HHS Public Access

Author manuscript

Nicotine Tob Res. Author manuscript; available in PMC 2016 March 28.

Published in final edited form as:

Nicotine Tob Res. 2015 November ; 17(11): 1324–1330. doi:10.1093/ntr/ntu344.

A High Throughput Method for Estimating Mouth-Level Intake of Mainstream Cigarette Smoke

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Abstract

Introduction—We developed a high throughput method for estimating smoker's mainstream smoke intake on a per-cigarette basis by analyzing discarded cigarette butts. This new method utilizes ultraviolet/visible (UV-Vis) spectrophotometric analysis of isopropanol-soluble smoke particulate matter extracted from discarded cigarette filters.

Methods—When measured under a wide range of smoking conditions for a given brand variant, smoking machine delivery of nicotine, benzene, polycyclic aromatic hydrocarbons, and tobacco-specific nitrosamines can be related to the overall filter extract absorbance at 360 nm. Once this relationship has been established, UV-Vis analysis of a discarded cigarette filter butt gives a quantitative measure of a smoker's exposure to these analytes.

Results—The measured mainstream smoke constituents correlated closely (correlation coefficients from 0.9303 to 0.9941) with the filter extract absorbance. These high correlations held over a wide range of smoking conditions for 2R4F research cigarettes as well as popular domestic cigarette brands sold in the United States.

Conclusions—This low cost, high throughput method is suitable for high volume analyses (hundreds of samples per day) because UV-Vis spectrophotometry, rather than mass spectrometry, is used for the cigarette filter butt analysis. This method provides a stable and noninvasive means for estimating mouth-level delivery of many mainstream smoke constituents. The ability to gauge the mouthlevel intake of harmful chemicals and total mainstream smoke for cigarette smokers in a natural setting on a cigarette-by-cigarette basis can provide insights on factors contributing to morbidity and mortality from cigarette smoking, as well as insights on strategies related to smoking cessation.

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Supplementary Material

Supplementary 1–3 can be found online at http://www.ntr.oxford-journals.org

Declaration of Interests

None declared.

Introduction

Smoking continues to be the leading cause of preventable death in the United States.[1] Long-term exposure to thousands of mainstream cigarette smoke chemicals results in cumulative effects potentially leading to cardiovascular disease and cancer among smokers. Individual smokers smoke differently, and even an individual smoker's consumption pattern can change on a per cigarette basis and from day to day.[2] Changes in smoking consumption patterns influence the mainstream smoke intake of nicotine and other chemical constituents. Gaining a better understanding of the levels of toxicants to which people are exposed, as they smoke during their normal day-to-day activities, may improve insight in the overall impact that select toxic compounds have on morbidity and mortality from smoking.

Accurately assessing a smoker's exposure to the toxic compounds in mainstream cigarette smoke is a challenging problem. Machine smoking, while a useful tool for product comparison, is a poor predictor of a smoker's exposure. Unlike machines, two smokers do not necessarily smoke a particular brand in the same manner, nor does an individual smoke each cigarette identically. Differences in smoking likely reflect an individual's situational needs at the time of smoking. Smokers can alter their intake of smoke constituents by changing their puff volume, time between puffs, number of puffs, and filter ventilation-hole obstruction (deliberate or inadvertent). While tobacco smoke biomarkers, such as urinary nicotine metabolites, salivary cotinine, serum thiocyanate, and exhaled carbon monoxide, can provide information on smoke intake, biomarkers provide time averaged information rather than describing individual cigarette consumption patterns.[3–6] Most biomarker techniques are invasive, require sophisticated and expensive equipment, require special storage and handling, and often involve complex sample preparation steps prior to analysis. Additionally, genetic differences influence the smoker's metabolism and elimination rates, thus complicating the interpretation of individual results.[7]

Several techniques have been used to examine discarded cigarette filters as a way to estimate smoke deliveries.[8–12] We previously reported a noninvasive method for estimating cigarette smoke exposure using solanesol trapped on spent cigarette filter butts[8,9] and correlated those levels with mainstream smoke deliveries using standardized machine smoking techniques. Estimates of dry particulate matter delivery and filter efficiencies based on the spectrophotometric absorbance from cigarette filter's methanol extract at 310 nm, have been previously used to compare various biomarkers of exposure to cigarette smoke.[13,14]

Building on our prior solanesol work, we developed an alternative spectrophotometric assay to analyze isopropanol (IPA) extracts of trapped mainstream smoke particulate matter from cellulose acetate filter butts. We related the overall absorbance of this extract at 360 nm to smoking machine delivery of nicotine, benzene, four polycyclic aromatic hydrocarbons, and two tobacco-specific nitrosamines, giving a quantitative measure of a smoker's exposure to these analytes on a cigarette-to-cigarette basis. For this quantitative measure to work, each individual analyte does not necessarily have to exhibit absorbance in the ultraviolet (UV) region. The overall UV absorbance is related to the overall total particulate matter (TPM) level, which, in turn, is related to mouth-level deliveries of individual analytes.

Experimental

Cigarettes are machine smoked over a wide range of conditions (including different puff numbers and different smoking regimens). Nicotine, benzene, four polycyclic aromatic hydrocarbons, and two tobacco-specific nitrosamines are measured in mainstream smoke. The "tar" from each cigarette butt is also extracted with IPA and analyzed spectrophotometrically. Correlation curves relate the absorbance at 360 nm of the filter butt extract and specific analyte deliveries.

Materials and Reagents

Isopropyl alcohol was obtained from Fisher Scientific. Methanol and cyclohexane were obtained from Lab Depot Inc. Benzene and benzene-d_6 were purchased from Aldrich Chemical Co. N′-nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and nicotine were purchased from Toronto Research Chemicals. ^{13}C-labeled analogues for NNN and NNK as well as the US-EPA 16 PAH Cocktail were obtained for use as internal standards from Cambridge Isotope Laboratories. The 44-mm glass fiber Cambridge filter pads (CFPs) were obtained from Whatman. 1-L Tedlar® brand polyvinylfluoride (PVF) bags, carboxen-polydimethylsiloxane (Carboxen-PDMS) solid-phase microextraction (SPME) fibers, and Mininert® septum caps were purchased from Supelco. All chemicals and solvents were used without further purification. Cigarettes were obtained from the Massachusetts Department of Public Health or local retail outlets in Atlanta, GA. Cigarettes were stored in their original packaging at −20 °C until needed for testing.

Smoking Conditions

Cigarettes and CFPs were conditioned at 22 °C and 60% relative humidity for at least 24 hr before smoking according to International Standards Organization (ISO) 3402:1999. All cigarettes were smoked using an ASM-500 16-port linear smoking machine (Cerulean) according to either the ISO (35-ml puff volume, 60-s puff internal, 2-s puff duration) or Canadian Intense (55-ml puff volume, 30-s puff internal, 2-s puff duration, filter holes 100% blocked) regimens. Additionally, systematic variation of puff numbers (i.e., 2, 4, 6 …) using both regimens provided a wide range of smoke deliveries. After smoking, the cigarette filters were detached from the residual tobacco column and stored in cryovials (Nalgene) at −20 °C until further processing.

Ultraviolet/Visible Sample Preparation and Analysis

After smoking each cigarette, a 1-cm length of cigarette filter was cut from the mouth end and the paper wrapper was removed.[13] These 1-cm filter segments and a 1-cm portion of unused filter tow (for reference blank) were placed in separate wells of a 48-well plate (E&K Scientific) along with 4 ml of IPA added to each well using a Quadra 96 multichannel automatic pipetting system (Tomtec). The plates were covered and agitated for 30 min at 200 rpm on an orbital shaker. After agitation, the plate was further mixed using the Quadra 96 by repetitively pipetting 200 μl in and out of each well. A 200-μl aliquot was transferred, using the Quadra 96, to a Costar® UV transparent flat bottom 96-well plate (Corning Incorporated). Samples were analyzed with a uQuant 96-well plate reader (BIO-TEK

Instruments) at wavelengths of 360, 380, and 400 nm with background subtraction of the blank cell. The background-corrected absorbance at 360 nm was used for all calculations. All samples were analyzed within 5 min of transfer to minimize solvent evaporation. Data were collected and analyzed using the plate reader software (KC4, BIO-TEK Instruments).

Nicotine and Tobacco-Specific Nitrosamine Determination

The TPM collected on the standard glass-fiber CFP, generated with varying puff numbers collected using the ISO or modified intense smoke regimens, was analyzed for nicotine and tobacco-specific nitrosamine (TSNAs) using an Agilent 1100 liquid chromatograph (LC) coupled with an API 4000 (Sciex) tandem mass spectrometer (MS/MS) as previously published.[15] Sample preparation was streamlined with direct extraction of the CFPs in the LC mobile phase using 20 mM ammonium acetate containing 5% methanol.[16] One hour before smoking, the CFPs were treated with 2 ml of 50 mM ascorbic acid in methanol to reduce TSNA artifact formation.[17] Mainstream smoke nicotine deliveries were determined by adding nicotine to our LC/MS/MS TSNA method described above.[15] Isotopically-labeled nicotine was included in the TSNA internal standard panel for nicotine quantification. Nicotine used the transitions 163 to 130, 163 to 117, and 166 to 130 for the quantitative, confirmation, and internal standard transitions, respectively. This approach allows quantification of nicotine and TSNAs in the same smoke particulate matter collected from the same cigarette, removing cigarette-to-cigarette variability that would exist otherwise. The corresponding ultraviolet/visible (UV-Vis) analyses of the filter extracts were correlated with the mainstream smoke deliveries of both nicotine and TSNAs.

Benzene Determination

The vapor-phase portion of mainstream cigarette smoke was collected in individual 1-L PVF bags attached directly to individual ASM 500 puffing engines as previously described and analyzed by gas chromatography/mass spectrometry (GC/MS).[18] Internal standard (benzene-*d*) was added to each 1-L PVF bag prior to smoking. Following smoking, the PVF bags were sealed and a portion of the smoke sample was transferred via cannula to an evacuated 20-ml headspace vial (Microliter Corporation). Vials, containing the analytical samples, were loaded on a Combi-Pal auto sampler (LEAP Technologies) equipped with a SPME sampling arm for quantitative analysis using a 6890/5973 GC/MS (Agilent).

Polycyclic Aromatic Hydrocarbon Determination

Mainstream smoke TPM was analyzed for Polycyclic Aromatic Hydrocarbons (PAHs) using an Agilent 6890/5793 GC/MS as previously published.[19] The levels of four PAHs (benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, and benzo[a]pyrene) were selected and measured based on their inclusion on the International Agency for Research on Cancer monographs as either Group 1 (“carcinogenic to humans”) or Group 2B (“possibly carcinogenic to humans”).[20] The particulate phase of mainstream smoke was collected on a CFP, which was subsequently spiked with an isotopically enriched ^{13}C-PAH mixture. Filter pads were extracted with methanol followed by reduction of the solvent volume. The extract was then loaded, washed, and eluted using a C-18 solid phase extraction (SPE) cartridge

(Varian, Inc.). Four PAHs were then quantified by selected ion monitoring GC/MS using the isotopically enriched ^{13}C compounds as internal standards.

Sample Stability

To evaluate sample stability under a wide range of storage conditions, a large pool of 2R4F cigarettes were smoked and the butts collected. These cigarette butts were stored in bags under three conditions: room temperature (RT) exposed to ambient light, RT in the dark, and in a freezer at −20 °C. All samples were analyzed in triplicate at 1, 2, 3, 4, 13, and 26 weeks.

Quality Control Materials

The 2R4F research cigarette (University of Kentucky) served as the quality control (QC) material for this study. The 2R4F cigarettes were smoked according to the ISO or modified Canadian Intense smoking regimens and the corresponding butts were saved as low and high QC samples, respectively, for UV-Vis analysis. Absorbance levels from the extract of these butts, smoked over three weeks, were characterized to determine the mean and the 95th and 99th confidence intervals for the low and high QC values. Each analytical run included QC cigarettes and acceptance was based on criteria prescribed by Taylor.[21]

Data Analysis

Data analysis was performed using Excel Office 2003 (Microsoft). Non-weighted curves relating different measured machine-smoked analyte levels from mainstream smoke with absorbance of the cigarette butt extract were fit using Excel's built in functions, including logarithmic fit for PAHs and linear least squares fit for other analytes. Non-zero y-intercepts were allowed.

Results and Discussion

Wavelength Selection

IPA extracts of used cigarette butts yielded sufficient recovery of particulate matter trapped in the filter during smoking for UV measurement. This yellow-tinged extract yielded a strong UV absorbance, saturating the detector at approximately 250 nm and steadily decreasing to baseline level around 600 nm. While previous researchers have used extractions in methanol with analysis at 310 nm,[11,13] our reduced solvent volume yielded a saturated 310 nm absorbance response. Therefore, a 360 nm wavelength was chosen for quantitative analysis because of its absorbance range of 0–1.5 absorbance units on extracts of several high tar test cigarettes smoked under the more intense machine smoking regimen. Identifying the exact chemicals responsible for this strong absorbance at 360 nm is beyond the scope of the current work, given the thousands of compounds in cigarette smoke.[22] However, because each cigarette brand is calibrated individually, brand-to-brand differences in filter efficiencies, filler blend composition, or additives that could influence the deposition of UV-absorbing chemicals in the TPM are minimized.

In essence, we measure "tar," a complex mixture of chemical as an overall smoke marker rather than measuring the UV absorbance of each individual constituent. The importance of this approach is the high throughput nature which is deemed more important than individual

analyte selectivity. As each individual brand is calibrated and correlated for specific smoke deliveries of target analytes, differences in “tar” composition between brands are taken into account.

Butt Storage Stability

Collecting cigarette butts over time coupled with uncertain storage conditions in a clinical or laboratory area required that we characterize the time-dependent stability of smoked cigarette butts under different storage conditions. Previously, we reported a method based on the analysis of solanesol deposited in the cigarette filters and found excellent stability.[8] However, because the IPA extract contains a complex mixture of tobacco smoke constituents, verifying the absorbance stability was required to demonstrate consistency with the solanesol method. To investigate the stability of the UV signal from stored cigarette butts, batches of used cigarette butts were generated by smoking 2R4F research cigarettes under both ISO and Canadian Intense smoking regimes. Samples were stored in commercial “zip-lock” baggies under three conditions (RT in the light, RT in the dark, and at −20 °C in the dark) and retrieved as needed for analysis. Subsequent analysis of replicates demonstrated good stability for at least 26 weeks for all storage protocols. The relative standard deviations of the sample means for the different storage conditions were similar to those seen for within-day runs, typically being around 20%. No statistical differences were seen at the 95% confidence level in the means of the absorbance levels for the three different storage conditions. Good sample stability facilitates the collection, handling, and analysis of discarded cigarette butts with minimal concern regarding storage over this timeframe. For consistency in our study, we store used cigarette butts in freezers at −20 °C until needed.

Reproducibility

Method reproducibility was determined by repeated analyses of low and high QC materials over 6 months. During this time period the values for both the high and low QC samples showed good reproducibility. The low QC point (2R4F smoked using the ISO regime) had an average absorbance value of 0.606 at 360 nm with a relative standard deviation (RSD) of 12%. The high QC point (2R4F smoked using the Canadian Intense regime) had similar reproducibility, with an average absorbance of 1.23 and RSD of 14%. These data demonstrate similar reproducibility to our previously reported LC/MS solanesol method for butt analysis.[9]

Curve Fitting

A linear relation was found for absorbance and nicotine (Figure 1), and the TSNAs (NNN and NNK), (Figures 2 and 3, respectively), and benzene (Supplementary 1). In contrast, a logarithmic relationship was observed between absorbance and the summed PAHs (Supplementary 2). Correlation coefficients for the curve fits of cigarette butt extract absorbance versus machine-smoked analyte levels ranged from 0.9396 for NNK to 0.9941 for benzene.

Comparison of Predicted With Published Values

Using the results of our low QC smoked under ISO conditions and with an average absorbance reading of 0.606, we are able to estimate the delivery of these analytes for the 2R4F cigarette when smoked under ISO conditions. As shown in Table 1, the predicted delivery is similar to previously published values for nicotine,[23] benzene,[18,23] TSNAs,[23] and the four carcinogenic PAHs.[19] While this method may slightly overestimate or underestimate the deliveries of these constituents, the magnitude of the absolute error is similar to previously determined errors in estimating smoke intake using smoking machine values.[8] The agreement between predicted delivery and published values is particularly good considering long-term analytical variation between measurements, some of which were performed in other laboratories.

Additional Brands

While simple correlations for research cigarettes are useful for method development, determining the feasibility of this method on commercial cigarettes is more important. Due to the wide range of cigarettes produced worldwide, variations in design parameters may add additional variability to relations between butt extract absorbance and chemical delivery in mainstream smoke. Variations in cigarette lengths, styles, tobacco blends, filter lengths, and filter efficiencies may cause significant changes in the absorbance of the cigarette-butt extract relative to mouth-delivered smoke constituents. In an effort to identify key design parameters, 10 cigarette brand variants from one popular U.S. manufacturer were selected to compare how changes in filter length, cigarette length, tobacco weight, and filter ventilation affect the relation of extract absorbance to nicotine delivery. Additionally, two brand variants from another manufacturer were also analyzed to see whether manufacturer-to-manufacturer differences would be readily observed.

Response Factors for Different Brands

Brand comparison was performed by analyzing how nicotine delivery varied with UV absorbance. Ideally, a single calibration curve would relate each analyte level (nicotine, TSNAs, PAHs, etc.) with the UV absorbance for different cigarette brand variants. We found, however, that this was not the case. Different calibration curves were needed for each brand variant. As shown in Supplementary 3, the response in terms of the correlation curve slope between measured nicotine levels and the absorbance of the cigarette butt extract varies considerably among brands from a single manufacturer. Additionally, based on this limited sample set, similarly designed cigarettes from different manufacturers also vary significantly in their respective response factors. Previous researchers have shown that using the last 1 cm of the cigarette filter butts for analysis removes filter ventilation as a source of variability.[11] Filter ventilation can play a dominant role in overall total smoke deliveries. We did find a weak correlation between filter length and analyte delivery in manufacturer A’s brands. This is consistent with prior results investigating how differences in filter design affect cigarette filtration efficiency for trapping mainstream smoke nicotine.[24] The slope of the correlation curves can vary substantially between brands and become even more pronounced for TSNAs due to the differences between tobacco blends (and resulting differences in TSNA levels in the respective types of tobacco fillers) found in Virginia and

American blended cigarettes.[15] Because of such possible variability, each brand variant needs to be individually analyzed to determine the relation between filter extract absorbance and mouth-level exposure to each analyte.

Conclusions

We developed a low cost, high throughput method using the absorbance of cigarette filter extracts for estimating mouth-level intake of selected mainstream smoke analytes on a per-cigarette basis. After establishing the initial correlations between filter butt extract absorbance and machine yields of various compounds, subsequent measurements of individual filter butts provide estimates of mouth exposure on a cigarette-to-cigarette basis over a wide range of smoking conditions. We observed excellent correlations (correlation coefficients from 0.9303 to 0.9941) between the UV absorbance of the IPA extracts of spent cigarette filter butts and nicotine, benzene, PAHs, and TSNAs. While this work focused on these select analytes, additional specific chemicals could be included with appropriate calibration.

Since large quantities of cigarette butts are generated from even modestly sized smoking studies, a high throughput method such as this study is critical for cost-effective research. The combination of high throughput, low cost per sample, and relatively inexpensive equipment can expand the applicability and accessibility of this technique.

Like previous methods based on analyzing spent cigarette butts, this method provides a noninvasive means for estimating mouthlevel intake of mainstream cigarette smoke constituents. By analyzing discarded cigarette filter butts, artificial biasing of naturalistic smoking conditions such as might occur with external flow devices, such as the CReSS flow meter, is minimized.

Data from the collected butts provides useful insights into smoking behavior of subjects in their respective familiar settings and on their own schedules.[25,26] Additionally, summing the contributions from each cigarette for 24-hr periods allows the daily intake to be estimated. By combining mouth-level exposure with biomarker studies we hope to better characterize how cigarette design and individual smoker’s behavior influences uptake of addictive and harmful smoke constituents that maintain addiction and contribute to disease risk in smokers.

The analysis of discarded cigarette filter butts provides several advantages over tobacco exposure biomarker studies. Because this technique allows measurements on a cigarette-to-cigarette basis, variations in exposure between cigarettes can be seen on a fine scale rather than the time-averaged information that biomarker studies provide. Also, this technique allows the levels of exposure to these analytes through smoking to be estimated quantitatively; using a biomarker approach to estimation of exposure levels, on the other hand, may include exposures from multiple sources. Cigarette butt collection is much less invasive than collection of body fluids, so recruitment of subjects to studies and compliance may be enhanced. Because of matrix effects and analyte levels, biomarker studies tend to be much more expensive due to instrumentation requirements. This study provides significantly

lower cost as well as greatly reduced instrumentation requirements. Finally, genetic differences in metabolism and the lack of a biomarker amenable to analysis may limit exposure measurements in biomarker studies, whereas this technique provides a more direct measure of exposure on a per-cigarette basis.

Cigarette butts involved in studies such as this are now being seen in a different light, moving from the liability category as environmental trash to a valuable asset to probe smoking behavior and smoke level intake. The information they provide will be a valuable tool in understanding factors that influence consumption patterns and possibly helping to tailor improved cessation strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Use of trade names is for identification only and does not imply endorsement by the Centers for Disease Control and Prevention or by the U.S. Department of Health and Human Services. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

Funding

This study was supported financially by internal CDC funding.

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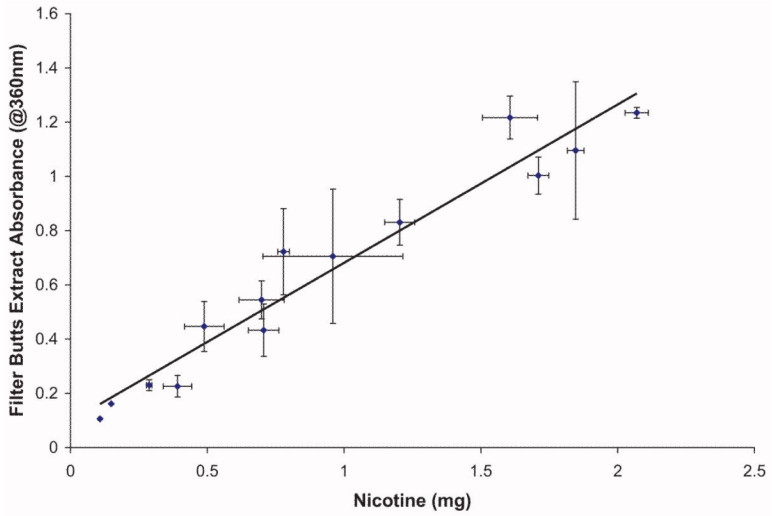

Figure 1.

Excellent correlation exists between filter butt extract absorbance at 360 nm and the mainstream smoke delivery for 2R4F research cigarettes collected under a range of smoking conditions. Errors bars represent 95% confidence levels; solid line is the linear least squares fit. $N = 14$; $R^2 = 0.9537$.

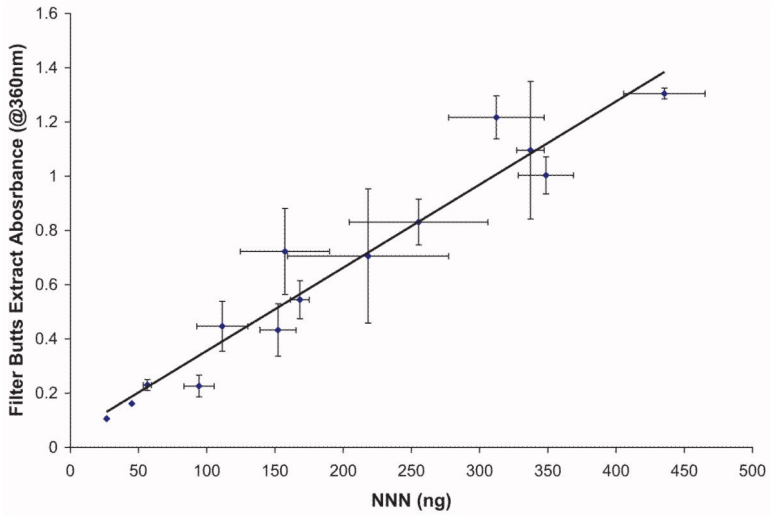

Figure 2.

Excellent correlation exists between filter butt extract absorbance at 360 nm and the mainstream smoke deliveries of the carcinogenic tobacco-specific nitrosamine N′-nitrosonornicotine (NNN) for 2R4F research cigarettes. Errors bars represent 95% confidence levels; solid line is the linear least squares fit. For NNN, $N = 14$; $R^2 = 0.9517$.

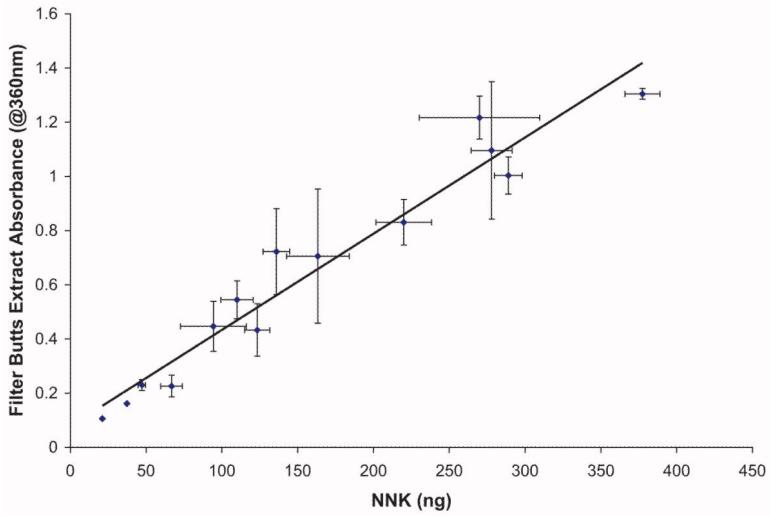

Figure 3.

Excellent correlation exists between filter butt extract absorbance at 360 nm and the mainstream smoke deliveries of the carcinogenic tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) for 2R4F research cigarettes. Errors bars represent 95% confidence levels; solid line is the linear least squares fit. For NNK, N = 14; R2 = 0.9396.

Table 1

Comparison of Estimated International Standards Organization (ISO) Deliveries for the 2R4F Reference Cigarette Determined Using Absorbance of Isopropanol Extracts of Cigarette Filters To Previously Published Results

Analyte	Estimated delivery (ISO conditions)	Previous results	Difference from previous results
Benzene	48 mg/cig	43 mg/cig[23]	12%
		44 mg/cig[18]	9%
Nicotine	0.86 mg/cig	0.75 mg/cig[23]	15%
NNN[a]	148 ng/cig	133 ng/cig[23]	11%
NNK[b]	133 ng/cig	116 ng/cig[23]	14%
PAHs[c]	42 ng/cig	40 ng/cig[19]	5%

Note. PAHs = polycyclic aromatic hydrocarbons.

[a] N′-nitrosonornicotine.

[b] 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

[c] Average summed levels of benz[a]anthracene, benzo[b]fluoranthene, benzo[k] fluoranthene, and benzo[a]pyrene.