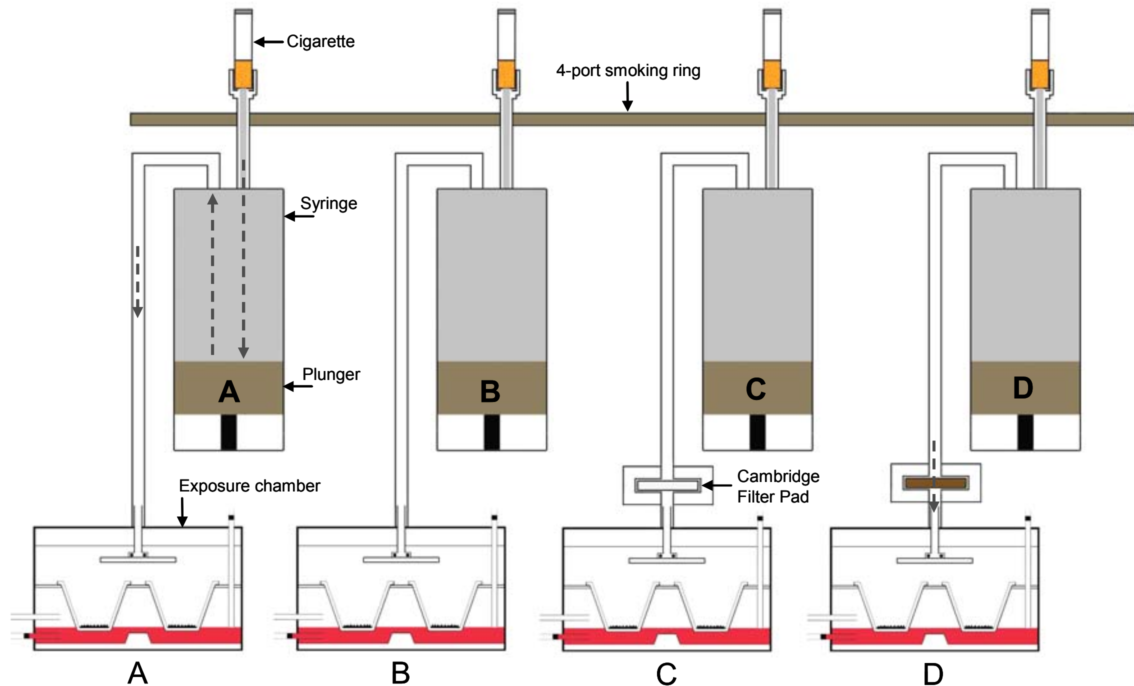

Figure 4-1: Schematic of the exposure of cell or tissue culture to whole cigarette smoke or vapor phase using the Borgwaldt RM20S® in combination with BAT's exposure chamber. The RM20S® can smoke up to eight cigarettes simultaneously, which correspond to a specific port, syringe and exposure chamber (A-D). The machine smokes the cigarettes, dilutes the smoke and delivers it into the exposure chambers (chambers were not used in this study). *Exposure chambers A & B exposed to whole smoke versus C & D exposed to vapor phase only.*

This study reports the repeatability, reproducibility and accuracy of whole smoke generation and dilution from the Borgwaldt RM20S® smoking machine using two gases (CH_4 and CO) and a cigarette smoke particulate matter marker (solanesol). The two reference gases were chosen based on their presence in the vapor phase of cigarette smoke and facile quantification of CH_4 by flame ionization detection (FID) and CO by infrared spectroscopy. To corroborate our findings, the CH_4 reproducibility measurements were compared between two different laboratories (Montreal, Canada and Southampton, UK) using the same method. Secondly, for the particulate component of cigarette smoke, the repeatability of the smoke generated by the Borgwaldt RM20S® was assessed by high

performance liquid chromatography coupled to an ultraviolet absorbance detector (HPLC/UV) for solanesol ($C_{45}H_{74}O$) extracted from the cigarette filter tip of a reference cigarette (3R4F). Solanesol naturally occurs in tobacco leaves and has been used as a direct marker of the amount of cigarette smoke particulate phase generated during combustion (Armitage *et al.*, 2004). Finally, the repeatability and reproducibility of the dilution of generated particulate phase were assessed based on extracted solanesol quantification for various dilutions of cigarette smoke. The precision and accuracy results obtained from this study will provide a reliable tool for dose delivery from the Borgwaldt RM20S® smoking machine, in combination with BAT's exposure chamber and Transwell® inserts, for future studies of exposure of cells and tissue cultures to whole cigarette smoke.

4.3 Materials and Methods

4.3.1 Whole Smoke Exposure System: the Borgwaldt RM20S® Smoking Machine and BAT's Exposure Chamber

The Borgwaldt RM20S® (Borgwaldt KC GmbH, Hamburg, Germany) is an automatic smoking machine that generates and dilutes cigarette smoke for *in vitro* cell culture investigations (Borgwaldt KC, 2010). It has a rotary based engine that can simultaneously smoke four types of cigarettes for several hours, depending on the smoking regime used. The instrument has an incorporated anemometer allowing for correct air flow, as well as electrical lighter, butt detector and butt extractor. The Borgwaldt RM20S® was designed in collaboration with BAT (Southampton, UK) and can be used with BAT's exposure chamber to enable cells or tissues to be exposed to the diluted smoke generated by the Borgwaldt RM20S®. Within the chamber, the cells or tissues lie on a 24 mm

diameter Transwell-Clear® insert (Corning, NY, USA) which is a microscopically transparent porous polyester membrane, and are exposed to smoke at the air-liquid interface (Thorne *et al.*, 2009, Phillips *et al.*, 2005).

Prior to the study, the entire instrument, including the syringes[20], was thoroughly cleaned by repeated rinsing with 70 % (v/v) ethanol in water until all residues were visibly removed. The cleaning solvent was allowed to evaporate thoroughly before conducting experiments At the beginning of each day, the instrument was tested as follows: the air velocity was verified to be 20 ± 3 cm/sec; checks were performed for air leaks (acceptable range of pressure between 2750-3000 Pa); and the puff volumes were adjusted to 35 mL. In between trials on the same day, clearing puffs were performed to reduce the potential effect of carry over. Following usage, the smoking ring, cigarette holders, butt length detector, airflow lid and ashtray were cleaned using the same ethanol-water mixture described above to ensure that all tar, ashes and loose pieces of tobacco did not build up and hinder the operation of the instrument. This cleaning procedure was done within 12 h before the next usage, always permitting sufficient time for the solvent to evaporate afterwards.

4.3.2 Vapor Phase Measurements-General Procedure

Two reference gas standards, CH_4 and CO, were chosen to assess the precision and accuracy of the smoke diluted by the Borgwaldt RM20S® instrument using a recovery-based study at several dilution levels. The general procedure was as follows: a sealed bag was filled with one of the two standard gases and placed at the inlet of the Borgwaldt RM20s® smoking machine (cigarette smoking position). The gas was then diluted in

[20] Syringes were calibrated with a similar technique as described below for CH_4 measurements and pistons were manually adjusted in order for the syringes to produce given predicted values of CH_4.

compliance with the International Standard Organization (ISO) puffing profile, consisting of a 35 mL puff volume, 2 s puff duration and 60 s puff interval (International Organization for Standardization, 1999) for a total of 10 or 15 min. A clean, pre-evacuated gas bag was connected to the exhaust position to collect the diluted standard gas. All four syringes (A-D) were assessed. The CH_4 measurements were compared between the two laboratories.

4.3.3 CH_4 Measurements in the Canadian Laboratory

The methane reference standard, comprised of 10% CH_4 in argon (Praxair, Danbury CT, USA), was used to assess dilutions corresponding to reference standard gas concentrations in air of 0.1, 0.2 and 0.52% (v/v) (*i.e.*, dilution factors of 1000-, 500- and 193-fold, respectively). Quantification of CH_4 for each syringe (A-D) was made with a 3010 MINIFID portable heated flame ionization detector (FID) total hydrocarbon analyzer (Signal Instruments, Willow Grove, PA, USA). This FID has a stated accuracy of better than ±0.2 ppm (or ±1%, whichever is greater) with linearity better than ±0.05 ppm (0.5%) for an analyte range of 0-100 000 ppm hydrocarbon, which was thus well suited for this study because the measured CH_4 concentrations ranged from 100 to 520 ppm. Triplicate runs were performed for each syringe at each dilution level, which took a period of three days to complete. The FID was calibrated each day using hydrocarbon-free air and a 500 ppm span gas (external reference, CH_4 in air (Praxair)). Given the specifications for the detector, it was presumed that 520 ppm CH_4 was still within the linear dynamic range even though it lies slightly above the external calibration point. The FID was fueled by a mixture of hydrogen with helium (40/60) (Praxair).

4.3.4 CH_4 Measurements in the UK Laboratory

The procedure in the UK laboratory was the same as that carried out in Canada with the following exceptions: the modified Borgwaldt RM20S® instrument contained eight syringes (syringes 1-8); the reference standard was 10% CH_4 in nitrogen (Air Products PLC, Cheshire, UK); runs were performed in quadruplicate; the 3010 MINIFID portable heated FID (same as above) used for quantification was from Signal Group Ltd, Surrey, UK.

4.3.5 CO Measurements

The carbon monoxide reference standard, comprised of 9.98% CO (Praxair), was used to assess dilutions corresponding to reference standard gas concentrations in air of 1, 2, 5 and 10% (v/v) (*i.e.*, dilution factors of 100-, 50-, 20- and 10-fold, respectively). Quantification of CO for syringes A-D (n=3/syringe) was made using a COA 205 infrared CO analyzer (Cerulean, Milton Keynes, UK). According to the manufacturer, the infrared analyzer has an accuracy of better than ±0.1 ppm (or ±1%) for a 0-100 000 ppm dynamic range. The general procedure described above was changed slightly to improve the method. The first modification included sending off the first puff to waste and collecting the second to sixteenth puff (15 puffs total). This was implemented to exhaust the air in the tubing to obtain a more accurate measurement of CO. Secondly, the connectors used for the gas bags were changed to reduce the dead volume. The infrared analyzer was calibrated each day using air and a 0.5% CO span gas (external reference, Praxair), which was deemed adequate for the targeted CO concentration range (*i.e.*, 0.1 to 1%) and given the instrument's wide dynamic range.

4.3.6 Particulate Phase Measurements-General Procedure

The cigarettes used in this study were Kentucky reference cigarettes (3R4F, University of Kentucky, USA) and were conditioned at 22°C and 60% relative humidity for 48 h prior to smoking. Cigarettes were smoked with compliance to ISO puffing profiles as described above. Cigarettes were smoked simultaneously (corresponding to syringes A-D) for 30 min for a total of 30 puffs/port (1 puff/min). For individual ports, this corresponded to four cigarettes being smoked per port. The cigarette smoke was evaluated directly in the filter tips (n=10/syringe) and also after dilution in air to concentrations of 1, 2 and 5% (v/v) (*i.e.*, dilution factors of 100-, 50- and 20-fold, respectively and n=3/syringe/dilution). Three runs were performed daily and the order in which dilutions were made was changed over the days to reduce potential bias. All four syringes were assessed.

Quantitation of solanesol, a cigarette smoke particulate phase marker, was used to assess the precision of smoke delivery in the Borgwaldt RM20S® instrument before and after smoke dilution. Standard solutions of solanesol (Sigma-Aldrich, St. Louis, MO; ≥ 95%) were prepared to construct a calibration curve consisting of concentrations ranging from 0.45 to 150 µg/mL in methanol (Thermo Fisher Scientific, Whitby, Canada, 99.9%). Solanesol was quantified by liquid chromatography with absorbance detection (at 210 nm) using an Agilent 1200 HPLC/UV system equipped with ChemStation software version B.03.02, (Agilent Technologies, Waldbronn, Germany). Separations were made on a Luna C_{18} analytical column (3 µm, 50 mm x 2.0 mm) with C_{18} guard column (Phenomenex, Torrance, CA, U.S.A.) under isocratic elution conditions in methanol:acetonitrile (70:30 (v/v)) at a flow rate of 0.3 mL/min. The total run time was 10 min. The column temperature was set to 30 °C and the autosampler cooler was set to 5 °C. Injections of 10 µL of all standards and samples were performed in duplicate. Peak areas were used for quantitation

and the extraction solvent was used as a blank. The instrumental detection limit (LOD) was determined using the standard deviation (σ) in the background noise of the solvent blank and the slope of the external calibration curve (*i.e.*, LOD = (3σ)/slope), whereas the method detection limit was determined using the peak height for solanesol at 1% (v/v) smoke dilution and the corresponding baseline noise to calculate 3σ.

4.3.7 Solanesol Measurements Prior to Dilution

The solanesol content in the cigarette filter tips was used to assess the smoke delivery precision prior to (*i.e.*, up-stream of) the dilution. From the individual filter tips (butts), 10 mm portions were cut from the mouth end and the four butts for a given port- corresponding to a 30 min smoking duration- were pooled to represent one sample and stored at -20 °C until analysis. The TPM from each pooled sample was extracted in 16 mL methanol with agitation using a flat bed shaker at 200 rpm for 20 min. Ten smoke samples were generated for each port (A-D) and analyzed for solanesol content by HPLC in order to evaluate the repeatability of smoke generation prior to the dilution step.

4.3.8 Solanesol Measurements Following Dilutions

To assess the repeatability and reproducibility of smoke delivery following dilution by the instrument, solanesol was quantified after its collection on a 44 mm diameter Cambridge filter pad (CFP) inserted after the dilution syringe (*i.e.*, in place of the exposure chamber, (Fig. 4-1). Each CFP was transferred to a 125 mL Erlenmeyer flask for extraction. To prevent any residue loss, the filter holder was wiped down with a quarter of a clean CFP, which was added to the Erlenmeyer flask. The “diluted” TPM on the CFP was extracted with 8 mL of the following solvent mixture under agitation for 20 min: 1% (v/v)

anethol (Acros Organics, Geel, Belgium, 99%), 0.25% (v/v) ethanol (Les Alcools de Commerce, Boucherville, Canada, 95%) and 0.1% (v/v) methanol in isopropanol (Thermo Fisher Scientific, 99.9%).

4.3.9 Statistical Analysis

Statistical analyses were carried out using MINITAB® v. 15.1.30 statistical software. The precision figures of merit calculated were relative standard deviation (RSD) across syringes at each dilution level, repeatability (r) and/or reproducibility (R). Linear regression was used to compute predictive equations and correlation coefficients for CO, CH_4 and solanesol. For gas phase measurements of CO dilutions, RSD and repeatability within the Canadian laboratory only were calculated. The repeatability limit, r, in this case is defined as the difference that can be expected between two individual measurements carried out on the same syringe with a probability of 0.95.

The inter-laboratory study was carried out for CH_4 dilution measurements only. In the case of CH_4, r is defined as the difference that can be expected between two individual measurements carried out within either laboratory with a probability of 0.95. Reproducibility, R, is the difference that can be expected between two measurements carried out between the two laboratories, two operators and two instruments, with a probability of 0.95. For this study, r and R were calculated by taking into account all of the results obtained from both Canada and UK laboratories.

For particulate phase measurements, repeatability, r, was calculated for solanesol dilutions in the same way as for CO for the Canadian laboratory only. In addition, a control chart (Shewhart chart) was used to visually compare the smoke delivery from the four ports

measured using solanesol. This tool also allowed assessment of the stability for smoke delivery from each port over time.

4.4 Results

4.4.1 Vapor Phase Measurements

The accuracy of dilution by the instrument's syringes was estimated by introducing a known, fixed concentration of CH_4 at the smoking port (10% CH_4 in argon gas reference standard) and then measuring the CH_4 concentration in the gas collected in each syringe for the three levels of dilution tested: 0.1, 0.2 and 0.52% (v/v) in air. For 100% accuracy[21] at each dilution level, the expected concentrations of collected methane should be 0.01% (100 ppm), 0.02% (200 ppm) and 0.052% (520 ppm). The precision of dilution was estimated based on triplicate CH_4 measurements for each syringe at each dilution level. The overall accuracy (reported as percent error) for the Canadian laboratory CH_4 measurements across the three dilution levels and four syringes (A-D) was between 6.4-19.5%, of which only 1% or less is attributed to error in the FID instrument used for the measurements, and the precision (reported as RSD) was between 2.2-4.5% (Table 4-1). The correlation between the selected CH_4 dilution level and measured CH_4 concentrations after dilution was evaluated by linear regression, giving an equation of $C_{CH4} = 35.54 + 0.86 \cdot \text{Dilution}$ and correlation coefficient of >0.98, which indicated good linearity across the dilution range studied (Fig. 4-2). According to the instrument specifications, error in the linearity for this analyte range due to the FID alone is ±0.5 % and is thus not a significant contribution. The

accuracy and precision were best in the middle range of dilutions (Table 4-1, last two columns). There was a positive bias at lower measurements and underestimation of the highest selected CH_4 concentration since the y-intercept was greater than zero and the slope was 14 % less than the predicted value of 1.00[22].

Table 4-1: Summary of the Borgwaldt RM20S®'s dilution precision: r, RSD and error values from measured CH_4 for selected levels of dilution for syringes A-D used in the Canadian laboratory (n=3).

Dilution (%)	CH_4 Target (ppm)	Mean CH_4 (ppm)	Repeatability r	RSD (%)	Error (%)
0.52	520	480.0	29.0	4.5	8.0
0.2	200	208.8	22.5	2.2	6.4
0.1	100	119.5	37.0	3.5	19.5

[21] Originally published as "For a recovery of 100% (i.e., an accuracy approaching 0% error)" and amended as "For 100% accuracy" in the thesis.
[22] The achieved data values (y axis) should be as close to the predicted data values (x axis), thus, ideally attaining 1.00.

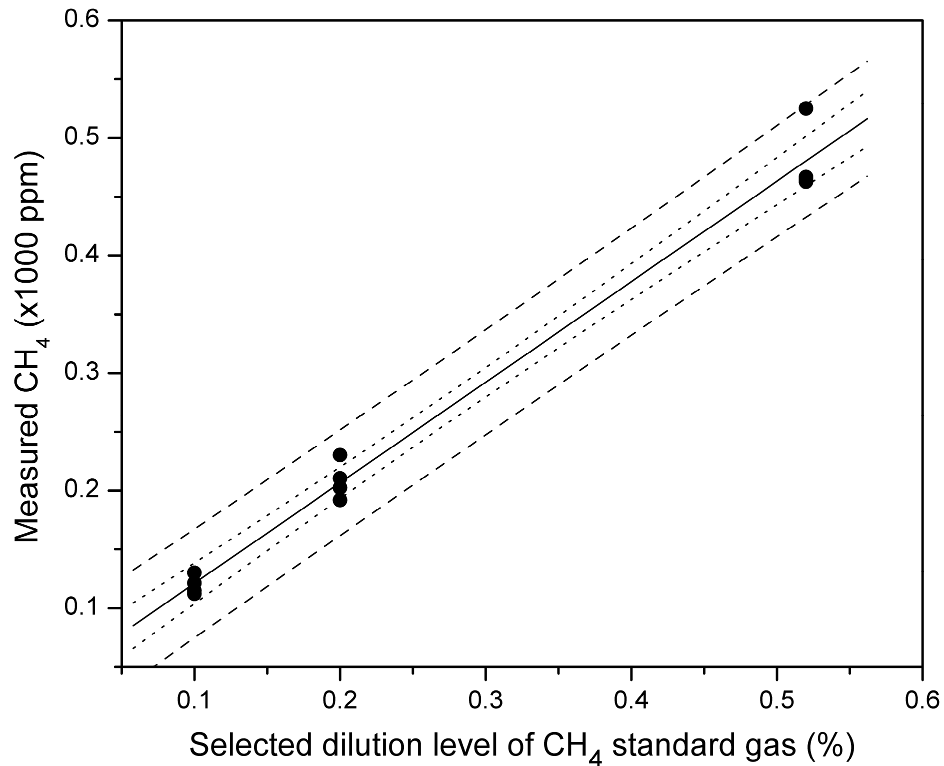

Figure 4-2: Assessment of the Borgwaldt RM20S® dilution precision and accuracy: correlation between the FID-based quantification of collected CH_4 as a function of the selected level of dilution for syringes A-D for a 10% CH_4 standard reference gas (Canadian laboratory), *i.e.*, CH_4 doses of 0.1, 0.2 and 0.52 % (v/v) in air. Data were acquired in triplicate for all dilution levels (n=3). Linear fit analysis: $y = 35.5 + 0.8557x$ (R^2 = 0.987) with upper and lower confidence intervals (dotted lines) and predicted intervals (dashed lines) calculated at 95% probability.

The CH_4 dilution measurements taken in the Canadian laboratory were compared to those taken in the laboratory located in the UK with a similar instrument having 8 syringes (syringes 1-8) for the same dilution levels (n=4/syringe) (Table 4-2). Contrary to results for the Canadian laboratory alone, accuracy was marginally better at the highest selected dilution level for the combined laboratories (Table 4-2, last column), even when taking into

consideration up to ±1% measurement error in the FID. The combined accuracy and precision data for all 12 syringes-four in the Canadian laboratory instrument (syringes A-D) and the eight in the UK laboratory instrument (syringes 1-8) were plotted (Fig. 4-3). The slopes, which reflect the accuracy of CH_4 measurements across the dilution range, were 0.86 and 0.93 for the Canadian and UK laboratories, respectively, and 0.91 for the pooled data (Fig. 4-3). By plotting the accuracy and precision of dilution of CH_4 for the 12 individual syringes (in the two laboratories) and the individual dilution levels, syringe bias depending on dilution level was observed (Fig. 4-4). At the target level of 100ppm, or dilution of 0.1% selected (top panel, Fig. 4-4), the variability between syringes is lower in the Canadian than in the UK laboratory. On the contrary, at the target level of 520ppm for dilution of 0.52% (bottom panel, Fig. 4-4), the variability between syringes is lower in the UK than in the Canadian laboratory. Despite these differences, the data are still consistent across groups; the combined repeatability and linearity of the dilution range studied had an RSD between 6.3-17.3% with R^2 >0.98 and accuracy between 4.3-13.1% (in percent error). From the RSD values, the lowest target dilution level gave the least favorable precision (Table 4-2). However, based on the r and R values calculated, the inter-lab variability was low and similar to inter-run variability for only the 0.1% (v/v) sample.

Table 4-2: Overall precision figures of merit of r, R, RSD and error values from measured CH_4 for three dilution levels for syringes A-D used in the Canadian laboratory (n=3), syringes 1-8 used in the UK laboratory (n=4).

Dilution (%)	CH_4 Target (ppm)	Mean CH_4 (ppm)	Repeatability r	Reproducibility R	RSD (%)	Error (%)
0.52	520	497.8	45.8	88.4	6.3	4.3
0.2	200	210.2	21.3	52.9	9.0	5.1
0.1	100	113.1	46.4	54.7	17.3	13.1

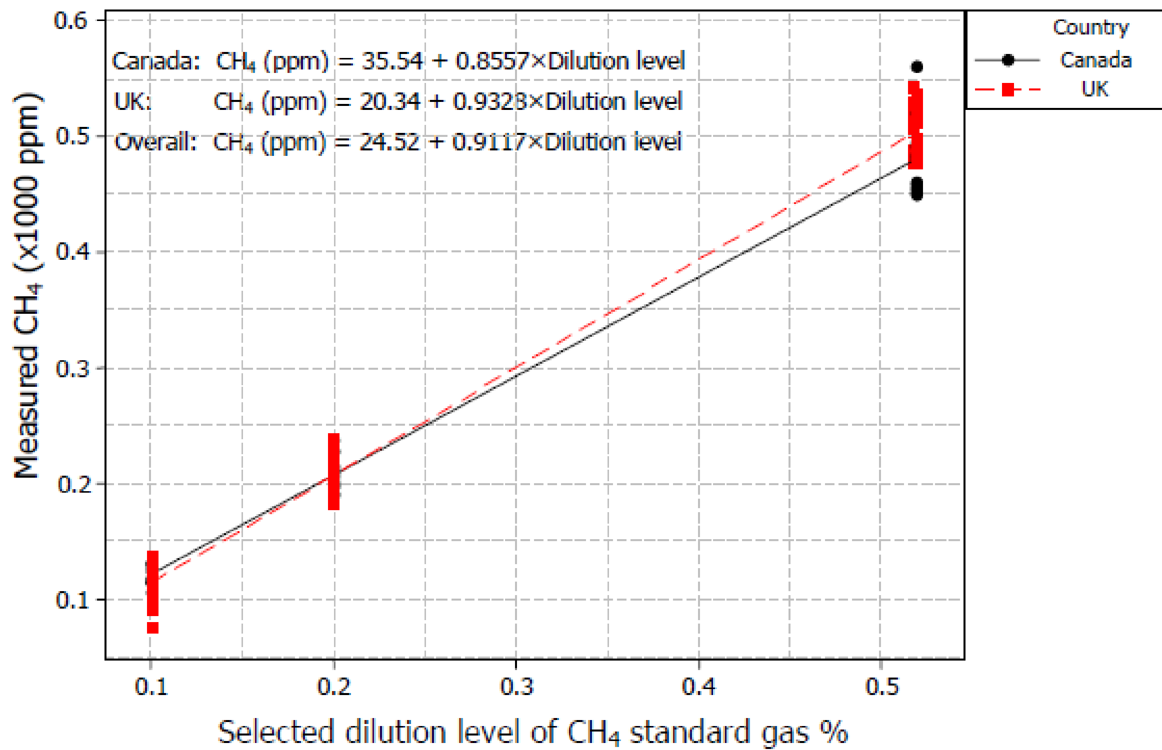

Figure 4-3: Assessment of the Borgwaldt RM20S® dilution precision and accuracy: correlation between the FID-based quantification of collected CH_4 as a function of the selected level of dilution by any given syringe for a 10% CH_4 reference standard. Data were acquired for all dilution levels and 12 syringes, *i.e.*, for syringes A-D used in the Canadian laboratory (n = 3/syringe/dilution) and syringes 1-8 used in the UK laboratory (n = 4/syringe/dilution). Calculated slopes were for each laboratory and combined measurements.

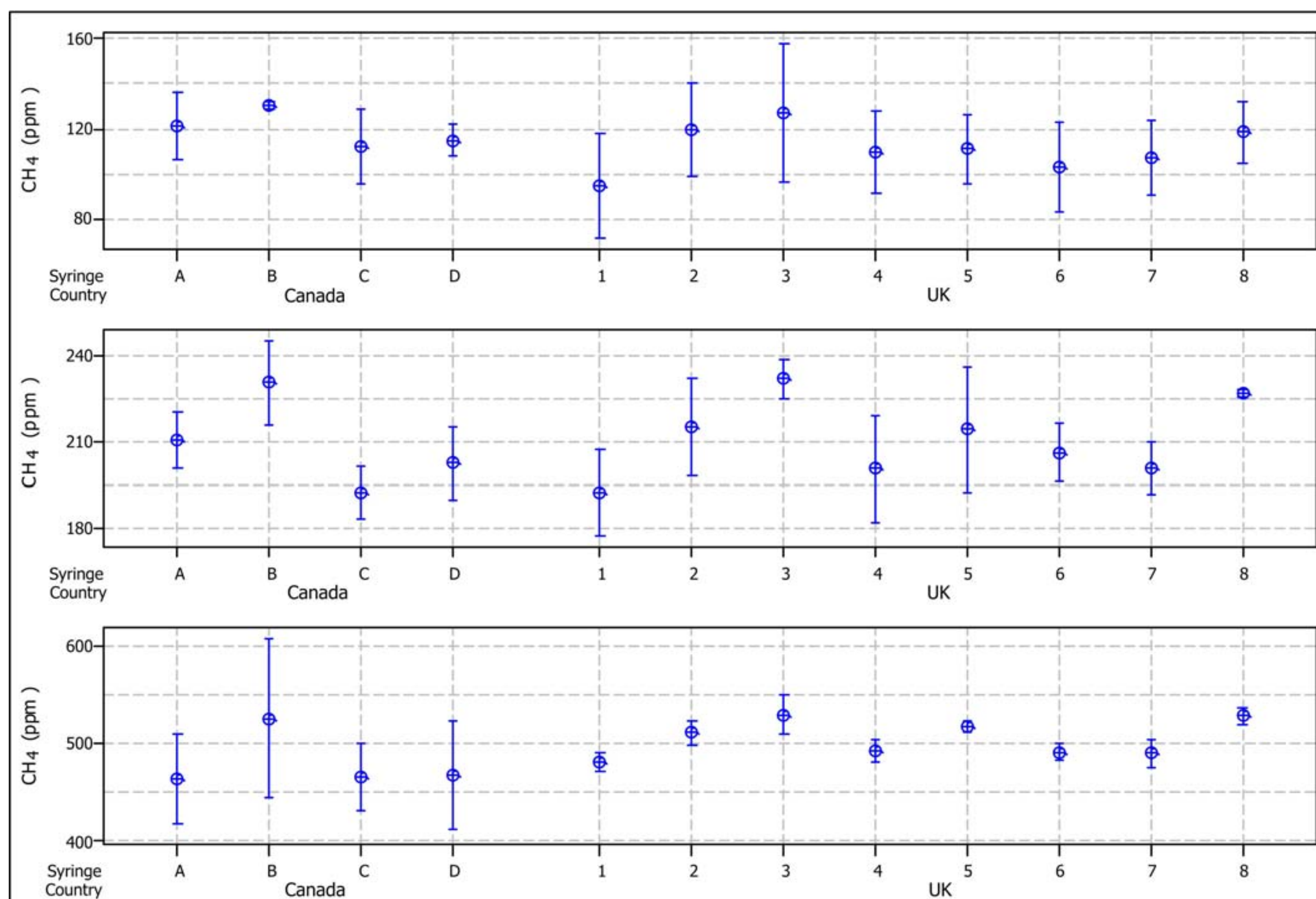

Figure 4-4: Dilution precision for individual syringes as a function of the desired level of dilution. Graphs showing the concentration of CH_4 collected after dilution of a 10% CH_4 gas standard by the syringes in both Borgwaldt RM20S® instruments (used in laboratories in Canada and the UK). Top, middle and bottom panels represent dilutions to 0.1%, 0.2% and 0.52%, respectively. Mean CH_4 concentrations are plotted with standard deviations (n=3) for each syringe.

Similar to the methane experiments, quantification of carbon monoxide (9.98% CO gas reference standard) collected at each syringe in the Canadian laboratory instrument, but for four dilution levels (1, 2, 5 and 10%), was also used to asses the accuracy and precision of dilution. For 100% accuracy[23] at each dilution level, the expected concentrations of collected CO would be 0.0998% (998 ppm), 0.1996% (1996 ppm), 0.499% (4990 ppm) and 0.998% (9980 ppm). Based on triplicate runs at each dilution level, the overall precision was determined to be between 0.7-3.7% RSD, of which 1% error can be attributed to the measurement system according to the manufacturer, and accuracy was between 5.8-6.4% relative error (Table 4-3). A good correlation between the targeted and the measured CO concentration across the 4 levels of dilution was observed (Fig. 4-5). The slope was 0.9137[24] compared to the predicted value of 0.998—an absolute error of 8.4%—but linearity was good with $R^2 = 0.99$. The CO detection system contributes only 1% to the total error. Having attained values within acceptable criteria (RSD < 10% and $R^2 > 0.95$) for both reference gases studied indicates that the equipment reliably generates and delivers accurate doses of whole smoke.

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[23] Originally published as "For 100% recovery (i.e., 100% accuracy)" and amended as "For 100% accuracy" in the thesis.
[24] Originally published as "913.7 compared to the predicted value of 998" but amended as "0.9137 compared to the predictied value of 0.998".

Table 4-3: Summary of the Borgwaldt RM20S®'s dilution precision: r, RSD and error values from measured CO for selected levels of dilutions for syringes A-D used in the Canadian laboratory (n=3).

Dilution (%)	CO Target (ppm)	Mean CO (ppm)	Repeatability r	RSD (%)	Error (%)
10	9980	9341.7	204.26	0.7	6.4
5	4990	4691.7	235.84	1.7	6.0
2	1996	2100.0	166.77	2.8	5.9
1	998	1058.3	166.77	3.7	5.8

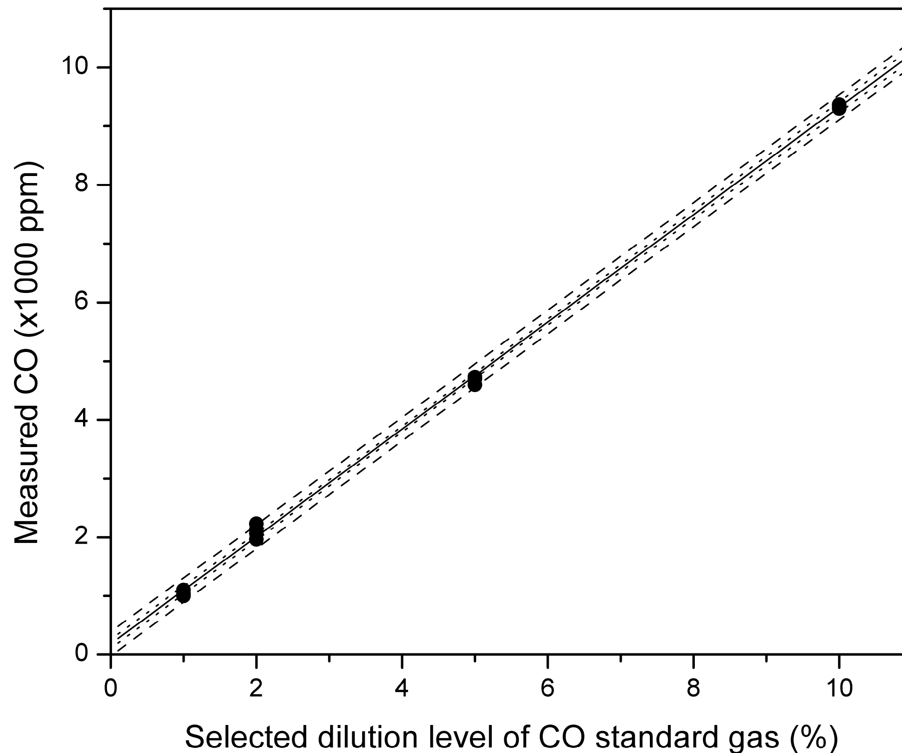

Figure 4-5: Assessment of the Borgwaldt RM20S® dilution precision and accuracy: correlation between the non-dispersive infrared (NDIR) based quantification of collected CO as a function of the selected level of dilution for syringes A-D for a 9.98% CO standard reference gas, *i.e.*, CO doses of 1, 2, 5 and 10 % (v/v) in air. Data were acquired in triplicate for all dilution levels (n=3). Linear fit analysis: $y = 186.3 + 0.9137x$ ($R^2 = 0.999$) with upper and lower confidence intervals (dotted lines) and predicted intervals (dashed lines) calculated at 95% probability.

4.4.2 Particulate Phase Measurements

Solanesol was quantified by HPLC/UV in cigarette filter tips prior to dilution as well as on CFPs placed after the dilution syringe (Fig. 4-1) to evaluate the repeatability of smoke delivery in the Borgwaldt RM20S®. First, ten cigarette smoke samples, each corresponding to 30 min smoking periods (4 tips pooled/port), were analyzed for solanesol content in the filter tips for each syringe port (A-D). The results are presented in the form of a Shewhart control chart for each port (Fig. 4-6). Shewhart charts are used for monitoring the stability of measurement systems. Their utility lies in predicting the temporal variation in precision based on short term variations, which can be easily measured. Individual concentrations of solanesol for the 40 samples (10 per port) ranged from 6.8 µg/mL (sample 3, syringe A) to 11.9 µg/mL (sample 8, syringe D) and the mean solanesol concentration across the four syringe ports was 9.5 ± 1.2 µg/mL between samples (n=40) for pooled samples (top panel Fig. 4-6). Upper (UCL) and lower (LCL) control limits, represented by a solid line, correspond to 3σ and indicate the threshold above which data points are statistically unlikely to exceed. The stability of the variation between successive samples, is represented by the moving range (MR), *i.e.,* the mathematical difference between successive samples for a given port (middle panel Fig. 4-6). The average MR is calculated for the 9 differences; the smaller this value, the lower the temporal variation. The difference (absolute value) between injection replicates related to HPLC performance was plotted as the range (R) within a measurement (bottom panel Fig. 4-6). Ideally, the range should approach 0, which is the default LCL. The worst value in the range chart was for sample 2, syringe port B, where duplicate HPLC injections resulted in a

difference of 1.25 μg/mL solanesol. The UCL and LCL bands tend to be the narrowest for syringe C data for all three charts (Fig. 4-6), indicating that syringe port C was the most stable and reliable at delivering the same dose of smoke. The overall precision associated with the smoke generated by the Borgwaldt RM20S® corresponded to an RSD of 12%, which is slightly above the expected precision tolerance of 10%. However, random errors associated with the extraction and analysis steps were expected to contribute to the overall precision of the method and thus 12% RSD was deemed acceptable for operation of the Borgwaldt RM20S®.

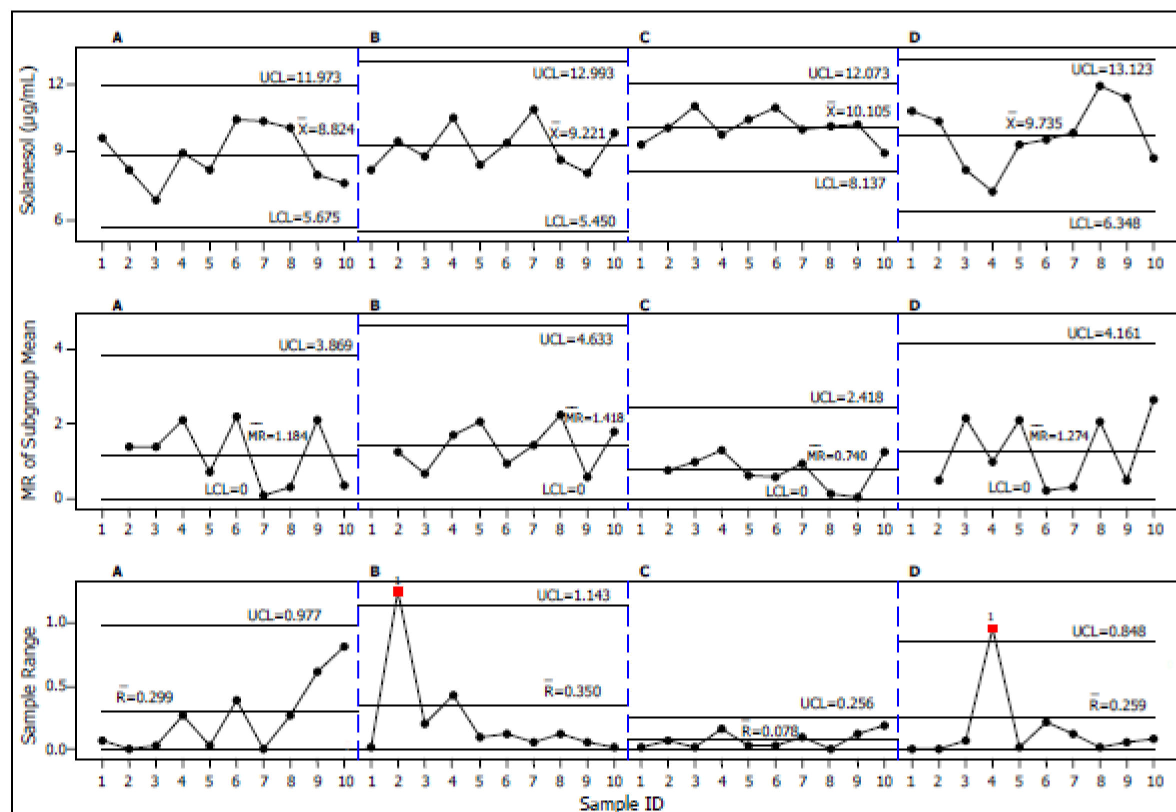

Figure 4-6: Assessment of the smoke delivery of the Borgwaldt RM20S®: Shewhart control charts showing the HPLC-based quantification of collected solanesol from cigarette filter tips. Data were acquired for the smoke delivery at all syringe ports: A-D (n=10 samples each) for a 30 min period (4 tips were pooled/port/sample). The top panel represents the mean solanesol concentrations (µg/mL) from replicates (n=2 injections) obtained for each pooled sample. The combined mean is shown as a solid line for each port (A-D). The middle panel represents the moving range (MR), or differences between successive samples. The bottom panel represents the range (R) within a measurement and is the difference between replicates with respect to HPLC determinations. Upper (UCL) and lower (LCL) control limits, represented by dotted lines, correspond to 3σ. The LCL label in the bottom panel was removed for clarity as it corresponds to 0.

Secondly, to assess the dilution reliability of each syringe, a CFP was used to collect the TPM of cigarette smoke following dilution and solanesol in the TPM was quantified. The cigarette smoke was diluted in air to concentrations of 1, 2 and 5% (v/v) (n=3/syringe), which would correspond to the dose when cells or tissue culture are placed in the exposure chambers (Fig. 4-1). The overall precision across the four syringes associated with the smoke generated and diluted by the Borgwaldt RM20S® was between 8.8-12% RSD (Table 4-4). The effect of run day and run order on the precision of dilutions showed no statistically significant difference. However, there was a borderline syringe effect ($p=0.047$ for the Bartlett's test for equality of variances). The solanesol concentration (µg per mL of extraction solvent) was plotted as a function of the selected dilution level (% of whole smoke) (Fig. 4-7) and linearity of the solanesol content was $R^2 > 0.95$ across the dilution range. The instrumental and method LODs were determined to be 3.4×10^{-4} and 1.4×10^{-2} µg/mL, respectively. Therefore, the solanesol concentrations investigated were at least 100 times higher than the quantitation limit (3.3·LOD) and thus free of errors associated with measurements at trace levels.

Table 4-4: Summary of the Borgwaldt RM20S®'s smoke dilution precision: r and RSD values for measured solanesol for selected dilutions for syringes A-D used in the Canadian laboratory (n=3).

Dilution (%)	Mean Solanesol (µg/mL)	Repeatability r	RSD (%)
5	19.8	4.53	12
2	8.66	1.69	9.0
1	4.34	0.98	8.8

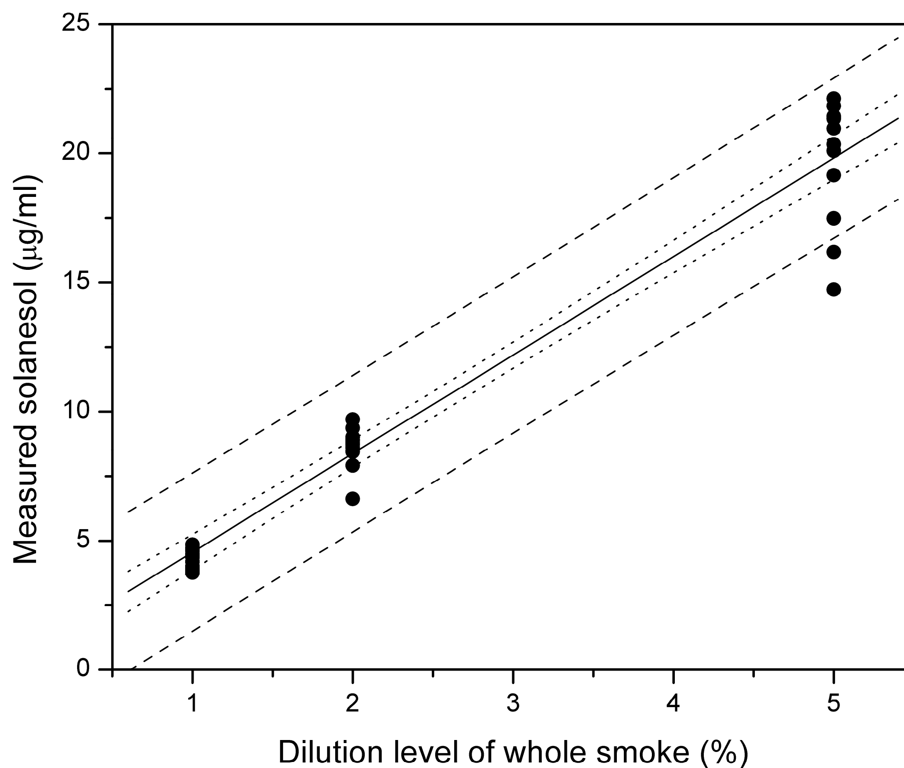

Figure 4-7: Assessment of the dilution precision of whole smoke in the Borgwaldt RM20S®: LC/MS based quantification of solanesol collected on a CFP as a function of the selected level of dilution of smoke for syringes A-D, *i.e.*, whole smoke doses of 1, 2 and 5% (v/v) in air. Data were acquired in triplicate for all dilution levels (n=3). Linear fit analysis: $y = 0.742 + 3.816x$ ($R^2 = 0.954$) with upper and lower confidence intervals (dotted lines) and predicted intervals (dashed lines) calculated at 95% probability.

4.5 Discussion

A rigorous evaluation of the precision and accuracy of smoke generation and dilution by the Borgwaldt RM20S® smoking machine is an essential step before any cell culture studies can be carried out because of the importance of dosimetry. We used the standard reference gases CH_4 and CO, as well as quantification of solanesol in the particulate phase of diluted and undiluted cigarette smoke, to carry out a detailed evaluation of the Borgwaldt RM20S®. In addition, inter-laboratory variation was assessed using the CH_4 data.

The use of two different standard reference gases used "as is" allowed us to assess the dilution repeatability of the instrument over a wide range of dilution levels: 0.1 to 10% (v/v) in air. Precision values ranged from 0.7 to 4.5% RSD for measurements carried out on the Canadian laboratory instrument only (Tables 4-1 and 4-3) and linearity of the measured standard gases after dilution versus the target dilution levels was better than R^2 = 0.98 (Figs. 4-2 and 4-5). Accuracy of the eight target dilution levels tested ranged from 5.8 to 19.5% relative error (Tables 4-1 and 4-3), where the latter value corresponded to dilution to 0.1% of the methane standard, in other words a 1000-fold dilution of the CH_4 introduced at the syringe port. It is important to note that this error value was more than twice that of any of the other dilution levels investigated. Given the good accuracy and precision expected for detection of CH_4 at 100ppm by the MINIFID system and the positive bias observed at this selected level (Table 4-1), it seems the syringes in the Canadian laboratory machine may not fully reach 1000-fold dilution. This explains the observed lower slope versus the predicted value of 1.00 (Fig. 4-2). Nonetheless, for dilutions between 0.2-10% (v/v) in air,

the accuracy of dilution of the Borgwaldt RM20S® was excellent, particularly considering that the FID alone contributed up to 1% of the total relative error.

As described in the Materials and Methods section, the procedure for CO standard gas experiments was slightly different than that for CH_4; improved connectors for gas bags were used and the first puff was directed towards the waste to reduce the effects of the first puff being diluted by air in the tubing. These modifications may have contributed slightly to the lower RSD values for CO (Table 4-3) and lower relative error.

The effect of syringe number was monitored in the inter-laboratory CH_4 study, where both precision and accuracy varied visibly, depending on the dilution level (Fig. 4-4). In the UK laboratory instrument, precision and accuracy were superior for the highest standard gas concentration tested, 0.52% (equivalent to 520 ppm CH_4, 1:193 whole smoke dilution)(Fig. 4-4c, syringes 1-8). The RSD values between laboratories for targeted CH_4 dilutions in air of 0.1, 0.2 and 0.52% (v/v) were 17.3, 9.0 and 6.3%, respectively. Between laboratories, no statistically significant differences ($p>0.05$) were observed between standard deviations for target values of 100ppm and 200ppm (dilution to 0.1% and 0.2% of the CH_4 standard, respectively). However, at the target value of 520ppm CH_4 reference gas, significant differences ($p = 0.025$) were observed between the Canadian and UK laboratories. The variation within syringes in the Canadian lab was observed to increase at the lowest standard gas concentration tested, 0.1% (equivalent to 100ppm CH_4, 1:1000 whole smoke dilution (v/v)), which is not commonly used for *in vitro* cell culture analyses.

Achieving an overall precision of between 2.2-4.5% RSD for the standard reference gases (Tables 4-1 and 4-3), which includes the inherent errors in the CH_4 and CO detectors, was deemed satisfactory in terms of repeatability for further use of the Borgwaldt RM20S®

instrument. Moreover, the reproducibility between labs for dilutions down to 0.2 and 0.52% CH_4 standard in air (v/v) was 6.3 and 9.0% RSD, which indicated a comparable reproducibility between laboratories for these commonly used dilutions for dosing cells and tissue cultures.

Measuring solanesol in the filter tips of the cigarettes allowed for the assessment of the precision of the smoke generation prior to dilution (based on particulate phase measurements), which was 12% RSD on average across 4 syringes. The measurements were found to be within upper and lower confidence levels for each port, within their respectable limits (middle panel Fig. 4-6). The RSD of the solanesol quantification following dilution (*i.e.*, from TPM collected on the Cambridge filter pad) was between 8-12% (Table 4-4) and the relationship between quantity and dilution level was linear (R^2 >0.95) for the dilution range selected (Fig. 4-7). Although the solanesol data yielded a poorer repeatability than CH_4 and CO, as well as a slight syringe effect, it was still considered acceptable when taking into account the accuracy of the smoke generation (potential deposition on tubing and syringe surfaces or inherent variability of cigarettes[25]), the additional sample handling (*e.g.*, extractions) compared to gas phase measurements and error involved in the analytical measurement by HPLC. The syringe effect could possibly be reduced by improving the piston adjustment of the syringes, or by planning the experimental design to include alternating syringes, or by applying a weighting factor. On the other hand, the residual plots (data not shown) and percent error for CO data indicated

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[25] The variability for 3R4F cigarettes is 1.5 % (n=11), based on TPM measurements (University of Kentucky, 2010b).

that the points were random *i.e.*, no effect of syringes, which is likely because particulate phase deposition was not a factor for the gas standard measurements.[26]

The precision figures obtained for solanesol measurements following dilution were similar to those obtained from the standard gas measurements and demonstrate that the particulate marker values were within acceptable limits in the measurement system, indicating the equipment is suitable for cell exposure studies.

Table 4-5: Summary of the Borgwaldt RM20S®'s smoke dilution precision: r and RSD values for measured solanesol for selected dilutions for syringes A-D used in the Canadian laboratory (n=3).

Dilution (%)	Mean Solanesol (µg/mL)	Repeatability r	RSD (%)
5	19.8	4.53	12
2	8.66	1.69	9.0
1	4.34	0.98	8.8

4.6 Conclusions

Overall, the values obtained from CH_4, CO and solanesol repeatability experiments were within acceptable limits in measurement systems (RSD < 10% and R^2 >0.95) indicating the equipment can reliably generate accurate doses of cigarette smoke suitable for cell exposure and dosimetry studies. Furthermore, having assessed the precision of the smoking machine with CH_4 from two different laboratories and with different operators provides us with even greater confidence in the reliability of dose.

These results suggest the Borgwaldt RM20S® system provides a reliable and repeatable method for generating and delivering whole smoke to *in vitro* cell cultures using exposure systems, which to our knowledge, has never been assessed or specified by the supplier. We propose to further characterize the dose within the chamber following exposure to whole smoke as a function of dilution factor, in terms of specific smoke constituents. This system could also be used for evaluation of cigarette design modifications intended to potentially reduce the harmful effects of smoke.

4.7 Acknowledgements

The authors thank M. Gaudreau, J. Dumont, J. Verreault and M. Barber for their technical support. N. Kaur was partially funded by the Natural Sciences and Engineering Council of Canada (NSERC) and the Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA).

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[26] Another factor that may have contributed to the variability in gas and particulate measurements was the instrumental air velocity having up to 15% RSD (20 ± 3 cm/sec).

5 Headspace Stir-bar Sorptive Extraction-Gas Chromatography/Mass Spectrometry Characterization of the Diluted Vapor Phase of Cigarette Smoke Delivered to an *In Vitro* Cell Exposure Chamber.

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Note on my contribution

My participation in research tasks: I carried out most of the experimental work and data analysis. Statistical analyses were carried out by Michel Guillet (Statistician).

Publication: I wrote the article, which took into account comments/corrections provided by Jean-Louis Cabral, André Morin (co-supervisor) and Karen C. Waldron (supervisor). Jason Adamson was responsible for illustrating Fig. 5-1.

5.1 Abstract

Advanced smoke generation systems, such as the Borgwaldt RM20S® smoking machine used in combination with the BAT exposure chamber, allow for the generation, dilution and delivery of fresh cigarette smoke to cell or tissue cultures for in vitro cell culture analyses. Recently, our group confirmed that the Borgwaldt RM20S® is a reliable tool to generate and deliver repeatable and reproducible exposure concentrations of whole smoke to *in vitro* cultures (Kaur *et al.*, 2010). However, the relationship between dose and diluted smoke components found within the exposure chamber has not been characterized. The current study focused on the development of a headspace stir bar sorptive extraction (HSSE) method to chemically characterize some of the vapor phase components of cigarette smoke generated by the Borgwaldt RM20S® and collected within a cell culture exposure chamber. The method was based on passive sampling within the chamber by HSSE using a Twister™ stir bar. Following exposure, sorbed analytes were recovered using a thermal desorption unit and a cooled injection system coupled to gas chromatography/mass spectrometry for identification and quantification. Using the HSSE method, sixteen compounds were identified. The desorption parameters were assessed using ten reference compounds and the following conditions led to the maximal response: desorption temperature of 200 °C for 2 min with cryofocussing temperature of -75 °C. During transfer of the stir bars to the thermal desorption system, significant losses of analytes were observed as a function of time[27]; therefore, the exposure-to-desorption time interval was kept at the minimum of 10 ± 0.5 min. Repeatability of the HSSE method was

assessed by monitoring five reference compounds present in the vapor phase (10.1 - 12.9 % RSD) and n-butyl acetate, the internal standard (18.5 % RSD). The smoke dilution precision was found to be 17.2, 6.2 and 11.7 % RSD for exposure concentrations of 1, 2 and 5 % (v/v) cigarette vapor phase in air, respectively. A linear response of analyte abundance was observed as a function of dilution. Extrapolation to 100 % (v/v) cigarette vapor phase, *i.e.*, undiluted smoke, gave yields for the five compounds ranging from 6 to 450 ng for 10 min exposure.

5.2 Introduction

Recent advancements have led to the development of several smoke generation systems such as the Borgwaldt RM20S® (Phillips *et al.*, 2005), the Burghart Mimic Smoker-01® (Scian *et al.*, 2009a, Scian *et al.*, 2009b) and the Vitrocell Smoking Robot VC 10® (Aufderheide *et al.*, 2000). Also, this has led to the development of novel *in vitro* exposure systems such as British American Tobacco's (BAT) exposure chamber (Phillips *et al.*, 2005) and the CULTEX system (Aufderheide *et al.*, 2000). These systems generate fresh cigarette smoke over a wide range of dilutions *(i.e.,* exposure concentrations) required for *in vitro* cell culture investigations.

The Borgwaldt RM20S® in combination with BAT's exposure chamber using Transwell® inserts enables direct exposure of *in vitro* cellular cultures to whole cigarette smoke at the air-liquid interface (Fig. 5-1) (Massey *et al.*, 1998, Phillips *et al.*, 2005, Thorne *et al.*, 2009). This smoking machine, first commercialized in 2005, can smoke up to four cigarettes simultaneously with the smoke collected into four independent

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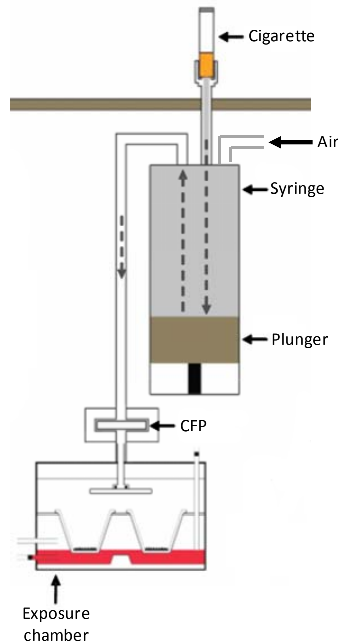
[27] Losses of 19-31 % were observed for the 5 reference compounds.

syringes. Each syringe can dilute the cigarette smoke with air in ratios ranging from 1:1.14 to 1:4000 (smoke volume : air volume), which corresponds to a range of 87 - 0.025 % (v/v) cigarette smoke in air. For biological exposures, doses tend to be in the range of 0.4 - 5% (v/v) cigarette smoke in air (Phillips *et al.*, 2005). The dose at this range of whole smoke dilution has only been correlated to the mass of total particulate matter (TPM) deposited on a Cambridge filter pad (CFP) placed either before the exposure chamber or within it, on a Transwell® insert (Massey *et al.*, 1998, Phillips *et al.*, 2005).

a)



b)

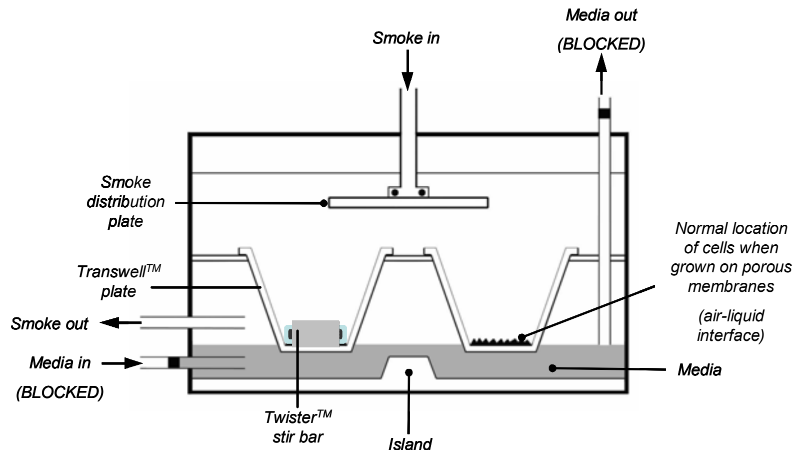


Figure 5-1: a) Schematic of the Borgwaldt RM20S® in combination with the BAT exposure chamber showing one of the four smoking ports connected to a dilution syringe. The machine smokes the cigarette, dilutes the smoke and delivers it to the exposure chamber (Thorne *et al.*, 2009). Insertion of a CFP to trap particulate matter downstream of the syringe allows exposure of cell or tissue culture to the diluted vapor phase only. b) Cross-section of the exposure chamber (Thorne *et al.*, 2009) showing the location for the Twister™ stir bar for HSSE experiments. For *in vitro* cell culture assays, medium flows in and out of the chamber. In this work, no cells and no culture medium were used

Our group recently carried out a study to determine the precision and accuracy of dilution of the smoke dose generated by the Borgwaldt RM20S® and delivered to the exposure chamber by measuring two reference standard gases (CH_4 and CO) introduced at the smoking port and a cigarette particulate phase marker (solanesol) from whole smoke (Kaur *et al.*, 2010). The repeatability of vapor phase dilution was ≤ 4.5 % RSD for dilutions of 0.1 - 0.52% (v/v) CH_4 in air and was ≤ 3.7 % RSD for dilutions in air of 1 - 10% (v/v) CO. The accuracy of CO measurements was 5.8 – 6.4 % error for the dilution range studied. The repeatability of dilution of the particulate phase in air ranged from 8.8 – 12 % RSD when quantifying solanesol. Overall, the findings suggested that the Borgwaldt RM20S® is a reliable tool to generate and deliver repeatable and reproducible doses of whole smoke to *in vitro* cultures (Kaur *et al.*, 2010). Scian *et al.* (Scian *et al.*, 2009b) measured in detail the chemical constituents of the particulate phase and reported recoveries at the exposure chamber of < 40 % in the Burghart smoking system for most of the compounds monitored, with repeatability of the measurements reaching over 35 % RSD for smoke diluted to 50% (v/v) in air. To date, no studies have reported the chemical characterization of the vapor phase smoke components within the exposure chamber itself. Evaluation of the dosimetry linearity of gaseous compounds present in the cigarette smoke following dilution and transfer to the exposure chamber is important to complete the characterization of this type of *in vitro* cell system.

Cigarette smoke is an extremely complex aerosol mixture composed of over 5000 chemical compounds (Rodgman *et al.*, 2009a, Rodgman *et al.*, 2009b) found distributed between the particulate and vapor phases. The vapor phase of cigarette smoke contains

volatiles and semivolatiles that play a major role in the *in vitro* toxicological responses (Bombick *et al.*, 1997b, CORESTA, 2005, Phillips *et al.*, 2004, Wieczorek *et al.*, 2006, Bombick *et al.*, 1997c). In this study, only the vapor phase of cigarette smoke was targeted. Techniques used for the collection and extraction of vapor phase constituents include vapor-liquid extraction, simultaneous distillation extraction (SDE), solid phase microextraction (SPME) and stir bar sorptive extraction (SBSE). The first two techniques tend to be time consuming and has resulted in poor repeatability (Zhong *et al.*, 2005) compared to SPME and SBSE. SPME using poly(dimethylsiloxane) (PDMS) as the absorptive phase has been used for the analysis of the volatile phase of tobacco flavors (Zhong *et al.*, 2005). Among other problems with SPME. is the reduced sensitivity due to limiting volumes of PDMS that can be used. SBSE, which also relies on PDMS as the sorptive phase, may resolve some of the issues faced when using SPME.

SBSE, which was introduced in 1999 (Baltussen *et al.*, 1999), is based on the theory of SPME and has been used extensively for environmental, food and biological applications (Bicchi *et al.*, 2009). SBSE was explored for extraction of cigarette vapor phase components due to its higher mass loading compared to traditional SPME, as it uses a thicker film of adsorbent together with an increased surface area (Ochiai *et al.*, 2008). For the moment, PDMS is the only commercially available SBSE coating. It provides low detection limits; in the sub-ppb level (Baltussen *et al.*, 1999, Benijts *et al.*, 2001). Typical SBSE devices, such as the Twister™ from Gerstel, consist of a magnetic stir bar encased in glass and coated with a ≤ 1 mm film of PDMS, which can contain between 55-219 μL of PDMS (compared to ≤ 0.5 μL in SPME) depending on the length of the stir bar. SBSE used to sample the vapor phase is referred to as headspace stir bar sorptive extraction (HSSE)

(Tienpont *et al.*, 2000, Bicchi *et al.*, 2000, Hou *et al.*, 2006, Bicchi *et al.*, 2002). Following exposure to a liquid or gaseous sample, the stir bar is placed within a thermal desorption unit coupled to a GC for separation and analysis. Advantages of HSSE and SBSE include robustness, ease of handling volatile compounds, automation, stability, good reproducibility and application to many types of analytes, heterogeneous samples and vapor, liquid and solid samples. A major drawback associated with both techniques is the difficulty to perform true quantitation even when using an internal standard, particularly in HSSE when only liquid phase standards are available.

The objective of the current study was to develop a solvent-free method to chemically characterize the vapor phase components (volatiles and semivolatiles) of diluted 3R4F cigarette smoke, generated by the Borgwaldt RM20S® and collected within an exposure chamber. The method chosen is based on passive sampling within the chamber (where cells or tissue cultures would be placed) by HSSE using a Twister™ stir bar. Following exposure to diluted cigarette smoke, sorbed analytes are recovered using a thermal desorption unit (TDS) and a cooled injection system (CIS) coupled to GC/MS for identification and quantification. A central composite experimental design based on three factors was proposed to determine the optimal desorption temperature, desorption time and CIS cryofocussing temperature needed to maximize the peak areas of ten selected reference compounds. Each factor was assessed at three levels. Using the experimental center-point, the repeatability of the HSSE method for diluted cigarette smoke was assessed for five of the vapor phase reference compounds. Recovery of the vapor phase components was measured as a function of time. Lastly, the dilution precision was measured for a range of

smoke dilutions typically used for cell culture assays (range of 1 - 5 % (v/v) cigarette smoke in air).

5.3 Materials and Methods

5.3.1 Whole Smoke Exposure System: the Borgwaldt RM20S® Smoking Machine and BAT's Exposure Chamber

The Borgwaldt RM20S® (Borgwaldt KC GmbH, Hamburg, Germany) is an automatic smoking machine that generates and dilutes cigarette smoke for *in vitro* cell culture investigations (Borgwaldt KC, 2010). It has a rotary based engine that can simultaneously smoke four types of cigarettes for several hours, depending on the smoking regime used. The instrument has an incorporated anemometer allowing for correct air flow, as well as electrical lighter, butt detector and butt extractor. The Borgwaldt RM20S® was designed in collaboration with BAT (Southampton, UK) and can be used with BAT's exposure chamber to enable cells or tissues to be exposed to the diluted smoke generated by the Borgwaldt RM20S® (Fig. 5-1a). Within the chamber, the cells or tissues lie on a 24 mm diameter Transwell® clear insert (Corning, NY, USA), which is a microscopically transparent porous polyester membrane, and are exposed to smoke at the air-liquid interface (Thorne *et al.*, 2009, Phillips *et al.*, 2005). However, no cells/tissues were used in the current study. At the beginning of each day and following each usage, the machine was run through a thorough maintenance routine, as this can affect the performance of the instrument (Kaur *et al.*, 2010). The cigarettes used in this study were Kentucky Reference

cigarettes (3R4F) (University of Kentucky, USA) and were conditioned at 22°C and 60 % relative humidity for 48 hours prior to smoking. Cigarettes were smoked in compliance to International Standard Organization (ISO) puffing profiles, consisting of 35mL puff volume over a 2 sec puff duration and 60 sec puff interval (International Organization for Standardization, 1999) for a total of 10 min unless otherwise indicated. Despite having four dilution syringes available for use, the same syringe (syringe C) was used throughout this study to eliminate any potential bias that could have been generated by the effect of a given syringe (Kaur *et al.*, 2010). Cigarette smoke was generated with the Borgwaldt RM20S® and a 44 mm diameter CFP was placed at the inlet of the exposure chamber to capture the particulate phase allowing only the vapor phase of the smoke to enter the exposure chamber. Some semi-volatiles are distributed between both the particulate and vapor phases (*i.e.*, phenols), and a portion of these compounds can be retained on the CFP (Baker, 1999, Adam *et al.*, 2006). Within the chamber, the PDMS-coated Twister™ stir bar was placed on a 24 mm diameter Transwell™ plate (Corning, NY, USA) for the duration of the smoke run (Fig. 5-1b). During exposure, no liquid/medium was used within the exposure chamber, where it would normally be if cells were present. Following exposure to the diluted smoke vapor phase (ranging from 1 to 10 % (v/v) in air), the stir bar was removed and transferred to the TDS.

5.3.2 HSSE Sampling and Collection

Commercially available Twister™ stir bars from Gerstel (Mülheim an der Ruhr, Germany) were 2 cm in length coated with a 0.5 mm thick PDMS film, which corresponds to 47 μL of PDMS. Conditioning was carried out according to the manufacturer;s direction

as follows: stir bars were placed in HPLC grade acetonitrile (Fisher Scientific, Whitby, Ontario, Canada) for 2 days and transferred into a specialized thermal conditioning unit for stir bars (Gerstel) at 280 °C for 4 h with a helium flow rate of 40 mL/min. This procedure provided for removal of residual acetonitrile and other impurities present in the PDMS phase. After conditioning, then cooling to room temperature, the stir bars were either exposed to a sample or stored in sealed glass tubes to prevent contamination from the surrounding environment. Following exposure, the stir bar was removed using a piece of stainless steel wire and transferred into an empty glass thermal tube of 4 mm ID and 177 mm length, blocked at both ends (Gerstel). The minimum transfer time between the end of smoke exposure and insertion into the TDS was 10 ± 0.5 min. Using the optimized desorption method, the stir bar was immediately ready for the next extraction. According to the manufacturer, stir bars may be used for hundreds of extractions with little or no deterioration, as long as temperatures are below 325 °C and solvents are not used for extraction of compounds. Multiple carry-over tests were performed to confirm that stir bars would not contribute to carry-over if re-used. During the experiments involving the use of higher than normal thermal desorption temperatures, stir bars were not re-used if deterioration of the PDMS phase was observed; *e.g.,* if siloxane background peaks were seen in the chromatogram, corresponding to hexamethylcyclotrisiloxane (m/z 207), octamethylcyclotetrasiloxane (m/z 281) or decamethylcyclopentasiloxane (m/z 73, 267 and/or 355).

5.3.3 Thermal Desorption and GC/MS Analysis

Analyte determination by GC/MS was carried out with an Agilent 6890N/5973N System (Agilent technologies, Waldbronn, Germany) equipped with a TDS (Gerstel) connected to a programmable temperature vaporization cooled injection system (CIS-4 model, Gerstel) using a transfer line heated at 300 °C. Desorption was carried out at 200, 250 or 300 °C for 1, 2 or 3 min under a helium flow of 60 mL/min. On the CIS-4 injector, the cryofocussing temperature was set to -100, -75 or -50 °C using liquid nitrogen (Praxair, Danbury, CT, USA). Following cryofocussing, the temperature of the CIS-4 was ramped up to 300 °C at 10 °C /min and held for 2 min during which time the analytes were transferred to the GC column with a split ratio of 37.3:1, unless otherwise indicated. Deactivated quartz wool liners were used (Gerstel). Separation was carried out in a DB-5MS column (60 m × 0.25 mm ID, 0.25 μm film thickness, from Agilent Technologies) with a constant column head pressure of 25 psi helium as the carrier gas. The oven temperature was set to 60 °C for 0 min and increased by 2 °C/min to 108 °C, then held for 1 min, for a total run time of 25 min. Finally, the analytes were detected by the mass selective detector (MSD) in scan mode from 41 to 300 m/z at 5.29 scans/sec, in positive ion mode. For all quantitative work, peak areas were obtained from integration of peaks in the extracted ion chromatograms (XICs).

5.3.4 Reference Compounds

From the compounds identified in the vapor phase of cigarette smoke using the methods described above, five were chosen for use as reference compounds based on their abundances, their wide distribution across the chromatographic elution window and their

peak resolution: benzene, 2,5-dimethyl furan, toluene, ethyl benzene and limonene (Thermo Fisher Scientific, ≥ 95 %).

5.3.5 Preparation of n-Butyl Acetate Internal Standard (IS)

A 7.6 µM standard solution of n-butyl acetate (Thermo Fisher Scientific) was prepared in HPLC grade methanol (Thermo Fisher Scientific). The IS concentration was selected based on the abundance range of the analyte ions in our sample run. The standard solution was aliquotted into amber GC vials with crimped aluminum caps (Chromatographic Specialties, Brockville, ON, Canada) for single use and stored at 4 °C until use.

In each case, 1 µL of the IS was transferred directly onto the 2 cm stir bar using a micropipette and left in a closed dish for 15 min to partition into the bulk PDMS. To assess the repeatability of the internal standardization procedure, the stir bar was placed in the center of a TDS tube and transferred to the TD system using the center point conditions (*i.e.,* desorption at 250 °C for 2 min and cryofocussing at -75 °C) of the experimental design as described in the next section. The repeatability was calculated as the percent relative standard deviation (RSD) obtained for the mean XIC peak area of the IS (n=12, using twelve different stir bars) by GC/MS analysis.

For smoke exposure experiments, stir bars spiked with IS were exposed to diluted vapor phase (10% (v/v) cigarette smoke in air). Following exposure, the stir bar was transferred to the TDS within 10 ± 0.5 min. Repeatability was calculated as the percent RSD (n=6, using six different stir bars) in mean XIC peak areas of the IS, of each of the five reference compounds listed above, as well as for the ratio reference peak-to-IS.

5.3.6 Optimization of Desorption Parameters and Statistical Analysis

Three factors (TDS desorption temperature, desorption time and CIS-4 cryofocussing temperature) were selected for maximization of the GC/MS peak area response using a face-centered composite experimental design. Three different levels of each factor were selected: desorption temperatures of 200, 250 and 300 °C; desorption times of 1, 2 and 3 min; cryofocussing temperatures of -50, -75 and -100 °C. Other than these parameters, all other conditions were the same as those previously described for exposure of the stir bar to 5 % (v/v) vapor phase in air for HSSE-GC/MS experiments. The XIC peak areas from the five reference compounds and an additional five analytes (2-methyl-1,3-butadiene, 3-methyl-2-butanone, p-xylene, styrene and 1-methyl-4-(1-methylethylidene)cyclohexane), were analyzed using a quadratic regression with second order interactions to determine the maximal response for the three experimental factors.

5.3.7 Stability of Sorbed Compounds on the Stir Bar

Following exposure to 5 % (v/v) vapor phase in air, the stir bar (*i.e.,* with sorbed sample) was transferred to the TDS at various time intervals to assess the effect of the delay time between vapor phase exposure and thermal desorption on recovery of the sorbed analytes. The times selected for this study were 10, 40, 160 and 1440 min (24 hours), where 10 min represented the fastest possible transfer from the exposure chamber to the TDS due to instrumental constraints. For stir bars not immediately analyzed, the desorption tubes were placed in individual plastic containers with caps (having inert inserts), wrapped in foil and stored at 4 °C. Using the optimized TDS and cryofocussing parameters, the XIC peak areas of the five reference compounds were used to assess their sorption persistence on the

stir bar after exposure. Each time point was analyzed in triplicate (*i.e.,* n=3, using three different stir bars).

5.3.8 Measurement of Dilution Precision

Stir bars were exposed to various smoke dilution levels typically used for cell culture assays (1, 2 and 5% (v/v) cigarette vapor phase in air) for a 10 min smoking period. The optimized HSSE method and TDS parameters were used for GC/MS measurement of the XIC peak areas of the five reference compounds for each dilution level. Each dilution was analyzed in triplicate (*i.e.,* n=3, using three different stir bars).

5.3.9 Semi-Quantitative Analysis of Diluted Smoke Vapor Phase

Standard solutions of the five reference compounds were prepared in HPLC grade hexane (Thermo Fisher Scientific) and calibration curves comprising 4 to 7 points covering the following ranges were prepared: benzene, 0 – 250 μM; 2,5-dimethyl furan, 0 – 50 μM; toluene, 0 – 100 μM; ethylbenzene, 0 – 10 μM; limonene, 0 – 10 μM. These concentration ranges were selected to match the XIC peak areas obtained in the smoke vapor samples. In each case, 1 μL of diluted standard was directly transferred onto the 2 cm stir bar using a micropipette and left in a closed dish for 10 min (same duration as smoke exposure). Following exposure, the stir bar was transferred to the TDS within 10 ± 0.5 min. This quantification procedure was used so that the sorption/desorption processes were taken into account. The optimized HSSE method and TDS parameters were used for GC/MS measurement of the XIC peak areas of each standard. Each concentration was analyzed once (*i.e.*, n=1). The linear equations obtained from the calibration curves (Table 5-3) were

used to estimate the minimum quantities of each reference compound present after exposure to 1, 2 and 5% (v/v) cigarette vapor phase in air (Table 5-3).

5.4 Results and Discussion

5.4.1 Vapor Phase Characterization using HSSE, Thermal Desorption and GC/MS Analysis

Our long term goal is to correlate the chemical composition with the toxicological response of cells or tissue exposed to diluted whole cigarette smoke. These experiments complement previous work carried out on the reliability of the smoke generation and dilution in the Borgwaldt RM20S® where repeatability, reproducibility and accuracy for solanesol, a particulate phase marker, and the standard reference gases CH_4 and CO were determined (Kaur *et al.*, 2010).

Following a 30 min exposure of the stir bar to 10 % (v/v) vapor phase in air, 16 compounds were identified by thermal desorption-GC/MS using the NIST Scientific and Technical Database Library with a match criterion of ≥80 % (Fig. 5-2). All were confirmed to be present in cigarette smoke based on open literature (Rodgman *et al.*, 2009b, Adam *et al.*, 2006, Chen *et al.*, 2003, Polzin *et al.*, 2007, Dong *et al.*, 2000, Darrall *et al.*, 1998, Bartle *et al.*, 1969, Baggett *et al.*, 1974, Stedman, 1968, Mauldin, 1976). Of the compounds identified, which included primarily esters, ketones, aldehydes or hydrocarbons, five were chosen as reference compounds for further study and method optimization (benzene, 2,5-dimethyl furan, toluene, ethyl benzene and limonene) based on their distribution across the

elution window. The remaining eleven compounds detected in the sample are identified in the GC/MS chromatogram (Fig. 5-2).

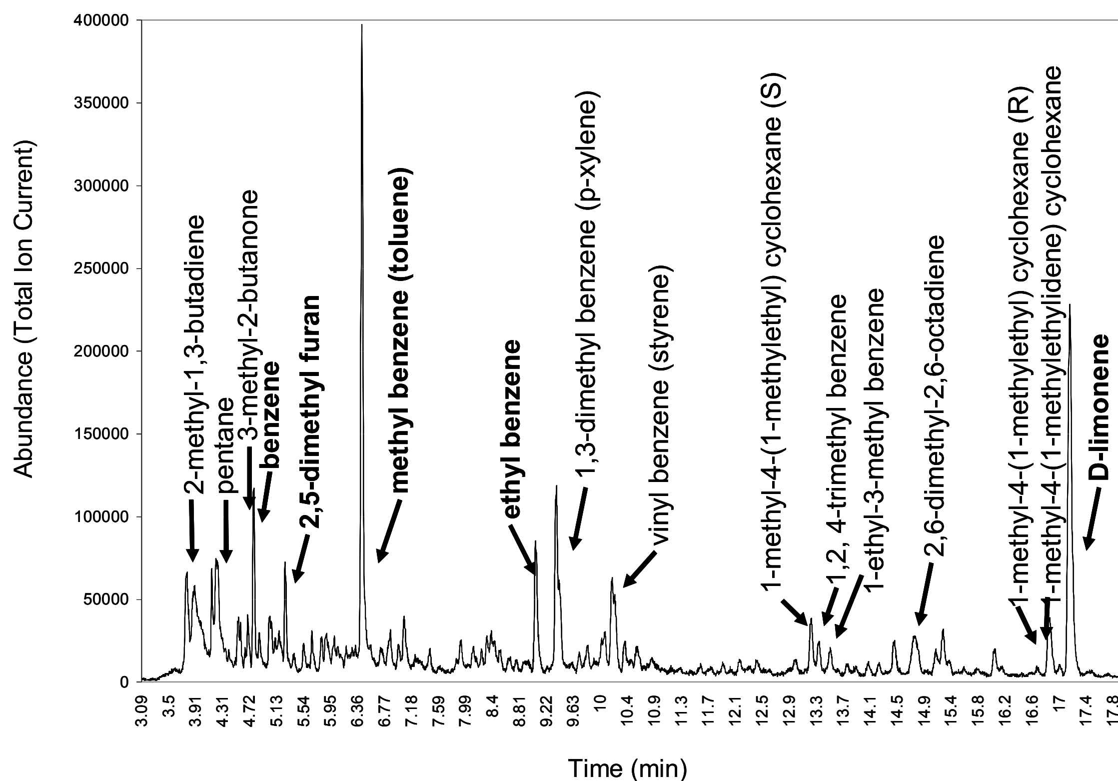


Figure 5-2: HSSE-GC/MS chromatogram showing the identification of 16 compounds found in the vapor phase sample. Sorption was carried out using a 2 cm PDMS-coated stir bar (Twister™) exposed to 10% (v/v) smoke vapor phase dilution in air for a smoking period of 30 min. The five compounds chosen for reference purposes, shown in bold type, were benzene, 2,5-dimethyl furan, toluene, ethyl benzene and limonene.

Prior to assessing the repeatability[28] of the method, the use of an IS was explored. Ideally, the IS should be a volatile species exposed in a similar way as the vapor phase to

[28] Recall, repeatability measurements correspond to the agreement of individual results obtained from testing the same sample, in the same laboratory, having a difference in one of the following: analyst, apparatus or the day whereas, reproducibility measurements correspond to the agreement of individual results obtained from testing one sample, in different laboratories, by a different analyst, on a different apparatus, on a different day (Centre d'expertise en analyse environnementale du Québec, 2007). The combination of repeatability and reproducibility data adds to the robustness of the method.

the stir bar during the smoke exposure process and should not interfere with the analytes in the chromatogram. However, due to limitations of the sample type, smoking machine and health and safety concerns, the IS had to be applied directly to the stir bar as a liquid prior to exposure. n-Butyl acetate was chosen for its volatility, which was similar to the reference compounds, its absence in the cigarette vapor phase sample and its elution time, which was well separated from the most abundant analytes. Its purpose was to eliminate response variability due to the thermal desorption-GC/MS analysis steps, but not due to the smoke generation, dilution, or volatile sorption processes. The IS peak areas obtained from XICs were compared following a 15 min sorption period in a closed vessel. The peak area repeatability for the IS was 25.8 % RSD (n=12)[29]. Despite its poor precision, the IS was used during collection of data following smoke vapor phase exposure. Sources of precision error associated with the use of the IS may include pipetting and transferring at the 1 µL level, volatility of the IS over the sorption period, variability in the stir bar retention capacity and/or TDS and GCMS steps.

The repeatability of the HSSE method was assessed by comparing the peak areas of the five reference compounds and the spiked IS after exposure to 5 % (v/v) cigarette vapor phase in air. The repeatability of the IS following exposure improved[30] to 18.5 % RSD (n=6) (Table 5-1), which was surprising because it included additional handling steps and a longer total exposure period compared to the closed vessel experiment. The repeatability of the five reference compounds varied from 10.1 - 12.9 % RSD (n=6) (Table 5-1, column 4),

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[29] Q test was performed on internal standard repeatability data values and indicated that all points should be retained (no outliers).
[30] F test performed on both data sets (IS repeatability), variances were not significantly different because F_{calc} (1.62) < $F_{critical}$ (4.70) at $\alpha = 0.05$.

which represents errors associated with the combined steps of smoke generation, smoke dilution, sorption by the stir bar, desorption by the TDS, cryofocussing and analysis by GC/MS[31]. The poorer precision for the IS compared to the five reference compounds could be due to it not having been sorbed in the same way (*i.e.,* applied to the stir bar from the liquid versus vapor phase) as well as due to errors associated with pipetting only 1 μL at each replicate. Application of the IS to correction of analyte peak areas resulted in higher RSDs (Table 5-1, column 6) than for the analytes alone and thus was not used for quantitative purposes. As a result, peak areas for the reference compounds without IS correction were compared in subsequent studies. Due to the complexity of the sample, XICs were used for integration of the peak areas to avoid measurement of co-eluting peaks and for accurate quantification.

[31] The RSDs obtained in our study were comparable to those obtained from similar studies: 5-20 % RSD (Darrall *et al.*, 1998), 10.2-37.1 % RSD (Polzin *et al.*, 2007).

Table 5-1 : Repeatability data for exposure of a PDMS-coated stir bar (Twister™) to 5% (v/v) vapor phase in air from 3R4F cigarettes for a 10 min smoking period. Integration results were obtained from the peak areas of the 5 reference compounds and the IS by GC/MS using the XICs.

Peak #	Peak ID	Mean Peak Area	RSD (%) (n=6)	Ratio $A_{analyte}/A_{IS}$	RSD (%) of Ratio
1	Benzene	56816.0	11.2	0.939	17.6
2	2,5-Dimethyl furan	20759.7	12.9	0.342	17.3
3	Toluene	235373.8	12.2	3.900	19.7
4	Ethylbenzene	35956.2	10.1	0.596	19.1
5	Limonene	34796.5	12.2	0.578	20.9
IS	n-Butyl acetate	61921.2	18.5	-	-

5.4.2 Optimization of Desorption Parameters and Statistical Analysis

A face-centered composite experimental design was used to obtain the maximal response for 10 analytes as a function of three desorption parameters. The 3-factor, face-centered design can be visualized as a cube with a star centered inside having its six points at the center of each face of the cube. Fifteen combinations of the three levels of each factor—desorption temperature, desorption time and cryofocussing temperature—were used, corresponding to those at the 8 “corners” of the cube, at the centers of the 6 faces (*i.e.,* the star points) and at the single, center point. The order of the runs was randomized to

reduce bias and the center point condition (desorption at 250°C for 2 min, cryofocussing at -75°C) was repeated at the beginning, the end and throughout the series of runs (*e.g.,* runs 1, 9, 11, 15, 16 and 20), which is typical in face-centered composite design. Quadratic regression analysis of the XIC peak area responses for each analyte did not yield any significant factor effects or interactions in the parameters assessed for the measured analytes. Low precision—in excess of 10 % RSD in peak areas (Table 5-1)—may have been responsible for this result. However, a slight trend in two of the factors could be seen graphically for the five main reference compounds (Fig. 5-3 b and c) with the best responses occurring at a desorption temperature of 250 °C for 2 min with a cryofocussing temperature of -75 °C[32]. It is important to note that at higher desorption temperatures (> 250°C), deterioration of the PDMS phase was observed by the presence of siloxane background peaks. Therefore, 200 °C was deemed the best desorption temperature to avoid damage to the stir bar.

[32] The trend observed for peak area as a function of desorption time (Fig. 5-3b) does not follow the expected trend predicted, increasing over time, and thus remains unclear.

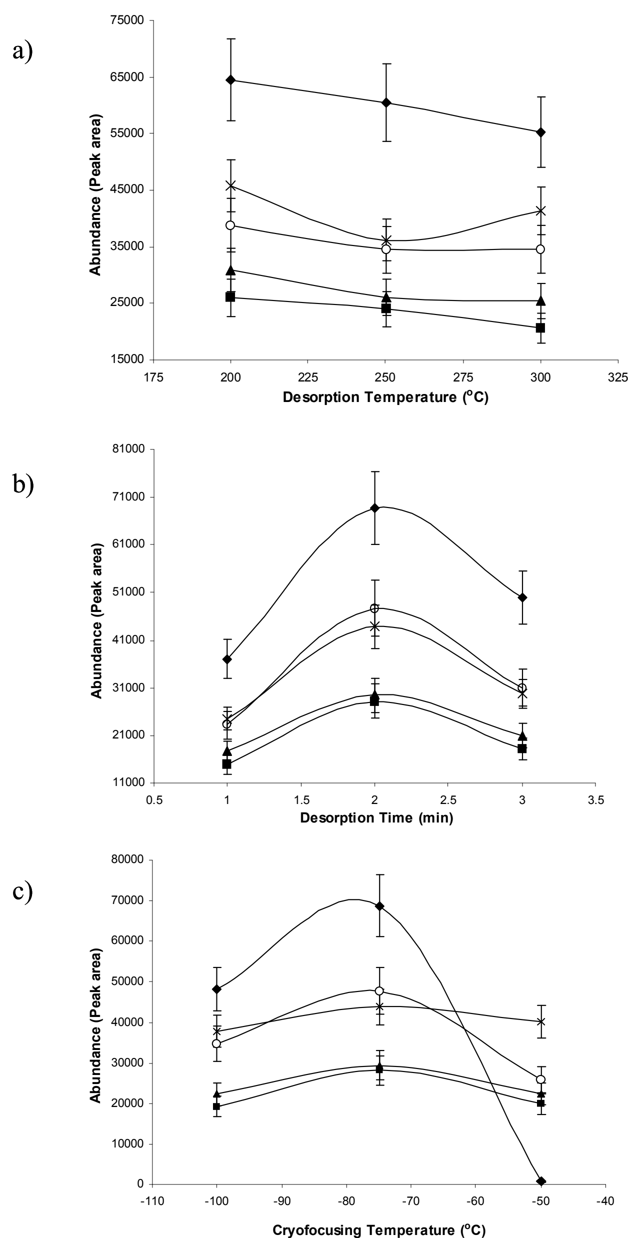

Figure 5-3: Effect of the three optimization factors on the abundances of five reference compounds exposed to 5 % (v/v) vapor phase in air. a) Peak area as a function of stir bar desorption temperature for a 2 min desorption time and cryofocussing at -75°C; b) Peak area as a function of stir bar desorption time for desorption at 250°C and cryofocussing at -75°C; c) Peak area as a function of cryofocussing temperature when desorption was held at 250°C for 2 min. Integrated peak areas of the reference compounds were obtained from the GC/MS XICs: Benzene, ─◆─; 2,5-Dimethyl furan, ─■─; Toluene (peak area /10 to aid plotting), ─▲─; Ethyl benzene, ─╳─; Limonene, ─○─ . Each data point represents an average of three individual runs using three different stir bars and error bars indicate relative standard deviation.

Arrangement of the runs in decreasing order of cryofocussing temperature followed by increasing desorption time (Table 5-2) also showed that the highest peak area responses for six of the ten analytes occurred at the desorption temperature of 200 °C for a 2 min desorption time and cryofocussing at -75 °C, and thus these maximal conditions were used for all further studies. These desorption conditions are similar to those used in another tobacco flavor study of volatile and semi-volatile components (Hou *et al.*, 2006). Some analytes were not efficiently cryofocussed, and thus absent, when using -50 °C (Table 5-2).

Table 5-2: Optimization of the HSSE desorption parameters representing 10 vapor phase components found on a stir bar following exposure to 5 % (v/v) cigarette vapor phase in air. The 10 compounds identified by GC/MS are listed in order of retention time, t_r. Relative peak areas were calculated from the XIC peak areas for each analyte

Desorption Parameters					Relative Peak Area[a]									
					2-Methyl-1,3-butadiene	3-Methyl-2-butanone	Benzene	2,5-Dimethyl furan	Toluene	Ethyl-benzene	p-Xylene	Styrene	1-Methyl-4-(1- methyl-ethylidene) cyclohexane	Limonene
Run #	Desorption Temp (°C)	Desorption Time (min)	Cryofocussing Temp (°C)	t_r	3.77 min	4.72 min	4.78 min	5.27 min	6.42 min	9.06 min	9.36 min	10.20 min	16.80 min	17.14 min
				b.p.	34 °C	94 °C	80 °C	93 °C	110 °C	136 °C	138 °C	145 °C	~172 °C [b]	176 °C
12	200	1	-50		0	0	0	0.80	0.70	0.75	0.66	0.63	0.54	0.58
8	300	1	-50		0.04	0	0.09	0.88	0.90	0.96	0.94	0.95	0.88	0.80
5	250	2	-50		0.02	0	0.01	0.76	0.72	0.88	0.84	0.77	0.50	0.52
19	200	3	-50		0	0	0	0.69	0.68	0.77	0.29	0.70	0.78	0.84
6	300	3	-50		0	0	0.06	0.92	0.94	0.91	0.92	**1.00**	**1.00**	**1.00**
4	250	1	-75		0.74	0.73	0.57	0.57	0.57	0.53	0.54	0.54	0.35	0.47
7	200	2	-75		0.94	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**	0.90	0.74	0.78
Mean [b]	250	2	-75		0.90	0.86	0.91	0.79	0.73	0.80	0.70	0.74	0.63	0.67
10	300	2	-75		0.91	0.76	0.86	0.79	0.82	0.90	0.87	0.75	0.58	0.70
14	250	3	-75		0.82	0.75	0.78	0.70	0.68	0.65	0.30	0.62	0.60	0.63
3	200	1	-100		0.82	0.68	0.82	0.65	0.56	0.73	0.74	0.77	0.60	0.62
2	300	1	-100		**1.00**	0.81	0.86	0.72	0.65	0.68	0.67	0.69	0.57	0.62
13	250	2	-100		0.86	0.74	0.75	0.74	0.72	0.83	0.37	0.77	0.62	0.70
17	200	3	-100		0.88	0.87	0.82	0.62	0.64	0.84	0.39	0.78	0.71	0.67
18	300	3	-100		0.76	0.65	0.56	0.63	0.55	0.61	0.59	0.58	0.47	0.50

[a] Per compound, i.e., data were normalized vertically with respect to the highest abundance (peak area) in a given column.

[b] Boiling point estimated based on relative retention time.

[c] Mean response of six center point runs: 1, 9, 11, 15, 16 and 20.

5.4.3 Stability of Sorbed Compounds on the Stir Bar

Using the optimized desorption conditions, the stability of the vapor phase components sorbed on the stir bar (*i.e.*, their resistance to spontaneous desorption) was studied. Four time intervals representing transfer of the stir bar from the exposure chamber to the TDS were assessed: 10 min, which was the minimum transfer time, 40, 160 and 1440 min (24 hours). The stability was estimated by comparing the peak areas of the five reference compounds as a function of transfer time following exposure to 5 % (v/v) cigarette vapor phase in air (Fig. 5-4). A 19 - 31 % loss in the reference compounds was observed within 40 min followed by only a 0 - 9 % loss over the next two hours. The losses were independent of the compounds' volatility[33]. As predicted, these results indicate the importance of considering the volatile, or semi-volatile, nature of the sample in such studies. Therefore, for all further studies, stir bars were consistently transferred to the TD system as soon as possible (*e.g.,* 10 ± 0.5 min) to maintain consistency between runs and to maximize sensitivity. This study did not take into account potential degradation of vapor phase compounds, or reactions and interactions between various smoke components (Rodgman, 2000).

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[33] The desorption rates were not similar for all compounds, but a function of their respective partition coefficients with the PDMS phase and concentration gradients. Log $K_{o/w}$ and losses were for; benzene (2.13 and 19%); 2,5-dimethyl furan (2.24 and 27%); toluene (2.73 and 31%); ethylbenzene (3.15 and 24%) and D-limonene (4.38 and 31%), respectively. The only reference compound that did not follow this trend was ethylbenzene. Based on error bars (SDs) for the measurements, losses between 19-31 % do not indicate significant losses and are most likely related to error on measurements.

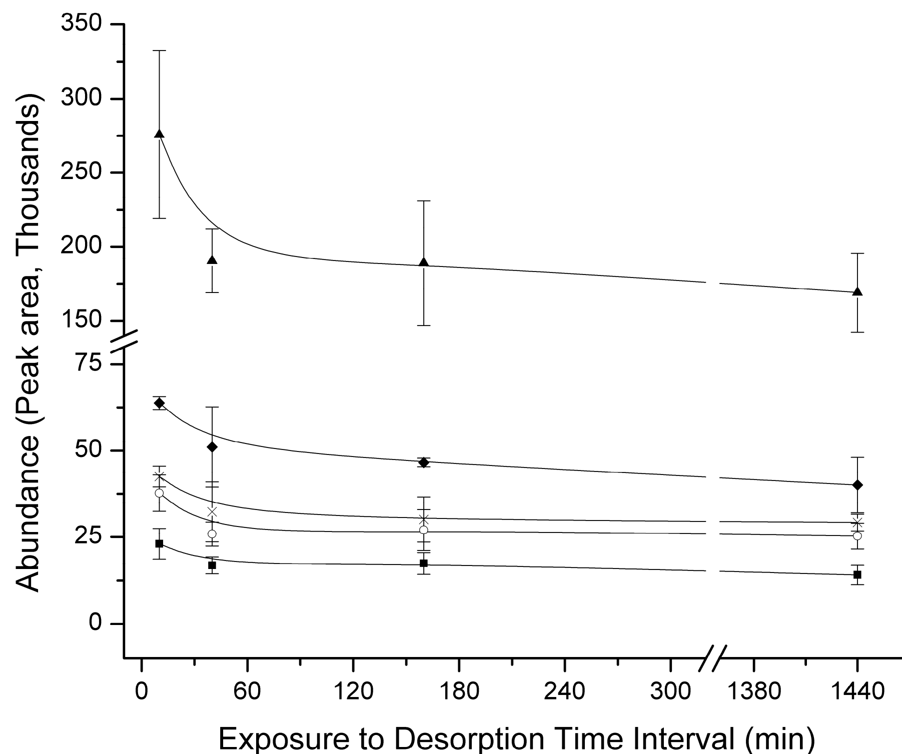

Figure 5-4: Stability measurements of five vapor phase reference compounds desorbed from a Twister™ stir bar, following exposure to 5 % (v/v) cigarette vapor phase in air for HSSE experiments. The transfer time intervals (*i.e.,* between exposure and desorption) selected for the analysis were 10, 40, 160 and 1440 min (24 hours). Integration results were obtained from the XIC peak areas of the 5 reference compounds by GC/MS: Benzene, ─◆─; 2,5-Dimethyl furan, ─■─; Toluene, ─▲─; Ethylbenzene, ─✕─; Limonene, ─○─ . Each data point represents an average of 3 individual runs using 3 different stir bars and error bars indicate standard deviation.

5.4.4 Measurement of Dilution Precision

Using the optimized HSSE-GC/MS method, the dilution precision of the vapor phase was measured by monitoring the peak areas of the five reference compounds as a function of the exposure concentration (*i.e.,* dose equivalent): 1, 2 and 5 % (v/v) cigarette

vapor phase in air (Fig. 5-5). The average RSDs associated with the vapor phase generated and diluted by the Borgwaldt RM20S® were 17.2, 6.2 and 11.7 % (n=3) at 1, 2 and 5 % (v/v) smoke, respectively. The linearity was good with correlation coefficients (r^2) of >0.99 for each compound. Previous work in our laboratory indicated that the repeatability of the dilution of reference standard gases (CH_4 and CO) was between 0.7 - 4.5 % RSD with r^2 >0.99 for a similar dilution range (Kaur *et al.*, 2010). From the same study, it was also shown that the precision of the smoke generation itself was only 12 % RSD based on measuring solanesol in the particulate phase over the same smoke dilution range: 1 – 5 % (v/v) in air. The lower precision seen in the current study at 1 % smoke dilution (*i.e.,* 17.2 % average RSD for 5 compounds) is likely due to high variability in the minute quantities of analyte sorbed on the stir bar at this dilution level since all other factors affecting the mass sorbed, *i.e.,* PDMS film thickness, sample volume, partition coefficient, etc., were unchanged. Taking this into account, the results obtained in the current study corroborate the conclusion that the Borgwaldt RM20S® presents acceptable limits for repeatability measurements across dilutions.

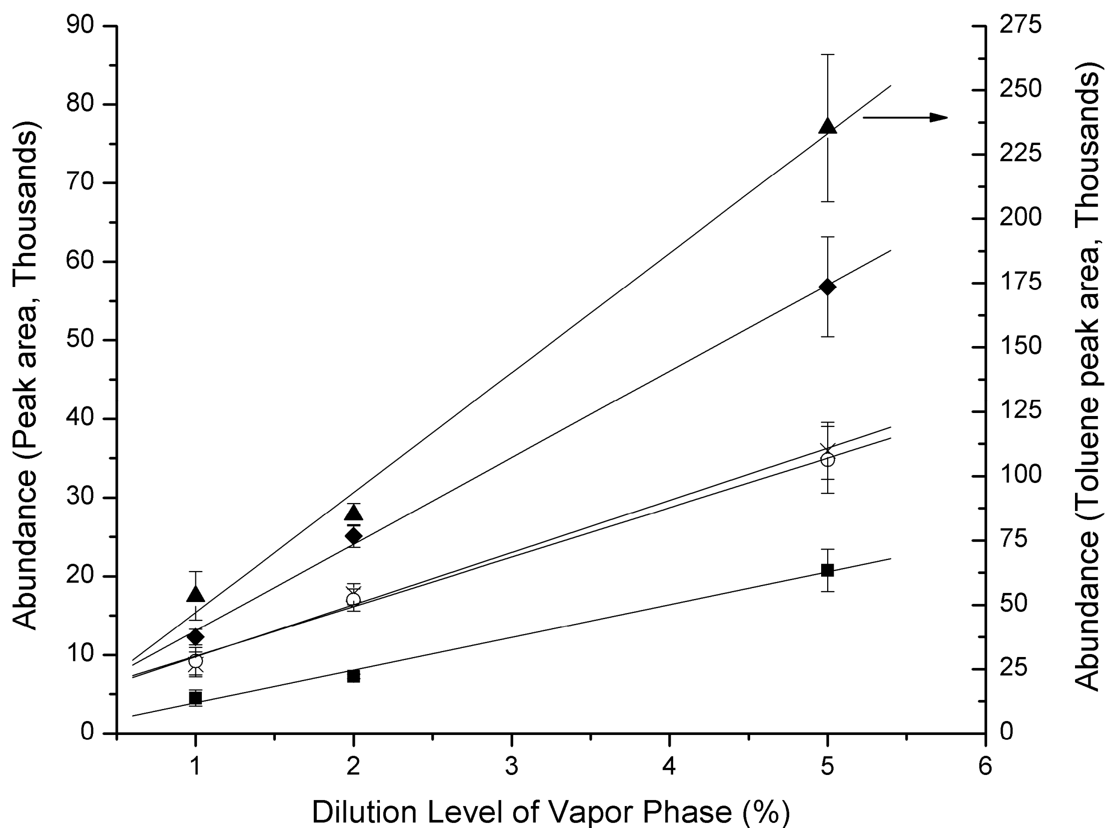

Figure 5-5: Dilution precision data representing the vapor phase components found on a PDMS coated stir bar (Twister™), following exposure to 1, 2 and 5 % (v/v) cigarette vapor phase in air from 3R4F cigarettes for HSSE experiments. Integration results were obtained from the peak areas of the 5 reference compounds by GC/MS using the XICs: Benzene, —◆—; 2,5-Dimethyl furan, —■—; Toluene, —▲— (abundance shown on right vertical axis); Ethylbenzene, —✕—; Limonene, —○— . Each data point represents an average of 3 individual runs using 3 different stir bars and error bars indicate standard deviation.

5.4.5 Semi-Quantitative Analysis of Diluted Smoke Vapor Phase

External calibration was achieved using standard solutions in hexane applied to the stir bar. The same TDS-GC/MS parameters as with the vapor phase measurements were used.

Relative response factors calculated from the XIC peak areas for each standard at 10 µM showed that ethylbenzene and limonene gave large responses (Table 5-3), presumably because of their stronger partition into the PDMS phase on the stir bar. Based on the smoke vapor phase peak areas at each dilution level (*e.g.*, Fig. 5-5 data), yields ranging from 0.1 to 22 ng were estimated (Table 5-3). It should be noted that the values in Table 5-3 represent minimum amounts as it is unlikely that equilibrium was reached for each puff. Quantitative HSSE is typically carried out in a closed system whereas in this experiment, each puff remained in the exposure chamber for 1 min and was then pushed out by a subsequent puff of fresh cigarette vapor phase. In addition, 100 % recovery was likely not attained for the sorption/desorption process with the PDMS phase, which is compound dependent. Extrapolation to 100% vapor phase, *i.e.*, undiluted smoke, gives estimated yields in the range of 6 to 450 ng for the reference compounds (Table 5-3), which is 2 – 3 orders of magnitude less than reported amounts per cigarette (one full cigarette = 7 – 10 puffs). Other studies on the vapor phase components of cigarette smoke have reported quantities of benzene ranging from 23 to < 70 µg/cig (Darrall *et al.*, 1998, Adam *et al.*, 2006, Chen *et al.*, 2003, Baggett *et al.*, 1974, Polzin *et al.*, 2007), 2,5-dimethylfuran at 58 µg/cig (Baggett *et al.*, 1974), toluene ranging from 57 to < 200 µg/cig (Darrall *et al.*, 1998, Adam *et al.*, 2006, Chen *et al.*, 2003, Baggett *et al.*, 1974, Polzin *et al.*, 2007), ethylbenzene ranging from 4.4 – 5.5 µg/cig (Darrall *et al.*, 1998, Polzin *et al.*, 2007) and D-limonene at 64 µg/cig (Baggett *et al.*, 1974). The fact that previous studies were carried out on different tobacco blends (*e.g.*, 1R4F, 2R4F reference cigarettes, etc.) with different smoking apparatuses and assessed using different sampling methods (*e.g.*, cold or cryo traps (Darrall *et al.*, 1998, Baggett *et al.*, 1974, Polzin *et al.*, 2007); collection bags (Darrall *et al.*, 1998, Polzin *et al.*,

2007); solvent trap (Darrall *et al.*, 1998); on-line analysis (Baggett *et al.*, 1974, Adam *et al.*, 2006) does not fully account for the large differences in quantities obtained compared to the present study. Besides the HSSE factors mentioned above, underestimation of the yields may also result from the procedure used to introduce the liquid-phase standards (*i.e.*, error in pipetting 1 μL; differences between the PDMS/liquid versus PDMS/gas phase sorption processes), the known dilution error of up to 6.4% for the smoking machine (Kaur *et al.*, 2010) and the large error in the calibration curve intercept values (Table 5-3).

Table 5-3: Semi-quantitative analysis of the five reference vapor phase 1 compounds found on a stir bar following 10 min exposure to 1, 2 and 5 % (v/v) cigarette vapor phase in air for HSSE experiments.

Reference compound	t_r of standard (min)	t_r of sample (min)	Relative response factor[a]	Calibration curve[b]	Estimated minimum amount for 10 min exposure to diluted smoke vapor phase (ng)[c]			Extrapolation to undiluted smoke vapor phase (ng)
					1 %	2 %	5 %	(100 %)
Benzene	4.79	4.78	1.0	y = (189 ± 30)x + (3401 ± 4013) r^2 = 0.9529	3.7	8.9	22.0	450
2,5-Dimethyl furan	5.26	5.27	1.9	y = (364 ± 65)x + (1626 ± 1660) r^2 = 0.9405	0.76	1.5	5.1	110
Toluene	6.41	6.42	8.5	y = (2257 ± 121)x + (1300 ± 5145) r^2 = 0.9858	2.1	3.4	9.6	190
Ethylbenzene	9.03	9.06	27	y = (11896 ± 634)x - (3712 ± 3252) r^2 = 0.9915	0.11	0.19	0.35	6
Limonene	17.1	17.1	15	y = (48004 ± 2200)x - (1006 ± 1538) r^2 = 0.9937	0.21	0.38	0.75	13

[a] Based on XIC peak areas for standards at 10 μM.
[b] Linear regression curves have slope units of Peak area/μM; r^2: correlation coefficient.
[c] Calculated from average peak areas (Fig. 5) then converted to ng based on 1 μL standard applied to stir bar. Exposure of 10 min = 10 puffs. Error is estimated to be on the order of 18 – 25 % RSD based on results for the IS, n-butyl acetate.

5.5 Conclusions

The HSSE method was successfully applied to the characterization of the vapor phase of diluted cigarette smoke collected in an exposure chamber. This procedure allowed for the components sorbed on the stir bar to be desorbed, re-focused and analyzed in one integrated/automated experimental step, without the use of extraction solvents. This technique, when coupled to GC/MS, allowed for the rapid and direct quantitative analysis of volatile and semi-volatile smoke vapor phase components in the exposure chamber of the Borgwaldt RM20S® and provided a linear response across smoke dilutions. The IS, n-butyl acetate, did not prove useful for improving precision associated with the method. Semi-quantitative analysis of five smoke vapor phase components showed systematic underestimation of the yields compared to previously published values. Improvements in quantification and precision might be achieved by using deuterated standards or a gas-phase internal standard (*i.e.*, hexane) introduced at each puff via a switching valve inserted before the dilution syringe. Nonetheless, the results obtained in this study were within acceptable limits for repeatability measurements between dilutions.[34]

The HSSE technique is simple, cost effective, can be easily implemented in most laboratories and can be applied to a wide range of analyses, *e.g.,* environmental, food and automotive. Moreover, these results show that although there are a wide range of volatiles and semi-volatiles with different physical properties, dilution and delivery in the Borgwaldt RM20S® is achieved through a non-selective manner. In addition, this study provided

[34] A linear response was observed for dilutions of 1- 10 % smoke vapour phase in air, and thus, was considered acceptable.

additional knowledge about a whole smoke exposure system to give us a more complete picture of the exposure concentration applied to cell cultures for future toxicology studies.

5.6 Acknowledgements

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6 Conclusion

6.1 General Remarks: Summary of Principle Results

Tobacco smoke is one of the most challenging mixtures to analyze based on its complexity, from the number and types of constituents to its aerosol nature involving a wide range of particles mixed with the vapor phase. Our work was based on the development of reliable and robust tobacco smoke fractionation methods to allow for the comparisons of toxicological effects. Specific analytical separation and detection techniques in combination with *in vitro* toxicological assays were used for these types of analyses. The uniqueness of our approach lies in the union of chemical identification in parallel with toxicological characterization. Our approach began with designing the methodology to work with an individual tobacco component, which could then be applied to a more complex sample, *e.g.*, the vapor phase of cigarette smoke.

Our first objective was to chemically characterize the most bioactive fractions associated with the particulate phase combustion products of a single tobacco constituent, chlorogenic acid. By combining analytical techniques in parallel with *in vitro* toxicological testing, specifically the IVMNT, we developed a method to fractionate and identify the groups of compounds responsible for the cytotoxicity observed by the IVMNT. Catechol, a co-carcinogen and known product of the combustion of chlorogenic acid, was identified to be the major component in the most bioactive fraction assessed by the IVMNT.

The practical complexity of assessing tobacco smoke and vapor phase toxicity was simplified by using the Borgwaldt RM20S® whole smoke generation instrument and BAT *in vitro* exposure chamber, which became available in our laboratory in 2007. In previous reports using the same system, cell culture studies had been carried out and a positive dose

response relationship was observed for cells exposed to cigarette whole smoke and cell viability measured by the NRU assay (Phillips *et al.*, 2004). *In vitro* biological assays have a high degree of variability associated with the use of heterogeneous cell samples, thus, all other sources of variability must be reduced or accounted to accurately assess toxicological endpoints. However, to our knowledge, there was no manufacturer's specifications or data generated on the reliability of the cigarette smoke dose generated and delivered within the exposure chamber. Our second objective was to assess the reliability of the dose delivery from this type of instrumentation. Using reference gas standards (CO and CH_4, introduced at the smoking port) as well as a particulate phase marker (solanesol, in actual cigarette smoke), we were able to assess the machine's reliability for smoke generation and dilution in terms of repeatability, reproducibility and accuracy; the delivery system was deemed reliable for cell exposure studies based on our results in Chapter 4. Moreover, our results were comparable to those obtained from another laboratory for a similar study (*i.e.* for CH_4 repeatability studies, our laboratory gave an overall precision of 2.2-4.5% and combined precision from both labs, gave a RSD of 6.3-17.3%).

Our third objective was to chemically characterize the vapor phase constituents delivered to the exposure chamber using HSSE (Chapter 5). HSSE has been used for the collection of volatiles in the environment/air, food and flavors (Hou *et al.*, 2006, Bicchi *et al.*, 2002, Koester, 2003, Bicchi *et al.*, 2009) and to our knowledge has not been specifically used for the enrichment of tobacco smoke components generated by a smoking machine. Thus, we developed a sensitive HSSE technique to extract the vapor phase components present in the chamber during cigarette smoke exposure, at a dilution or dose commonly used in cell culture exposures. The major components were identified by mass

spectrometry to evaluate the dosimetry of gaseous compounds present in the exposure chamber.

Recently Scian *et al.*, carried out similar types of characterization studies for another type of smoke exposure system, the Burghart Mimic Smoker-01 (Scian *et al.*, 2009a, Scian *et al.*, 2009b). Their study was solely based on particulate phase measurements to assess the reproducibility of the dose delivered to the exposure chamber and the deposition (*i.e.* loss) between undiluted and diluted smoke (50 ± 10 % loss observed). The authors concluded that significant changes occur between smoke generation and delivery to the exposure chamber based on the analysis of the particulate phase. Overall, their study confirms the importance of chemical characterization for these types of systems in order to accurately assess toxicological endpoints. Other than these mentioned studies, only biological studies have been carried out with whole smoke exposure systems.

Traditionally, the approach used by the tobacco industry targeted the solvent extracts of the particulate phase or vapor phase using indirect methods (Lestari *et al.*, 2006). The general trend is now heading towards generation and subsequent analysis of whole cigarette smoke, as a holistic and more direct way to approach smoke (Phillips *et al.*, 2005, Scian *et al.*, 2009a, Scian *et al.*, 2009b, Thorne *et al.*, 2009, Aufderheide *et al.*, 2000, Massey *et al.*, 1998). More specifically, the new techniques developed can be used to assess relative toxicities between different types of cigarettes and perhaps to the assessment of a harm-reduced product. The techniques developed in this work can also be applied to other industries that face similar problems based on the complexity of samples and the combination of sophisticated type of instrumentation required for biologically active samples, including, environmental/air quality, diesel exhaust, food, fragrance.

6.2 Successes and Drawbacks of the Project

In the field of tobacco science, it is rare to find multi-disciplinary research tying together refined analytical chemistry techniques with toxicological assays. Our objectives of being able to analyze a biologically relevant complex mixture in a reliable way were met. From our study on chlorogenic acid combustion products (Chapter 3), the most toxic sub-fraction was identified and contained catechol, 3,4-dihydroxybenzoic acid and an isomer of cis-benzeneglycol (structure unconfirmed). Catechol and 3,4-dihydroxybenzoic acid have been identified as tobacco smoke constituents. Catechol is a "Hoffmann analyte" (Rodgman *et al.*, 2009b), genotoxic and blocks cell proliferation (Kaur *et al.*, 2009). 3,4-Dihydroxybenzoic acid has not been reported to be genotoxic or an inhibitor of cell proliferation. The characterization of the cigarette smoke vapor phase components presented in Chapter 5, involved the use of 5 reference markers: benzene, 2,5-dimethyl furan, ethylbenzene, toluene and D-limonene. These markers have also been identified in tobacco smoke (Rodgman *et al.*, 2009b). Benzene and toluene are both "Hoffmann analytes" (Rodgman *et al.*, 2009b); benzene, toluene and ethylbenzene are commonly used as markers for smoke volatile organic compounds (Hinwood *et al.*, 2006, Sanchez *et al.*, 2006); and 2,5-dimethyl furan has been used as an effective tobacco smoke biomarker in human breath and as an environmental tobacco smoke marker (Gordon *et al.*, 2002, Bi *et al.*, 2005). Our objectives were not to identify all of the compounds present in our samples, but, to develop the analytical methods to characterize tobacco smoke components at biologically relevant concentrations. By confirming the presence of the compounds listed

above, we can conclude that the methodology developed is viable for analysis of complex mixtures, such as tobacco smoke.

Although simplification of the sample matrix is required for the analysis of tobacco smoke, it is important to note that synergistic/additive effects may have been disrupted and/or not accounted for (Chapter 3).

The method used for quantitation (Chapter 5) underestimated product yields and had many sources of error. In addition, some of the non-volatiles and semi-volatiles not being extracted/enriched using the HSSE method; assessment of both the particulate and vapor phases can not be carried out simultaneously using this method. In terms of the semi-quantitative analysis performed on the cigarette smoke vapor phase components (Chapter 5), the method of analysis can largely influence the results obtained because of the many variables involved. Despite the use of reference cigarettes, collection and extraction techniques for vapor and particulate phases are not consistent throughout the literature and standardization is quite challenging. Sorptive phases and solvents preferentially extract compounds that have high respective selectivity to "get what you select for". The methodology developed (Chapters 4-5) can potentially serve as standardized methods for the assessment of instrumentation (Chapter 4) or screening of tobacco products (Chapter 3, 5) for the Tobacco Industry.

6.3 Future Challenges

In Chapter 5, we used *n*-butyl acetate as our internal standard for the analysis of vapor phase components in cigarette smoke by HSSE. Subsequent to publication, an F test

was performed on the internal standard data (18.5 -25.8 % RSD) to assess variances; variances were not significantly different because F_{calc} (1.62) < $F_{critical}$ (4.70) at $\alpha = 0.05$. Future work to improve quantitation, could include investigating the use of a internal standard (*i.e.*, deuterated benzene or a hexane gas phase standard) introduced at each puff via a switching valve inserted before the dilution syringe. This could eliminate the high variability associated with pipetting low volumes of 1μL. It is important to consider that fresh smoke analytes were present in the exposure chamber, sent to the exhaust while subsequent puffs were generated and not allowed to equilibrate. Further studies could include allowing for the extraction by HSSE following exposure in a closed vessel for a fixed period of time.

Furthermore, other work could include, simultaneous chemical characterization of the particulate and vapor phases in the chamber during cell culture exposure and also working towards an online method to monitor the particulate and vapor phase components in real time or puff-by-puff, to reduce losses, increase sensitivity and to monitor the smoke as it changes over time (*i.e.* aging process and generation of artifacts) (Borgerding *et al.*, 2005, Adam *et al.*, 2006).

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8 Appendix A - Experimental Flow Chart

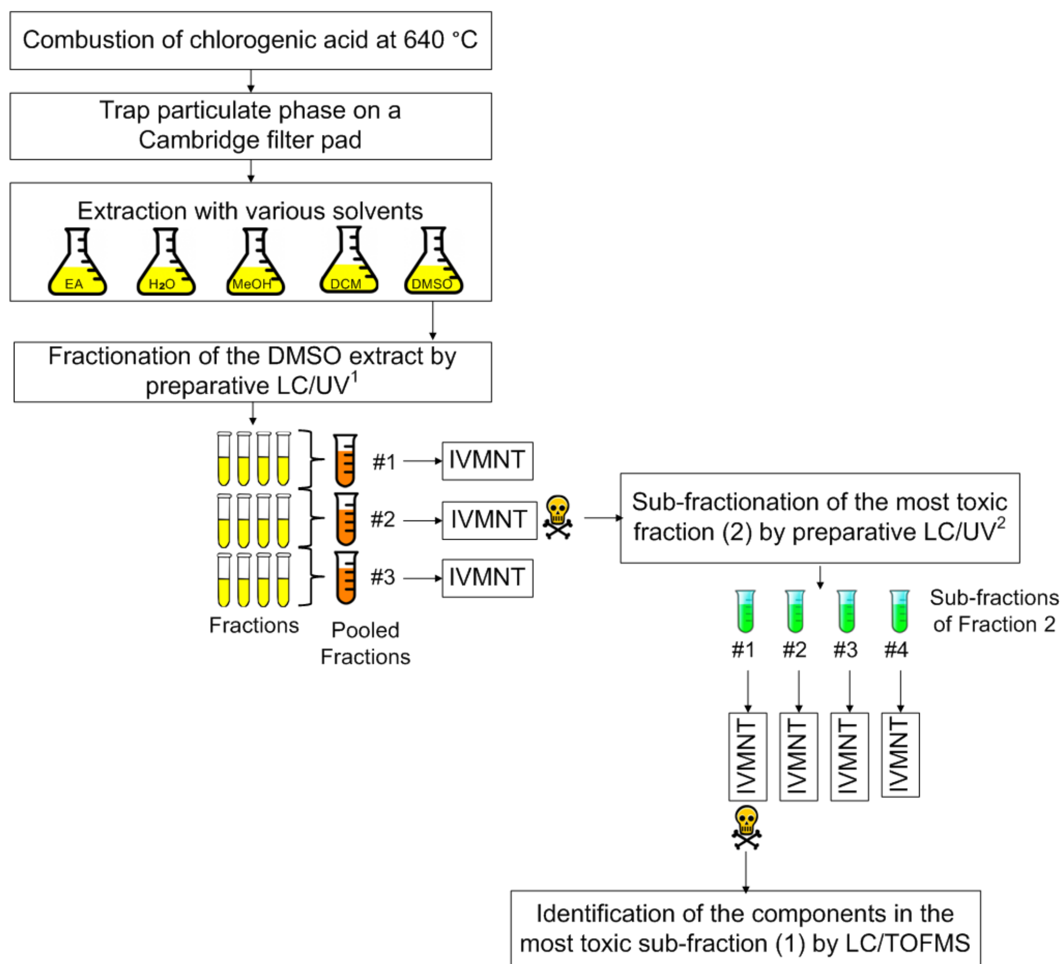

Figure 8-1: Experimental flow chart of the chlorogenic acid combustion product study. [1]Corresponds to LC/UV reversed-phase experimental conditions described in section 3.3.9 [2]Corresponds to LC/UV reversed-phase experimental conditions described in section 3.3.11

9 Appendix B - Maximization of the HPLC-MS Separation of a Standard Mixture of Seven Compounds found in the Combustion of Chlorogenic Acid

The DMSO extract of the combustion products of chlorogenic acid was separated and collected in three major fractions by preparative LC/UV. The second fraction was identified as the most bioactive fraction by the IVMNT. Many compounds were identified within this fraction by HPLC-MS. The objective of this set of experiments was to maximize the analytical separation of this second fraction using seven standard reference compounds to represent this fraction. This appendix is based on work done and reported by a summer student, Mari Bratberg, that I co-supervised with Karen C. Waldron (Bratberg, 2006).

In total, four different stationary phases (Table 9-1) and nine different mobile phase compositions (Fig. 9-1 and 9-2) were tested. **Table 9-1:** Description of HPLC separation columns used (Phenomenex, Torrance ,CA)

Column number	Name	Phase	Description	Particle size/ µm	Pore size/Å	Diameter (mm)	Length (mm)
1	Synergi	Polar-RP	Ether-linked phenyl with polar end-capping	4	80	4,6	150
2	Gemini	C6-Phenyl	R=C_6-phenyl (no endcapping)	5	110	4,6	150
3	Synergi	Hydro-RP	C_{18} with polar end-capping	4	80	4,6	150
4	Gemini	C18	R=C_{18} (no endcapping)	5	110	4,6	150

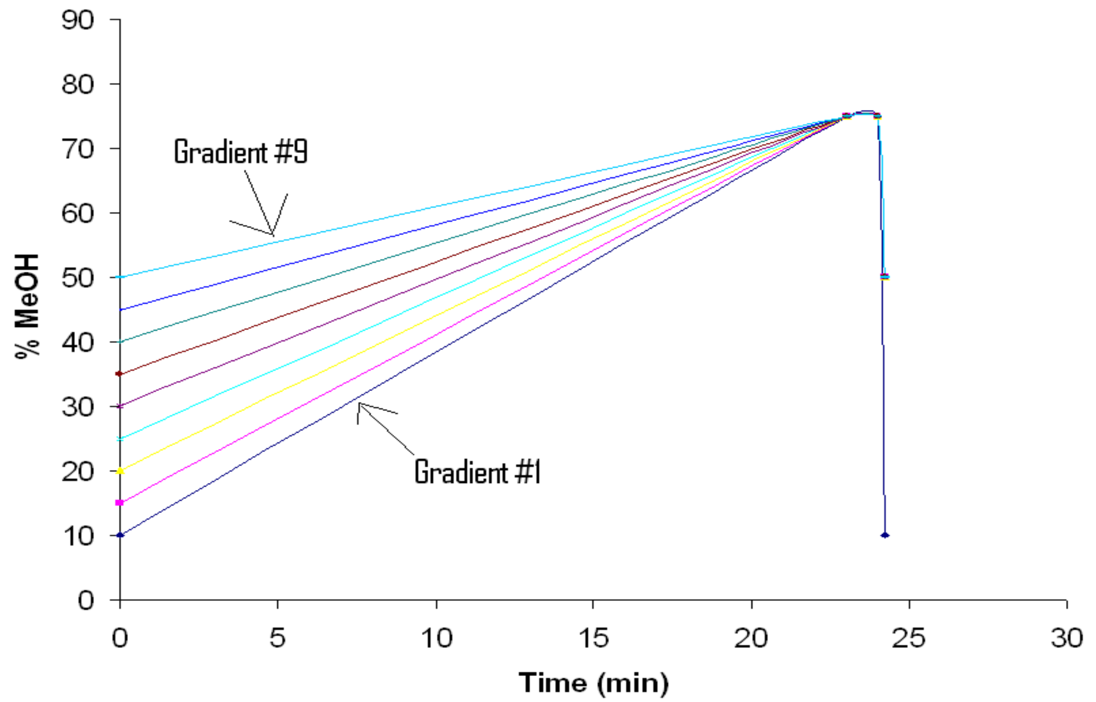

Figure 9-1: Representation of the nine types of mobile phase gradient elutions used. The solvent consisted of MeOH in 0.1% aqueous formic acid (pH 3-4), with concentrations of MeOH of 10-50 to 75% increasing over 24 min.

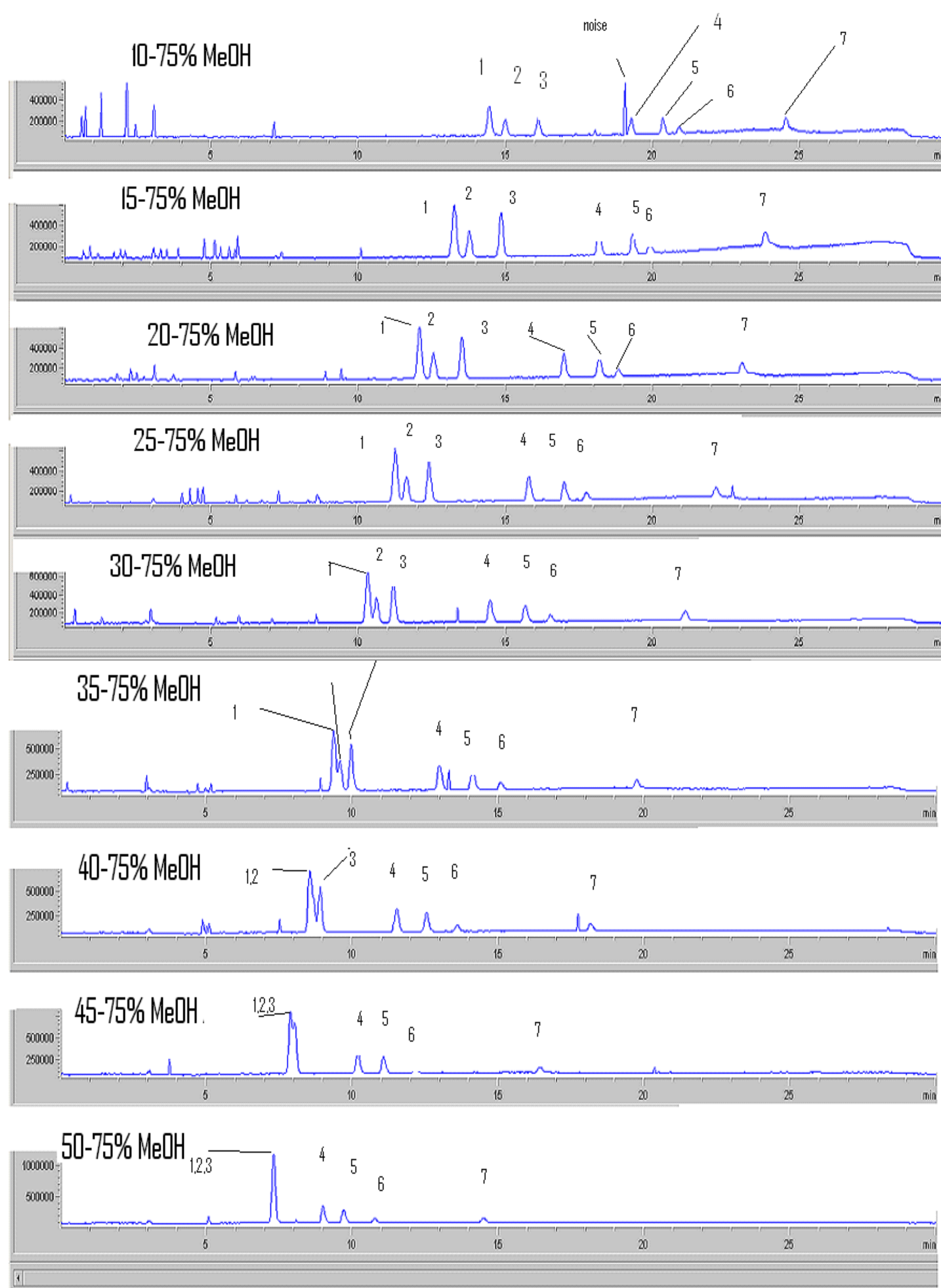

Figure 9-2: Total Ion Chromatograms (m/z 50-1000) of the comparison of the nine types of mobile phase gradient elutions used MeOH of 10-50 to 75% in 0.1 % aqueous formic acid, increasing over 24 min.

All columns run with mobile phase gradient of 20 to 75% MeOH were well separated (Fig. 9-3), and the best resolution was obtained with the Synergi Polar-RP and the Gemini C_6-Phenyl column.

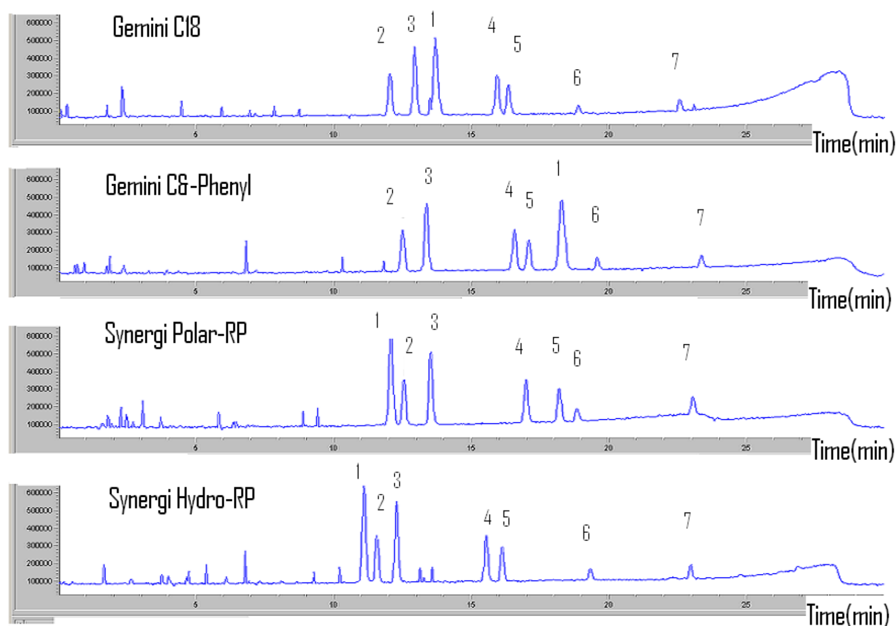

Figure 9-3: Total Ion Chromatograms (m/z 50-1000) representing the separations of the standard mixture using a gradient of 20-75% MeOH in 1% aqueous formic acid. Mixture contained: 2,5-dihydroxybenzoic Acid (1), *p*-hydroxybenzoic acid (2), caffeic acid (3), *p*-coumaric acid (4), 4-hydroxy-3-methoxycinnamic acid (5), benzoic acid (6) and trans-cinnamic acid (7).

In terms of elution order, the same relative elution order was obtained using the four columns except for one of the standard reference compounds, 2,5-dihydroxybenzoic acid. 2,5-dihydroxybenzoic acid should be the most polar compound thus the least retained on all columns. Instead, 2,5-dihydroxybenzoic acid was the first compound to elute in the more

polar columns, Synergi Polar-RP and Hydro-RP, third to elute in the Gemini C_{18} and fifth in the Gemini C_6-Phenyl column. The interaction of this compounds with the stationary phase of the Gemini C_6-Phenyl column could be explained by interactions between benzene ring and the stationary phase phenyl groups, since the analyte contains OH groups in ortho- and meta-positions to the carboxylic group, making the benzene ring more accessible for interactions with column (compared to benzene rings with their constituents in para-position)

Considering gradient elution conditions, the larger the initial amount MeOH used, the higher eluent strength. This led to shorter time of analysis and dramatic loss of resolution. Using inital conditions of 20% MeOH (gradient #3), with an ether-linked phenyl stationary phase with polar end-capping (Synergi Polar-RP) resulted in a good compromise between time of analysis and resolution. These conditions were used for subsequent separations of the second fraction of the combustion products of chlorogenic acid.

Finally, to evaluate the repeatability of the LC/MS separation, the RSD was calculated from 10 identical runs on one column (Gemini C_{18}) was determined and the repeatability was assessed for retention time, 0.11 % RSD, and peak area, 1.28 % RSD. The overall RSD of the LC/MS system was determined to have excellent repeatability at 1.28%.

10 Contributions to Research for N. Kaur

Publications:

1. Kaur N, Cabral J-L, Waldron KC & Morin A **(2010)** Headspace Stir Bar Sorptive Extraction–Gas Chromatography/Mass Spectrometry Characterization of the Diluted Vapor Phase of Cigarette Smoke Delivered to an *in Vitro* Cell Exposure Chamber. J Chrom A, (*accepted*).

2. Kaur N, Lacasse M, Roy J-P, Cabral J-L, Adamson J, Errington G, Waldron KC, Gaca M & Morin A **(2010)** Evaluation of precision and accuracy of the Borgwaldt RM20S® smoking machine designed for *in vitro* exposure. J Inhal Toxicol, 22 (14): 1174-1183.

3. Kaur N, Lacasse M, Furtos A, Waldron KC & Morin A **(2009)** Sequential fractionation with concurrent chemical and toxicological characterization of the combustion products of chlorogenic acid. J Chrom A, 1216**,** 8270-8276.

Presentations:

1. Kaur, N., Morin, A., Waldron, K.C. and Fürtös A. (2006) "The Fractionation, Chemical Characterization and Biotoxicity Study of the Chlorogenic Acid Combustion Extracts" and proposal on "Fractionation of Whole Tobacco Smoke". *50th Annual CORESTA Congress*, Paris, France, October 15-20, 2006 (Oral presentation—International Conference).

2. Kaur, N., Cabral J-L., Waldron, K.C. and Morin, A. (2009) "Stir-bar sorptive extraction (SBSE) coupled with gas chromatography/mass spectrometry (GC/MS) to

characterize the diluted tobacco smoke generated from the Borgwaldt RM20S® whole smoke exposure system". *Annual Society of Toxicology of Canada Symposium (STC)* Montréal, Québec, November 29- December 1, 2009. (Poster presentation).

3. Kaur, N., Lacasse, M., Waldron, K.C., Cabral, J-L. and Morin, A. (2008) "Chemical Assessment of a Whole Tobacco Smoke Exposure System". *Annual Society of Toxicology of Canada Symposium (STC)* Montréal, Québec, December 1-3, 2008. (Poster presentation).

4. Lin, J., Roy, J-P., Poirier, N., Kaur, N., Lacasse, M., and Morin, A. (2008) "The Use of Lung Slice Culture in the Assessment of Cigarette Smoke Toxicity". *Annual Society of Toxicology of Canada Symposium (STC)* Montréal, Québec, December 1-3, 2008. (Poster presentation).

5. Kaur, N., Lacasse, M., Waldron, K.C., Fürtös A. and Morin, A. (2007) "Toxicological, Chemical Characterization and Fractionation of the Combustion Products of a Single Tobacco Component: Chlorogenic Acid". *11th International Congress of Toxicology (ICT)* Montréal, Québec, July 15-19, 2007. (Poster presentation—International Conference).

6. Kaur, N., Morin, A., Waldron, K.C. and Fürtös, A. (2006) "The Fractionation, Chemical Characterization and Biotoxicity Study of the Chlorogenic Acid Combustion Extracts". *60th Tobacco Science Research conference (TSRC)*, Montréal, Québec, September 17-20, 2006 (Poster, presented orally—International Conference; resulting from doctoral work in progress).