

Summary of Regulatory Methods and Procedures for Determination of Harmful and Potentially Harmful Components in Tobacco Smoke*

by

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SUMMARY

Tobacco smoke consists of over 5000 chemical components, most of them are carcinogenic, respiratory toxicants, cardiovascular toxicants, addictive, etc. In 2006 the Working Group on Tobacco Control identified substances in tobacco smoke, which are harmful and potentially harmful for human health (Priority List) and encouraged the development of methods for the analysis of these components. This review focuses on the different methods of evaluation of harmful and potentially harmful components in tobacco smoke used by the International Agency for Research on Cancer (IARC) and the U.S. Food and Drug Administration (FDA) and furthermore on the official methods proposed by the World Health Organization (WHO) and the Cooperation Center for Scientific Research Relative to Tobacco (CORESTA) for analysing these constituents. The various methods for the determination of the substances in tobacco smoke included in the Priority List are compared. In addition, the different evaluation of components from the Priority List is presented. The authors hope that this review will acquaint readers with the harmful components in tobacco smoke. Also, that it will help accredited or scientific laboratories to compare the different methods and to choose appropriate methods depending on their laboratory equipment and laboratory chemicals available. [Contrib. Tob. Nicotine Res. 33 (2024) 112–135]

KEYWORDS

Harmful components; tobacco smoke; official methods; analysis; determination

ZUSAMMENFASSUNG

Tabakrauch besteht aus über 5000 chemischen Bestandteilen, von denen die meisten krebserregend, atemwegstoxisch, kardiovaskulär toxisch, suchterzeugend usw. sind. Die Arbeitsgruppe zur Eindämmung des Tabakkonsums hat 2006 die für die menschliche Gesundheit schädlichen und potenziell schädlichen Stoffe im Tabakrauch identifiziert (Prioritätenliste) und die Entwicklung von Methoden zur Analyse dieser Bestandteile angeregt. Diese Übersicht konzentriert sich auf die unterschiedliche Bewertung der schädlichen und potenziell schädlichen Bestandteile im Tabakrauch gemäß der Internationalen Agentur für Krebsforschung (IARC) und der US-amerikanischen Food and Drug Administration (FDA) sowie auf die von der Weltgesundheitsorganisation (WHO) und dem Cooperation Center for Scientific Research Relative to Tobacco (CORESTA) vorgeschlagenen offiziellen Methoden zur Analyse dieser Bestandteile. Die verschiedenen Methoden zur Bestimmung der in der Prioritätenliste aufgeführten Stoffe im Tabakrauch werden verglichen. Darüber hinaus

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wird die unterschiedliche Bewertung von Bestandteilen aus der Prioritätenliste angeregt. Die Autoren hoffen, dass diese Übersicht die Leser mit den schädlichen Bestandteilen im Tabakrauch vertraut machen wird. Außerdem wird sie den akkreditierten oder wissenschaftlichen Laboratorien helfen, die verschiedenen Methoden zu vergleichen und je nach ihrer Laborausstattung und den verfügbaren Laborchemikalien die geeignete Methode zu wählen. [Contrib. Tob. Nicotine Res. 33 (2024) 112–135]

RESUME

La fumée de tabac contient plus de 5 000 composants chimiques, dont la plupart sont cancérigènes, toxiques pour les voies respiratoires, toxiques pour le système cardiovasculaire, addictifs, etc. En 2006, le groupe de travail sur la lutte antitabac a identifié les substances présentes dans la fumée de tabac qui sont nocives et potentiellement nocives pour la santé humaine (Liste Prioritaire) et a encouragé le développement de méthodes d'analyse de ces composants. Cette étude se concentre sur les différentes évaluations des composants nocifs et potentiellement nocifs de la fumée de tabac selon le Centre international de recherche sur le cancer (CIRC) et la Food and Drug Administration (FDA) des États-Unis, ainsi que sur les méthodes officielles d'analyse de ces composants, proposées par l'Organisation mondiale de la santé (OMS) et le Centre de coopération pour la recherche scientifique relative au tabac (CORESTA). Les différentes méthodes de détermination des substances présentes dans la fumée de tabac, incluses dans la liste prioritaire, sont comparées. En outre, les différentes évaluations des composants de la liste prioritaire sont encouragées. Les auteurs espèrent que cette étude permettra aux lecteurs de se familiariser avec les composants nocifs de la fumée de tabac. Elle aidera également les laboratoires accrédités ou scientifiques à comparer les différentes méthodes et à choisir la méthode appropriée, en fonction de l'équipement du laboratoire et des produits chimiques disponibles. [Contrib. Tob. Nicotine Res. 33 (2024) 112–135]

ABBREVIATIONS

B[a]P	Benzo[a]pyrene
CORESTA	Cooperation Center for Scientific Research Relative to Tobacco
CRM	CORESTA Recommended Method
DAD	Diode array detector
FLD	Fluorescence detection
GC	Gas chromatography
HCI	Health Canada Intense
HPLC	High-performance liquid chromatography
ISO	International Organization for Standardization
MS	Mass spectrometry
SOP	Standard Operating Procedure
SPE	Solid-phase extraction
TNCRI	Tumour non-cancer risk index
TSNA	Tobacco-specific nitrosamine

INTRODUCTION

Tobacco smoke consists of over 5000 chemical components, some of them are cancerogenic, co-cancerogenic, etc. (1).

In order to establish comparable measurements for testing tobacco products globally, consensus methods are required for measuring specific contents and emissions of cigarettes. Using the criteria for prioritization set at the third meeting of the World Health Organization (WHO) Framework Convention Working Group on Tobacco Control in Ottawa, Canada, in October 2006, the Working Group identified the following emissions in mainstream smoke for which methods for testing and measurement (analytical chemistry) should be validated as a priority (2).

The WHO stated that in regard to emissions, the Working Group wishes to underscore that “tar”, nicotine, and carbon monoxide should not be the sole emissions measured for product monitoring purposes as they do not provide a sufficiently broad chemical profile. Using the general criteria of relevance to public health, the Working Group believes that the list of 44 “Hoffmann Analytes” forms a sound basis for such purposes (2).

The most common measurements for cigarettes have been machine-measured “tar”, nicotine, and CO yields per cigarette, based on the International Organization for Standardization (ISO) and Intense Smoking Regimes. Such results do not provide valid estimates of human exposure or toxicity and thereby may cause harm by misleading smokers and regulators alike (3).

Regulation of tobacco products requires the establishment of a metric or a set of metrics by which tobacco products can be assessed. New product assessment approaches for setting regulatory measures are introduced. The new approach proposed by the WHO Study Group on Tobacco Product Regulation (TobReg) requires the quantification of the levels of known harmful toxicants that accompany a specified amount of nicotine, which is the principal addictive substance in smoke sought by smokers, as measured in a standardized machine testing regimen.

The current Priority List of toxic contents and emissions of combustible tobacco products was drawn up by TobReg, during the ninth meeting of TobReg in 2017.

The components of different chemical classes in both the particle and the gas phase of the smoke were taken into account. The substances included in the Priority List were selected on the basis of various criteria:

- The presence of specific chemicals in cigarette smoke at levels that are toxic for smokers, as determined by well-established scientific toxicity indices;
- Variations in concentration among cigarette brands that are substantially higher than the variations in repeated measurements of the toxicant in a single brand;
- The availability of technology to reduce the concentration of a given toxicant in smoke, should an upper limit be mandated (3).

The WHO provided a provisional list of cigarette emissions of interest that can be divided into several groups (3).

- Alkaloids: nicotine;
- Aldehydes: acetaldehyde, acrolein, formaldehyde, crotonaldehyde, propionaldehyde, n-butyraldehyde;
- Aromatic amines: 3-aminobiphenyl, 4-aminobiphenyl,

- 1-aminonaphthalene, 2-aminonaphthalene;
- Hydrocarbons: benzene, 1,3-butadiene, isoprene, styrene, toluene;
- Polycyclic aromatic hydrocarbons (PAHs): benzo[*a*]-pyrene (B[*a*]P);
- Tobacco-specific nitrosamines (TSNAs): 4-(*N*-nitroso-methyl-amino)-1-(3-pyridyl)-1-butanone (NNK), *N*'-nitrosonornicotine (NNN), *N*'-nitrosoanabasine (NAB), *N*'-nitrosoanatabine (NAT);
- Phenols: catechol, *m*-cresol, *p*-cresol, *o*-cresol, phenol, hydroquinone, resorcinol;
- Other organic compounds: acetone, acrylonitrile, quinoline, pyridine;
- Metals and metalloids: arsenic, cadmium, chromium, lead, mercury, nickel, selenium;
- Other constituents: ammonia, carbon monoxide, hydrogen cyanide, nitrogen oxides.

The aim of this study was to review the official methods for the determination of harmful and potentially harmful components in tobacco smoke.

A detailed search using the internet databases *Scopus* and *Web of Science* for published papers and reports dealing with the constituents of tobacco smoke and assessments of their toxic hazards was performed. Four different classifications of toxicity of substances in tobacco smoke were selected – according to the International Agency for Research on Cancer (IARC) (5) monographs, Food and Drug Administration (FDA)(6), Seventh report of a WHO study group (3) and FOWLES and DYBING (4).

EVALUATION OF HARMFUL AND POTENTIALLY HARMFUL COMPONENTS IN TOBACCO SMOKE

IARC Monographs on the identification of carcinogenic hazards to humans

The IARC Monographs Programme evaluates the carcinogenic risk of chemicals (e.g., formaldehyde), complex mixtures (e.g., air pollution), occupational exposures (e.g., work in coke production), physical agents (e.g., solar radiation), biological agents (e.g., hepatitis B virus), pharmaceuticals (e.g., diethylstilbestrol), and other important factors (e.g., tobacco smoking) and assigns them to different groups (5):

- Group 1: The agent is carcinogenic to humans
This category is used when there is sufficient evidence of carcinogenicity in humans. In other words, there is convincing evidence that the agent causes cancer in humans. The evaluation is usually based on the results of medical studies showing development of cancer in exposed humans. Agents can also be classified in Group 1 on the basis of sufficient evidence of carcinogenicity in experimental animals supported by strong evidence in exposed humans that the agent exhibits one or more of the recognized key characteristics of human carcinogens.
- Group 2: This category includes agents with a range of evidence for carcinogenicity in humans and in experimental animals. At one extreme of the range are agents with positive but not conclusive evidence in humans. At the other extreme are agents for which evidence in

humans is not available but for which there is sufficient evidence of carcinogenicity in experimental animals. There are two subcategories, which indicate different levels of evidence:

- Group 2A: The agent is probably carcinogenic to humans

This category is used when there is limited evidence of carcinogenicity in humans and

- either sufficient evidence of carcinogenicity in experimental animals or
- strong mechanistic evidence, showing that the agent exhibits key characteristics of human carcinogens.

Limited evidence of carcinogenicity means that a positive association has been observed between exposure to the agent and cancer but that other explanations for the observations (technically termed “chance”, “bias”, or “confounding”) could not be ruled out with reasonable confidence. This category may also be used when there is inadequate evidence regarding carcinogenicity in humans but both sufficient evidence of carcinogenicity in experimental animals and strong mechanistic evidence in human cells or tissues.

- Group 2B: The agent is possibly carcinogenic to humans

This category is generally used when only one of the following evaluations has been made by the Working Group:

- Limited evidence of carcinogenicity in humans;
- Sufficient evidence of carcinogenicity in experimental animals;
- Strong mechanistic evidence, showing that the agent exhibits key characteristics of human carcinogens.

- Group 3: The agent is not classifiable as to its carcinogenicity to humans.

This category is used most commonly when the evidence of carcinogenicity in humans is inadequate, the evidence of carcinogenicity in experimental animals is limited (or inadequate), and the mechanistic evidence is limited (or inadequate). Limited evidence of carcinogenicity in experimental animals means that the available information suggests a carcinogenic effect but is not conclusive.

Food and Drug Administration (FDA)

Furthermore, the U.S. FDA has established a list of 93 harmful and potentially harmful constituents in tobacco products. FDA classified components into 5 groups: carcinogen (CA), respiratory toxicant (RT), cardiovascular toxicant (CT), reproductive or developmental toxicant (RDT), and addictive (AD) (6).

Seventh report of the WHO Study Group on Tobacco Product Regulation

Quantitative data for characterizing the hazards of the reviewed toxicants are generated by calculating “toxicant animal carcinogenicity indices (TACIs)” and “toxicant non-cancer response indices (TNCRIIs)”. For these calculations,

published toxicant yields (obtained with the modified Intense Smoking Regime) are normalized per milligram of nicotine and multiplied by cancer and non-cancer potency factors (3).

Cancer potency factors are defined as T_{25} per milligram ($1/T_{25}$), where T_{25} is the long-term daily dose that will produce tumours in 25% of animals above the background rate at a specific tissue site. T_{25} is determined by linear extrapolation from the lowest dose that results in a statistically significant increase in tumours. The pertinent data from the cancer bioassays underlying calculation of the T_{25} values are converted into cancer potency factors per milligram.

For non-cancer potency factors, the long-term reference exposure levels of a chemical at or below of which no adverse health effects are anticipated for individuals with long-term exposure to that level is used. Reference exposure levels were derived from both human and animal toxicological data and presented for the target system for each substance.

Fowles and Dybing

A process of prioritisation (ranking) of these chemical hazards was applied (4). This process involved ranking the identified chemicals in terms of their so-called “comparative risk indices”. The comparative risk ranking was based on published analytical results for mainstream cigarette smoke combined with published toxicological potency information for cancer and non-cancer health effects.

They have assumed that the most significant toxicants in cigarette smoke are those that have been identified and studied toxicologically. For yield estimates per cigarette, reports using standard smoking machine conditions and International Organization for Standardization (ISO) methods are taken in preference to those focusing on “intense” smoking conditions.

The cancer risk indices (CRIs) for mainstream smoke are calculated on a per-cigarette per-day basis by multiplying the yield per cigarette (mg) with the published cancer potency factor, assuming complete absorption (in the case of smokers) of the chemicals in the reported yield. It is also assumed in the calculations that exposure takes place for an average of 60 years out of a 75-year lifespan, for an average person.

Table 1 (page 116) presents the chemical components in tobacco smoke included in the Priority List and evaluated for their harmfulness according to IARC and FDA. Most of these substances are in the IARC and FDA lists (3–6).

COMPOUNDS IN TOBACCO SMOKE AND OFFICIAL METHODS FOR DETERMINATION

To ensure implementation of Articles 9 and 10 of the WHO FCTC, laboratory capacity must be available that meets the highest standards of excellence, transparency, reliability, and credibility (7). Laboratories need standardized, reliable, and accurate analytical methods to conduct the scientifically rigorous testing required for tobacco products globally (8).

A set of analytical methods for selected priority emissions

were developed and validated by the WHO Tobacco Laboratory Network (TobLabNet) – Standard Operating Procedures (SOPs) (9). CORESTA also developed Priority List analysis methods (10). In addition, ISO (11) and Health Canada (12) have published their own methods.

To measure the amount of harmful substances in tobacco smoke, a smoking machine is used to generate smoke. This machine smokes cigarettes in accordance with an established method (13). There are ISO and Intense Smoking Regimes. Among them, the ISO smoking regime is not a more representative of actual human smoking behaviour though.

Another smoke generation method developed by Health Canada and validated and recommended by the WHO involves more intense puffing and combustion conditions and also takes into account the covering of ventilation holes. These regimes are characterized by an increased puff volume and double the number of puffs per minute, which is close to the manner of smoking in modern people. The parameters of different smoking regimes are presented in Table 2 (14).

Smoke generation for most of the published methods, standards, and procedures for the analysis of substances of the Priority List is performed under both smoking modes – standard ISO regime and Health Canada Intense Smoking Regime (HCI).

Table 2. Parameters of different smoking regimes (8).

Parameters	Smoking regime		
	Standard ISO	Intensive	
		Massachusetts	Canada
Puff volume (mL)	35	45	55
Puff duration (s)	2	2	2
Puff frequency (/min)	1	2	2

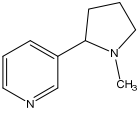
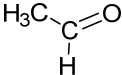
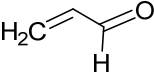
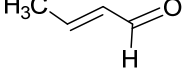
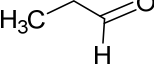
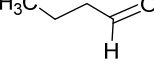
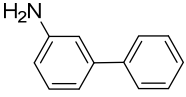
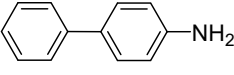
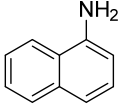
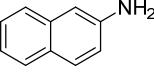
Aldehydes (carbonyl compounds)

In the Priority List the group of carbonyl compounds is represented by 6 components - acetaldehyde, acrolein, formaldehyde, crotonaldehyde, propionaldehyde, *n*-butyraldehyde. Aldehydes are toxic to the respiratory system (15). IARC classified formaldehyde in Group 1 (the agent is carcinogenic to humans), acrolein in Group 2A (the agent is probably carcinogenic to humans), while acetaldehyde and crotonaldehyde in Group 2B (the agent is possibly carcinogenic to humans). *n*-Butyraldehyde is not included in the IARC list.

FDA classified formaldehyde, acetaldehyde, and crotonaldehyde as carcinogenic; formaldehyde, acetaldehyde, acrolein, propionaldehyde as respiratory toxicant; and acrolein and propionaldehyde as cardiovascular toxicant. *n*-Butyraldehyde is not included in the FDA list (Table 1). Only acetaldehyde has a toxicant animal carcinogenicity index (TACI). The study indicates that a long-term daily dose of 6.1 mg acetaldehyde will produce tumours in 25% of animals above the background rate at a specific tissue site.

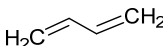
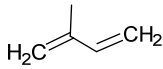
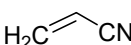
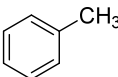
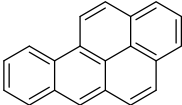
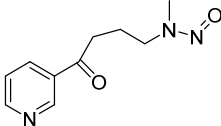
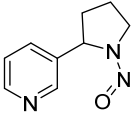
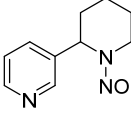
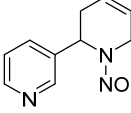
The cancer risk index (CRI) is for formaldehyde

Table 1. Chemical components in tobacco smoke and evaluation of their harmful effects according to IARC (5), FDA (6), WHO Study Group (3), and FOWLES and DYBING (4).

Group	Compound	Chemical structure	IARC ^a (group, year)	FDA ^b	WHO study group ^c Carcinogenicity or toxicity data		FOWLES AND DYBING ^d
					TACI	TNCRI	CRI
Alkaloids	Nicotine CAS 54-11-5		–	–	–	–	–
Aldehydes	Acetaldehyde CAS 75-07-0		Group 2B, 1999	CA, RT, AD	6.1	67.1	9.18×10 ⁻⁵
	Acrolein CAS 107-02-8		Group 2A, 2021	RT, CT	–	1099	–
	Formaldehyde CAS 50-000-0		Group 1, 2012	CA, RT	–	19.8	9.90×10 ⁻⁶
	Crotonaldehyde CAS 4170-30-3		Group 2B, 2021	CA	–	–	–
	Propionaldehyde CAS 123-38-6		–	CT, RT	–	–	–
	n-Butyraldehyde CAS 123-72-8		–	–	–	–	–
Aromatic amines	3-Aminobiphenyl CAS 2243-47-2		–	–	–	–	–
	4-Aminobiphenyl CAS 92-67-1		Group 1, 2012	CA	–	–	3.60×10 ⁻⁷
	1-Amino-naphthalene (1-Naphthylamine) CAS 134-32-7		Group 3, 1987	CA	–	–	–
	2-Aminonaphthalene (2-Naphthylamine) CAS 91-59-8		Group 1, 2012	CA	–	–	1.80×10 ⁻⁷

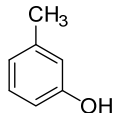
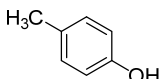
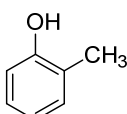
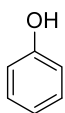
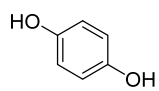
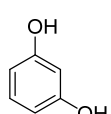
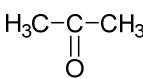
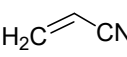
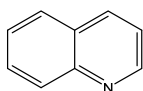
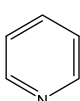
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Table 1. Chemical components in tobacco smoke and evaluation of their harmful effects according to IARC (5), FDA (6), WHO Study Group (3), and FOWLES and DYBING (4) - continued.

Group	Compound	Chemical structure	IARC ^a (group, year)	FDA ^b	WHO study group ^c Carcinogenicity or toxicity data		FOWLES AND DYBING ^d CRI
					TACI	TNCRI	
Hydrocarbons	Benzene CAS 71-43-2		Group 1, 2018	CA, CT, RTD	2.6	0.64	6.71×10 ⁻⁵
	1,3-Butadiene CAS 106-99-0		Group 1, 2012	CA, CT, RTD	9.9	2.4	3.02×10 ⁻⁴
	Isoprene CAS 78-79-5		Group 2B, 1999	CA	—	—	—
	Styrene CAS 100-42-5		Group 2A, 2019	CA, RT	—	0.01	—
	Toluene CAS 108-88-3		Group 3, 1999	RT, RTD	—	0.22	—
Polycyclic aromatic hydrocarbons	Benzo[a]pyrene CAS 50-32-8		Group 1, 2012	CA	0.0086	—	1.93×10 ⁻⁶
Tobacco-specific nitrosamines	4-(N-nitrosomethyl-amino)- 1-(3-pyridyl)-1-butanone (NNK) CAS 64091-91-4		Group 1, 2012	CA	3.4	—	7.80×10 ⁻⁶
	N'-Nitrosornicotine (NNN) CAS 16543-55-8		Group 1, 2012	CA	0.29	—	3.80×10 ⁻⁵
	N'-Nitrosoanabasine (NAB) CAS 37620-20-5		Group 3, 2007		—	—	—
	N'-Nitrosoanatabine (NAT) CAS 71267-22-6		Group 3, 2007		—	—	—
Phenols	Catechol CAS 120-80-9		Group 2B, 1999	CA	0.58	1.2	—

continued

Table 1. Chemical components in tobacco smoke and evaluation of their harmful effects according to IARC (5), FDA (6), WHO Study Group (3), and FOWLES and DYBING (4) - continued.

Group	Compound	Chemical structure	IARC ^a (group, year)	FDA ^b	WHO study group ^c Carcinogenicity or toxicity data		FOWLES AND DYBING ^d
					TACI	TNCRI	CRI
Phenols	<i>m</i> -Cresol CAS 108-39-4		–	CA, RT	–	0.01	–
	<i>p</i> -Cresol CAS 106-44-5		–	CA, RT	–	0.01	–
	<i>o</i> -Cresol CAS 95-48-7		–	CA, RT	–	0.01	–
	Phenol CAS 108-95-2		Group 3, 1999	CT, RT	–	0.07	–
	Hydroquinone CAS 123-31-9		Group 3, 1999	–	1.2	–	–
	Resorcinol CAS 108-46-3		Group 3, 1999	–	–	–	–
Other organic compounds	Acetone CAS 67-64-1		–	RT	–	–	–
	Acrylonitrile CAS 107-13-1		Group 2B, 1999	CA, RT	1.4	2.1	1.29×10 ⁻⁴
	Quinoline CAS 91-22-5		Group 2B, 2019	CA	–	–	–
	Pyridine CAS 110-86-1		Group 2B, 2019	–	–	–	–
Metal and metalloids	Arsenic CAS 740-38-2	As	Group 1, 2012	CA, CT, RT	–	0.16	1.16×10 ⁻⁴

continued

Table 1. Chemical components in tobacco smoke and evaluation of their harmful effects according to IARC (5), FDA (6), WHO Study Group (3), and FOWLES and DYBING (4) - continued.

Group	Compound	Chemical structure	IARC ^a (group, year)	FDA ^b	WHO study group ^c Carcinogenicity or toxicity data		FOWLES AND DYBING ^d CRI
					TACI	TNCRI	
Metal and metalloids	Cadmium CAS 7440-34-9	Cd	Group 1, 2012	CA, RT, RDT	1.7	2.6	2.16×10 ⁻⁵
	Chromium CAS 7440-47-3	Cr	Group 3, 1990	CA, RT, RDT	–	–	3.15×10 ⁻⁵
	Lead CAS 7439-92-1	Pb	Group 2B, 1987	CA, CT, RDT	0.00	–	7.68×10 ⁻⁹
	Mercury CAS 7439-97-6	Hg	Group 3, 1993	CA, RDT	–	0.02	–
	Nickel CAS 7440-02-0	Ni	Group 2B, 1990	CA, RT	–	–	1.43×10 ⁻⁷
	Selenium CAS 7782-49-2	Se	Group 3, 1987	RT	–	–	–
Other constituents	Ammonia CAS 7664-41-7	NH ₃	–	RT	–	0.07	–
	Carbon monoxide CAS 630-08-0	CO	–	RDT	–	1.3	–
	Hydrogen cyanide CAS 74-90-8	HCN	–	RT, CT	–	17.2	–
Other constituents	Nitrogen oxides CAS 10102-43-9	NO _n	–	–	–	3.1	–

^a Group 1: The agent is carcinogenic to humans

Group 2A: The agent is probably carcinogenic to humans

Group 2B: The agent is possibly carcinogenic to humans

Group 3: The agent is not classifiable as to its carcinogenicity to humans

^b Carcinogen (CA), respiratory toxicant (RT), cardiovascular toxicant (CT), reproductive or developmental toxicant (RDT), addictive (AD).

^c Toxicant animal carcinogenicity index (TACI); toxicant non cancer response index (TNCRI)

^d Cancer risk index (CRI), CRI per cigarette/day

9.90×10^{-6} per cig/day, while for acetaldehyde it is 9.18×10^{-5} per cig/day, which corresponds with the IARC classification (Table 1).

There are two official methods for determining selected carbonyls in tobacco smoke:

- SOP 08 (*Determination of aldehydes in mainstream cigarette smoke under ISO and intense smoking conditions*); and
- CORESTA Recommended Method (CRM) No. 74 (*Determination of selected carbonyls in mainstream cigarette smoke by HPLC*) (16, 17).

SOP 08 is a method for the determination of carbonyl compounds in the total particulate matter of tobacco smoke, while CRM No. 74 determines carbonyls in the gas-phase of tobacco smoke (Table 3).

SOP 08 is developed for the determination of formaldehyde, acetaldehyde and acrolein as their 2,4-dinitrophenylhydrazones, while CRM No. 74 is suitable for the determination of 8 selected carbonyl compounds: formaldehyde, acetaldehyde, acetone, acrolein, propionaldehyde, crotonaldehyde, 2-butanone and *n*-butyraldehyde as their 2,4-dinitrophenylhydrazones.

In SOP 08, mainstream cigarette smoke is produced according to the WHO Intense Regime or the ISO regime. One cigarette is smoked per smoke trap, which contains 300 mg CX-572 particles and a glass fibre filter pad. Mainstream smoke total particulate matter from the cigarette test sample is trapped onto a smoke trap containing 300 mg CX-572 particles and a glass-fibre filter pad. The aldehydes are extracted by adding a solution containing a mixture of carbon disulphide and methanol onto the Carboxen particles and glass fibre filter pad. The extracted aldehydes are derivatized with 2,4-dinitrophenylhydrazine (DNPH). The derivatized aldehydes are determined by high-performance liquid chromatography (HPLC) and equipped with ultraviolet (UV) or diode array detector (DAD).

The World Health Organization, together with the Tobacco Laboratory Network developed the standard operating procedure SOP 08. An international collaborative study was conducted in 2012, involving testing of three reference cigarettes (1R5F, 3R4F, and CM6) and two commercial brands by eight laboratories. From the date of the collabora-

Table 3. Determination of selected carbonyls in mainstream cigarette smoke.

SOP 08. Standard Operating Procedure for Determination of Aldehydes in Mainstream Cigarette Smoke Under ISO and Intense Smoking Conditions. 31 August 2018 (16)	CRM No. 74. Determination of Selected Carbonyls in Mainstream Cigarette Smoke by HPLC (August 2019) (17)
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Compounds

Formaldehyde, acetaldehyde, acrolein (acrylaldehyde)

Formaldehyde, acetaldehyde, acetone, acrolein, propionaldehyde, crotonaldehyde, 2-butanone and *n*-butyraldehyde as their 2,4-dinitrophenylhydrazones

Method summary

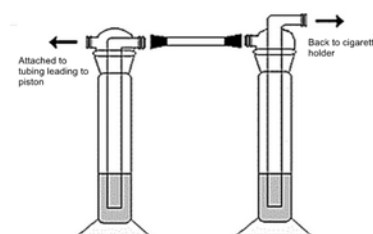
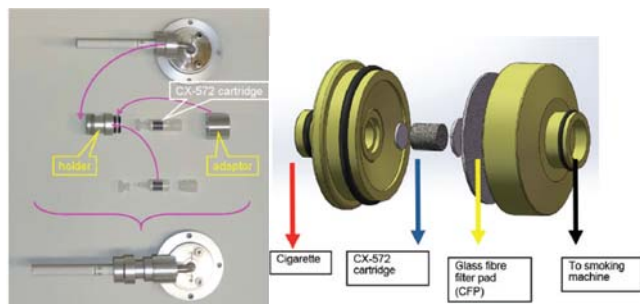
Cigarettes are smoked on a smoking machine according to the ISO or intense regime. One cigarette is smoked per smoke trap, containing 300 mg CX-572 particles and a glass fibre filter pad. This standard operating procedure is suitable for the quantitative determination of the following three aldehydes in mainstream (MS) cigarette smoke: formaldehyde, acetaldehyde, and acrolein to be analyzed by liquid chromatography. The aldehydes in mainstream tobacco smoke are extracted by adding a solution containing a mixture of carbon disulphide and methanol and are derivatized with a solution of 2,4-dinitrophenylhydrazine (DNPH). The samples are subjected to analysis using high-performance liquid chromatography (HPLC), equipped with ultraviolet (UV) or diode array detector (DAD).

Cigarettes are smoked on a smoking machine that has been fitted with impingers, but without the specified filter pad holder containing the glass fiber filter. The carbonyls in mainstream tobacco smoke are trapped by passing each puff through an impinger device containing an acidified solution of 2,4-dinitrophenylhydrazine (DNPH) in acetonitrile. An aliquot of the smoke extract is then syringe-filtered and diluted with 1% Trizma™ base in aqueous acetonitrile. The samples are subjected to analysis using reverse phase HPLC/UV or HPLC/DAD.

Apparatus and equipment

Linear smoking machine
Rotary smoking machine
HPLC system with a UV and/or DAD detector
Carboxen 572 particles, 20/45 mesh size

Linear smoking machine
Rotary smoking machine
HPLC system with a UV and/or DAD detector



continued

Table 3. Determination of selected carbonyls in mainstream cigarette smoke - continued.

SOP 08. Standard Operating Procedure for Determination of Aldehydes in Mainstream Cigarette Smoke Under ISO and Intense Smoking Conditions. 31 August 2018 (16)			CRM No. 74. Determination of Selected Carbonyls in Mainstream Cigarette Smoke by HPLC (August 2019) (17)			
<i>Derivatization solution</i>						
DNPH solution: Approximately 1 g DNPH-HCl is added to a 50-mL volumetric flask, then 10 mL of 85% phosphoric acid is carefully added.			DNPH solution: Approximately 150 mL deionized water is added to a 200-mL volumetric flask, then 28 mL of 85% phosphoric acid is carefully added. The solution is made up to a volume of 200 mL with deionized water. 6.8 g (0.024 mole) of DNPH (approximately 30% water) is weighed into a 2-L amber volumetric flask and 1 L of acetonitrile is added. 58 mL of the diluted phosphoric acid solution is added and gently mixed. The volume is diluted with deionized water.			
			Trizma™ base dilution solution: MeCN : 1% aqueous Trizma™ (80:20 v/v) - 2.00 g of Trizma™ base is dissolved in 200 mL of deionized water in a 1-L volumetric flask. The volume is diluted with acetonitrile.			
<i>Linear range</i>						
The calibration should cover the concentration range of interest. The linear range is approximate to the aldehyde content in tobacco smoke.			The calibration should cover the concentration range of interest. The linear range is approximate to the carbonyl content in tobacco smoke.			
<i>Number of cigarettes to be smoked</i>						
ISO / Intense smoking regime Linear smoking machine: 1 Rotary smoking machine: 1			ISO / Intense smoking regime Linear smoking machine: 2 Rotary smoking machine: 5			
<i>Sample preparation</i>						
The aldehydes mainstream apparatus is assembled on the smoking machine using Carboxen 572 and glass fibre filter pad. The method is used for simultaneous determination of aldehydes (SOP 08) and volatile organics (SOP 09) in tobacco smoke. Transfer 0.5 mL to 5 mL flask and add 200 µL of DNPH solution and diluted to 5 mL. An aliquot of solution is analyzed by HPLC/UV or HPLC/DAD.			The carbonyl mainstream apparatus is assembled on the smoking machine without using the filter pads and filter holders and cigarettes are smoked. The DNPH smoke extract solution is allowed to sit for five to thirty minutes. After that 6 mL of 1% Trizma™ base solution is pipetted into a 10 mL volumetric flask. An aliquot of solution is analyzed by reverse-phase HPLC/UV or HPLC/DAD.			
<i>Sample analysis</i>						
High performance liquid chromatography with UV or DAD Detector			High performance liquid chromatography with UV or DAD Detector (RP HPLC - UV or RP HPLC - DAD).			
<i>HPLC equipment and its operating conditions</i>						
LC column:	Ascentis RP-Amide (150 mm × 4.6 mm, 3 µm) or equivalent		LC column:	250 mm x 4 mm, Reversed Phase (RP) C18 (5 µm), or equivalent		
Column Temperature:	30 ± 5 °C		Disposable Guard Column:	4 mm x 4 mm, RP C18 (5 µm), or equivalent		
Injection volume:	20 µL		Column Temperature:	30 °C		
UV detection:	360 nm or maximum wavelength at 300–400 nm		Injection volume:	20 µL		
Degasser:	On		UV or DAD detection:	365 nm		
Binary pump flow:	1.0 mL/min		Flow rate:	1.5 mL/min		
Mobile Phase A:	water		Mobile Phase:	- Solvent A: Prepare 2 L of 30% Acetonitrile, 10% THF, 1% IPA in Type I water, filter and degas (UHP Helium sparged) - Solvent B: Prepare 2 L of 65% Acetonitrile, 1% THF, 1% IPA in Type I water, filter and degas (UHP Helium sparged) - Solvent C: Acetonitrile (UHP Helium sparged)		
Mobile Phase B:	acetonitrile					
Total analysis time:	45 min					
Gradient conditions:			Gradient conditions:			
0.0	100% A	0% B	0.0	100% A	0% B	0% C
10.0	55% A	45% B	8.0	70% A	30% B	0% C
25.0	55% A	45% B	20.0	47% A	53% B	0% C
30.0	0% A	100% B	27.0	0% A	100% B	0% C
30.0	0% A	100% B	30.0	0% A	0% B	100% C
31.0	55% A	45% B	32.0	0% A	0% B	100% C
40.0	55% A	45% B	34.0	95% A	5% B	0% C
			Equilibration 10.0	100% A	0% B	0% C

tive study this method shows that it is fit for purpose of testing and measuring aldehydes in mainstream cigarette smoke and that is suitable for a range of concentrations of interest. The method for this study is less eligible for the collection of formaldehyde due to its highly volatile and hydrophilic nature. The relative comparative repeatability is less than 30%. Due to the low number of participating laboratories, the inter-laboratory precision values are accompanied with relatively high variations, nevertheless this method can be considered conditionally validated for formaldehyde (16).

CORESTA developed another official method for determining the carbonyl compounds in the gas-phase of tobacco smoke by high-performance liquid chromatography with UV detector (HPLC/UV) or high performance liquid chromatography with DAD detector (HPLC/DAD): CRM No. 74 (*Determination of selected carbonyls in mainstream cigarette smoke by HPLC* (17)). Cigarettes are smoked on a smoking machine that is fitted with impingers, but without the specified filter pad holder, containing the glass fiber filter. The carbonyls in mainstream tobacco smoke are trapped by passing each puff through an impinger device containing an acidified solution of 2,4-dinitrophenylhydrazine (DNPH) in acetonitrile. The carbonyls are determined using derivatization with DNPH followed by HPLC/UV or HPLC/DAD detection. An aliquot of the smoke extract is then syringe-filtered and diluted with 1% Trizma™ base in aqueous acetonitrile. The samples are subjected to analysis using reverse-phase HPLC/UV or HPLC/DAD (Table 3).

A collaborative studies was conducted assessing the methodology. In 2010, mean selected carbonyl yields, repeatability (r) and reproducibility (R) values were determined from a collaborative study involving 15 laboratories and 5 replicate analyses of two reference cigarettes (3R4F and 1R5F), the CORESTA Monitor CM6 and 5 commercial cigarettes covering a wide range of blends and designs (18).

The Health Canada Official Method T-104 (*Determination of Selected Carbonyls in Mainstream Tobacco Smoke*) is identical to the CRM No. 74, but while in the CRM No. 74 cigarettes are smoked on a smoking machine that has been fitted with two impingers, in the Health Canada Official Method, cigarettes are smoked on a smoking machine that has been fitted with one impinger (19).

Aromatic amines

From the group of aromatic amines are included 3-aminobiphenyl, 4-aminobiphenyl, 1-aminonaphthalene, 2-aminonaphthalene, but in the development of methods for their determination are added also 2,6-dimethylaniline, *o*-anisidine and *o*-toluidine.

According to the IARC classification (5), 2-aminonaphthalene and 4-aminobiphenyl are carcinogenic to humans (Group 1), while 1-aminonaphthalene is not classifiable as to its carcinogenicity to humans (Group 3).

According to the FDA list (6), 3-aminobiphenyl, 4-aminobiphenyl, 1-aminonaphthalene, and 2-aminonaphthalene are classified as carcinogens. Only 3-aminobiphenyl is not

included in the IARC or the FDA list.

Exposure to aromatic amines is associated with bladder cancer, and 2-aminonaphthalene and 4-aminobiphenyl are known human bladder carcinogens (20, 21).

The cancer risk index (CRI) for 4-aminobiphenyl is 3.6×10^{-7} per cigarette/day, while for 2-aminonaphthalene it is 1.80×10^{-7} per cigarette/day, which corresponds with the IARC classification (Table 1).

The aromatic amines in tobacco smoke are not equally investigated. There are ISO methods for determining aromatic amines in indoor air, leather, and textiles, but for tobacco smoke there is only one published – the CORESTA Recommended Method No. 95 (*Determination of aromatic amines in mainstream cigarette smoke by gas chromatography/mass spectrometry with negative chemical ionization (GC/MS (NCI))* (22)). The method requires a specific gas chromatograph configuration that is not applicable to most other methods for testing components in tobacco smoke, which may lead the laboratory to refrain from offering this analysis (Table 4).

The GC/MS with negative chemical ionization and methane as gas reagent are expensive considering that they support only one analysis. The method is applicable for ISO and Intense Smoking Regime. The ISO method requires more cigarettes for the test samples and a smaller aliquot of reagents than the intensive regime, whereas the procedure for sample preparation for the two regimes of smoking is the same.

CRM No. 95 is suitable for quantitative determination of 7 aromatic amines in mainstream cigarette smoke: 1-aminonaphthalene, 2,6-dimethylaniline, 2-aminonaphthalene, 3-aminobiphenyl, 4-aminobiphenyl, *o*-anisidine, and *o*-toluidine. An international collaborative study was conducted in 2016, involving 11 laboratories. The study included 7 cigarette products covering a wide range of blends and cigarette design constructions, two reference cigarettes from the University of Kentucky (3R4F and 1R6F) and the CORESTA Monitor Test Piece CM8 (22). The method includes cigarette smoking and collecting mainstream smoke on a glass-fibre filter pad. The aromatic amines are extracted with dichloromethane, followed by derivatization with heptafluorobutyric anhydride (HFBA) and are purified on a Florisil SPE. Quantification of aromatic amines is performed using GC/MS (NCI) (Table 4). The Health Canada method T-102 (*Determination of 1- and 2-aminonaphthalene and 3- and 4-aminobiphenyl in mainstream tobacco smoke* (23)) involves collection of the amines on a fibre filter disc, extraction with 5% hydrochloric acid solution, liquid-liquid extraction with dichloromethane with sodium hydroxide solution, and extraction with hexane. The hexane extracts are dried with sodium sulphate, derivatized with pentafluoropropionic acid anhydride and trimethylamine, followed by solid-phase extraction (SPE) with Florisil column, and are quantified by GC/MS. This method is more complicated compared to CRM No. 95, because it involves an additional liquid-liquid extraction with dichloromethane before derivatization, but the recovery is high: 98.4% for 4-aminobiphenyl and 96.8% for 2-aminonaphthalene.

Table 4. CRM No. 95 – Determination of aromatic amines in mainstream cigarette smoke by gas chromatography mass spectrometry with negative chemical ionization (22).

Method description		
Compounds		
1-Aminonaphthalene, 2,6-dimethylaniline, 2-aminonaphthalene, 3-aminobiphenyl, 4-aminobiphenyl, o-anisidine, o-toluidine		
Internal standard		
2,6-Dimethylaniline- <i>d</i> ₁₁ , 2-aminonaphthalene- <i>d</i> ₇ , 4-aminobiphenyl- <i>d</i> ₉ , o-toluidine- <i>d</i> ₉		
Method summary		
Cigarettes are smoked on a standard smoking machine. The mainstream smoke is collected on a glass-fibre filter pad (GFP). After addition of internal standards, the GFP is extracted with dichloromethane using a laboratory shaker for 20 min. The extract is filtered, derivatized with HFBA, purified on a Florisil SPE and analysed by GC/MS-NCI.		
Apparatus and equipment		
Linear smoking machine or Rotary smoking machine GC/MS-NCI		
Extraction solution		
Dichloromethane		
Linear range		
1-Aminonaphthalene:	0.4 – 5.0 ng/mL	
3-Aminobiphenyl:	0.07 – 0.9 ng/mL	
2,6-Dimethylaniline:	0.1 – 3.1 ng/mL	
4-Aminobiphenyl:	0.07 – 0.9 ng/mL	
2-Aminonaphthalene	0.4 – 5.0 ng/mL	
o-Anisidine	0.1 – 1.1 ng/mL	
o-Toluidine	1 – 16 ng/mL	
Number of cigarettes to be smoked		
ISO smoking regime	Intense smoking regime	
• Linear smoking machine: 5	• Linear smoking machine: 2–3	
• Rotary smoking machine: 10	• Rotary smoking machine: 5	
Sample preparation		
Extraction of filter pads: The pads are removed from the holders, trans- 5 mL aliquot is derivatized with HFBA (25 µL ferred into the Erlenmeyer flask and 50 mL for ISO regime and 50 µL for intense regime) dichloromethane + 1 mL internal standard for 40 min. intermediate solution is added. The extraction of aromatic amines from the pad is for 40 min.	Sample derivatization: The pads are removed from the holders, trans- 5 mL aliquot is derivatized with HFBA (25 µL ferred into the Erlenmeyer flask and 50 mL for ISO regime and 50 µL for intense regime) dichloromethane + 1 mL internal standard for 40 min. intermediate solution is added. The extraction of aromatic amines from the pad is for 40 min.	Sample clean-up: SPS cartridge (Florisil) is pre-conditioned with 12 mL of dichloromethane (discard). Whole sample extract is transferred into the cartridge and is allowed to pass through the cartridge (collect). The cartridge is eluted with 8.5 mL dichloromethane (collect). The both eluents are combined, mixed well and an aliquot is transferred to a GC vial for GC/MS-NCI analysis.
Sample analysis by GC/MS-NCI		
GC equipment and its operating conditions:	Mass detection conditions:	
GC column:	low/mid-polarity, with a (14%-cyanopropyl-phenyl)-methylpolysiloxane stationary phase, MS source temperature:	240 °C (ion trap 200 °C)
	a 30-m column with a 0.32-mm internal MS quadrupole temperature:	150 °C
	diameter and 1-µm film thickness or equivalent. MS mode:	106 °C
		NCI
Injector temperature:	250 °C	Data acquisition mode:
Injection mode:	Splitless	Selected Ion Monitoring (SIM)
Injection volume:	3 µL	Reagent gas:
Mode:	Constant flow	Methane at 40% flow
Flow rate:	1.5 mL/min	
Column temperature:	40 °C (0.5 min), 15 °C/min to 240 °C (hold for 5 min), 50 °C/min to 270 °C (hold for 10 min)	
		Ion peak for identification (m/z):
		o-Toluidine: 283
		2,6-Dimethylaniline: 297
		o-Anisidine: 299
		1-Aminonaphthalene: 319
		2-Aminonaphthalene: 319
		3-Aminobiphenyl: 345
		4-Aminobiphenyl: 345
		o-Toluidine- <i>d</i> ₉ : 290
		2,6-Dimethylaniline- <i>d</i> ₁₁ : 306
		2-Aminonaphthalene- <i>d</i> ₇ : 326
		4-Aminobiphenyl- <i>d</i> ₉ : 354

Hydrocarbons (volatile organic compounds, VOCs)

Benzene, 1,3-butadiene, isoprene, styrene, and toluene are included in the Priority List. According to IARC (5), benzene and 1,3-butadiene are carcinogenic for humans (Group 1), styrene is probably carcinogenic to humans (Group 2A), while isoprene is possibly carcinogenic to humans (Group 2B). Toluene is not classifiable as to its carcinogenicity to humans (Group 3). FDA has classified benzene, 1,3-butadiene, isoprene, and styrene as carcinogens (6). Benzene and 1,3-butadiene are cardiovascular toxicants, whereas benzene, 1,3-butadiene, and toluene are reproductive or developmental toxicants. In addition, toluene is a respiratory toxicant.

Benzene, 1,3-butadiene, and isoprene have toxicant animal carcinogenicity indices (TACIs). The study shows that long-term daily doses of 2.6 mg benzene, 9.9 mg 1,3-butadiene, and 3.7 mg isoprene will produce tumours in 25% of animals above the background rate at a specific tissue site (6).

The reference exposure levels of benzene, 1,3 butadiene, styrene, and toluene – at or below of which no adverse health effects are anticipated for individuals with long-term exposure – are 0.6 mg, 2.4 mg, 0.01, and 0.22, correspondingly (TNCRI index).

The cancer risk index (CRI), for benzene is 6.71×10^{-5} per cigarette/day, while for 1,3-butadiene it is 3.02×10^{-4} per cigarette/day, which corresponds with the IARC classification (Table 1).

Benzene and butadiene cause cancers of the haematolymphatic organs (24). Isoprene causes tumours at various sites in laboratory animals (25). Toluene is toxic to the central nervous system. These compounds are present in high amounts in cigarette smoke and probably play a role in lung cancer in smokers (26–28).

There are two official methods for the determination of volatile organic compounds in mainstream cigarette smoke, presented in Table 5 (29, 30):

- SOP 09 (*Standard Operating Procedure for Determination of Volatile Organics in Mainstream Cigarette Smoke under ISO and Intense Smoking Conditions*) (29). This method was developed by the WHO, together with the Tobacco Laboratory Network. An international collaborative study was conducted from 2015–2017, involving 10 laboratories and 5 samples (3 reference cigarettes and 2 commercial brands), and
- CRM No. 70 (*Tobacco – Determination of Selected Volatile Organic Compounds in Mainstream Cigarette Smoke by GC-MS – September 2019*), which was developed by CORESTA (30).

The SOP 09 is suitable for quantitative determination of two VOCs in mainstream cigarette smoke: benzene and 1,3-butadiene by combined gas chromatography/mass spectrometry. CRM No. 70 is applicable to the quantification of five selected VOCs (1,3-butadiene, isoprene, acrylonitrile, benzene, and toluene) in mainstream tobacco smoke from cigarettes, smoked following either ISO 3308 or ISO 20778. Use of benzene- d_6 as an internal standard in both methods is recommended (29, 30).

The CORESTA Special Analytes Task Force (renamed CORESTA Smoke Analytes Sub-Group in 2017) carried out a study in 2005 to compare smoke analyte yield data

obtained from different laboratories using their own preferred methodologies. This study had shown significant and unacceptable differences in volatiles yields, especially for 1,3-butadiene and acrylonitrile and suggested that further work was required to understand factors influencing the yield variability. The Task Force reviewed the key parameters of existing methodologies and further studies were carried out on selected volatiles between 2008 and 2009 (31).

These studies investigated critical method steps that required optimisation before incorporation into the CORESTA Recommended Method No. 70 (30).

CRM No. 70 was initially published after a 2009 collaborative study involving 20 laboratories from 12 countries using the ISO 3308 smoking regime (31). Further data were provided for the same selected volatile substances from 10 samples with different “tar” yields from a 2012 collaborative study using both ISO 3308 and HCI smoking regimes, which involved 17 laboratories from 11 countries (30).

According to SOP 09, the VOCs are collected on Carboxen particles and extracted with an extraction solution containing carbon disulfide and methanol. SOP 09 proposes two extraction solutions depending on sample preparation. An aliquot of the eluate solution is analyzed by gas chromatography/mass spectrometry (GC/MS) (29).

In CRM No. 70, VOCs are collected by passing the mainstream smoke of cigarettes into cryogenic traps (impingers) containing methanol. An extraction is not performed. An aliquot of the combined impinger solutions using GC/MS is analysed (30).

The linear range in SOP 09 is dependent on the type of smoking machine (linear or rotary) and extraction solution. For 1,3-butadiene, the linear range varies between 10–80 µg/mL (Option A - rotary smoking machine), 10–100 µg/mL (Option B - rotary smoking machine) and 20–160 µg/mL (Option A and Option B - linear smoking machine). For benzene, the linear range varies between 4–40 µg/mL (Option B - rotary smoking machine), 4–32 µg/mL (Option A - rotary smoking machine), and 8–64 µg/mL (Option A and Option B - linear smoking machine) (29).

The linear range for 1,3-butadiene in CRM No. 70 is 5–50 µg/mL and it is lower than in SOP 09, while the linear range for benzene is higher in SOP 09 than in CRM No. 70. Injection mode in both methods is split, but in a different split ratio 1:10 (SOP 09) and 1:30 (CRM No. 70). Column temperature is approximately the same. In SOP 09 transfer line temperature and ion source are lower than in CRM No. 70 (29, 30).

More substances can be analysed with CRM No. 70 than with SOP 09. Although the CRM No. 70 method requires a larger number of cigarettes to perform the analysis, it is much simpler compared to the method proposed in SOP 09. The development of both methods by scientific groups involves the use of reference cigarette material from the University of Kentucky (1R5F and 3R4F) and the CORESTA Monitor Test Piece CM6 and allows the methods to be compared in terms of their mean yield, repeatability and reproducibility of the identified 1,3-butadiene. The mean yield for 1,3-butadiene comparable between the two methods: CRM No. 70 (CM6: 60.30 g/cig, 1R5F: 12.20 g/cig, and 3R4F: 41.40 g/cig) and SOP 09 (CM6:

Table 5. Comparison between SOP 09 and CRM No. 70 for the determination of volatile organics in mainstream cigarette smoke.

SOP 09. Standard Operating Procedure for Determination of Volatile Organics in Mainstream Cigarette Smoke under ISO and Intense Smoking Conditions 31.08.2018 (29)	CRM No. 70. Determination of Selected Volatile Organic Compounds in Mainstream Cigarette Smoke by GC-MS - September 2019 (30)
<i>Compounds</i>	
1,3-Butadiene, benzene	1,3-Butadiene, isoprene, acrylonitrile, benzene, toluene
<i>Internal standard</i>	
Benzene- <i>d</i> ₆	Benzene- <i>d</i> ₆
<i>Method summary</i>	
Mainstream cigarette smoke according to the Health Canada Intense (HCI) or the ISO regime is performed. One cigarette per smoke trap is smoked, containing 300 mg CX-572 particles and a glass fibre filter pad. The volatile organic components are extracted by adding a solution containing a mixture of carbon disulfide and methanol onto the Carboxen particles and glass fibre filter pad. The analytical determination of VOCs (1,3-butadiene and benzene) is performed by GC/MS with electron ionization mode.	VOCs are collected by passing the mainstream smoke of cigarettes through a GFP as specified in ISO 3308 or in ISO 20778 into cryogenic traps (impingers) containing methanol. The impinger solutions are fortified with benzene- <i>d</i> ₆ and analysed by GC/MS.
<i>Apparatus and equipment</i>	
Linear smoking machine or Rotary smoking machine GC/MS	Linear smoking machine or Rotary smoking machine, Dewar flasks, Two impingers connected in series, GC/MS
<i>Extraction solution</i>	
Option A: Carbon disulfide and methanol Option B: Carbon disulfide and methanol (1:4) mixed solution, containing internal standard solution	10 mL of methanol is added to each impinger and placed into the Dewar flasks containing the dry ice / isopropanol solution (the temperature must be at or below -70 °C).
<i>Linear range</i>	
Option A – Linear smoking machine	1,3-Butadiene: 5 – 50 µg/mL
• 1,3-Butadiene: 20 – 160 µg/mL	Isoprene: 12 – 600 µg/mL
• Benzene: 8 – 64 µg/mL	Acrylonitrile: 4 – 200 µg/mL
Option A – Rotary smoking machine	Benzene: 4 – 200 µg/mL
• 1,3-Butadiene: 10 – 80 µg/mL	Toluene: 4 – 200 µg/mL
• Benzene: 4 – 32 µg/mL	
Option B – Linear smoking machine	
• 1,3-Butadiene: 20 – 160 µg/mL	
• Benzene: 8 – 64 µg/mL	
Option B – Rotary smoking machine	
• 1,3-Butadiene: 10 – 100 µg/mL	
• Benzene: 4 – 40 µg/mL	
<i>Number of cigarettes to be smoked</i>	
ISO smoking regime	ISO smoking regime
• Linear smoking machine: 3	• Linear smoking machine: 5 or 10
• Rotary smoking machine: 3	• Rotary smoking machine: 10
Intense smoking regime	Intense smoking regime
• Linear smoking machine: 3	• Linear smoking machine: 3 or 6
• Rotary smoking machine: 3	• Rotary smoking machine: 5
<i>Sample preparation</i>	
Option A: 2 mL (for linear) or 4 mL (for rotary) of carbon disulfide is added into the vial or flask. 8 mL (for linear) or 16 mL (for rotary) methanol is added slowly. Carboxen CX-572 is added quickly into the two-phase solution. After the bubbling stops, the glass fibre filter pad is placed into the vial or flask. The mixture is shaken gently on a rotary shaker for 30 min at 120 cycles per min. 5 mL of the supernatant solution is transferred into a vial and 100 µL (for linear) or 50 µL (for rotary) of internal standard solution is added. 1 mL portion of the eluate solution is transferred to a 1.5 mL autosampler vial and analyzed by GC/MS.	After all samples are smoked following ISO regime, each impinger is spiked with 100 µL of benzene- <i>d</i> ₆ spiking solution. For the intense regime, each impinger is spiked with 200 µL of benzene- <i>d</i> ₆ spiking solution. The impingers are stoppered and gently mixed. An aliquot of the combined impinger solutions is transferred into an amber GC vial and analyzed for volatiles using GC/MS.
Option B: The Carboxen 572 particles and the glass fibre filter pad are transferred into the vial or flask. 10 mL (for linear) or 20 mL (for rotary) of solvent mixture is added slowly into the vial or flask. The mixture is shaken gently on a rotary shaker for 30 min at 120 cycles or rounds per min. 1 mL portion of the eluate solution to a 1.5 mL autosampler vial is transferred and analyzed by GC/MS.	

continued

Table 5. Comparison between SOP 09 and CRM No. 70 - continued.

SOP 09. Standard Operating Procedure for Determination of Volatile Organics in Mainstream Cigarette Smoke under ISO and Intense Smoking Conditions 31.08.2018 (29)		CRM No. 70. Determination of Selected Volatile Organic Compounds in Mainstream Cigarette Smoke by GC-MS - September 2019 (30)	
GC/MS		Sample analysis GC/MS	
		GC equipment and its operating conditions	
GC Column:	InertCap AQUATIC-2 with a 60-m column with 0.25-mm internal diameter and 1.4-µm film thickness is suitable.	GC Column:	Fused silica capillary column with a 60-m column with 0.25-mm internal diameter and 1.4-µm film thickness is suitable.
Injector temperature:	240 °C (ion trap: 200 °C)	Injector temperature:	150 °C
Injection mode:	Split	Injection mode:	Split
Injection split ratio:	1:10	Injection split ratio:	1:30
Injection volume:	1 µL or 2 µL (Ion trap 1:5; after 1.5 min 1:120)	Injection volume:	3 µL
Mode:	Constant flow	Injection split flow:	30 mL/min
Flow rate:	1.0 mL/min (ion trap 1.0 mL/min)	Column temperature:	40 °C (6 min); 20 °C / min to 225 °C (hold for 6 min)
Column temperature:	40 °C (6 min); 6 °C/min to 250 °C		
		Mass detection conditions	
Interface/transfer line temperature:	180 °C (ion trap 200 °C)	Interface/transfer line temperature:	240 °C
MS source temperature:	200 °C (ion trap 180 °C)	MS source temperature:	240 °C
Dwell time:	50 ms	Acquisition mode:	SIM (or scan)
Ionization mode:	Electron ionization (ionizing voltage at 70 eV)	Solvent delay:	Column dependent
Detection:	Full-scan m/z 30 – 300	Detection:	Full-scan m/z 40 – 200
Ion peak for identification (m/z):		Ion peak for identification (m/z):	
Benzene:	78		Quantification Confirmation
Benzene- <i>d</i> ₆ :	84	1,3-butadiene	54 53
1,3-Butadiene:	54	Isoprene	67 68
		Acrylonitrile	52 53
		Benzene	78 77
		Benzene- <i>d</i> ₆	84 83
		Toluene	91 92

60.86 g/cig, 1R5F: 11.06 g/cig, and 3R4F: 39.23 g/cig). The limits for repeatability (r) and reproducibility (R) of the two methods are very different: (CM6 with r = 1.74, R = 2.18 for SOP 09 and r = 21.20, R = 37.9 for CRM No. 70; 1R5F with r = 1.96, R = 4.27 for SOP 09 and r = 5.30, R = 8.20 for CRM No. 70; 3R4F with r = 7.20, R = 16.34 for SOP 09 and r = 13.30, R = 29.60 for CRM No. 70). In addition, in 2018 and 2020 two ISO methods for the determination of selected volatile organic compounds were published based on the CRM No. 70 method:

- ISO 21330:2018 Cigarettes – Determination of Selected Volatile Organic Compounds in the Mainstream Smoke of Cigarettes Method Using GC/MS (32); and
- ISO 23923:2020 Cigarettes – Determination of Selected Volatile Organic Compounds in the Mainstream Smoke of Cigarettes with An Intense Smoking Regime. Method using GC/MS (33).

Health Canada Official Method - T116 (*Determination of 1,3-butadiene, isoprene, acrylonitrile, benzene and toluene in mainstream tobacco smoke*) is identical to the CRM No. 70 (34).

Polycyclic aromatic hydrocarbons (PAHs)

According to the IARC and the FDA, B[a]P, from the PAH group, is carcinogenic to humans. B[a]P has a toxicant animal carcinogenicity index (TACI). The studies show that long-term daily dose of 8.6 µg will produce tumours in 25% of animals above the background

rate at a specific tissue site. The cancer risk index (CRI) is higher: 1.93×10^{-6} per cigarette/day (35–39).

B[a]P along with other substances from the PAH group are formed during incomplete combustion of organic matter and always occur as mixtures. Many PAHs are potent carcinogens or toxicants in laboratory animals. PAHs are accepted to be major contributors to lung cancer in smokers (35–39).

There are two official methods for determining the PAHs, in particular B[a]P, in tobacco smoke (Table 6) (40, 42):

- SOP 05 (*Determination of benzo[a]pyrene in mainstream cigarette smoke under ISO and intense smoking conditions*). The World Health Organization, together with the Tobacco Laboratory Network developed the standard operating procedure. An international collaborative study was conducted in 2012, involving testing of three reference cigarettes (1R5F, 3R4F, and CM6) and two commercial brands by eight laboratories (40). Mainstream smoke total particulate matter from the cigarette test sample is trapped onto a glass-fibre filter pad. A solution containing an isotope-labelled internal standard is spiked onto the pad, which is extracted with cyclohexane. The cyclohexane extract is eluted through a silica solid-phase extraction cartridge. The eluent collected is analysed by gas chromatography/mass spectrometry (GC/MS) with electron ionization detection (40); and
- CRM No. 58 (*Determination of benzo[a]pyrene in mainstream cigarette smoke by GC-MS*) developed by

Table 6. Comparison between SOP 05 and CRM No. 58 for the determination of benzo[a]pyrene in mainstream cigarette smoke.

SOP 05. Standard Operating Procedure for Determination of Benzo[a]pyrene in Mainstream Cigarette Smoke under ISO and Intense Smoking Conditions, February 2015 (40)	CRM No. 58. Determination of Benzo[a]pyrene in Mainstream Cigarette Smoke by GC-MS, November 2019 (41)
<i>Compounds</i>	
Benzo[a]pyrene	Benzo[a]pyrene
<i>Internal standard</i>	
B[a]P- <i>d</i> ₁₂	B[a]P- <i>d</i> ₁₂
<i>Method summary</i>	
Mainstream smoke total particulate matter from the cigarette sample is trapped onto a GFP. Internal standard is spiked onto the GFP, which is extracted with cyclohexane. The cyclohexane extract is purified using SPE by passing through a pure silica unbound phase SPE cartridge, and the eluent is collected. The sample is analyzed by GC/MS with electron ionization detection.	Mainstream smoke total particulate matter from the cigarette sample is trapped onto a GFP. Internal standard is spiked onto the GFP, which is extracted with methanol, and the methanol extract is diluted with water. Crude water/methanol smoke extract is purified using SPE by passing through a cyclohexyl bonded silica (CH) SPE cartridge, followed by the elution of B[a]P with cyclohexane. The sample is analyzed by GC/MS operated in the SIM data acquisition mode for identification, confirmation and quantification of B[a]P .
<i>Apparatus and equipment</i>	
Linear smoking machine or Rotary smoking machine GC/MS	Linear smoking machine or Rotary smoking machine GC/MS
<i>Extraction solution</i>	
Cyclohexane	Methanol
<i>Linear range</i>	
2 – 60 ng/mL	1 – 200 ng/mL
<i>Number of cigarettes to be smoked</i>	
ISO smoking regime	ISO smoking regime
• Linear smoking machine: 5	• Linear smoking machine: 5 – 10
• Rotary smoking machine: 20	• Rotary smoking machine: 10 – 20
Intense smoking regime	Intense smoking regime
• Linear smoking machine: 3	• Linear smoking machine: 5 – 10
• Rotary smoking machine: 10	• Rotary smoking machine: 10 – 20
<i>Sample preparation</i>	
Extraction of filter pads: The pads from the holders are removed, transferred into Erlenmeyer flask and cyclohexane and internal standard (40 mL cyclohexane + 40 µL of the B[a]P- <i>d</i> ₁₂ , for 44-mm pads, or 100 mL cyclohexane+ 100 µL of the B[a]P- <i>d</i> ₁₂ , for 92-mm pads) are added to the flask. The extraction of B[a]P from the pad is for 60–80 min.	Extraction of filter pads: The pads from the holders are removed, transferred into Erlenmeyer flask and methanol and internal standard (20 mL + 200 µL of the B[a]P- <i>d</i> ₁₂ for 44-mm pads, or 50 mL + 200 µL of the B[a]P- <i>d</i> ₁₂ for 92-mm pads) are added to the flask. The flask is shaken vigorously until the filter pad disintegrates. The solution is filtered through a glass suction filter or using paper filtration and the volume is -supplemented to at least 40 mL for a 44-mm filter pad, or to at least 80 mL for a 92-mm pad. Distilled water is added into the flask in order to obtain a solution containing 60% water and 40% methanol.
Sample clean-up: SPE cartridge is pre-conditioned with 10 mL cyclohexane (discard). 10 mL of sample extract is pipetted onto the cartridge and is allowed to pass through the cartridge (collect). The cartridge is eluted with two further 15-mL aliquots of cyclohexane, allowing the cartridge to run dry after the last aliquot is passed through. The cyclohexane solution is evaporated almost to dryness and is dissolved with 1 mL cyclohexane.	Sample clean-up: SPE cartridge is pre-conditioned with 10 mL methanol and 10 mL of a mixture of water and methanol (60:40, v/v). The extraction solution is passed through the SPE cartridge and the cartridge is dried (discarded). The cartridge is eluted with 15 mL cyclohexane. The volume of the cyclohexane solution is reduced to 1 mL. If necessary, the cyclohexane solution can be dried from the water. The obtained solution is transferred into an amber autosampler vial and capped with a PTFE lined septum cap.
<i>Sample analysis</i>	
GC/MS	GC/MS
<i>GC equipment and its operating conditions</i>	
GC Column - Fused silica capillary column with a methylphenyl (5%) polysiloxane stationary phase, a 30-m column with a 0.25-mm internal diameter and 0.25-µm film thickness is suitable	GC Column - Fused silica capillary column with a methylphenyl (5%) polysiloxane stationary phase, 30-m column with a 0.25 mm internal diameter and 0.25 µm film thickness is suitable.
Injector temperature: 280 °C (ion trap: 200 °C)	Injector temperature: 290 °C
Injection mode: Splitless	Injection mode: Splitless
Injection volume : 1 µL or 2 µL	Injection volume: 1 µL
Mode: Constant flow	Mode: Constant flow
Flow rate: 1.2 mL/min (ion trap 1.0 mL/min)	Flow rate: 0.9 mL/min
Column temperature: 150 °C for 0 min; 6 °C/min to 260 °C, hold at 260 °C for 7 min 50 °C/min to 290 °C, hold at 290 °C for 20 min	Column temperature: 80 °C (hold for 3 min); 5 °C/min to 290 °C (hold for 20 min)

continued

Table 6. Comparison between SOP 05 and CRM No. 58 - continued.

SOP 05. Standard Operating Procedure for Determination of Benzo[a]pyrene in Mainstream Cigarette Smoke under ISO and Intense Smoking Conditions, February 2015 (40)	CRM No. 58. Determination of Benzo[a]pyrene in Mainstream Cigarette Smoke by GC-MS, November 2019 (41)	
<i>Mass detection conditions</i>		
Interface/transfer line temperature: 280 °C (ion trap 200 °C)	Interface/transfer line temperature: 270 °C	
MS source temperature: 230 °C	MS source temperature: 230 °C	
Dwell time: 50 ms		
Ionization mode: Electron ionization		
Detection: Full-scan m/z 30-300	Ion peak for identification (m/z):	
Ion peak for identification(m/z):	Quantification	Confirmation
B[a]P 252	B[a]P 252	126
B[a]P-d ₁₂ 264	B[a]P-d ₁₂ 264	132

CORESTA. The first collaborative study supporting this method was conducted in 2003 and involved 13 laboratories and 5 replicate analyses of 7 cigarette samples including the University of Kentucky reference cigarette 2R4F (42). In 2012, it was obtained from a second collaborative study involving 12 laboratories. This study provided data on the measurement of B[a]P in 10 cigarette samples (7 commercial products and 3R4F, 1R5F, and the CORESTA Monitor CM6) smoked under both the ISO 3308 and ISO 20778 regimes. There are 5 versions February 2004 (Version 1), March 2013 (Version 2), July 2014 (Version 3), January 2018 (Version 4 - formatting changes only), November 2019 (Version 5 - editorial changes for ISO 20778 Intense Smoke Regime and provide citation for supporting collaborative study) (41).

The compound B[a]P and the internal standard B[a]P-d₁₂ are the same in both methods. Mainstream smoke total particulate matter from the cigarette sample is trapped onto a glass fiber filter pad. The B[a]P is extracted from the pads. Using solid phase extraction (SPE) the crude extracts are cleaned-up and analyzed by GC/MS (40, 41).

The numbers of cigarettes smoked in both methods are different. The SOP 05 strictly fixes the number of cigarettes smoked, depending on the smoking regime (ISO or Intense) and the smoking machine (linear or rotary smoking machine), while in CRM No. 58 the number of cigarettes smoked may vary. Therefore, the linearity in CRM No. 58 has a wider range (1–200 ng/mL) compared to the SOP 05 (2–60 ng/mL) (40, 41).

While in the SOP 05 the extraction of B[a]P is with cyclohexane, in the CRM No. 58 the extraction is carried out with methanol and the extraction solution is diluted with water in order to obtain a solution containing 60% of water and 40% of methanol (40, 41).

The SPE cartridges and the procedure of sample clean-up are different. SOP 05 recommended pure silica unbound phase and elution of B[a]P with cyclohexane, while in CRM No. 58 cyclohexyl bonded silica (CH) SPE cartridge is used, followed by the elution of B[a]P with cyclohexane (40, 41).

The determination of B[a]P mainstream cigarette smoke is by GC/MS in both methods. GC equipment, its operating conditions and MS detection conditions are approximately the same. For the GC, a fused silica capillary column with a methylphenyl (5%) polysiloxane stationary phase, a 30-m column with a 0.25-mm internal diameter and 0.25-µm film

thickness is suitable for both methods (Table 6) (40, 41).

Health Canada's method T-103 is completely different from SOP 05 and CRM No. 58 (43). The quantification of B[a]P is carried out with reverse-phase HPLC via fluorescence detection.

The procedure is simplified and involves extraction with cyclohexane, sample clean-up with silica cartridge and elution with hexane, evaporation and reconstitution with acetonitrile, reverse-phase HPLC and quantitation via fluorescence detection. Recovery is between 85 and 110%.

Tobacco-specific nitrosamines (TSNAs)

NNK, NNN, NAB, and NAT are included in the Priority List. NNK and NNN are carcinogenic to humans, according to the IARC and the FDA, while NAB and NAT cannot be classified as carcinogenic to humans.

NNK and NNN have toxicant animal carcinogenicity indices (TACIs). The study shows that long-term daily doses of 3.4 mg acetaldehyde and 0.29 mg NNN will produce tumours in 25% of animals above the background rate at a specific tissue site.

The cancer risk index (CRI) for NNK is 7.8×10^{-6} per cigarette/day, which is lower compared to NNK (3.8×10^{-5} per cigarette/day, Table 1).

There are two official methods for determining tobacco-specific nitrosamines in tobacco smoke:

- SOP 03 (*Determination of tobacco-specific nitrosamines in mainstream cigarette smoke under ISO and intense smoking conditions*) (44), and
- CORESTA CRM No. 63 (*Determination of tobacco specific nitrosamines in cigarette mainstream smoke - GC/TEA method*) (Table 7) (45).

SOP 03 was developed by the World Health Organization together with the Tobacco Laboratory Network. An international collaborative study was conducted in 2012, involving testing of three reference cigarettes (1R5F, 3R4F, and CM6) and two commercial brands by eight laboratories (44). The method is suitable for quantitative determination of four tobacco-specific nitrosamines in mainstream cigarette smoke: NNN, NAB, NAT, and NNK. Mainstream smoke from the cigarette test sample is trapped onto a glass-fibre filter pad. A solution containing a mixture of two (or four) isotope-labelled internal standards is spiked onto the pad, which is extracted with ammonium acetate. The extract is filtered and analysed by high-performance liquid chromatography/tandem mass spectrometry

Table 7. Determination of tobacco-specific nitrosamines in cigarette mainstream smoke by SOP 03 and CRM No. 63.

SOP 03. Standard Operating Procedure for Determination of Tobacco-Specific Nitrosamines in Mainstream Cigarette Smoke Under ISO and Intense Smoking Conditions – June 2014 (44)		CRM No. 63. Determination of tobacco specific nitrosamines in cigarette mainstream smoke – GC-TEA method, January 2019 (45)	
NNN, NNK, NAT, and NAB		<i>Compounds</i> NNN, NAB, NAT, and NNK	
<i>Internal standard</i> Deuterium-labelled 3-(1-nitrosopyrrolidin-2-yl)pyridine(NNN-<i>d</i>₄) Deuterium-labelled 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK-<i>d</i>₄) Deuterium-labelled <i>N</i>-nitrosoanatabine (NAT-<i>d</i>₄) Deuterium-labelled <i>N</i>-nitrosoanabasine (NAB-<i>d</i>₄)		<i>Internal standard</i> <i>N</i>-Nitrosopentyl-(3-picoly)-amine (NNPA)	
<i>Method summary</i> Conditioned cigarettes are smoked using standard procedures. Mainstream smoke is trapped on a glass fiber filter pad. After addition of an internal standard, the filter pad is extracted with ammonium acetate. The extract is filtered and analysed by high-performance liquid chromatography–tandem mass spectrometry (HPLC/MS-MS) with electrospray ionization. Analyte ions are detected in the MS-MS mode.		<i>Method summary</i> Conditioned cigarettes are smoked using standard procedures. Mainstream smoke is trapped on a glass fiber filter pad. After addition of an internal standard, the filter pad is extracted with dichloromethane. Sample clean-up of the extraction solution is accomplished with one of the following methods: - Elution of the extract through an alumina column, followed by the elution of the TSNAs with an acetone/dichloromethane (50:50 v/v) mixture; - Elution of the extract through a combined silica-alumina column, followed by the elution of the TSNAs with an 8% methanol in dichloromethane solution. The extract is concentrated followed by quantitative analysis using gas chromatography with a thermal energy analyser for detection (GC-TEA).	
<i>Apparatus and equipment</i> Linear smoking machine or Rotary smoking machine HPLC system interfaced to MS-MS (triple quadrupole mass spectrometer)		<i>Apparatus and equipment</i> Linear smoking machine or Rotary smoking machine Gas chromatography with a thermal energy analyser	
<i>Extraction solution</i> Ammonium acetate		<i>Extraction solution</i> Dichloromethane	
<i>Linear range</i> NNN: 0.5 – 200 ng/mL NAB: 0.5 – 200 ng/mL NAT: 0.5 – 200 ng/mL NNK: 0.5 – 200 ng/mL		<i>Linear range</i> NNN: 50 – 2000 ng/mL NAB: 50 – 2000 ng/mL NAT: 50 – 2000 ng/mL NNK: 50 – 2000 ng/mL	
<i>Number of cigarettes to be smoked</i> ISO smoking regime - Linear smoking machine: 5 - Rotary smoking machine: 20 Intense smoking regime - Linear smoking machine: 3 - Rotary smoking machine: 10		<i>Number of cigarettes to be smoked</i> ISO smoking regime - Linear smoking machine: 10 - Rotary smoking machine: 20	
<i>Sample preparation</i> Extraction of filter pads The pads from the holders are removed, transferred into the 60-mL amber bottle and added 100 mmol/L aqueous ammonium acetate extraction solution (20 mL for 44-mm pads, 50 mL for 93-mm pads) + internal standard (200 µL for a 44-mm pad, 500 µL for a 92-mm pad). The extraction of nitrosamines from the pad is for 30 min. Remove aliquots (e.g. 1–2 mL) of the extract, filter the aliquots with at most 0.45 µm membrane filter, and place in an autosampler vial.		<i>Sample preparation</i> Extraction of filter pads The pads from the holders are removed, transferred into the Erlenmeyer flask and added 100 mL dichloromethane + 400 µL internal standard (10 cigarettes) or 200 mL dichloromethane + 800 µL internal standard (20 cigarettes). The extraction of nitrosamines from the pad is for 30 min. The volume is completed with dichloromethane to 150 mL (10 cigarettes) and 300 mL (20 cigarettes). An aliquot of the solution is concentrated to approximately 5 mL. The elution solution is concentrated to a volume 2 mL. Sample clean-up - 1: In the liquid chromatographic column alumina slurry (50 mL of dichloromethane and 10 g of basic alumina) is placed. The extract is added. 30 mL of dichloromethane is added and is passed through the column (waste). The TSNAs are eluted with 100 mL of a solution of acetone – dichloromethane (50 : 50 v/v). The elution solution evaporated and dissolved to a volume of 2 mL. Sample clean-up - 2 : In the liquid chromatographic column is placed approximately 2 g of anhydrous sodium sulphate, 15 g of alumina, 15 g of silica and 2 g of anhydrous sodium sulphate. The extract is added. 100 mL of dichloromethane is added and passed through the column (waste). The TSNAs are eluted with 160 mL of a solution of 8 % methanol in dichloromethane. The elution solution evaporated and dissolved to a volume of 2 mL.	

continued

Table 7. Determination of tobacco specific nitrosamines in cigarette mainstream smoke by SOP 03 and CRM № 63 - continued.

SOP 03. Standard Operating Procedure for Determination of Tobacco-Specific Nitrosamines in Mainstream Cigarette Smoke Under ISO and Intense Smoking Conditions – June 2014 (44)			CRM No. 63. Determination of tobacco specific nitrosamines in cigarette mainstream smoke – GC-TEA method, January 2019 (45)		
			<i>Sample analysis</i>		
HPLC system interfaced to MS-MS			Gas chromatograph – Thermal Energy Analyser (GC-TEA)		
<i>Chromatography equipment and its operating conditions</i>					
HPLC column capable of distinct separation of TSNA and isotope labelled TSNA peaks from those of other cigarette emission components, e.g. Agilent Zorbax Eclipse XDB-C18 (2.1 x 150 mm, 3.5-µm particle size)			GC Column: Fused silica capillary column with a 50 % methyl/50 % phenyl polysiloxane stationary phase, a 30-m column with a 0.53-mm internal diameter and 1-µm film thickness is suitable for this analysis.		
Injection volume:	5 µL		Injector temperature:	230 °C	
Column oven temperature:	40 ± 1 °C (Alter as appropriate for the column used.)		Injection mode:	Splitless	
Degasser:	On		Injection volume:	2 µL	
Binary pump flow:	0.2 mL/min		Mode:	Constant flow	
Mobile phase A:	0.1 % acetic acid in water (v/v)		Column temperature:	150 °C for 2 min, 3 °C/min to 230 °C, 20 °C/min to 250 °C, hold at 250 °C for 3 min	
Mobile phase B:	0.1 % acetic acid in methanol (v/v)				
Gradient:	Shown in Table 3				
Total analysis time:	12 min		TEA conditions		
Mobile Phase A:	0.1% acetic acid in water (v/v)		TEA interface temperature: 240 °C		
Mobile Phase B:	0.1% acetic acid in methanol (v/v)		TEA pyrolysis temperature: 500 °C		
Gradient conditions:					
0.0	50 % A	50 % B			
3.0	10 % A	90 % B			
4.0	0 % A	100 % B			
5.0	0 % A	100 % B			
5.5	50 % A	50 % B			
12.0	50 % A	50 % B			

(HPLC/MS-MS) with electrospray ionization. Analyte ions are detected in the MS-MS mode. SOP 03 is applicable and tested for ISO and Intensive Smoking Regime with analytical recovery over 90%.

CRM No. 63 is suitable for the quantitative determination of four tobacco-specific nitrosamines in mainstream cigarette smoke: NNN, NAB, NAT, and NNK (45).

Between 1999 and 2005, a Task Force composed of CORESTA members studied the existing methodologies for the determination of the TSNAs in the mainstream smoke of cigarettes. Several methods have been proposed for this determination, which are mainly based on two types of analytical methodologies: gas chromatography/thermal energy analyzer (GC/TEA) and liquid chromatography with tandem mass spectrometry (LC/MS-MS) (45).

The Task Force decided in the first instance to develop a method using GC/TEA, because this methodology was the most widely used in laboratories analysing nitrosamines, and only in that case it was possible to obtain the collaboration of a sufficient number of experienced laboratories to develop a recommended method.

A major international study involving nine laboratories and seven cigarette samples including the 2R4F (a reference cigarette previously available from the University of Kentucky) and covering a wide range of blends and construction was conducted in 2005 (47).

The main difference between the two methods (SOP 03 and CRM No. 63) is the use of the technical equipment - HPLC system interfaced to MS-MS for SOP 03 and gas chromatography/thermal energy analyzer (GC/TEA) for CRM No. 63. The sample preparation for CRM No. 63 requires an additional sample clean-up of the extraction solution, which prolongs the procedure, whereas with

SOP 03 only an extraction of 30 minutes is required. The linear range of SOP 03 is between 0.5 and 200 ng/mL and is lower than that of CRM No. 63 (50–2000 ng/mL).

The official method for determining the tobacco-specific nitrosamines in cigarette mainstream smoke (CRM No. 63) includes cigarette smoking. The mainstream smoke is trapped on a glass fiber filter pad. The tobacco-specific nitrosamines are extracted with dichloromethane. Sample clean-up of the extraction solution can be carried out with two methods, either with a liquid chromatographic column with alumina and elution with a solution of acetone and dichloromethane (50:50 v/v) or with a liquid chromatographic column with combined silica-alumina column and elution with 8% methanol in dichloromethane. The summary of CRM No. 63 is presented in Table 7.

Health Canada Official Method T-111 for the determination of nitrosamines in mainstream tobacco smoke includes cigarette smoking, concentrating the TSNAs by extraction with dichloromethane, followed by column chromatography onto basic alumina. The quantitative analysis is performed by combined gas chromatography/thermal energy analysis (GC/TEA) (46). The procedure repeats the CRM No. 63, but the difference is the amount of eluent acetone/dichloromethane (50:50 v/v) in the sample clean-up.

Phenolic compounds

The phenolic compounds in the Priority List include catechol, *m*-cresol, *p*-cresol, *o*-cresol, phenol, hydroquinone, and resorcinol.

According to the IARC, catechol is classified in Group 2B (the agent is possibly carcinogenic to humans), while

phenol, resorcinol, and hydroquinone are classified in Group 3 (the agent is not classifiable as to its carcinogenicity to humans). *o*-Cresol, *m*-cresol, and *p*-cresol are not included in the IARC list. On the other hand, they are classified as respiratory toxicants according to the FDA list, catechol, *m*-cresol and *p*-cresol are carcinogens, and phenol is a cardiovascular toxicant. Hydroquinone and resorcinol are not included in the FDA list. Catechol and hydroquinone have toxicant animal carcinogenicity indices (TACI). The study shows that long-term daily doses of 0.58 mg catechol, and 1.2 mg hydroquinone

will produce tumours in 25% of animals above the background rate at a specific tissue site. The reference exposure levels of *o*-cresol, *m*-cresol, *p*-cresol – at or below of which no adverse health effects are anticipated for individuals with long-term exposure – are 0.01, while for phenol it is 1.2 (TNCRI index) (Table 1).

The official method for determining the phenolic compounds in cigarette mainstream smoke by HPLC-FLD was developed by CORESTA (CRM No. 78 *Determination of selected phenolic compounds in mainstream cigarette smoke by HPLC-FLD*) (Table 8) (47).

Table 8. CRM No. 78 – Determination of Selected Phenolic Compounds in Mainstream Cigarette Smoke by High Performance Liquid Chromatography with Fluorescence Detector.

Method description				
Compounds				
Hydroquinone, resorcinol, catechol, phenol, <i>p</i> -cresol, <i>m</i> -cresol, and <i>o</i> -cresol				
Method summary				
Cigarettes are smoked on a routine smoking machine and mainstream smoke is trapped on a glass-fibre filter pad (GFP). The GFP is extracted with aqueous acetic acid. An aliquot of the extract is syringe filtered and analysed by HPLC/FLD.				
Apparatus and equipment				
Linear smoking machine or Rotary smoking machine HPLC/FLD				
Extraction solution				
1% acetic acid solution				
Linear range				
The calibration should cover the concentration range of interest. The linear range is approximate to the phenolic compounds in tobacco smoke.				
Number of cigarettes to be smoked				
ISO smoking regime Linear smoking machine: 5 Rotary smoking machine: 10				
Sample preparation				
Extraction of filter pads: The pads from the holders are removed, transferred into an Erlenmeyer flask and 40 mL 1% acetic acid solution (5 cigarettes) and 80 mL 1% acetic acid solution (10 cigarettes) is added. The extraction of phenolic compounds from the pad is until the CFP has disintegrated. The CFP extracts may need to be diluted.				
Sample analysis				
HPLC/FLD				
HPLC equipment and its operating conditions				
HPLC column with pentafluoro phenylpropyl (PFP) stationary phase; an example of the column dimensions are 3-µm, a 150-mm column and 4.6 mm internal diameter or equivalent.		Wavelength programmable fluorescence detector settings (example):		
Column Temperature:	Ambient	Time (min)	Excitation (nm)	Emission (nm)
Auto-sampler Tray Temperature:	4 °C	0.0	280	310
Injection volume:	10 µL or 20 µL	12.4	280	310
		12.5	274	298
		23.0	274	298
		24.0	280	310
Mobile Phases:		28.0	280	310
Mobile phase A:	Prepare 2 L of 1% acetic acid in deionized water and degas			
Mobile phase B:	Prepare 2 L of 1% acetic acid in methanol and degas			
Flow rate:	0.8 mL/min			
The following is an example of gradient conditions:				
Time (min)	% A	% B		
0	78	22		
8	78	22		
8.5	55	45		
21	55	45		
22	0	100		
28	0	100		

The official CORESTA method was a basis for publishing ISO 23904:2020 (*Cigarettes – Determination of selected phenolic compounds in cigarette mainstream smoke with an Intense Smoking Regime using HPLC-FLD*) and ISO 23905:2020 (*Cigarettes – Determination of selected phenolic compounds in cigarette mainstream smoke using HPLC-FLD*). The two methods divide the analysis into two documents according to the regime of smoking the cigarettes, either ISO regime or the intensive one. Both ISO methods use CORESTA Technical Report 2013 Collaborative Study for Selected Phenolic Compounds in Mainstream Cigarette Smoke data. The method is easily applicable due to the accessible apparatus requirements and inexpensive reagents.

The CRM was produced through a full collaborative study involving 18 laboratories from 11 countries smoking 10 samples with a range of blend styles (Virginia flue-cured, American blend, and dark air-cured) and ISO “tar” yields (1–13 mg) under both the ISO and HCI regimes. There are two versions: Version 1 from July 2014 and Version 2 with updated r&R tables (47).

The method is suitable for the determination of 7 selected phenolic compounds such as hydroquinone, resorcinol, catechol, phenol, *p*-cresol, *m*-cresol, and *o*-cresol in cigarette mainstream smoke by HPLC/FLD. The method is simple and involves smoking cigarettes. The total particulate matter from the cigarette sample is trapped onto a glass-fibre filter pad. The phenolic compounds are extracted from the pads with 1% acetic acid solution. An aliquot of the extract is syringe filtered, diluted and analysed by HPLC/FLD. The summary of CRM NO. 78 is presented in Table 8.

The Health Canada Official Method T-114 (*Determination of phenolic compounds in mainstream tobacco smoke*) describes the extraction and determination of phenolic compounds of mainstream tobacco smoke by reverse-phase HPLC and it is identical to the CORESTA Recommended Method No. 78 (48).

“Tar”, nicotine, and CO (TNCO)

According to Directive 40, the emission levels from cigarettes placed on the market or manufactured in the European Member States (‘maximum emission levels’) shall not be higher than 10 mg of “tar” per cigarette, 1 mg of nicotine per cigarette, and 10 mg of carbon monoxide per cigarette (49).

Determination of TNCO in tobacco smoke is conducted according to ISO 4387, ISO 10315, and ISO 8454 (50–52). The automatic process of smoking cigarettes is performed on a linear or rotary smoking machine according to ISO 3308:2012 (53). The total particulate matter (TPM) is collected in a glass fiber filter trap. The mass of the TPM is extracted from the trap with isopropanol, containing internal standard for determination of the water and nicotine content. The nicotine content of an aliquot of the smoke extract is determined by gas chromatography. The vapor phase of cigarette smoking is collected for measurement of the CO by using a non-dispersive infrared (NDIR) analyser (50–52).

CONCLUSION

The 44 harmful and potentially harmful components in the tobacco smoke and Priority List set at the third meeting in WHO Framework Convention Working Group on Tobacco Control in Ottawa, Canada, in October 2006, is summarised. The International Agency for Research on Cancer and the U.S. Food and Drug Administration compared the toxicity of the substances included in the Priority List. In this review the different official methods for components of the Priority List are compared. The authors hope that this review will help the accredited and/or scientific laboratories to compare the different methods and to choose the appropriate method, depending on laboratory equipment and available laboratory chemicals.

CONFLICTS OF INTEREST

No potential conflict of interest is reported by the authors. The authors have sole responsibility for the writing and content of the paper.

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