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Standardized methods for the regulation of cigarette-smoke constituents

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ABSTRACT

This article includes a summary of the development of existing standardized methods to test cigarette smoke, and a review of both the capability of current methods for testing cigarette-smoke constituents and current performance standards relevant to regulatory testing. There is a comparison of the reproducibility of some currently approved methods to determine volatile constituents and tobacco-specific nitrosamines in cigarette smoke with the Horwitz prediction of reproducibility. There is discussion of appropriate activities to support the development and the implementation of more reproducible testing methods and an indication of the tasks that should be prioritized to achieve optimal inter-laboratory agreement of data.

Contents

1. Introduction

Organizations in different regions of the world, including Parties to the World Health Organization Framework Convention on Tobacco Control (WHO FCTC) and the US Food and Drug Administration (FDA), are working to increase the regulation of tobacco and cigarette-smoke constituents. A principal requirement for the implementation of regulatory controls is the capability to measure substances of regulatory interest at relevant levels in a reproducible manner. For substances in cigarette smoke that the FCTC Parties and the FDA have proposed should be reported, most existing testing methods have been developed by individual interest groups (i.e., a single regulatory body, an industry research association or an independent laboratory) and thus may not have been internationally harmonized.

At the third Conference of the Parties (COP) in November 2008, the COP mandated the WHO Tobacco Laboratory Network (TobLabNet) to validate test methods within five-and-a-half years for a number of tobacco and smoke constituents. These include methods to determine "tar" (defined as the mass of particulate matter remaining on a filter pad after subtraction of the measured nicotine and water content), under two machine-smoking regimes:

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- methods to determine constituents of tobacco smoke, including tobacco-specific nitrosamines, benzo[a]pyrene, aldehydes, volatile organic compounds and carbon monoxide [1]; and,
- three methods to determine five constituents of tobacco.

The choice of constituents to be determined is based upon recommendations by the WHO Study Group on Tobacco Product Regulation (TobReg) [2]. In 2012, WHO published a documented procedure for the intense smoking of cigarettes [3], which, although similar in structure to the procedures published by Health Canada [4] and the International Organization for Standardization (ISO) [5], differed in specific dimensions and tolerances. Because the documented WHO procedure has not been subject to wider peer review, it is impossible to evaluate the potential impact of these differences on inter-laboratory agreement of data.

Historically, the constituents measured and reported in cigarette smoke have comprised tar, nicotine and, more recently, carbon monoxide, for which test methods have been validated and harmonized [6]. The relative abundance of these substances in cigarette smoke and their amenability to gravimetric or spectroscopic measurement have facilitated robust measurement procedures. However, the number of additional substances that the FDA and COP/TobReg have proposed should be reported [7,8], their relatively low abundances in cigarette smoke and the chemical complexity of the tobacco-smoke matrix are significant technical challenges to the development of robust, efficient analytical methods.

The aim of this review is to document briefly the historical development of cigarette-smoke testing methods, to summarize the capability of current cigarette-smoke-constituent testing methods and performance standards relevant to regulatory testing, and to discuss an approach to development and implementation of appropriate cigarette-smoke-constituent testing methods. Because there are no international standards for the testing of smokeless tobacco products, discussion is confined to the measurement of constituents of cigarette smoke.

2. Historical testing of cigarette smoke

According to Rodgman and Perfetti [9], by 2012, over 6000 substances had been identified in cigarette smoke. The number of discrete chemical species present in cigarette smoke is unknown

but, according to Wakeham [10], may be as many as 100,000. Cigarette pyrogenesis, pyrolysis and the resulting chemical composition of smoke are strongly influenced by variables in both cigarette design [11] and the smoking conditions employed. Cigarette smoke is a concentrated aerosol containing thousands of substances distributed dynamically between aerosol particles and the surrounding gaseous phase. An understanding of the chemical composition of cigarette smoke requires appropriate consideration of several factors, including the design of the product, the method of smoke generation, the smoking parameters used, how the constituents are collected for analysis, and the reactivity of many smoke constituents [12].

2.1. Generation and collection of mainstream smoke samples

When a cigarette is lit, the temperature of the ignited tobacco rises and a hot carbonaceous coal is formed, with peak temperatures exceeding 900°C during a 2-s puff [13], after which the temperature rapidly declines to 600–700°C and the relative abundance of oxygen adjacent to the coal reduces. Within the tobacco rod, there is a steep temperature gradient, such that, a few millimetres from the coal, the remainder of the tobacco rod is largely at ambient temperature. Adjacent to the hot coal, thermolytic processes (including distillation, pyrolysis and combustion) act on components of the tobacco to form the smoke constituents, which are subject to further processes, such as condensation, elution and filtration.

Standardized procedures have been developed for the reproducible generation of cigarette-smoke samples and the representative collection of constituents of cigarette smoke [6]. A requirement of these procedures is the reproduction of the basic phenomena of human smoking in the form of short puffs with longer delays between puffs. Cigarette-smoking machines deliver a reproducible puff of defined volume and peak shape in accordance with relevant testing standards (Table 1). However, because of the range of human smoking behavior, no regime can be considered representative of human smoking. Also important is control of the moisture content of the tobacco, atmospheric humidity, temperature and airflow around the cigarettes during testing, because these parameters can affect the composition of smoke [12]. Mainstream cigarette smoke is the smoke that is drawn through the filter during puffing, whereas sidestream smoke is the smoke released between puffs from the lit

Table 1
Smoking parameters specified in various standards {Reproduced from [14]}[j]

Parameter	Standard method					
	FTC[a]	UK[a]	DIN[a]	Canada[a], Australia[a], New Zealand[a], Japan	Original CORESTA/ISO[b]	Revised CORESTA/ISO[c]
Puff volume (mL)	35 ± 0.5	35 ± 0.5	35	35	35 ± 0.3	35 ± 0.25
Puff duration (s)	2 ± 0.2	2	2	2	(1.8-2.2) ± 0.03	2 ± 0.05
Puff frequency (puff/min)	1	1	1	1	1[d]	1[e]
Butt length – plain cigarette (mm)	23	20	23	30	23	23
Butt length – filter cigarette (mm)	T + 3[f]	T + 3[g] T + 5[j]	T + 3[h]	30[i]	T + 3[h]	T + 3[h]
TPM trapping system	C	C	E	C	C or E	C
Water analysis	GC/TCD	KF or GC/TCD	KF or GC/TCD	KF or GC/TCD	KF or GC//TCD	KF or GCTCD
Nicotine analysis	GS or GC/FID	GC/FID	GC/FID	GC/FID	GC/FID	GC/FID
Ambient temperature (°C)	23.9	22	22	22	22	22
Ambient RH (%)	60	60	60	60	60	60

C, Cambridge filter pad; E, Electrostatic precipitation; F, Filter length; FTC, Federal Trade Commission; GC, Gas chromatography; GS, 'Griffith still' procedure; KF, Karl Fischer titration; RH, Relative humidity; T, Filter tipping overwrap; TPM, Total particulate matter, TCD, Thermal conductimetric detection; FID, Flame-ionization detection.

[a] Superseded by the revised ISO method.
[b] Before 1991.
[c] After 1991. Air flow conditions around the cigarette are also specified, including their distribution and how they should be measured.
[d] One puff every 60 ± 1 s.
[e] One puff every 60 ± 0.5 s.
[f] 23 mm or (T + 3) mm, whichever is longer.
[g] For cigarettes ≤ 75 mm, 20 mm or (T + 3) mm, whichever is longer.
[h] 23 mm or (T + 3) mm, or (F + 8) mm, whichever is longer.
[i] 30 mm or (T + 3) mm, whichever is longer.
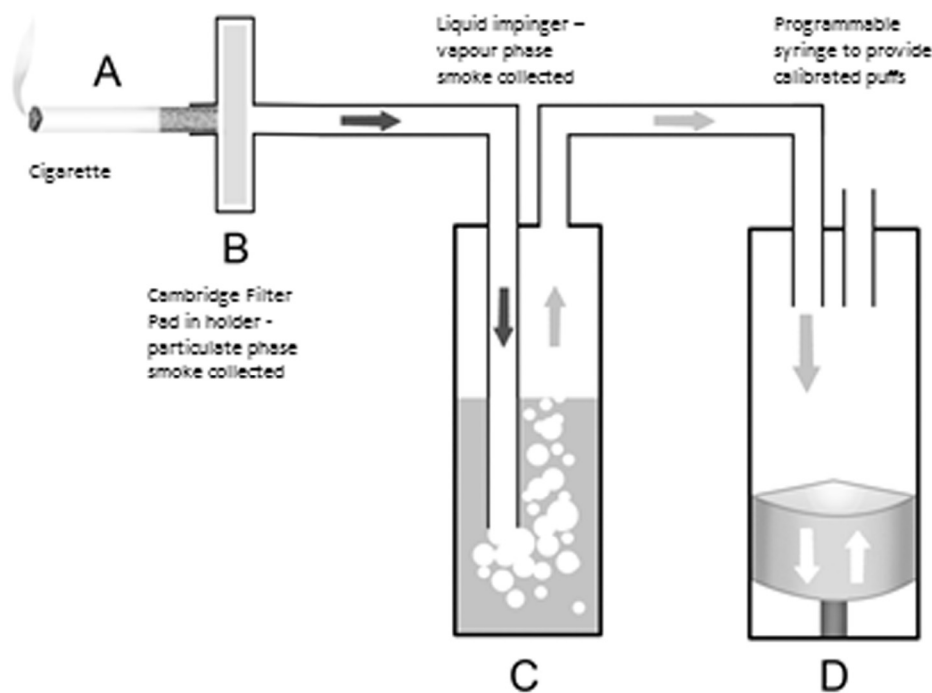[j] For cigarettes > 75 mm, 20 mm or (T + 5) mm, whichever is longer.

Fig. 1. Collection of a sample of mainstream smoke using a Cambridge filter pad and liquid impinger.

end of the cigarette. The smoke collected on a Cambridge filter pad [15] is commonly referred to as total particulate matter (TPM) and comprises water, nicotine and nicotine-free dry particulate matter (NFDPM or "tar"). Figs 1 and 2 illustrate common approaches to the collection of mainstream cigarette smoke for analysis.

2.2. Standardization of machine-based testing methods for nicotine and tar

During the 1960s, to facilitate the comparison of product-test data provided for cigarettes from different manufacturers' testing laboratories, the US Federal Trade Commission (FTC) sought the standardization of machine-based testing for nicotine and tar yields in cigarette smoke [16]. The test conditions – which included smoking the tobacco rod to a prescribed length, drawing a series of puffs of 35-mL volume as first described by Bradford et al. [17], and the collection of TPM on a Cambridge filter pad as described by Wartman et al. [15] – were evaluated in an inter-laboratory collaborative study reported by Ogg [18]. From 1966, these conditions were applied to the testing of cigarettes by the FTC's own laboratory [19] in order to provide a relative ranking of tar and nicotine yields under standardized conditions.

A similar test method was developed in the UK, and, in 1970, the standard puff volume in the UK changed from 25 mL to 35 mL, and the butt length for machine smoking was changed from 18 mm to 20 mm. The standard method was published by the UK Tobacco Research Council in 1972 [20]. An informative review of the development and the significance of standards for smoking-machine methodology was documented by Baker in 2002 [14]. Table 1 summarizes the machine-smoking conditions previously employed in the standards.

2.3. Development of standards for cigarette-smoke testing

In 1968, the International Organization for Standardization (ISO) established a Technical Committee to develop standards relating to the testing of tobacco and tobacco products, including machine smoking. Between 1978 and 1986, ISO Technical Committee 126

developed standards relating to the testing of cigarette smoke [21] including:

- ISO 3400 [22] (Cigarettes - Determination of alkaloids in smoke condensates - Spectrometric method);
- ISO 4387 [23] (Cigarettes - Determination of total and nicotine-free dry particulate matter using a routine analytical smoking machine); and,
- ISO 8454 [24] (Cigarettes - Determination of carbon monoxide in the vapor phase of cigarette smoke - NDIR method).

The development of each standard occurred over several years, and involved the participation of numerous testing laboratories and the scientific co-operation of CORESTA (Cooperation Centre for Scientific Research Relative to Tobacco). At the present time, ISO Technical Committee 126 has developed 64 international standards (including revisions) relating to the testing of tobacco and tobacco products, many of which have been revised to reflect continuing technical developments in this field [25]. Information on the CORESTA website (accessed November 2014) demonstrates that CORESTA Recommended Methods correlate with no fewer than 36 of the ISO standards [26].

3. Chemical constituents of smoke proposed for regulation

The establishment of standardized procedures for the machine smoking of cigarettes, together with the reproducible preparation of cigarette-smoke samples [6], has facilitated more detailed investigations into the chemical composition of cigarette smoke. From the early studies of Wynder and Hoffmann [27] to the present time, research into the constituents of tobacco smoke has led to the identification of more than 5600 discrete substances in mainstream cigarette smoke [28–31].

The relative abundance and the biological activity of some constituents of mainstream cigarette smoke have led to their inclusion in lists of smoke toxicants that potentially should be measured and/ or controlled in cigarette smoke, including those proposed by
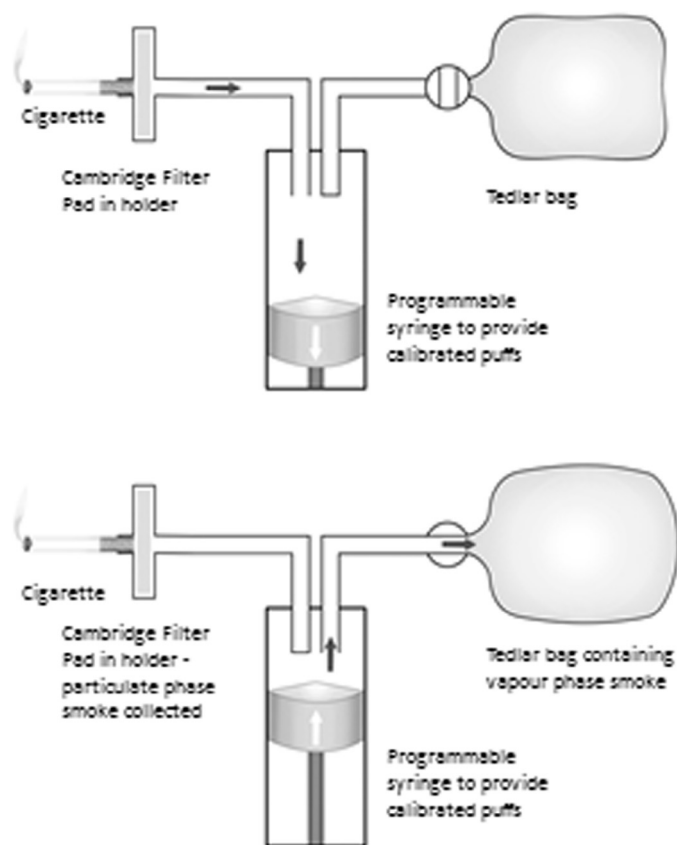
Fig. 2. Collection of a sample of mainstream smoke using a Cambridge filter pad and Tedlar bag.

Hoffmann et al. [32], Smith and Hansch [33], and Fowles and Dybing [34]. During this time, regulatory bodies in different regions have identified suites of constituents of tobacco and tobacco smoke that should be monitored and reported. Many of the substances that are required to be monitored are common across regions (Table 2).

3.1. Variations in analyses of smoke constituents

With the exception of standards developed for the measurement of tar, nicotine, carbon monoxide and benzo[a]pyrene in smoke, at present, there are no internationally harmonized methods for measurement of cigarette-smoke constituents. Methods developed and validated independently [42] and by Health Canada [43] have been adopted by laboratories in Canada and the USA, whereas technically equivalent methods have been developed and evaluated by CORESTA under collaborative trial [26] and are applied in other regions, such as Europe. In some cases (e.g., the determination of benzo[a]pyrene in cigarette mainstream smoke), the analytical techniques differ: high-performance liquid chromatography (HPLC) with fluorescence detection is mandated by Health Canada [44], whereas gas chromatography (GC) with mass selective detection is supported by CORESTA [45] and ISO [46].

Inter-laboratory studies [47,48] have indicated that results are not always directly comparable between laboratories, thus complicating the potential interpretation of data for regulatory purposes. For example, the coefficient of variation among six laboratories using different analytical techniques was found to be as high as 100% for the levels of some smoke constituents in University of Kentucky Reference Cigarettes (Table 3) [49]. It seems probable that the divergence of methods between laboratories contributes to the variability observed. In this context, it is notable that the COP to

the FCTC has not proposed the direct adoption of established chemical-testing methods, such as those published by Health Canada, and that the FDA is engaged in ongoing consultation with regard to appropriate technical standards for cigarette and cigarette-smoke-constituent testing. A summary of methods applied to the measurement of Hoffmann toxicants in smoke is presented in Table 4.

3.2. Variations in smoking conditions

In addition to the analytical techniques used for measurement, other testing parameters may also be varied, including machine-smoking conditions (see also sub-section 2.2). The standardized conditions of the FTC and other methods (Table 1) derive from the requirement for reproducibility and comparability of data and, as such, are not representative of human smoking. Because of the observed variation in cigarette smoking between smokers and for individual smokers over time, a standard smoking method representing all smokers and conditions is impossible [50].

To evaluate the yield of cigarette-smoke constituents under more intense smoking conditions, several agencies have proposed different combinations of puff volume, puff frequency and ventilation-hole blocking. The ventilation holes in the filter allow air to be drawn in to mix with, and thus to dilute, the mainstream smoke [13]. Table 5 summarizes the intense smoking-machine parameters used or proposed by various regulatory authorities.

4. Evaluating the performance of existing methods for measuring cigarette-smoke constituents

Many, if not all, of the cigarette-smoke constituents required to be reported are subject to regulation in other consumer products and notably in food. Peer-reviewed and regulatory analytical methods for the determination of many of the same substances in food have been in existence for many years [53,54]. Methods specific for the determination of constituents of cigarette smoke other than tar, nicotine and carbon monoxide have been developed and reported by organizations that include Health Canada, CORESTA, ISO and independent laboratories. The methods have been validated in accordance with standards of good practice {e.g., the IUPAC guidelines for single laboratory validation [55] and ISO 5725-2 [56]}, but the scope of use of the methods has not always been defined and the methods contain different supporting information. For example, Recommended Methods published by CORESTA include a summary of repeatability and reproducibility (e.g. [45],), whereas methods published by Health Canada do not (e.g. [44],).

4.1. Effect of inter-laboratory reproducibility

Horwitz [57] noted that the important question to be answered in the evaluation of methods of analysis is how much allowance must be made for between-laboratory variability when interpreting the values produced by different laboratories. If the variability or error produced by the method does not permit effective regulation as required by the statute (according to Horwitz [57]), the method is unacceptable for the intended purpose. Similarly, methods with high variability would not be considered appropriate for reporting data to regulators. Few analytical methods for the determination of cigarette-smoke constituents seem to have been developed with the intent to assure adequate reproducibility for regulatory use. At the time they were developed, many cigarette-smoke test methods were not evaluated against defined performance criteria but they have been assessed retrospectively, most frequently by the industry research body, CORESTA. In a special communication published in 2008 [58], the WHO Study Group on Tobacco Product Regulation proposed a strategy for cigarette regulation by applying performance standards for selected toxicants.

Table 2
Smoke constituents reporting requirements of different regulatory authorities {Reproduced from [12]}

Constituent	BC	HC	ANVISA	Taiwan	Massachusetts	Australia*	UK**	FDA
Tar	✓	✓	✓	✓	✓	✓	✓	–
Nicotine	✓	✓	✓	✓	✓	✓	✓	✓
CO	✓	✓	✓	✓	✓	✓	✓	✓
Ammonia	✓	✓	✓	–	✓	✓	✓	✓
HCN	✓	✓	✓	✓	✓	✓	✓	–
NO	✓	✓	–	–	✓	✓	✓	–
NOx	–	✓	✓	–	–	✓	–	–
N'-nitrosoanabasine (NAB)	✓	✓	✓	–	✓	✓	✓	–
N'-nitrosoanatabine (NAT)	✓	✓	✓	–	✓	✓	✓	–
N'-nitrosonornicotine (NNN)	✓	✓	✓	–	✓	✓	✓	✓
4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)	✓	✓	✓	–	✓	✓	✓	✓
Benzo[a]pyrene (BaP)	✓	✓	✓	✓	✓	✓	✓	✓
Formaldehyde	✓	✓	✓	✓	✓	✓	✓	✓
Acetaldehyde	✓	✓	✓	–	✓	✓	✓	✓
Propionaldehyde	✓	✓	✓	–	✓	✓	✓	–
Butyraldehyde	✓	✓	✓	–	✓	✓	✓	–
Acrolein	✓	✓	✓	–	✓	✓	✓	✓
Crotonaldehyde	✓	✓	✓	–	✓	✓	✓	✓
Acetone	✓	✓	✓	–	✓	✓	✓	–
Methyl ethyl ketone	✓	–	✓	–	✓	✓	✓	–
Arsenic	–	✓	✓[a]	–	✓	–	✓	–
Cadmium	✓	✓	✓[a]	–	✓	✓	✓	–
Chromium	✓	✓	✓[a]	–	✓	–	✓	–
Lead	✓	✓	✓[a]	–	✓	✓	✓	–
Mercury	✓	✓	✓[a]	–	✓	✓	✓	–
Nickel	–	✓	✓[a]	–	✓	–	✓	–
Selenium	–	✓	✓[a]	–	✓	–	✓	–
Acrylonitrile	✓	✓	✓	–	✓	✓	✓	✓
1,3-Butadiene	✓	✓	✓	–	✓	✓	✓	✓
Isoprene	✓	✓	✓	–	✓	✓	✓	✓
Benzene	✓	✓	✓	✓	✓	✓	✓	✓
Toluene	✓	✓	✓	–	✓	✓	✓	✓
Styrene	✓	✓	✓	–	✓	✓	✓	–
Pyridine	✓	✓	✓	–	✓	✓	✓	–
Quinoline	✓	✓	✓	–	✓	✓	✓	–
1-Aminonaphthalene	✓	✓	✓	–	✓	✓	✓	✓
2-Aminonaphthalene	✓	✓	✓	–	✓	✓	✓	✓
3-Aminobiphenyl	✓	✓	✓	–	✓	✓	✓	–
4-Aminobiphenyl	✓	✓	✓	–	✓	✓	✓	✓
Phenol	✓	✓	✓	–	✓	✓	✓	–
o-Cresol	✓	✓	✓	–	✓	✓	✓	–
m-Cresol	✓	✓	✓	–	✓	✓	✓	–
p-Cresol	–	–	✓	–	–	–	✓	–
Hydroquinone	✓	✓	✓	–	✓	✓	✓	–
Catechol	✓	✓	✓	–	✓	✓	✓	–
Resorcinol	✓	✓	✓	–	✓	✓	✓	–
Eugenol	–	✓[a]	✓	–	–	–	–	–
Menthol	–	✓[a]	–	–	–	–	–	–
Reference	[b]	[35]	[36]	[37]	[38]	[39]	[40]	[41]

BC, British Columbia; FDA, Food and Drug Administration; HC, Health Canada; ANVISA, Agencia Nacional de Vigilancia Sanitaria, Brazil.
 * Voluntary; ** On one occasion.[a] Optional.[b] Available on request from HLTH.TobaccoInfo@gov.bc.ca.

The recommendation was to establish levels for selected toxicants in mainstream cigarette smoke per mg of nicotine and to prohibit the sale or the import of cigarette brands that have yields above these levels. In this context, it is informative to review the performance of selected methods in use and to evaluate the impact of method reproducibility on the variability of test results.

At present, there is no independent proficiency scheme for the determination of cigarette-smoke constituents. Reference or control cigarettes are available on a commercial basis {e.g., the University of Kentucky Reference Cigarettes [59]} and monitor products have been developed by CORESTA [60], which has reported the results of their evaluation in collaborative studies [47,48]. In their report of a collaborative study of the CORESTA Recommended Method for the determination of selected volatile substances in cigarette mainstream smoke, Intorp et al. [61] noted that the reproducibility (R) of measurement of the target constituents was poorer than that observed for the determination of "tar", nicotine and carbon monoxide. This would be expected because the relative abundances of

the trace constituents were several orders of magnitude lower than for "tar", nicotine and carbon monoxide and would be predicted by the Horwitz function [62,63], which estimates the precision of measurement that can be achieved at a specified concentration, to be subject to greater intra- and inter-laboratory measurement variability.

Regarding the yield of isoprene in mainstream smoke of eight different test cigarettes smoked under ISO conditions, for example, the mean concentration was 58–553 μg per cigarette, the value of R 31–198 μg per cigarette, and the ratio of R to the mean concentration was 0.36–0.62 [61]. For five substances measured in cigarette smoke in the same analysis (1,3-butadiene, isoprene, acrylonitrile, benzene and toluene), the ratio of R to the mean concentration was 0.27–1.16. The reproducibility of measurement of "tar", nicotine and carbon monoxide were not reported but are assumed to satisfy the performance criteria stated in the relevant ISO standards (e.g., for the measurement of "tar", the reproducibility RSD should be in the range 73% at 0.82 mg to 11% at 17.4 mg, according to ISO 4387:2000). Intorp et al. [61] stated their findings factually and, other than

Table 3
Measured concentrations of constituents in 2R4F Kentucky Reference cigarette smoke
{Adapted from [49]}

Analyte	Unit	No. of labs	Average	CV_R (%)
Ammonia	µg/cig	5	11.0	11
1-Aminonaphthalene	ng/cig	4	15.1	23
2-Aminonaphthalene	ng/cig	4	10.3	22
3-Aminobiphenyl	ng/cig	4	2.97	9
4-Aminobiphenyl	ng/cig	4	1.73	21
Benzo[a]pyrene	ng/cig	6	6.96	27
2-Butanone	µg/cig	6	62.7	25
Acetaldehyde	µg/cig	6	560	15
Acetone	µg/cig	6	265	5
Acrolein	µg/cig	6	58.8	14
Butyraldehyde	µg/cig	6	29.6	9
Crotonaldehyde	µg/cig	6	16.2	43
Formaldehyde	µg/cig	6	21.6	14
Propionaldehyde	µg/cig	6	43.9	13
Hydrogen cyanide	µg/cig	5	109	9
1,3-Butadiene	µg/cig	5	29.9	25
Acrylonitrile	µg/cig	5	8.28	11
Benzene	µg/cig	5	43.4	17
Isoprene	µg/cig	5	298	26
Toluene	µg/cig	5	64.9	33
Mercury	ng/cig	4	3.82	50
NO	µg/cig	2	223	13
NOx	µg/cig	2	269	9
Catechol	µg/cig	6	37.9	7
Hydroquinone	µg/cig	6	32.4	14
m + p Cresol	µg/cig	6	5.84	25
o-Cresol	µg/cig	6	1.89	14
Phenol	µg/cig	6	7.32	42
Resorcinol	µg/cig	5	0.91	54
Pyridine	µg/cig	5	7.02	36
Quinoline	µg/cig	5	0.23	19
Styrene	µg/cig	5	5.11	45
Arsenic	ng/cig	3	10.3	108
Cadmium	ng/cig	4	47.8	26
Chromium	ng/cig	2	73.0	
Lead	ng/cig	4	32.9	100
Nickel	ng/cig	1	5.12	
Selenium	ng/cig	3	34.9	109
NAB	ng/cig	6	16.3	18
NAT	ng/cig	6	119	15
NNK	ng/cig	6	116	9
NNN	ng/cig	6	133	12
TPM	mg/cig	1	11.3	
Carbon monoxide	mg/cig	6	11.9	5
Nicotine	mg/cig	6	0.75	6
"Tar"	mg/cig	6	8.91	6

Data rounded to 3 significant figures.

remarking upon the relative reproducibility, did not compare the observed inter-laboratory agreement with a fitness-for-purpose criterion, such as a target reproducibility standard deviation.

4.2. The Horwitz ratio

The Horwitz Ratio (HorRat) is a normalized parameter indicating the performance of methods of analysis with respect to between-laboratory precision (reproducibility) [64]. It is the ratio of the observed relative standard deviation among laboratories calculated from the performance data, RSD_R, to the corresponding predicted relative standard deviation, $PRSD_R$, which is calculated from the Horwitz equation as follows:

$$PRSD_R (\%) = 2\,C^{-0.1505}$$

where C is the concentration found or added, expressed as a mass fraction. The Horwitz ratio is largely independent of analyte, matrix, method and time of publication (as a surrogate for the state of the art of analytical chemistry). It is one of the acceptability criteria for many chemical methods of analysis recently adopted by organizations dealing with food

analysis, including AOAC International, the European Committee for Standardization and the Nordic Analytical Committee. If there is no method with which to compare the precision parameters, theoretical repeatability and reproducibility values can be calculated from the Horwitz equation [65].

Assigning a nominal analyte "concentration" of 100 microgram per cigarette and assuming a sample mass of 1 g (the mass of tobacco varies between ~500 mg and 800 mg depending upon the design of the cigarette), the value of C was 0.0001 and the value of $PRSD_R$ was about 8%. Applying the Codex method selection criterion of $RSD_R \le 2\ PRSD_R$ [66] generated an acceptable upper value for reproducibility (RSDR) of around 16%. The ratios of the reproducibility standard deviation for all five target constituents reported by Intorp et al. [61] to the Horwitz predicted reproducibility standard deviation (i.e., the HorRat values) were in the range 1–3.6 (Table 6), whereas the Joint FAO/WHO Codex Alimentarius Commission regards a ratio of ≤ 2 as acceptable.

A similar evaluation was conducted for reproducibility data reported in CORESTA Recommended Method 75 (Determination of tobacco-specific nitrosamines in mainstream cigarette smoke by LC tandem mass spectrometry (LC-MS/MS) [67]. For four substances measured in mainstream cigarette smoke under ISO and Canadian Intense smoking conditions at mean concentrations of 1.5–603 ng per cigarette, the HorRat values were 0.5–1.3 (Table 7).

5. Discussion

For methods for the determination of mainstream cigarette-smoke constituents the observed inter-laboratory reproducibility is variable and, for some methods, is greater than the reproducibility predicted using the Horwitz equation. Sub-optimal reproducibility of results may be related to procedural differences between laboratories where methods are not documented in detail or where more than one method is available. Poor reproducibility may also be associated with the possible heterogeneity of plant materials or potential variability in the cigarette-smoking process.

Table 8 summarizes mean concentrations and reproducibility of measurement of tar, nicotine and carbon monoxide in a recent collaborative study [60] and compares the observed reproducibility with the Horwitz predicted reproducibility. Comparison suggests that, for the measurement of nicotine, which is normally conducted by GC with internal standardization, the reproducibility is lower than that predicted by the Horwitz ratio, but is higher than was observed in ISO 10315 [68], whereas, for the measurement of carbon monoxide (by direct spectroscopic absorbance) and NFDPM (which depends on gravimetric analysis and subtraction of the masses of water and nicotine measured using internally standardized methods), the reproducibility is greater than that predicted by the Horwitz function.

As a further point of comparison, the ratio of observed to predicted reproducibility for the measurement of tobacco-specific nitrosamines in cigarette smoke at low-ng levels suggests that more can be done to enhance the reproducibility of other cigarette-smoke test methods. For example, some of the published methods for the determination of mainstream cigarette-smoke constituents do not include internal standardization. Furthermore, for some classes of smoke constituent, more than one method of analysis has been published, so that laboratories apply different methods. Where method reproducibility is poor, performance could be improved by using technical guidance from European Commission Decision 2002/657/EC [69], especially to address measurement selectivity and chromatographic separation, and to utilize the inherent ruggedness of stable-isotope dilution analysis with MS detection.

More frequent, independent proficiency studies for the measurement of mainstream cigarette-smoke constituents, especially those constituents prioritized for reporting by the WHO, FDA and

Table 4
Summary of analytical methods applied to the measurement of 'Hoffmann' toxicants in mainstream cigarette smoke

Analyte or class	ISO, HC or CORESTA recommended method	Principle of method	Observations
Ammonia	HC T101	Mainstream smoke is passed through a CFP and collected in a liquid impinger containing 0.1 M H_2SO_4. The pad is extracted with the impinger solution, and an aliquot is analyzed by cation exchange chromatography (mobile phase, 0.003 M methane sulfonic acid solution) coupled with suppressed conductivity detection.	No IS used.
Aromatic amines: 1- and 2-aminonaphthalene and 3- and 4-aminobiphenyl	HC T102	Mainstream smoke TPM is collected on a CFP, extracted using 5% hydrochloric acid solution, and internal standard (D9-4-aminobiphenyl) is added to the solution. The filtrate is washed with dichloromethane, the pH is adjusted with sodium hydroxide solution, and the filtrate is extracted with hexane. The dried hexane extract is derivatized with pentafluoropropionic acid anhydride and trimethylamine, passed through a Florisil column, and quantitated by GC-MS.	The deuterated IS is added to the acid solution after extraction of the pad.
Benzo(a)pyrene	HC T103	TPM collected on a CFP is extracted with cyclohexane. An aliquot of the extract is subjected to SPE using silica and NH_2 cartridges in series. The B[a]P is eluted with hexane, evaporated to dryness, reconstituted with acetonitrile, and subjected to reversed phase HPLC with fluorescence detection.	No IS used. Does cyclohexane fully extract B(a)P from wet TPM?
	CORESTA RM 58 ISO 22634:2008	Mainstream smoke is trapped on a CFP. Internal standard (B[a]P-D12, B[a]P-D12) is spiked onto the CFP, the CFP is extracted with methanol, and the methanol extract is diluted with water. The diluted extract is purified by SPE (cyclohexyl-bonded silica), and B[a]P is eluted with cyclohexane, concentrated, and analyzed by GC-MS.	Does methanol fully extract incurred B(a)P from TPM?
Carbonyl compounds: formaldehyde, acetaldehyde, acetone, acrolein, propionaldehyde, crotonaldehyde, 2-butanone and n-butyraldehyde	HC T104	Unfiltered mainstream tobacco smoke is scrubbed of volatile carbonyls by passing each puff through an impinger into a fritted Dreschel trap containing an acidified solution of 2,4-dinitrophenylhydrazine in acetonitrile. An aliquot of the reacted DNPH-smoke extract is syringe-filtered, diluted with 1% Trizma base in aqueous acetonitrile, and subjected to reversed-phase HPLC with UV detection.	No IS used. Syn/anti isomerism (acid catalyzed) may affect selectivity.
	CORESTA RM 74	Unfiltered mainstream smoke is passed through an impinger containing an acidified solution of 2,4-dinitrophenylhydrazine in acetonitrile. An aliquot of the smoke extract is syringe-filtered and diluted with 1% Trizma base in aqueous acetonitrile before analysis using reversed-phase HPLC/UV or HPLC/DAD.	No IS used. Syn/anti isomerism (acid catalyzed) may affect selectivity.
Hydrogen cyanide	HC T107	Mainstream smoke is passed through a CFP and into a trap containing 0.1 M NaOH. The CFP is extracted with 0.1 M NaOH, and both the pad extract and impinger solution are analyzed by an automated continuous flow colorimetric analyzer. Sodium cyanide in the extract is converted to cyanogen chloride by an aqueous solution of chloramine-T. The cyanogen chloride reacts with pyridine to form glutaconic aldehyde, which is reacted with a pyrazolone reagent to form a colored complex that is monitored spectrophotometrically and quantified by external standard calibration.	Method is not compatible with use of IS.
Trace metals: nickel, lead, arsenic, cadmium, chromium, selenium	HC T109	Mainstream smoke is electrostatically precipitated onto a glass EP tube. The TPM is extracted in 25 mL of methanol. The methanol extract is evaporated and subjected to microwave digestion using a mixture of hydrochloric acid, nitric acid and hydrogen peroxide. The gaseous phase metals are trapped in an impinger containing a 10% v/v nitric acid solution. The impinger solution is added to the same digestion vessel as the EP tube product and subjected to microwave digestion. The digests are analyzed by flameless atomic absorption spectroscopy (or graphite furnace atomic absorption).	No IS used.
Oxides of nitrogen: nitric oxide; total oxides of nitrogen (NO_x)	HC T110	Unfiltered mainstream smoke is exhausted into an evacuated SMC puff by puff. The gas is mixed and at intervals an aliquot of each puff is routed to a dual channel chemiluminescence analyzer. In channel A, the sample stream is reacted with ozone and the resultant chemiluminescent emission is directly proportional to the NO concentration in the sample. In channel B, the sample stream is chemically reduced by a catalyst and mixed with ozone, where the resultant chemiluminescent emission is due to NO_x ($NO + NO_2$). The NO_2 concentration is calculated by subtraction.	External calibration.
TSNAs: N-nitrosonornicotine (NNN), 4-(N-nitrosomethylamino)- I –(3-pyridyl)- 1 -butanone (NNK), N-nitrosoanatabine (NAT) and N-nitrosoanabasine (NAB)	HC T111	Mainstream smoke is trapped onto a CFP. The TSNAs are concentrated by extraction with dichloromethane, followed by column chromatography onto basic Alumina. The fraction containing TSNA is eluted and quantitatively analyzed by combined GC-TEA. *N*-nitrosoguvacoline is used as an internal standard.	GC-TEA with a single IS.
	CORESTA RM 75	Mainstream smoke is trapped on a CFP. After addition of internal standards (deuterated analogues of the target compounds), the CFP is extracted with 20 mL of 0.1 M ammonium acetate solution using by shaking. The extract is syringe filtered directly into an auto sampler vial. The samples are subjected to reversed-phase HPLC and quantified via tandem MS.	Stable isotope dilution HPLC/MS/MS.
Pyridine, quinolone, styrene	HC T112	Mainstream smoke TPM is collected on a CFP and the vapor phase into cryogenic traps containing methanol. The pad is placed into an Erlenmeyer flask and the deuterated internal standards are added. The pad is extracted by shaking with the impinger solution and the extracts are quantified by GC-MS.	Stable isotope dilution GC-MS.
Phenolic compounds: hydroquinone, resorcinol, catechol, phenol, m-cresol, p-cresol and o-cresol	HC T114	Mainstream smoke TPM is collected on a CFP. The pad is extracted with 1% acetic acid. An aliquot of the TPM extract is syringe filtered, diluted and subjected to reversed-phase gradient liquid chromatography. Phenols are monitored using selective fluorescence detection and quantified by comparison to an external standard calibration.	No IS used.

Table 4 (*continued*)

Analyte or class	ISO, HC or CORESTA recommended method	Principle of method	Observations
TNCO: tar, water, nicotine, carbon monoxide	HC T115	Mainstream smoke is collected onto a pre-weighed CFP. The gas phase is collected in a vapor phase collection bag, and then introduced into a NDIR analyzer and the % CO determined. The CFP is re-weighed and the difference is the TPM. The CFP is extracted with isopropanol containing internal standards, and the extract is analyzed for nicotine and water by gas chromatography using FID and TCD, respectively. The tar value is determined by subtracting the water and nicotine from the TPM.	External calibration for CO. The accuracy of measurement of 'tar' depends upon accuracy for TPM, water and nicotine.
	CORESTA RM 5/ISO 8454:2007	Carbon monoxide in mainstream cigarette smoke by NDIR analysis (CO measured as summarized above).	
	CORESTA RM 7/ISO 10315:2000	Nicotine in mainstream cigarette smoke by gas chromatography (nicotine is measured by GC/FID as above).	
	CORESTA RM 8/ISO 10362-1:1999	Water in mainstream cigarette smoke by gas chromatography (water is measured by GC/TCD as summarized above).	
	CORESTA RM 23/ISO 4387:2000	Determination of total and nicotine-free dry particulate matter using a routine analytical cigarette-smoking machine determination of TPM and preparation for water and nicotine measurements.	
Selected volatile compounds: 1,3-butadiene, isoprene, acrylonitrile, benzene, toluene	HC T116	Mainstream smoke is passed through a CFP and into cryogenic impinger traps containing methanol. The impinger solutions are spiked with D6-benzene and injected onto a gas chromatograph/mass spectrometer (GC-MS) for quantitation.	Single deuterated IS for substances with a wide volatility range.
	CORESTA RM 70	Selected volatiles are collected by passing the mainstream smoke of cigarettes through a glass fiber filter pad as specified in ISO 3308:2009 into cryogenic traps containing methanol. The impinger solutions are fortified with benzene-D6 and analyzed by GC-MS.	

B[a]P, Benzo(a)pyrene; CFP, Cambridge filter pad; EP, Electrostatic precipitation; FID, Flame-ionization detection; GC-MS; Gas chromatography-mass spectrometry; GC-TEA, Gas chromatography-thermal energy analysis; HC, Health Canada; HPLC, High-performance liquid chromatography; IS, Internal standard; NDIR, Non-dispersive infrared; SMC, Smoke-mixing chamber; SPE, Solid-phase extraction; TCD, Thermal conductivity detection; TPM, Total particulate matter; TSNA, Tobacco-specific nitrosamines.

other regulatory bodies, should facilitate the evaluation of inter-laboratory agreement of data and support the development of more robust methods, in accordance with the requirements of ISO 17025 [70]. The unavailability of certified reference materials is currently a major constraint to the evaluation of inter-laboratory precision of measurement. There is a significant need for a forum for discussion and sharing of good technical practice (similar to, for example, scientific workshops convened by the FDA Center for Tobacco Products), the production of reference materials for the evaluation of accu-

racy (trueness) of measurement and the establishment of appropriate proficiency-testing schemes. Many thousands of products may be tested annually and regulatory controls may be implemented shortly. Without collective investment in the development of robust quality-control processes, technical agreement between industry, commercial testing laboratories and regulatory organizations will be very difficult to demonstrate, and perhaps impossible.

6. Conclusion

The clarification and the adoption of technical standards and the international harmonization of test methods for tobacco products, especially mainstream cigarette-smoke constituents, require prioritization at an international level in order to establish analytical reproducibility that is consistent with data-reporting requirements. There is a need for:

a) a forum for discussion and sharing of good technical practice;
b) the production of reference materials for the evaluation of accuracy (trueness) of measurement; and,
c) the establishment of appropriate proficiency-testing schemes.

Table 5
Machine-smoking regimes proposed by various authorities {Adapted from [51] with the addition of specifications from WHO TLN SOP 01 [3]}

	FTC[a]	ISO 3308	Massachusetts method	Canadian "intense"	WHO TLN SOP 01
Puff volume (cm³)	35	35	45	55	55
Puff frequency (s)	60	60	30	30	30
Puff duration (s)	2	2	2	2	2
Ventilation blocking (%)	0	0	50	100	100

[a] The FTC rescinded its guidance in 2008 [52].

Table 6
Comparison of published and predicted reproducibility of the measurement of five volatile substances in mainstream cigarette smoke under ISO conditions {Published data for five commercial and three control cigarettes are taken from [61]}

Substance	1,3-Butadiene		Isoprene		Acrylonitrile		Benzene		Toluene	
	High	Low	High	Low	High	Low	High	Low	High	Low
Mean, µg/cig	60.3	7.53	553	58	12.3	0.98	60.3	6.73	85.4	8.53
R	37.9	6.98	98	31	5.9	1.08	21	4.69	41	9.93
sdR	13.68	2.52	35.36	11.19	2.13	0.39	7.58	1.69	14.79	3.58
RSD_R, %	22.7	33.5	6.4	19.3	17.3	39.8	12.6	25.1	17.3	42
Mass fraction	6×10^{-05}	7.53×10^{-06}	0.00055	6×10^{-05}	1×10^{-05}	9.8×10^{-07}	6×10^{-05}	6.7×10^{-06}	8.5×10^{-05}	8.5×10^{-06}
$PRSD_R$, %	8.6	11.8	6.2	8.7	11	16	8.6	12	8.2	11.6
HorRat	2.6	2.8	1	2.2	1.6	2.5	1.5	2.1	2.1	3.6

'High' and 'Low' indicate data for mean concentrations measured for each substance in the highest and lowest yield products.

Table 7

Comparison of published and predicted reproducibility for measurement of tobacco-specific *N*-nitrosamines in mainstream cigarette smoke {Published data are taken from [67]}

Smoking conditions	Tobacco-specific *N*-nitrosamine							
	NNN		NAT		NAB		NNK	
ISO	High	Low	High	Low	High	Low	High	Low
Mean, µg/cig	277	9.6	145	11	20	1.5	122	3.3
R	70	5.8	74	6.2	9	0.9	41	2.1
sdR	25.3	2.1	26.7	2.2	3.2	0.3	14.8	0.8
RSD_R, %	9.13	21.88	18.41	20	16	20	12.13	24.24
Mass fraction	2.77×10^{-07}	9.6×10^{-09}	1.45×10^{-07}	1.1×10^{-08}	2×10^{-08}	1.5×10^{-09}	1.22×10^{-07}	3.3×10^{-09}
$PRSD_R$, %	19.4	32.2	21.4	31.5	28.8	42.6	22	37.8
HorRat	0.5	0.7	0.9	0.6	0.6	0.5	0.6	0.6
Intense								
Mean, µg/cig	603	34.9	322	39.4	42.9	5.5	297	12.1
R	225	28.6	214	25.2	21.4	6.2	144	9.3
sdR	81.2	10.32	77.22	9.09	7.72	2.24	51.96	3.36
RSD_R, %	13.5	29.6	24	23.1	18	40.7	17.5	27.8
Mass fraction	6.03×10^{-07}	3.49×10^{-08}	3.22×10^{-07}	3.94×10^{-08}	4.29×10^{-08}	5.5×10^{-09}	2.97×10^{-07}	1.21×10^{-08}
$PRSD_R$, %	17.3	26.5	19	26	25.7	35	19.2	31.1
HorRat	0.8	1.1	1.3	0.9	0.7	1.2	0.9	0.9

HorRat, Horwitz ratio; R, Reproducibility; RSD_R, Relative standard deviation of the reproducibility; $PRSD_R$, Predicted RSD_R.

Table 8

Comparison of predicted and published reproducibility of measurement of "'tar", nicotine and CO in CORESTA monitor cigarettes {Published data are taken from [60]}

Substance	NFDPM		Nicotine		Carbon monoxide	
	ISO	HCI	ISO	HCI	ISO	HCI
Mean, mg/cig	13.3	28.5	1.24	2.53	12.9	24.9
R	1.88	5.5	0.15	0.32	1.9	2.9
sdR	0.678	1.985	0.054	0.115	0.686	1.046
rsdR %	5.1	7	4.4	4.5	5.3	4.2
PRSDR%	3.8	3.4	5.5	4.9	3.8	3.5
HorRat	1.3	2.1	0.8	0.9	1.4	1.2

HorRat, Horwitz ratio; R, Reproducibility; RSDR, Relative standard deviation of the reproducibility; PRSDR, Predicted RSDR; ISO [4]; HCI [3].

Otherwise, regulatory authorities may need to consider the practical limitations of tobacco-product testing data generated without the benefit of harmonized quality-control processes and with poorer inter-laboratory agreement of test data than is achieved in the regulation of other consumer products, such as foods. In this context, the recently announced cooperative agreement between the US FDA Center for Tobacco Products and the Kentucky Tobacco Research and Development Centre to develop certified reference tobacco products for instrument calibration, method validation and laboratory proficiency testing, as well as for non-clinical investigational purposes, offers a significant opportunity to harmonize analytical methods and to improve their reproducibility to standards consistent with gathering regulatory data.

Acknowledgements

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