

## Review

## Cigarette Filter–based Assays as Proxies for Toxicant Exposure and Smoking Behavior—A Literature Review

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## Abstract

**Background:** Cigarettes are being marketed with filters that differ in composition and design. The filters have different toxicant trapping efficiencies, and smoking stains reflect variations in smoking behavior. Presented herein are the results of a structured literature review that was done to identify cigarette filter–based assays that may serve as proxies for mouth-level exposure and assessing smoking methods.

**Methods:** A search of the published scientific literature and internal tobacco company documents from 1954 to 2009 was carried out.

**Results:** The literature search identified diverse schemes for assessing cigarette filters, including visual inspection and digital imaging of smoked-stained spent filters, and quantitative determinations for total particulate matter (TPM), nicotine, and solanesol. The results also showed that: (a) there are sufficient data to link filter-based chemical measures to stan-

dardized smoking machine–measured yields of tar and nicotine; (b) TPM eluted from filters or in chemical digest of filters can be used to estimate the efficiency of the filter for trapping smoke solids; (c) visual and digital inspection of spent filters is useful in finding indicators of variations in smoking behaviors; and (d) there is a correlation between solanesol and nicotine measured in filters and exposure biomarkers in smokers.

**Conclusions:** The cigarette filter may prove useful in estimating smoking behaviors such as filter vent blocking and puffing intensity, and may have utility as proxy measures of mouth-level smoke exposure in clinical trials. Additional investigations are needed to compare the different proposed assay schemes and the assay results with measurements of human biomarker assays of smoke exposure. (Cancer Epidemiol Biomarkers Prev 2009;18(12):3321–33)

## Introduction

The filtered cigarette gained prominence in response to the health fears surrounding smoking beginning in the mid-1950s, accompanied by extensive advertisements emphasizing safety (1, 2). Today, filtered cigarettes account for 99% of US cigarette sales (3), and the filter is a continuing target of product innovation by the cigarette industry as reflected in patents and new product introductions. For example, selective filtration technologies have been recently introduced and marketed on novel cigarettes that are being promoted as potential reduced-exposure products (PREP), such as B&W's Advance, Vector Tobacco's Omni, and Philip Morris' Marlboro UltraSmooth (4, 5). Cigarette filter materials (e.g., cellulose acetate and charcoal) and filter designs (filter ventilation and multisegment filters), and the effects of different filters on the

mechanical and chemical retention of mainstream smoke components have been reviewed previously (6).

The introduction of PREPs has led to a growth in diverse methodologies for assessing smokers' exposure to various smoke constituents. Also, different schemes have been proposed for measuring how a person actually smokes the product; this is known as smoking topography (1).

Determining the exposure reduction of a particular PREP relative to conventional cigarettes is complex, involving measurement of both smoke yield and behavior (7, 8). This represents an important lesson from the regular/full flavor to light/low-tar cigarette experience, where it was presumed that changes in cigarette tar yields would lead to changes in exposure. We now know that smoker-product interactions are extremely important in determining exposure to smoke constituents – a phenomenon known as compensation, where smokers adjust their smoking behavior to titrate their nicotine intake (9). More recently it has been established that biomarkers of tobacco smoke exposure show that levels of nicotine and carcinogen metabolites are no different among the full-flavor, light, and ultralight cigarette categories (10-13).

It is widely accepted that human exposure cannot be fully defined using smoke emissions derived from smoking machines (9, 14). By way of example, Hammond and

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coworkers undertook an investigation to compare measures of smoke volume and nicotine uptake among human smokers against the puffing variables and nicotine yields generated by five different machine smoking regimens (15). The results of the study documented that none of the four machine smoking regimens adequately represented human smoking behavior, nor did they generate yields associated with human measures of nicotine uptake. This can be carried to measures of biological effect. Anderson and coworkers (16) have reported the results of a study to define the influence of cigarette consumption and smoking machine yields of tar and nicotine on the nicotine uptake and oral mucosal lesions of smokers. They concluded that the nicotine uptake was significantly correlated to the number of cigarettes smoked per day but not to the smoking machine yields of tar and nicotine per cigarette.

There is a tradition in the social sciences of utilizing proxy measures to estimate other, harder-to-measure parameters (17). Although not a true substitute, the proxy measures can provide useful information, often in a more easily accessible form. It may be practical, for example, to use the cigarette filter as a potential proxy for exposure to tobacco smoke constituents. Because smoke is drawn into the filter by the smoker's puff, what exits the proximal (mouth) end of the filter should be proportional to what entered the distal (tobacco) end of the filter. In a ventilated filter, air drawn through the vents also enters the equation. Air from the vents changes the smoke flow patterns but does not alter the basic reality that what comes out has to be a function of what went in.

A number of research groups have explored various approaches, to be detailed below, for using the material trapped by the filter as an indicator of smoke exposure. In addition, several lines of investigation have used spent filters of smoked cigarettes to identify characteristics of smoker behavior (e.g., puff intensity and filter vent blocking).

For the assessment of PREPs, it is important to clarify whether a filter-based assay can be used to assess modern PREPs, such as B&W's Advance and Philip Morris' Marlboro UltraSmooth. The filter is an ongoing focus of active tobacco industry research and development. Indeed, a search of "cigarette filter" in the Google patents database returned 89 US patents issued to Philip Morris, 44 patents to R. J. Reynolds Tobacco Company, and 3 patents to Lorillard Tobacco Company; all patents had been issued since 2000.

Reported herein are the results of a structured review of the literature that was done to: (a) evaluate filter-based assays to measure filter trapping efficiency and variations in smoking behavior; (b) assess the advantages and disadvantages of the different technologies; (c) determine whether such assays can act as proxies for exposure to tobacco smoke; and (d) identify the research gaps that need to be filled to be able to use cigarette filter-based assays to assess human exposure.

## Materials and Methods

We reviewed scientific papers published in peer-reviewed journals, meeting abstracts, slide lectures, and poster presentations, as well as tobacco industry internal documents. A search of the scientific literature from 1957

through 2009 was done. Peer-reviewed papers were sought in the databases of PubMed<sup>5</sup> and Scopus.<sup>6</sup> Topic-specific writings were also sought in US patents; these were searched using the databases at Google<sup>7</sup> and the United States Patent and Trademark Office.<sup>8</sup> Tobacco industry documents, surrendered as a result of litigation, were retrieved from Tobacco Documents Online<sup>9</sup> and the Legacy Tobacco Documents Library.<sup>10</sup>

Company databases were also searched, including, by way of example, sites for Philip Morris USA,<sup>11</sup> RJ Reynolds Tobacco Company,<sup>12</sup> and the British American Tobacco Company.<sup>13</sup>

Additional writings were sought in the meeting abstracts of the Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA).<sup>14</sup> In addition, searches of the tobacco science publications *Beitrage zur Tabakforschung (Contributions to Tobacco Research)*,<sup>15</sup> *Recent Advances in Tobacco Science*, and *Tobacco Science Research Conference* abstracts were conducted.

Databases were searched using keywords and short-string phrases and basic Boolean operators. Keywords and phrases, either singularly or in combination, included, by way of example, filter, cigarette, tar, nicotine, filtration efficiency, staining, color, stain pattern, and other subject-specific terms. Efforts were made to avoid any bias. All papers published in peer-reviewed journals were included. All recovered documents were reviewed and examined. Notable, topic-specific abstracts that had not been subsequently published as papers were also included. References cited in the publications were used to identify additional papers not recovered in keyword searches. Each paper retrieved was read critically for soundness of methodology (e.g., study design, analytic methodology, benchmarks, and reproducibility).

## Results

**Results of Literature Search of Cigarette Filter-based Assays.** The literature search of filter-based assays identified 74 subject-specific writings (18-91). The literature sources from which these papers were obtained are shown in Table 1. Most of the papers ( $n = 44$  of 74; 60%) were peer-reviewed publications that were retrieved from Pub Med or Scopus. Some documents were abstracts and presentations at scientific meetings ( $n = 22$  of 74; 30%). Other writings were from diverse sources ( $n = 6$ ; 8%), a US patent ( $n = 1$ ), and a dissertation ( $n = 1$ ). The writings were from 1965 to 2009. Selected cigarette filter-based assays as proxies for smoking exposure and behavior are presented within in the chronology of the

<sup>5</sup> <http://www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed>

<sup>6</sup> <http://info.scopus.com/etc/citationtracker>

<sup>7</sup> <http://www.google.com/patents>

<sup>8</sup> <http://www.uspto.gov>

<sup>9</sup> <http://tobaccodocuments.org>

<sup>10</sup> <http://legacy.library.ucsf.edu>

<sup>11</sup> <http://philipmorrisusa.com/en/cms/Home/default.aspx>

<sup>12</sup> <http://www.rjrt.com>

<sup>13</sup> <http://www.bat.com>

<sup>14</sup> <http://www.coresta.org>

<sup>15</sup> <http://www.beitraege-bti.de/nxt/gateway.dll?f=templates&fn=default.htm&vid=btifi:btifi>

milestones of filter development from 1860 to 2009 (refs. 92-112; Table 2).

Our analysis of the literature identified different methods using spent cigarette filters as proxies for measuring smoking behavior and exposure to smoke constituents. These methods included (a) visual and (b) digital imaging of the filter cut surface, and the analyses of filters for (c) particulates, (d) nicotine, and/or (e) solanesol. Findings relevant to each of these five methods are reviewed below.

### Visual Inspection and Imaging

*Analytical Methods Based on Visual Inspection of the Filter.* Among the simplest methods for assessing smoke emissions and exposure is to visually examine the intensity and pattern of the tar stain (Fig. 1). This is used to assess how smokers block the vent holes on filters. The vents are generally located 12 to 15 mm from the mouth end of the filter, and are organized in a ring around the filter. When a filtered cigarette is smoked, smoke is drawn through the filter and some particulate matter is trapped by filter material such as on cellulose acetate fibers. Over the course of smoking a cigarette, the filter becomes "stained" with a brownish color due to the accumulation of smoke deposits. When the fingers of the smoker block the vent holes, the staining of the cut surface of the filter takes the shape of a "bulls-eye" pattern (Fig. 1).

Kozlowski and colleagues (28) first presented the concept of utilizing the filter observations in semiquantitative measurements, noticing that as more vents were blocked, "observable smoke deposits increase on the proximal [smoker] side of the perforations, and staining spreads toward the sides of the filter." The stain appears as a central tar stain surrounded by a ring of unstained filter, the size of which varies directly with the level of ventilation. The higher the level of ventilation, the more unstained area is found. Baker and Lewis (42) reported that the filter stain pattern depends on manufacturing variables such as degree of ventilation, number of vent holes, size of vent holes, number of rows of holes, and depth to which the vents extend into the filter. Thus, this assay is useful to determine the amount of vent hole blocking by smokers, which can vary from smoker to smoker and affect exposure (28). If one were to block some ventilation holes, the air flow through the outer edge would be reduced. Further, if one were to cover all of the holes, the stain would be uniform. Figure 1 also illustrates the filter stain pattern on the cut-end of a cigarette that has been smoked in which the vents had or had not been blocked.

Visualizing filter stain patterns is only semiquantitative because it relies on an observer's estimate for the percentage of the outer edge of the filter that was stained. Several studies have investigated the reliability and validity of the

technique on ultralight and light cigarette brands (29). Lombardo and colleagues (30) carried out the first controlled experimental studies of the stain pattern technique, finding that ratings for unblocked (accuracy, 79%) and completely blocked (90%) butts were equally accurate and better than for partially blocked butts (52%). When partial and full block were combined, 90% of partially blocked butts were categorized as blocked. The authors note that the rate of false positives was twice as great as the rate of false negatives.

Kozlowski et al. (29) examined filters collected from smokers at shopping malls and applied a three-level rating system: (a) no stain at the outside edge or stain <3 mm (not blocked); (b) light-to-moderate stain around outside (partially blocked); and (c) uniform stain (fully blocked). Three independent raters were used and scores were derived by averaging across raters: 58% of butts showed some blocking, 42% showed no blocking, and 19% of butts showed full blocking. No differences in blocking frequency were seen as a function of tar yield (i.e., blocking was as likely with 4-mg cigarettes as with 1-mg cigarettes). Interrater correlations ( $r = 0.86, 0.86, \text{ and } 0.91$ ) were high, as was concordance ( $r = 0.95$ ).

Pillitteri and colleagues (37) examined ratings of stain patterns and concluded that the method "is best suited to detect the presence versus absence of vent-blocking rather than the extent of vent-blocking" (p. 407). There are examples of utilizing this method for qualitative assessment of filter vent blocking in human studies (113, 114).

Sweeney (90) assessed the reliability and accuracy of visualizing butt staining patterns and found that the test-retest and interrater reliabilities were high ( $r \geq 0.90$ ), and the sensitivity and specificity were good ( $r \geq 0.80$ ) for a variety of light and ultralight brands. Prignot and Jamart (48) also reproduced the accuracy of stain pattern assessment of vent blocking in a sample of butts collected from a Belgian hospital. The published studies reviewed here indicate that a visual stain pattern test can be useful to identify cases when cigarettes go unblocked and can reliably distinguish between the presence and absence of blocking. However, the technique cannot discriminate degrees of blocking (i.e., number of puffs blocked or amount of ventilation occluded by lips or fingers). To this end, the technique is sufficient to estimate the prevalence of any degree of vent-blocking in the general public, but is likely unsuitable to estimate the increase in exposure received by smokers who block vents.

O'Connor and colleagues (49) extended and automated this assay to take advantage of digital image analysis technology. The system involves taking a color digital picture of the mouth end of the cigarette butt and segmenting it into edge and center portions. The mean color value

**Table 1. Literature source of documents addressing cigarette filter-based assays as proxies for toxicant exposure and smoking behavior**

Literature source	Number of documents	Reference citation
Peer-reviewed scientific papers identified in PubMed or Scopus	44	18-61
Abstracts, posters, and/or slide presentations at scientific meetings	22	62-83
Recommended methods	6	84-89
US patents	1	90
Dissertations	1	91
Total	74	

**Table 2. Milestones of cigarette filter development and published papers describing cigarette filter–based assays as proxies for smoking behavior and exposure**

Year	Discovery
1860-1920	The concept of cigarette filters is formulated; the intent is to keep tobacco particles from entering the mouth. Initially, the filter is a truncated cigarette holder that contains a plug of cotton. (92)
1927	Boris Aivaz files a patent for a cigarette filter made of crepe paper with or without cellulose wadding. Two years later, he promotes the idea to filter maker Filtrona of Vienna. (93, 94)
1931	Parliament (Benson and Hedges) cigarette is introduced as a premium-priced filtered cigarette. The filter consists of a cardboard mouthpiece with a tuft of cotton. It is considered the first major filter tip cigarette in the United States. (95)
1936	Viceroy originates in the United States as the world's first cork-tipped filter cigarette. In 1952 it becomes the first brand to add a cellulose acetate filter, establishing a new industry standard. (96)
1939	"Brown and Williams introduces Viceroy, the first national brand to feature a filter of cellulose acetate. Advertising increases the use of physicians to counter the claims that cigarettes are a major health problem." (97)
1939	Machinery is developed that binds the cigarette filter to the tobacco column, thus facilitating high-speed production of filter cigarettes. Notwithstanding, filter cigarettes generate little interest and contribute very little to the market.
1940	Cigarettes are smoked almost exclusively by men. Filters are promoted as a novelty item to attract female smokers to increase sales. (98)
1952	Lorillard introduces Kent with a "Micronite Filter" that "offers the greatest health protection in cigarette history." The filter is made of asbestos and is discontinued four years later. (99, 100)
1954	RJ Reynolds introduces Winston filter cigarettes, but promotes the taste benefit, not health risk reduction.
1954	Consumers seek facts about filter-tip cigarettes, "safer" cigarettes, lung cancer and the dilemma of the "problem smoker." (101)
1956	Fluorescent tars in smoke condensate of mechanically smoked cigarettes stain different types of cells as observed with a fluorescent microscope. This observed fluorescence of tobacco tar becomes the basis for subsequent assays, reported in 1960 (see below), of cigarette filter tar-trapping efficiency. (18)
1960	Cigarette filters efficiency is defined by photofluorometry. (20)
1963	Liggett and Myers introduce Lark with a trademark charcoal filter in an effort to halt a 5-year downward drift in sales. The distinguishing feature of the brand, both in terms of physical characteristics and taste, is the 3-piece "Keith" filter, the middle section of which contains small charcoal granules, reportedly reducing the harmfulness of the cigarette's smoke. (102)
1967	Philip Morris scientists express concern that smokers could block filter ventilation holes with lips while smoking. (103, 104)
1968	CORESTA methods are described for determining alkaloid (nicotine) retained by the cigarette filter. Nicotine content of steam condensate from the filter is defined by spectrophotometry. Spectro-photometric method is reported the following year for measuring tar and nicotine in cigarette smoke captured onto a Cambridge pad. (85, 105)
1970	Filters are made with vents that permit air to be introduced into mainstream smoke. This design feature generates misleadingly lower tar yields recorded by smoking machines, and yields a lighter and milder taste that may be misinterpreted by the smoker as being less dangerous and, for very heavily ventilated cigarettes, may lead to the smoker to compensatory smoking behavior. (106, 107)
1973	Factors influencing the retention of smoke constituents in cigarettes with cellulose filters with and without activated (estimated) carbon are reported by scientists in Switzerland.
1973	Scientists at the Tennessee Eastman Company describe a method for measuring the elution of nicotine and total particulate matter from a cigarette filter. The technique uses <sup>14</sup> C-labeled nicotine or <sup>14</sup> C randomly labeled tobacco. (26)
1976	The amount of nicotine entering the mouth of a smoker, defined as the mouth-level exposure, is estimated from a determination of the amount of nicotine trapped in the filter of each cigarette smoked. The results indicate a substantial variation in mouth-level exposure for the subjects studied, even among smokers of cigarettes that deliver similar amounts of nicotine when smoked on a machine under conditions that are programmed to mimic human smoking. (27)
1977	A method is described for the quantitative analysis of cigarette tars using fluorescence spectroscopy. (28)
1980	Almost all cigarettes sold have filters (93% market share, non-filter cigarettes, 7% market share).
1980	An automated method of measuring Federal Trace Commission tar of low-delivery cigarettes is described. 2-Propanol is used to extract TPM from Cambridge pads. The measurements are taken for extracts excited at 475 nm and emission recorded at 520 nm. (29) The same year, a spectrophotometric method is described for measuring the efficiency of cigarette filters. (30)
1980	A spectrophotometric method is described for measuring the filtration efficiency of cigarette filters. TPM on the cigarette filters and Cambridge pad is directly proportional to the absorbance of the methanol solution measured at 310 nm. (30)
1980	There are reports of studies that were undertaken to measure temporal patterns of mouth level nicotine exposure and smoking rates by subjects in a natural setting. The results show a great variability in individual smoking patterns over the day. (31)
1980	Studies show that smokers of low-yield, ventilated-filter ("less-hazardous") cigarettes sometimes defeat the purpose of the smoke-dilution holes by occluding the holes unintentionally with the fingers or lips, or intentionally with tape. (32) Other ventilation hole blocking studies have been presented. (35)
1982	A technique is described that enables individuals to detect the number of puffs taken on a filter cigarette. With the proposed method, the "color" of spent filters after smoking is compared with a color scale that simulates the appearance of the filters exposed to low-, standard-, or high-yield smoking regimens. (29)
1985	A liquid chromatography procedure is established for the analysis of nicotine on cellulose acetate filters. (33)
2002	Health concern is expressed for material released from the cigarette filter into mainstream smoke. These include tar-coated cellulose acetate filter fibers and particles, carbon granules from charcoal cigarettes (108), asbestos from the filters of Kent "Micronite" cigarettes, and, more recently, microbes and microbial toxins of tobacco flake. (109, 110)
2003	Philip Morris introduces Marlboro UltraSmooth, characterized as new cigarettes with a new technology that allows the flavors and nicotine through a new carbon filter so as to provide a smooth taste and nicotine, but with potentially less exposure to the carcinogens of conventional cigarettes. The cigarette, characterized as a potential reduced exposure product (PREP), fails to gain significant consumers' acceptance and is withdrawn in 2008. (94)
2005	RJ Reynolds introduces new cigarette brands with flavors in the filter to appeal to youths. (111)
2006	Comparison of smoke exposure as defined by analysis of cigarette filter, saliva and urine. (72)
2006	Validation is obtained for methods to determine consumer smoked cigarette yields from cigarette filter analysis. (73)

(Continued on the following page)

**Table 2. Milestones of cigarette filter development and published papers describing cigarette filter–based assays as proxies for smoking behavior and exposure (Cont'd)**

Year	Discovery
2008	Spectrofluorometric method is established for measuring smoke particulate matter on cigarette filters and Cambridge pads. (86)
2008	Camel Crush is a full flavor cigarette containing a capsule in the filter that, when crushed, releases a liquid that causes the smoke to be menthol flavored. (112)
2008	Comparative studies of different filter designs define the effect of charcoal containing cigarette filters on gas phase volatile organic compounds in cigarette smoke. (93–98)
2009	Polzin and coworkers' report expands the technologies to provide a lower-cost high-throughput method for estimating smokers' mouth-level of exposure to select mainstream smoke constituents from discarded filter butts. (88)
2009	Investigators at British American Tobacco (BAT) report the results of studies to estimate and correlate cigarette smoke exposure in smokers in Germany as defined with the use of spent filters and biomarkers of exposure. (113)

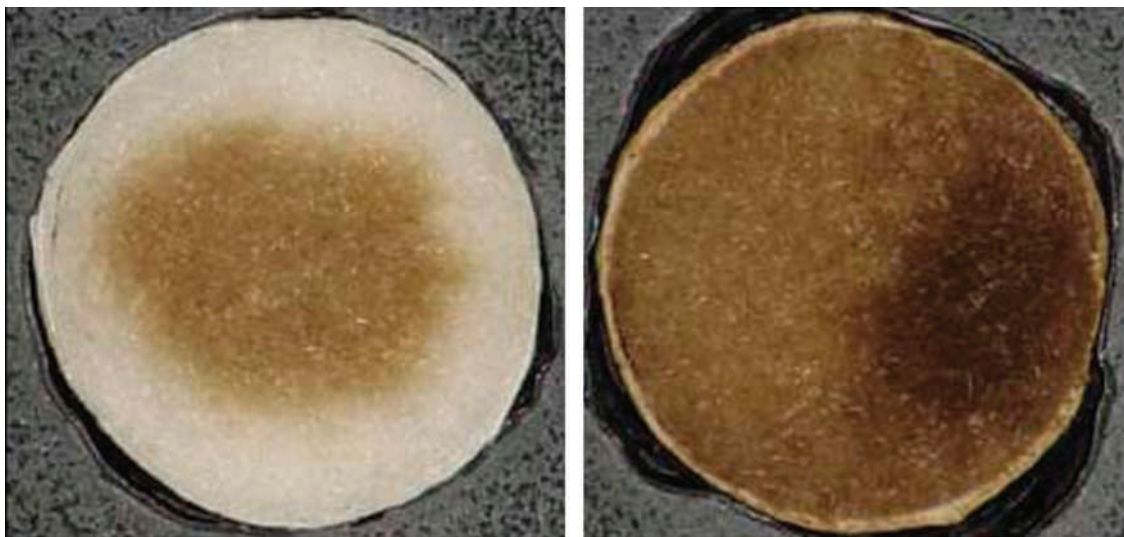
is then calculated for each region and the ratio of the two serves as an index of blocking. Two studies evaluated the effectiveness of three-color measures (i.e., hue, saturation, and value) at discriminating whether at least 50% blocking had occurred. In study 1, saturation showed perfect discrimination between unblocked Carlton butts and butts with at least 50% of the vents blocked during syringe smoking. In study 2, saturation showed 95% accuracy at identifying Marlboro Ultra Light butts with at least four puffs blocked by smokers' lips. The results indicated that the pattern of color saturation was related to vent blocking. Since this paper, the investigator has adopted the same international standard color system used by Rickert and colleagues (65, 68), but the basic principles remain consistent. This improved visualizing method also will need to be validated against a human biomarker exposure assessment.

*Estimation of Smoking Intensity.* In 1980 Kozlowski (28) proposed a visual color scale that was used by smokers to estimate the number of puffs taken on the cigarette. In this technique, spent cigarette butts were compared with three standardized colors placed along an 11-point scale. Butts had been generated by syringe-smoking and had between 5 and 16 puffs (35 mL) taken from the cigarettes. Overall,

the correlation between ratings and puff number was good ( $r = 0.97$ ; ref. 28).

This subjective technique was linked to filter nicotine by Devitt et al. (32), who reported that color ratings by smokers correlated well with analytical values ( $r \sim 0.88$ ). Husset and colleagues (41) noted a correlation between cigarette butt stain color and the estimated amount of tar yield on a smoking machine for 22 French cigarette brands (full flavor, lights, super lights, and ultra lights). Color measures taken from these cigarettes were used to create a color scale. The authors concluded that "There is a clear relation between the amount of tar and the stain diameter as well as its color intensity."

In 1994, Rickert and coworkers (65) extended the studies of Kozlowski et al. (28), obtaining measures of stain color (i.e., hue, lightness, and chroma) from particulate matter collected on Cambridge pads by or trapped by filters by reflectance spectrometry when the cigarettes were smoked on a smoking machine. The amount of tar was best predicted by stain lightness (i.e., the degree of grayness). This relationship was linear when the data were converted to a logarithmic scale, and predicted tar values to within 0.5 mg (28). Rickert and colleagues (68) have continued their work on tar color and smoke toxicant yields. Using a different color



**Figure 1.** View of filter stains from cigarettes that had been smoked with filter ventilation holes that had not been blocked (*left*) and had been blocked (*right*).

**Table 3. Summary of advantages and disadvantages of five different cigarette filter-based assays**

	Visual inspection	Digital imaging	Filter particulates	Filter nicotine	Filter solanesol
Key Reference(s)	Kozlowski et al., 1980 (28) Kozlowski et al., 1982 (29) Pillitteri et al., 1994 (37)	O'Connor et al., 2005 (49) O'Connor et al., 2007 (57)	Paszkievicz and Pauly, 2008 (59)	St. Charles et al. 2006 (52) Shepperd et al., 2006 (53) St Charles et al., 2009 (43)	Watson et al. 2004 (45, 46) Polzin et al. 2009 (60)
Basic methodology	Human raters inspect filter for color intensity compared with standards; and human raters estimate percentage of staining reaching outer edge of filter.	Computer software segments image of mouth end of filter into edge and center and computes average color scores for each portion.	Extraction of TPM from filter with solvent (alcohol) or digestion (DMSO) of filter, followed by ultraviolet spectrofluorometry.	Extraction of nicotine followed by quantitation by gas chromatography (GC) or HPLC	Extraction of solanesol followed by quantitation by liquid chromatography and mass spectrophotometry (LC/MS)
Advantages of method	Conceptually simple.  Does not require chemistry lab	Conceptually simple.  Does not require chemistry lab; color features are objective and can be standardized	Technically simple assay; known fluorescence standards available	Conceptual link to exposure; data on relationship to nicotine intake	Stable, high molecular weight target - newer methods less complex; data linking to NNK exposure
Disadvantages of method	Requires training of raters; and limits scope of measurement (vent blocking; puff count)	Limited relationship to exposure biomarkers; currently labor intensive	No data on relationship to exposure biomarkers	Nicotine semivolatile; only papers from BAT	LC/MS equipment needed. Expensive, and relatively time intensive

determining system (i.e., MacBeth Color-Eye 2020+ spectrophotometer), they have related filter stain color to selected smoke constituents such as nicotine, total particulate matter, tobacco-specific *N'*-nitrosamines such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and styrene. Rickert and colleagues (56) also noted that the "a\* dimension," which defines the relative amount of red or green in the image, was most sensitive to changes in smoking intensity.

O'Connor and colleagues (49, 57) have applied the earlier work of Kozlowski (28, 29, 35, 36) and Rickert (65) to examine the relationship between staining intensity and smoking topography. In a 2007 paper, O'Connor and coworkers (57) found that total puff vol-

ume per cigarette correlated  $-0.71$  with cigarette staining intensity. In multivariate modeling, central staining (indicating peripheral staining associated with vent blocking) could be used to predict  $>80\%$  of variance in total puff volume. Furthermore, Strasser et al. (47) and O'Connor et al. (49) showed that changes in staining scores correlated with changes in smoking topography, providing evidence that staining intensity could be indicative of smoking behavior, and could be useful to examine changes in smoking behavior.

Visual inspection techniques, including digital imaging, seem to hold promise for assessing behavioral features of smoking, such as filter vent blocking, total puff volume, and number of puffs taken. The rater-based stain pattern assessment can judge only the presence or

**Table 4. Summary of conclusions, research gaps and opportunities related to cigarette filter-based assays**

Conclusions from literature	Sufficient data exist to link filter-based chemical measures to machine-measured yields of tar and nicotine. Tar eluted from filters or in chemical digest of filters can be used to estimate the efficiency of the filter for trapping tobacco smoke solids. Digital inspection of filters can be used as indicators of smoking behaviors such as filter vent blocking, number of puffs taken, and total volume drawn. Limited data suggest a correlation between solanesol and nicotine measured in filters and exposure biomarkers in smokers, although there is an inherent upper limit to the relationship between the filter assay and a biomarker.
Key research gaps and opportunities	There is an absence of head-to-head comparison of the different assays in the published literature, so there is an absence of a scientific basis for determining which approach is superior and under what conditions. Research is required to ensure that a proposed assay is applicable across different filter designs (e.g., fiber type and size, charcoal, ion resins, and flavor pellets). Data on the loss rate for nicotine from stored filters would be desirable, as would data on stability under different storage conditions, such as room temperature, refrigerated (4°C), frozen (-20°C), and ultralow (-70°C). Linkage of filter-based assays to biomarkers of exposure other than nicotine and with biomarkers of biological effect is needed to test whether filter-based assays can serve as proxy measures of mouth-level exposure.

absence of vent blocking, but is unsuited for assessing the extent of vent blocking (i.e., proportion of vents blocked). It is hoped that the digital image assessment system might improve upon this limitation with further development. Consistent with early findings that visually assessed stain color was associated with puff number, later work using standardized color analysis shows promise for estimating puff number and total smoke volume drawn through a filter, although further developmental work is necessary. Additional research is needed to validate this method of assessing human exposure through biomarker studies.

### Tar Elution and Fluorescence

*Cigarette Filter Efficiency Assayed Using Eluted Tar.* Filter efficiency is a central concept for the “functionality of filters.” A 1980 internal Philip Morris dictionary of tobacco terms defined this term as “the percentage of the incoming smoke or smoke component that is removed by a filter.” (115). Therefore, measuring efficiency can be an important consideration in terms of the utility of filters as proxy measures of smoking behavior or smoke exposure. One simple method for measuring the smoke solid-trapping efficiency of filters has been to compare the weight of filters before and after smoking; this has been described as the gravimetric method (19).

A number of fluorescence-based methods have been applied to assess filter efficiency. Many of these technologies were introduced soon after the widespread adoption of filters in the 1960s. It is known that tobacco smoke microparticulate solids, such as total particulate matter (TPM), express a high level of fluorescence (18). The fluorescence has been attributed to cyclic compounds in tobacco smoke, particularly those that are classified as polycyclic aromatic hydrocarbons (18). In 1960, McConnell and coworkers at the Tennessee Eastman Company (19), a major commercial source of cigarette filter fibers (“tow”), reported using the fluorescence of TPM as a means of evaluating cigarette filter efficiency. In the investigation, they used five different nonfiltered cigarettes. Included also were six different cellulose acetate filters that were capable of removing 20% to 55% of TPM in the tobacco smoke. In the initial experiments, the investigators showed that there was a linear relationship between fluorometric reading and smoke solids from the smoke condensate. With this information in hand, they were able to measure the smoke solids passing through the cigarette filter (indirect method) and the smoke solids collected on the filter (direct method). The results of the fluorometric method compared favorably with the gravimetric (sample weight) method.

Following the studies reported by McConnell and coworkers (19), Charles Thomas (25), an investigator at Philip Morris, used the same concept to craft an automated fluorescence-based method for measuring the tar of low-delivery cigarettes. The approach used was one in which the TPM was collected from glass-fiber Cambridge filter pads of smoking machines. The Cambridge filters are used in smoking machines to collect TPM to evaluate the vast array of chemicals in cigarette smoke (25). The results of the studies by Thomas (25) confirmed and extended the findings reported by McConnell and coworkers (19) who had shown that there was a linear relationship between fil-

ter fluorescence (Ex 365 nm; Em 400 to 600 nm) and the Federal Trade Commission “tar” values.

In 1980, Sloan and Curran (26) reported a new method for the spectrophotometric determination of filtration efficiency of cigarette filters. The filtration efficiency was determined by dividing the absorbance, measured at 310 nm, of the cigarette filter extract by the combined absorbance of the cigarette filter and Cambridge filter. Once again, an independent group of investigators confirmed and extended prior observations that there was a good correlation between the spectrophotometric methods and the gravimetric method.

Paszkievicz and Pauly (59) have devised a scheme for measuring TPM on cigarette filters using a procedure in which the filters from smoked Marlboro, Marlboro Ultra-light, and 2R4F Reference Standard Cigarettes were dissolved rapidly with 10 mL of DMSO, manual inversion, then 3 hours of shaking. Contaminants such as titanium dioxide were removed by centrifuging for 5 minutes. Finally, 0.1 mL of the solution was combined with 1.9 mL of diluent (DMSO:MeOH at 1:2, v/v) for analysis. Scanning spectrofluorometry was conducted with an excitation wavelength ( $E_x$ ) of 475 nm, and the emission ( $E_m$ ) wavelength was read at 535 nm. The fluorescent dye acridine orange was used as a surrogate standard. The assay showed a relative SD of <10%. Good linearity (>99%) was observed between machine-measured tar and relative fluorescence of extracted filters. The advantage of this and other fluorescence-based procedures is that they provided a technically simple, inexpensive, and high-throughput assay suitable for large-scale studies. Also, dissolving the filter circumvented problems associated with the incomplete removal of TPM from Cambridge filters that had been observed when using various solvents (e.g., alcohols) in different extraction procedures.

*Considerations for the Efficiency of Carbon Filters.* The methods discussed up to this point have focused on efficiency for trapping particulate matter; however, filters can be modified to remove different toxicants and chemicals in tobacco smoke. Filter composition and design are known to affect mouth-level delivery. For example, considerable effort has been made to define the effect of filtration by activated charcoal on the toxicologic activity of cigarette mainstream smoke, particularly the gas-phase components, from conventional and experimental cigarettes (116). It is to be noted that despite the extensive interest in charcoal filters, to date, filter-based assays to estimate human exposures from carbon filters have not been developed.

Activated carbon in the filter acts as an adsorbent of the gas phase elements because of its high specific surface area of >1,000 m<sup>2</sup>/g. Activated charcoal has an internal structure composed of irregular cavities or pockets, which can be seen readily with a scanning electron microscope. It is effective in removing smoke compounds with boiling points above approximately 30°C. Charcoal is marketed with a range of performance activity (e.g. chemical retention activity). It is characterized further by surface area, pore volume, hardness, density, ash content, pH value, and particle size. The performance of the activated carbon is measured by the amount of certain test chemicals the product can absorb. In general, the higher the charcoal activity, the greater the propensity for the filter to retain

vapor-phase constituents. For activated carbon used for filtering air and gases, the test chemical used is carbon tetrachloride (CTC). The activated carbon efficiency is expressed as % CTC. Carbon used in cigarette filters may have CTC that ranges from 50% to 100%. Methods for assessing the utility of filters with carbon for removing volatile components have also been described by Coggins (117), Laugesen (118), Mola (119), Polzin (120), Sarkar (121), Xue (122), Gaworski (116), and by investigators at the British American Tobacco Company (123, 124).

Different designs have been used in distributing the carbon in the filters, and detailed schemes have been presented in US patents. Two designs are used frequently. One is the carbon-on-tow design in which the carbon granules are distributed relatively evenly in the cellulose acetate filter plug. This design has been referred to as the "Dalmatian" filter (119). The second design is one in which the charcoal is placed in a cavity of the cellulose acetate filter. In most instances, the cavity is positioned between two plugs to give a plug-space-plug configuration in which the space component is filled with charcoal. Views of these different designs have been presented recently (119).

Studies reported by Polzin et al. (119) have compared the mainstream smoke constituent deliveries of cigarettes having different filter designs that had been smoked using three different regimens [International Organization for Standardization (ISO), Massachusetts, and Health Canada]. No significant difference was observed for tar and nicotine in comparative studies of same-length filters of cellulose acetate without carbon granules and with carbon granules (45 mg). For the same-filters and for the same-smoking schemes, significant lower deliveries were recorded for the charcoal-containing filter for acetaldehyde, acrolein, benzene, and styrene, and for the sum of 22 volatile organic compounds. These studies document that charcoal is effective in removing volatile organic compounds from machine-generated mainstream smoke. Overall, the brands with the most charcoal were effective in reducing the volatile organic compounds under even intense smoking conditions (96). There seems to be a growing body of evidence that cigarettes with activated carbon are effective in reducing certain volatile components in mainstream cigarette smoke as measured with smoking machines, but there is inadequate information to conclude that the observed reduction will be associated with a reduction in the known risk of diverse smoke-associated maladies, including cancer, and pulmonary and cardiovascular diseases.

In recent studies reported by Philip Morris, activated charcoal filtration reduced vapor phase irritants, which correlated with a marked decrease in *in vitro* cytotoxicity and *in vivo* morphology of the nose and lower respiratory tract in rat inhalation studies (122).

**Assays Examining Deposition of Specific Chemicals.** The desire to determine with greater accuracy smokers' exposures to various toxicants is coupled with a need to account for individual variations in smoking patterns. No two smokers puff identically, although each individual's patterns may be relatively consistent (113, 114). A commercial apparatus for measuring smoke flow has proven useful for measure puffing behavior (e.g., CReSS Lab Products, Borgwaldt KC GmbH), and the reliability of these

devices has been studied (15, 89). However, these and related flow meters require the use of mouthpieces that may interfere with normal smoking behavior, and require that the smoker use the device. Assaying the spent cigarette filter provides a proxy that bypasses the need for a mouthpiece or mechanical flow meter. Chemically specific assays can be used to correlate levels of one chemical to yields of toxicants to estimate yield in use. Two approaches have been put forward, each of which is discussed in detail below.

*Assessing Nicotine in the Filter.* A logical measure of mouth-level exposure would be nicotine trapped in the filter. Indeed, CORESTA issued a recommended spectrophotometric method for testing this in 1968 (125), and scientists at Philip Morris (PM) used such assays for in-house studies of smoking behavior in the early 1970s (126-128). Industry scientists developed a liquid chromatography approach in the 1980s. A liquid chromatography procedure was described in 1985 for the analysis of nicotine on cellulose acetate filters by Green and colleagues of the R. J. Reynolds Tobacco Company (33). In this method, the cellulose acetate filter was dissolved in acetonitrile to release any trapped nicotine. The investigators showed that the nicotine was stable on the filter, and that the proposed method was successful in circumventing problems previously encountered in removing nicotine from aged cigarette filters.

The measurement of nicotine on filters has recently been reintroduced by Shepperd and coworkers (53) at the British American Tobacco Company who have described the validation of methods for determining consumer-smoked cigarette yields from cigarette filter analysis. Two methods have been detailed. The first method, a whole filter scheme, is based on the analysis of whole filters using average values of filtration efficiencies obtained from a range of machine smoke puffing regimens. Procedurally, the filters are split longitudinally, cut in half transversally and then solvent-extracted. The nicotine content of the extract is then determined by gas chromatography with flame ionization detection as per CORESTA Recommended Method No. 9 (84). The second method is a part filter scheme that analyzes a 10-mm section from the mouth end of the filter, where the filtration efficiency remains relatively constant irrespective of puff flow rates and butt lengths. The analysis methods are largely equivalent with the exception of the extraction solution. Here also extract tar content was estimated with UV light absorbance using capillary electrophoresis and UV detection. Relative SD averaged 5%, and no information regarding recovery or limit of detection was provided.

The investigators reported that both filter methods gave good correlations with nicotine and tar yields. However, the part filter method was reported to be less susceptible to the effect of nicotine condensation and gave a more accurate assessment of yields than the whole filter method. The findings showed that the filter can be used for measuring tar and nicotine yields from cigarettes by smokers in their normal smoking environments. In addition, the methodology may provide a better understanding of the relationship between yields recorded for smokers versus different machine smoking regimens such as the ISO and Federal Trade Commission protocol developed in 1967.



The same group of investigators at British American Tobacco Company (52) also reported a comparison in smokers of cigarette filter nicotine levels, salivary cotinine, and urinary excretion of nicotine and its metabolites. The primary objective of this investigation was to determine the suitability of these different methodologies for estimating nicotine exposure and absorption in smokers. The research scheme was a 5-day clinical study of 74 smokers who smoked their own brands *ad libitum*. Filters were assessed as in the earlier Shepperd publication (53). The results showed that each method showed a high correlation ( $P < 0.01$ ) with the other two methods. The best correlation, however, was obtained between the filter nicotine analysis and urinary nicotine and nicotine metabolites. The pooled coefficient of variation (CV) within subjects across the study was 17.8%, consistent with the CV for saliva cotinine, urinary nicotine, nicotine metabolites, and reported cigarette use. The authors concluded that the filter analysis was less complicated and intrusive, and that the filters can be collected easily and without supervision. The authors note also that biomarker measurement in blood/plasma and smoking behavior measurements require that sampling or measurements be done in a laboratory environment.

Recent data assessing filters and biospecimens collected from German smokers in an ambulatory study (13) confirmed the earlier findings with respect to nicotine exposure and further compared filter nicotine to urinary biomarkers, including tobacco-specific *N*-nitrosamine, 1-hydroxy pyrene, and acrolein. This study showed that estimates of exposure obtained by filter analysis and biomarkers of exposure correlated significantly over a wide range of smoke exposures and that filter analysis may provide a simple and effective alternative to biomarkers for estimating smokers' exposure.

*Assessing Solanesol in the Filter.* Another chemical that can be measured in the filter to act as a proxy to exposure is solanesol. Solanesol is a tobacco-specific compound that is found in leaf tobacco and commercial tobacco products (55). It is a nonvolatile trisesquiterpenoid alcohol and withstands degradation during the burning of cigarettes (85, 128, 129). Solanesol has been used to measure the deposition and retention of cigarette smoke, and different components therein, in the lung (reviewed in refs. 130-132). Although UV light and ozone have been shown to degrade solanesol (132), Tucker and Pretty (133) reported that solanesol within the cigarette filter is not extensively exposed to sunlight or ozone, so this issue does not affect a filter assay result. An initial investigation in 2004 reported initial steps to assess levels of solanesol in the whole cigarette filter (45).

In brief, the procedure requires the removal of the outer tipping paper from the filter and separating the filter fibers manually to expose more surface area. The filters are then solvent-extracted and the extract is analyzed by high performance liquid chromatography (HPLC) and tandem mass spectrometry (MS/MS) with atmospheric pressure chemical ionization. The authors report recovery of 99.85% and relative SD of 5.35%. The limit of detection was 1.5 ng of solanesol on the column. Experiments by the authors showed that loss of solanesol from filters due to storage at room temperature was minimal (approximately 0.4% per day). Filters of cigarettes smoked

on smoking machines under ISO conditions were assessed for filter solanesol levels (45). Marlboro Light cigarettes were smoked in triplicate with the total puff count increasing from 1 to 7. The assay showed good linearity for puff number and puff volume. Filter solanesol also was highly correlated with tar and nicotine yields. Finally, the authors also assessed vent hole blocking and the good linearity was maintained for tar and nicotine. The authors concluded that filter solanesol meets criteria for a good smoke marker, providing a noninvasive means for assessing mouth-level some exposure for individuals.

A recent paper (88) extended and modified the original filter solanesol method. First, rather than using the whole filter, only the final 10 mm nearest the mouth end was used, consistent with the work by Shepperd and colleagues (77), discussed above. Polzin and colleagues (88) explored the use of a high-throughput and low-solvent method, wherein filters were added to high-volume 48-well plates and loaded into an automated workstation that adds solvent. This was then followed by transfer to 96-well plates for LC/MS analysis. A surrogate internal standard, geranylgeraniol, which is structurally similar to solanesol, was added. Using this method, the amount of solanesol retained in the filter was determined to be a function of the mainstream smoke delivery of both nicotine and tar. The authors reported recovery of 96% and relative SD of 10% under the prior preparation, compared with 95% and 11% for the low solvent method, making them comparable. To test the utility of the methods, the authors generated butts from Kentucky Reference cigarettes (2R4F) under ISO and Canadian Intense smoking conditions, with variations in puff number from 2 to the full tobacco rod length. Excellent linearity was recorded for both the standard solvent and low solvent.

The authors concluded that the modified method offered substantial savings at negligible change to outputs. The authors report that the new preparation and analysis methods increased throughput 5-fold relative to the prior method, meaning the ability to examine up to 250 samples per day on a single mass spectrophotometer. Thus, the measurement of solanesol provides a noninvasive and sensitive measure of tobacco smoke exposure. Further, solanesol in cigarette butts is stable; thus, the filters can be collected, stored, and processed as a batch when convenient. Studies assessing filter solanesol to human exposure and biomarkers have not yet been done.

## Summary of Findings

Table 3 presents a brief summary of the advantages and disadvantages of the different identified cigarette filter-based assays. Research gaps and opportunities to utilize cigarette filter-based assays as a means to assess individual variation in exposure to tobacco smoke constituents are presented in Table 4. The ultimate question is whether the filter-based assays have utility as proxy measures. Validation attempts for the methods have focused on correlation with specific smoke emissions and/or biomarkers of exposure in humans.

**Correlation of Filter-based Assays with Specific Smoke Emissions.** Polzin and colleagues (88) validated their standard and modified methods for solanesol

measurement against tobacco-specific nitrosamines *N'*-nitrosonornicotine (NNN) and NNK emissions. They reported that, indeed, filter solanesol can be used to estimate machine delivery of both carcinogens, with good linearity ( $R^2 = 0.9673$  for NNN and  $0.9512$  for NNK). The estimated deliveries of both constituents (based on solanesol) were consistent with prior reports of deliveries in the literature. Shepperd and colleagues (53) did not report the correlation between filter nicotine and emissions of acrolein, NNK, or pyrene per se. Rickert showed that tar color components could model 10 smoke constituent yields generated across a range of smoking conditions, with relative SDs that ranged from 8.1% (1,3-butadiene) to 51.4% (NNK).

#### Correlation of Filter-based Assays with Biomarkers.

Biomarkers of exposure are an important consideration in the evaluation of PREPs, tobacco products, and, more broadly, disease prevention (112, 134, 135). Filters can impact the chemical composition and toxicity of cigarette mainstream smoke (reviewed in refs. 43, 136-139). Thus, showing a relationship between filter-based assays and biomarkers of exposure would add strength to its utility as a proxy measure of mouth-level exposure. Existing data thus far indicate that the part-filter nicotine assays show significant relationships to nicotine intake, and at least the part-filter method has data linking filter nicotine to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), (3-hydroxypropyl) mercapturic acid (HPMA), and 1-hydroxypyrene (1-HOP) levels in smokers. However, data on eluted tar and visual inspection/imaging are lacking in this area. O'Connor and colleagues (57), for example, showed a low correlation between salivary cotinine and staining intensity.

## Discussion

The results of this structured review have shown that: (a) sufficient data exist to link filter-based chemical measures to ISO yields of tar and nicotine; (b) tar eluted from filters or in chemical digest of filters can be used to estimate the efficiency of the filter for trapping smoke solids; (c) visual and/or digital inspection of filters can be used as indicators of smoking behaviors such as filter vent blocking, number of puffs taken, and total volume drawn; and (d) limited data suggest a correlation between solanesol and nicotine measured in filters and exposure biomarkers in smokers.

**Research Gaps and Future Directions.** Linkage of filter-based assays to biomarkers of exposure other than nicotine and to biomarkers of biological effect is needed to truly test whether filter-based assays can serve as proxy measures of mouth-level exposure. It is important to clarify that a filter-based assay cannot in itself be a measure of exposure; smokers are exposed to what is not trapped by the filter. That is, smokers are exposed to what passes through the filter, and conversely are unexposed to what was captured by the filter. There is a limit to the relationship between such a measure and a biomarker, which is a product of factors inherent to the individual (e.g., gender, age, metabolism, and genetic makeup).

There is a noticeable absence of head-to-head comparison of the different assays. That is, individual laborato-

ries and research groups have developed and favor specific assays. In principle, the methods should have some overlap. Future studies, however, should characterize intercorrelations among different filter-based assays – where do they overlap, and where do some outdo others? For widespread applicability, a multilaboratory validation process would be preferable to determine the interassay reliability of the various methods.

All the research we identified using filter-based assays has relied exclusively on conventional cellulose acetate filters. However, a major consideration not specifically addressed is the role filter design characteristics (e.g., density, fiber denier, length, and overall efficiency) may play in the interpretation of assay results. At a minimum, some measurement of the unsmoked filter would need to be considered as a potential covariate or adjustment factor. Also, no published studies have incorporated PREPs and other smoking articles (i.e., electrically heated cigarettes) that are being promoted as reduced-risk products. Likewise, the majority of studies did not document the application of the technology for assessing cigarettes with filters containing charcoal, ion resins, embedded flavor elements, or other agents that have been incorporated into the filter. Thus, further research is required to assure that a proposed assay is applicable across different filter designs, or to define those filter designs for which particular assays may be unsuited.

Some of the proposed assays are technically complex in that they require multiple-step processing of the sample or expensive analytical instrumentation, such as the measurement of chemical deposition on filters for nicotine or solanesol. Such assays might prove challenging to translate to laboratories in developing countries, and such assays may not be applicable for large-scale screening studies. High-throughput and inexpensive technologies that can be applied to both conventional and modified reduced-risk cigarettes are needed, and additional investigations, particularly those that incorporate biomarkers of exposure, are required.

Many of the reported studies did not systematically address issues relating to storage and stability of the filters, and problems that may arise in stored filters from previously conducted studies. This is a particular concern for an assay whose best utility might be in field studies where tight controls on sample storage and handling may be impossible. Watson and colleagues noted the semivolatility of nicotine as a reason that they chose solanesol as their marker (45, 46). Solanesol loss from filters stored at room temperature was estimated at 0.4% per day. Similar data on the loss rate for nicotine would be desirable, as would data on stability under different storage conditions, such as room temperature, refrigerated (4°C), frozen (–20°C), and ultralow freezing (–70°C). Similarly, the impact of storage conditions and time from smoking on visual inspection and image-based assays could be better characterized.

In conclusion, emerging literature suggests that assays making use of the cigarette filter may prove useful in estimating smoking behaviors such as topography and filter vent blocking, and may have utility as proxy measures of mouth-level smoke exposure. The limited studies to date have not compared the methods directly, so it cannot yet be said whether one approach is superior or inferior to

others. However, it may be possible to use filters as surrogates or estimators of exposure, depending on the sensitivity of the assay, correlations with established measures of exposure in smokers, product characteristics, and the specific research question(s) being posed.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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