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Nicotelline: A Proposed Biomarker and Environmental Tracer for Particulate Matter Derived from Tobacco Smoke

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Abstract

Particulate matter (PM) derived from tobacco smoke contains numerous toxic substances. Since the PM and gas phase of tobacco smoke may distribute differently in the environment, and substances in them may have different human bioavailability, multiple tracers and biomarkers for tobacco smoke constituents are desirable. Nicotelline is a relatively non-volatile alkaloid present in tobacco smoke, and therefore it has the potential to be a suitable tracer and biomarker for tobacco smoke-derived PM. We describe experiments demonstrating that nicotelline is present almost entirely in the PM, in both freshly generated cigarette smoke and aged cigarette smoke. An excellent correlation between the mass of nicotelline and the mass of the PM in aged cigarette smoke was found. We also describe experiments suggesting that the main source of nicotelline in tobacco smoke is dehydrogenation of another little-studied tobacco alkaloid, anatalline, during the burning process. We show that nicotelline metabolites can be measured in urine of smokers, and that nicotelline can be measured in house dust from homes of smokers and non-smokers. We conclude that nicotelline should be useful as a tracer and biomarker for PM derived from tobacco smoke.

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SUPPORTING INFORMATION AVAILABLE

Brief description and diagram the of cigarette smoke generation/aging system; data for PM and nicotelline mass from the smoke generation/aging system; recovery data for extraction of nicotelline and anataline from tobacco using various solvents and conditions; mass spectrometry parameters for determination of tobacco alkaloids and TSNA; determination of nicotelline and anatalline in PM and unburned tobacco by LC-MS/MS using 3 different SRM transitions and by GC-MS; recovery data for nicotelline spiked into clay soil; precision and accuracy data for determination of nicotelline *N*-oxides in urine; EI mass spectra of nicotelline and nicotelline-d_8; plots of PM mass vs. nicotelline mass for 5 brands of cigarettes. This information is available free of charge *via* the Internet at http://pubs.acs.org/.

Keywords

Cigarettes; Environmental Tobacco Smoke (ETS); Nicotelline; Nicotine; Particulate Matter; Secondhand Smoke (SHS); Settled House Dust; Thirdhand Smoke (THS); Tobacco Alkaloids; Tobacco Smoke

NOMENCLATURE

Various terms have been used for the particulate matter derived from tobacco smoke. These include particulate,[1] particulate phase,[2] particle phase,[2,3] particulate matter (PM),[4] tobacco smoke particles,[5] total particulate matter (TPM),[6] and total suspended particles (TSP).[3] For tobacco smoke, it has been stated that TSP is roughly equivalent to respirable suspended particles (RSP).[3] In this report we have chosen to use "particulate matter" (PM) as a general term for material collected on filters. Although TPM has been used widely for PM derived from various sources, including tobacco smoke,[6] for cigarette smoking machine studies TPM is considered to be all of the material trapped on a Cambridge filter pad when using a standard smoking protocol, such as those specified by the US FTC, Health Canada, ISO or CORESTA. ISO 10185 (Tobacco and tobacco products – Vocabulary) defines total particulate matter as: "That portion of the mainstream smoke which is trapped in the smoke trap, expressed as milligrams per cigarette (mg/cig.)" Therefore, we use the term "PM" for material collected on filters in all experiments, in contexts that make it clear how the smoke was generated and collected. It appears that gas phase[3] and vapor phase[1,7] have been used interchangeably in the literature. Since the term "vapor phase" is often used for systems that are at gas saturation with a particular compound, we feel the term "gas phase" is more appropriate in the context of this report. Tobacco smoke in the atmosphere where smoking has occurred has been referred to as "Environmental Tobacco Smoke" (ETS) or "Secondhand Smoke" (SHS) in the scientific literature. Since both terms are commonly used, we use SHS/ETS in this report. Recently, there has been concern about the possible health consequences of tobacco smoke residues remaining on surfaces and in dust after tobacco has been smoked. These residues have been termed "Thirdhand Smoke" (THS).[8]

INTRODUCTION

Tobacco smoking and SHS/ETS exposure are major causes of morbidity and mortality worldwide. Tobacco smoke contains numerous toxic substances,[9] including 73 compounds classified as carcinogens by the International Agency for Research on Cancer.[10] Tobacco smoke is a complex mixture of particles, vapors, and gases. The PM and gas phase have different fates in the environment. SHS/ETS undergoes chemical changes after generation,[11] as well as differential distribution of its components in the environment such as shifts in vapor-particle distributions[4] and sorption on surfaces.[12] The toxicity of the PM of cigarette smoke has been known for many years.[13] It is highly carcinogenic, and the higher molecular weight PAHs are associated with the PM. Most of the particles in SHS/ETS are below 2.5 μm in diameter and thus may deposit in the alveoli of the lungs, where they may pose a health risk independently of particular smoke toxins.[14,15]

Biomarkers and environmental tracers, ideally unique to a toxic mixture such as tobacco smoke, are useful for exposure assessment and for source apportionment. Due to the complex chemistry of tobacco smoke, differential distribution of the phases, and differences in bioavailability of the components, multiple tracers and biomarkers are desirable.[4,7,16–18] Nicotine, its metabolite cotinine, and the tobacco-specific nitrosamine metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) are the most specific of the commonly used biomarkers for tobacco smoke exposure.[16,19,20] Carbon monoxide,[16]

metabolites of volatile organic compounds,[21] and PAH metabolites[22,23] are also useful biomarkers, but they have sources other than tobacco smoke. Levels of these biomarkers are generally elevated in smokers as compared to non-smokers, but specificity may be inadequate to measure SHS/ETS exposure. There is considerable interest in specific tracers and biomarkers for tobacco smoke PM, but the currently used markers are not ideal.

A widely used tracer for tobacco smoke PM has been the trisesquiterpene alcohol solanesol (Chart 1).[5,24] Its attributes are high concentrations in tobacco, and high molecular weight so that it is present in the PM. However, solanesol reacts readily with ozone and decomposes in the presence of UV,[5,25,26] which limits its stability in the environment. We put considerable effort into developing a sensitive LC-MS/MS method for measuring solanesol in biofluids of smokers in an attempt to develop it as a biomarker, but were not able to detect it in plasma or urine, probably because of extensive metabolism and lack of urinary excretion due to insolubility. Solanesol is also present in other plants, particularly the family *Solanaceae*. Scopoletin[4,27] and long-chain hydrocarbons present in tobacco leaf, iso- and anteisoalkanes (C_{29}–C_{34})[28] have also been used as environmental tracers for tobacco smoke (Chart 1). Since scopoletin is a phenolic compound, it would be expected to be susceptible to oxidation in the environment, and it is present in numerous plant species. Hydrocarbons have the advantage of high stability in the environment, especially under dry conditions in which degradation by microorganisms would not be expected. However, their mammalian metabolism would be expected to be complex due to multiple sites that could be attacked by oxidative enzymes, and therefore hydrocarbons would probably not be suitable biomarkers.

Nicotine has been utilized as a tracer for the PM of SHS/ETS.[1,7,29,30] Nicotine may perform well as a tracer for tobacco smoke PM in venues where smoking at relatively constant levels has resulted in a steady state for partitioning between the PM, the gas phase, and the surfaces upon which it is adsorbed.[4] Nicotine is unlikely to provide a good estimate of tobacco derived PM in places where smoking is sporadic, as characteristics of the indoor space, such as composition of walls and floors, can affect the partitioning of nicotine between the surfaces and the atmosphere, and particles have different removal processes than gas phase nicotine.[7,31]

Nicotelline (Chart 1) is a tripyridine alkaloid found in tobacco and tobacco smoke.[2,32–35] The long retention time in a temperature programmed gas chromatographic run[2] suggested low volatility and led us to hypothesize that nicotelline would be mainly in the PM of tobacco smoke, and would therefore be a useful tracer and biomarker for tobacco smoke PM. With the possible exception of the saturated hydrocarbons, nicotelline would be expected to be more stable in the environment than previously used tracers for tobacco smoke PM. In this report we describe experiments demonstrating that:

1. Nicotelline is almost entirely in the PM of SS smoke and the PM of aged (3–30 min) cigarette smoke.
2. The mass of nicotelline in smoke generated by a smoking machine and aged (3–30 min) in a custom-built aging chamber is highly correlated with the mass of the particulate matter collected.
3. The amount of nicotelline on a per cigarette basis is higher in smoke than in the unburned tobacco, and this is likely due to dehydrogenation of the structurally-related tobacco alkaloid anatalline, which leads to the formation of nicotelline.
4. Nicotelline can be measured in house dust of smokers' and non-smokers' homes.
5. Nicotelline is metabolized to *N*-oxides that can be measured in urine of smokers.

EXPERIMENTAL PROCEDURES

Chemicals

Nicotelline and nicotelline-d_8 were synthesized as described below. Unlabeled nicotelline can be purchased from Toronto Research Chemicals (TRC), North York, ON, Canada and other suppliers. Anatalline was purchased from 3B Scientific Corporation Libertyville, IL, originating from 3B Pharmachem International Co., Wuhan, PR China. Myosmine, 2,3′-bipyridine, tobacco-specific nitrosamine (NNN and NNK) standards and deuterium-labeled internal standards (NNN-d_4, and NNK-d_4) were obtained from TRC. Solvents for LC separations, methanol and water, were obtained from Honeywell/Burdick-Jackson, distilled-in-glass. Reagents and solvents used for sample extractions were of analytical reagent grade or HPLC grade. Unless otherwise specified, chemicals used in the synthesis of nicotelline-d_8 were from commercial vendors. *Caution:* NNK and NNN are carcinogens and appropriate safety precautions should be taken.

Supplies

Filters for collecting PM were standard 44 mm Cambridge filters purchased from Borgwaldt GmbH, Germany for smoking machine studies, and Pallflex Emfab membrane filters were obtained from Pall Corp., Port Washington, NY, for studies of aged smoke. Pall Emfab filters are pure borosilicate glass microfibers reinforced with woven glass cloth and bonded with PTFE. Bisulfate-coated filters used for collection of volatile bases were prepared as described by Hammond *et al*.[36]

Instrumentation

LC-MS/MS analyses were carried out with a Thermo Accela UPLC pump and Pal Autosampler interfaced to a Thermo Vantage triple-stage quadrupole mass spectrometer, or with an Agilent 1200 HPLC interfaced to a Thermo-Finnigan TSQ Quantum Ultra triple-stage quadrupole mass spectrometer. A Varian 450 GC interfaced to a Varian 320 triple quadrupole mass spectrometer was used for GC-EI-MS analyses. An Agilent 6890 GC interfaced to an Agilent 5973 quadrupole mass spectrometer was used for GC-PICI-MS analyses. An Agilent 6890 GC was used for GC-Nitrogen-Phosphorus Detection analyses. Solvent evaporation was carried out using a Savant SpeedVac Model SC210A. MS and SS cigarette smoke was generated using a single port linear smoking machine model RM1/G, Borgwaldt GmbH, Germany.

Cigarettes

Commercial US (9 brands), Polish (5 brands), and Chinese cigarettes (10 herbal sub-brands of a cigarette brand popular in southern China) were purchased for the study. Chinese herbal cigarettes are prepared from tobacco, with powdered herbal medicines or their extracts added to the tobacco filler. The cigarettes were conditioned for 48 h in relative humidity of 60%, according to ISO standard conditions.[37] Tobacco for nicotelline determination was removed from the same cigarette brands used for smoke analysis.

House Dust Samples

The contents of vacuum cleaner bags from homes of smokers and non-smokers were passed through a strainer to remove large pieces of debris. House Dust Standard Reference Material® 2585 was used as received from the National Institute of Standards & Technology (NIST). A damp clay soil sample was collected from about 15 cm below the surface during the winter of 2010 in a suburb of the San Francisco area. It was dried in an oven at 120 °C for 1 h, and ground with a mortar and pestle. This sample was used for comparison with house dust, and for spiking with analytes for evaluating extraction procedures.

Synthesis of Nicotelline-d_8 Internal Standard and Unlabeled Nicotelline

The synthesis was accomplished by Suzuki coupling of 2,4-diiodopyridine with 3-pyridine-d_4-boronic acid as shown in Scheme 1. **(1) 2,4,5,6-tetradeutero-3-pyridineboronic acid.** The method described by Englert and McElvain for the synthesis of unlabeled 3-bromopyridine[38] was used to synthesize 3-bromopyridine-2,4,5,6-d_4, which was converted to the boronic acid as described by Li *et al*.[39] A solution of 3-bromopyridine-d_4 (1.7 g, 10 mmol) and triisopropyl borate (2.8 mL, 1.9 g, 10 mmol) in 16 mL toluene and 4 mL anhydrous THF was cooled in a dry-ice acetone bath under an atmosphere of argon. With vigorous stirring, 7.5 mL of *n*-butyllithium, 1.6 M in hexanes (12 mmol) was added over 2 min. Stirring was continued for 20 min in the cooling bath, after which it was removed, and while warming to room temperature 10 mL of 2 M HCl was added. The acidic aqueous layer was separated, and the pH was adjusted to 7.6–7.7 with 5 M NaOH, resulting in precipitation of a white solid. The precipitate was collected by filtration, washed twice with 2 mL portions of water, air dried under suction, and then vacuum-dried at 0.1 mm Hg for 15 min to give 1.1 g of white powder. **(2) 2,4-Diiodopyridine** was prepared by heating a mixture of 2,4-dichloropyridine (1.6 mL, 2.2 g, 15 mmol), flame-dried sodium iodide (9 g, 60 mmol), and acetyl chloride (4.5 mL, 5 g, 63 mmol) in 30 mL propionitrile at 80–90 °C for 24 h.[40] The reaction mixture was cooled and poured into 600 mL water containing 3 g of Na_2SO_3 and 5 mL of 1 M NaOH, and extracted twice with 150 mL ethyl acetate. The extracts were combined, washed with 50 mL water containing 0.5 g of Na_2SO_3 and 1 mL of 1 M NaOH, and then passed through silica gel (1.5 × 4 cm) in a fritted glass filter funnel by suction. The filtrate was evaporated using a rotary evaporator to give 4 g of crude product, which was recrystallized from methanol to give 1.8 g of light brown solid, mp 79–80 °C (lit[41] mp 78–80 °C) ; GC-MS methane CI, MH^+ 332 (100%). **(3) 2,4-di-(2,4,5,6-tetradeutero-3-pyridyl)pyridine (Nicotelline-d_8)**. The procedure is based on the Suzuki cross-coupling modification described by Kudo *et al*.[42] 2,4-Diiodopyridine (0.34 g, 1 mmol), 2,4,5,6-tetradeutero-3-pyridineboronic acid (0.36 g, 3 mmol), tris(dibenzylidineacetone)palladium(0) [$Pd_2(DBA)_3$], (46 mg, 0.05 mmol), tricyclohexylphosphine (Cy_3P, 32 mg, 0.12 mmol), and tetra-*n*-butylammonium bromide (0.1 g) were added to a 100 mL argon-flushed round bottom flask. 1,2-Dimethoxyethane (10 mL) and 1.4 g of tripotassium phosphate in 5 mL water were added, and the mixture was refluxed with stirring, under a static pressure of argon, for 2 h. The reaction mixture was cooled and extracted twice with 25 mL portions of ethyl acetate. The extracts were combined, and back extracted with two 20 mL portions of 1 M sulfuric acid. The combined acid extracts were washed twice with 25 mL portions of ethyl acetate, made basic by addition of 15 g tripotassium phosphate, and then extracted with two 40 mL portions of ethyl acetate. These extracts were combined, dried over about 0.5 g of anhydrous potassium carbonate, and the solvent was removed with a rotary evaporator to give 0.25 g of solid. This was sublimed at 0.05 mm Hg, 200 °C air bath temperature, using a Kugelrohr apparatus to give 0.084 g of white crystalline solid with a slight yellow tinge, mp 151–152 °C. The reported melting point of unlabeled nicotelline is 147.5–148.5 °C.[43] Anal. Calcd for $C_{15}H_3D_8N_3$: C, 74.65; N, 17.41. Found: C, 74.34; N, 17.3. Hydrogen could not be determined directly because the method does not distinguish deuterium, and reports all hydrogen as ^1H. Using a correction based on the relative masses of $^1H_3$ and $^2H_8$, a factor of 1.726 multiplied by the reported total H of 4.56 gives 7.87. Calcd for H_3D_8: 7.93. Isotopic purity was determined by isobutane positive ion chemical ionization (PICI) GC-MS analysis (Agilent System, see “Instrumentation,” above) in the selected ion monitoring (SIM) mode. Reconstructed ion chromatograms corresponding to d_8, d_7, d_6, d_5, and d_0 isotopomers (*m/z* 242, 241, 240, 239, and 234, respectively) were integrated and summed to determine the percentage of these isotopomers in the product. The composition was 86.8% d_8, 12.0 % d_7, 0.99% d_6, 0.21% d_5, and 0.005% d_0. The EI mass spectrum and TIC from GC-MS analysis (Varian System, see “Instrumentation,” above) is in the Supporting Information, Figure S1.

Unlabeled nicotelline was synthesized from commercially available 3-bromopyridine as described above for nicotelline-d_8, mp 151.5–152.5 °C; lit[43] mp 147.5–148.5 °C. Anal. Calcd for $C_{15}H_{11}N_3$: C, 77.23; H, 4.75; N, 18.01. Found: C, 77.05; H, 4.55; N, 17.9. The EI mass spectrum and TIC from GC-MS analysis is in the Supporting Information, Figure S1. Syntheses of nicotelline have been reported previously.[43,44]

Synthesis of Nicotelline-*N*-Oxides

A solution of nicotelline (40 mg, 0.17 mmol) and *m*-chloroperbenzoic acid (85%, 50 mg, 0.25 mmol) in 5 mL methylene chloride was allowed to stand at room temperature for 3 days. TLC (silica gel, eluting with 3:1 ethyl acetate/methanol containing about 5% concentrated aqueous ammonia) revealed 3 spots with Rfs of 0.1, 0.34, and 0.74. The spot with Rf = 0.74 corresponded to nicotelline. The largest spot had Rf = 0.34 and was presumably mono-*N*-oxide(s). The brownish solution was washed with 1 mL of saturated aqueous tripotassium phosphate and evaporated to dryness with a current of nitrogen to give a brownish solid. This was dissolved in 1 mL of 50:50 ethyl acetate/methanol and chromatographed on a silica gel column, 15 × 1 cm, eluting with first 20 mL of 95:5:2 ethyl acetate/methanol/ concentrated ammonia, then with 40 mL of 80:20:4 ethyl acetate/ methanol/ concentrated ammonia. The eluate was collected in 3 mL fractions. Fractions 8–14 were mainly nicotelline. Fractions 15 and 16 were presumed to be mono-*N*-oxides based on TLC as described above. These were combined and evaporated to a brownish solid, which was triturated with 0.5 mL of ethyl acetate. The supernate was removed and the solid was dried with a current of nitrogen to give 3.5 mg of beige powder, mp 198–200 °C with slight darkening. LC-MS analysis (APCI) indicated at least two isomeric mono-*N*-oxides, two peaks with similar spectra, *m/z* 250 (MH^+ for *N*-oxide, base peak) and *m/z* 234 (MH^+ - 16).

Characterization of Alkaloid Standards

To verify the identity of nicotelline, anatalline, and nicotelline *N*-oxides used as standards, mass spectra were recorded and compared to literature data if available. In addition, spectra from extracts of tobacco products, and a smoker's urine extract in the case of nicotelline *N*-oxides, were obtained and compared to those of the standards to verify identity.

LC-MS/MS Analysis of Nicotelline and Anatalline Standards and Cigarette Butt Extract—Because levels of nicotelline in unburned tobacco are low, cigarette butts including material deposited on filters were analyzed. Ten cigarette butts (filters with some tobacco remaining) were collected in a parking lot, and placed in a 60 mL polypropylene bottle along with 25 mL 0.5 M HCl. The mixture was sonicated for 30 min, the solids were allowed to settle, and liquid was decanted to another bottle. To 2 mL of the liquid were added 1 mL 50% (w/v) aqueous potassium carbonate containing 0.2% v/v concentrated aqueous ammonia and 5 mL 50:50 dichloromethane/pentane. The mixture was vortexed, centrifuged, and the organic phase was transferred to a tube containing 0.5 mL of 1 M sulfuric acid. This was vortexed, centrifuged, and the organic phase was removed and discarded. To the aqueous acid phase containing the extracted bases were added 0.5 mL 50% (w/v) aqueous potassium carbonate containing 0.2% conc aqueous ammonia and 2 mL 50:50 dichloromethane/pentane. After vortex mixing and centrifugation, the final extract was transferred to a new tube and evaporated to dryness at 60 °C with a current of nitrogen. The residue was dissolved in 0.5 mL of 10 mM aq ammonium formate for LC-MS/MS analysis. The Quantum Ultra System (see "Instrumentation," above) was used in the APCI mode. The column was a Supelco Discovery HSF5 15 cm × 4 mm, 5 μm particle size, heated to 40 °C. Samples (10 μL) were injected with an initial mobile phase composition of 25% aqueous methanol containing 10 mM ammonium formate. After injection the composition was changed to 100% methanol containing 10 mM ammonium formate over 2

min. The flow rate was 0.7 mL/min. This composition was maintained for 6 min before returning to the initial composition. Product ion spectra were obtained for anatalline MH^+ *m/z* 240 at 25 eV, argon collision gas 1.5 mT. Product ion spectra for nicotelline MH^+ *m/z* 234 were obtained at 35 eV, with argon collision gas pressure at1.5 mT (Figure 1).

GC-EI-MS Analysis of Anatalline Standard and Tobacco Extract—To the tobacco filler (0.69 g) from a cigarette (Marlboro Red) in a glass vial was added 10 mL of 0.5 M aqueous HCl, the mixture was sonicated for 15 min, and the liquid was decanted to a new vial. To a 1 mL aliquot in a glass culture tube was added 0.5 mL of 2 M NaOH and 3 mL 70:30 toluene/1-butanol (v/v). The mixture was vortexed, centrifuged, and the organic phase was transferred to a tube containing 0.5 mL of 1 M sulfuric acid. This was vortexed, centrifuged, and the organic phase was removed and discarded. The aqueous acid phase containing the extracted bases was washed with 3 mL 70:30 toluene/1-butanol, then 0.5 mL 50% (w/v) aqueous potassium carbonate containing 0.2% v/v concentrated aqueous ammonia and 0.1 mL 90:10 toluene/1-butanol (v/v) were added. After vortex mixing and centrifugation, the final extract in 90:10 toluene/1-butanol was transferred to an autosampler vial. The analysis was performed using the Varian GC-MS system (see "Instrumentation," above) operated in the EI mode, ionization energy 70 eV. The injections were done in the splitless mode, injection port temperature 275 °C. The column was a Bruker FS 30 m × 0.25 mm ID, 0.25 μm df (5% diphenyl/95% dimethylpolysiloxane). The sample, 1 μL, was injected at a column temperature of 70 °C, then after a 1 min hold programmed to 290 °C at 25 °C/min followed by 5 min at this upper limit. Data was collected in the full scan mode (Q1) scanning from *m/z* 50 to *m/z* 350 over 0.5 min. Chromatograms of the tobacco extract and the anatalline standard, and spectra of anatalline are in Figure 2. Integration of the total ion chromatogram of the anatalline standard indicated a purity of 85%. Major ions in the EI spectrum of the anatalline standard were *m/z* (rel. int.): 239 (M^+, 31), 120 (100), 119 (77), 106 (75), 105 (67), 160 (29), 161 (26). Häkkinen *et al*.[45] reported: 239 (M^+, 39), 120 (100), 119 (91), 105 (88), 106 (85), 161 (28), 160 (27).

Mass spectral characterization of nicotelline N-oxides—Product ion spectra from LC-MS/MS analysis (Quantum Ultra System, see "Instrumentation," above) are presented in Figure 4. Also shown are the corresponding spectra from a smoker's urine extract (described below under "Identification of Nicotelline N-Oxides in Human Urine"), and the respective LC chromatograms

Nicotelline in PM from Mainstream (MS) and Sidestream (SS) Cigarette Smoke

MS and SS cigarette smoke from single cigarettes was generated using a Borgwaldt RM1/G smoking machine using a smoking regimen similar to those specified in ISO Standards 3308:2012[46] and 4387:2000,[47] *i.e.* puff duration 2 s, puff interval 60 s, puff volume 35 cm^3, and smoked until the butt length was 3 mm past the mouthpiece (also called filter overwrap). SS smoke collection was similar to ISO Standard 20773:2007,[48] *i.e.* a fishtail chimney (Borgwaldt GmbH, Germany) was used with linear velocity of 2 cm s^{-1}. However, our methods deviated from current ISO standards[46] because the Borgwaldt RM1/G smoking machine is a single port model. PM was collected using 44 mm Cambridge filters. After a cigarette was smoked, both filter pads (for MS and SS PM) were removed from filter holders. Each filter was weighed before and after collection in order to determine particulate matter (PM). The filter pads were then spiked with 100 μL internal standard (ISTD) solution containing 100 μg/mL nicotelline-d_8, transferred into extraction flasks, 25 mL of ethyl acetate was added, and pads were extracted for 30 min by mechanical shaking. Aliquots (5 mL) of extract were transferred to glass culture tubes, and 1 mL 1 N sulfuric acid was added. The tubes were vortexed, centrifuged, and placed in a dry ice-acetone bath to freeze the aqueous layers. The organic layer was poured off and discarded, the aqueous layer was

thawed and washed with 5 mL 2:1 ethyl acetate/toluene by vortexing, centrifuging, freezing, and pouring off the organic layer. The aqueous phase was made alkaline with 1 mL 50% K_2CO_3, and then extracted with 5 mL 2:1 toluene/ethyl acetate using the freeze-pour technique. The extracts were evaporated to dryness using a SpeedVac, and the residues were dissolved in 0.1 mL 10% methanol containing 12 mM HCl, and then transferred to autosampler vials for LC-MS/MS. The Quantum Ultra System (see "Instrumentation," above) was used in the APCI positive ion mode. The vaporizer and capillary temperatures were 450 and 250 °C respectively and the sheath and auxiliary gasses were set at 35 and 10 L/min. The discharge current was 5 μamps. The column was a Supelco Discovery HSF5 15 cm × 4 mm, 5 μm particle size. Samples (10 to 50 μL) were injected with an initial mobile phase composition of 75% aqueous methanol containing 10 mM ammonium formate. After injection the composition was changed to 100% methanol containing 10 mM ammonium formate over 7.5 min. The flow rate was 0.9 mL/min. The following transitions were monitored: 234 →207, nicotelline-d_0 and 242 → 214, nicotelline-d_8.

Nicotelline in PM and in the Base Fraction of the Gas Phase of Sidestream Smoke

Single cigarettes were "smoked" by attaching a 60 mL syringe, clamped in a ring stand inside a fume hood. Sidestream smoke was collected with a fishtail chimney (see above "Nicotelline in PM from Mainstream (MS) and Sidestream (SS) Cigarette Smoke"), drawn through a Cambridge filter using suction to collect PM, and then passed through fritted glass dispersion tubes into 4 traps, connected in series, each containing 50 mL of 0.1 N HCl in 50% methanol. The filters were extracted as described below under "Extraction of PM collected on Filters." The extracts of the filters and the acidic solution of basic substances that passed through the filters were analyzed by LC-MS/MS as described under "LC-MS/MS Determination of Nicotelline, Other Alkaloids and TSNA in PM, in Tobacco Extracts, and in House Dust." below. For nicotine analysis, aliquots of the extracts were analyzed as described below under "GC Determination of Nicotine."

Nicotelline and Other Alkaloids in PM and Base Fraction of the Gas Phase of Aged Cigarette Smoke

PM and basic substances in gas phase samples were collected using a smoke aging system designed to generate and deliver cigarette smoke for controlled human exposure studies. The system, which consists of a smoking machine (TE10z, Teague Enterprises, Woodland, CA), ducts, pump, and aging chamber, allowing continuous generation, aging, and transport of smoke from multiple cigarettes over several hours, (Diagram in Supporting Information, Figure S2) is described in Schick *et al.*[49] Briefly, smoke is drawn into the aging system with an air amplifier (Vaccon Co., Medway, MA), diluted into conditioned, filtered air and conducted through stainless steel ducting into a 6 m^3 stainless steel aging chamber. The aging chamber contains 3 staggered baffles and 2 fans to insure mixing. Smoke aerosol samples were collected at sites that provided aging times of 3 min (Figure S2, Site 2) and 30 min (Figure S2, Sites 4 and 5). Samples were collected by drawing the smoke through two filters in series. The front filter was an untreated 47 mm Pallflex Emfab membrane filter, and the rear filter was a 37 mm Pallflex Emfab membrane filter impregnated with sodium bisulfate to trap nicotine and other basic substances in the gas phase.[1] These filters retain 99.95% of 0.3 micrometer particles in the standard ASMD 2986-95A test. The mass of PM collected was determined by weighing the filters before and after sample collection. Both filters were extracted as described below under "Extraction of PM Collected on Filters," and the extracts were analyzed as described below under "LC-MS/MS Determination of Nicotelline, Other Alkaloids and TSNA in PM, in Tobacco Extracts, and in House Dust." Nicotine in the extracts was determined as described below under "GC Determination of Nicotine." The flow rates during sample collection were used to calculate PM and alkaloid concentrations in $\mu g/m^3$.

The cigarette brands were Camel, Camel Blue, Marlboro Red, Marlboro Gold, and Newport. Each cigarette brand was studied on a separate day. The cigarettes were purchased by the carton on the open market, and stored at −20 °C prior to use. The cigarettes were conditioned, and smoke from multiple cigarettes was generated as previously described.[49] Smoke was generated for time periods ranging from 2 to 6 h during collection at the 3 sites described above. This resulted in the collection of 4 to 9 samples of PM for each of the brands. The samples (N = 34) from all 5 brands were used to construct Figure 3 and the TOC graphic. Separate plots for each brand were used to construct Figure S3 in the Supporting Information. The PM and nicotelline data for all brands, sites, and collection times are in the Supporting Information, Table S1.

Extraction of Nicotelline and Anatalline from Cigarette Tobacco

Tobacco was removed from cigarettes and weighed. It was then transferred to 100 mL volumetric flasks and spiked with a the internal standard nicotelline-d_8 prior to adding the extraction solvent. Various extraction solvents and conditions were tested for the best recovery of nicotelline from tobacco, including 1 M HCl, 5% acetic acid, 1 M NaOH with mechanical mixing and sonication, and temperatures up to 70 °C. The several extraction procedures yielded similar results. For the reported data, tobacco from a single cigarette was weighed, transferred to 100 mL volumetric flask, 25 mL 0.1 N HCl in 50% methanol were added, and the flask was sonicated for 60 min at 60 °C. The flasks were then cooled and brought to a final volume of 100 mL with 0.1 N HCl in 50% methanol. Aliquots of the tobacco extract were analyzed as described below under “LC-MS/MS Determination of Nicotelline, Other Alkaloids and TSNA in PM, in Tobacco Extracts, and in House Dust.”

Subsequently, several extraction procedures were compared for recovery of anatalline, nicotelline, and nicotine. Tobacco from 10 cigarettes (Marlboro Red) was pooled and mixed. The following procedures were used for 0.7 g portions, using 25 mL of solvent, sonicating for 90 min at 75 °C: (1) 50% MeOH 0.1N HCl; (2) 50% MeOH 0.1N HCl containing 20 mM ascorbic acid; (3) 5% acetic acid; (4) 1M NaOH; (5) 200 mM citrate-phosphate buffer, pH 4.5; (6) 200 mM citrate-phosphate buffer, pH 4.5 containing 20 mM ascorbic acid; (7) 1M HCl. We also reanalyzed a sample extracted with 50% MeOH 0.1N HCl (different lot of tobacco) that had been stored for 1 year at 4 °C. The extracts were analyzed as described below under “LC-MS/MS Determination of Nicotelline, Other Alkaloids and TSNA in PM, in Tobacco Extracts, and in House Dust.” The results are presented in Table S2 in the Supporting Information.

Conversion of Anatalline to Nicotelline During Combustion

An oregano “cigarette” was prepared as follows: Approximately 1 g of oregano was hand rolled into a cigarettes using commercial cigarette paper. One oregano cigarette was fortified with anatalline by pipetting 100 μL solution of 100 μg/mL anatalline in 90% methanol along the length of the cigarette 3 times using standard laboratory pipet. The total amount of anatalline added to the oregano cigarette was 30 μg. The cigarette was allowed to dry for 3 h before being combusted in a fishtail chimney apparatus (see above under “Nicotelline in PM of Mainstream (MS) and Sidestream (SS) Cigarette Smoke”) with a Cambridge filter connected to a vacuum source. The experiment was also run using a blank 90% methanol treated oregano cigarette. The PM that was collected was extracted as described below under “Extraction of PM Collected on Filters,” and analyzed as described below under “LC-MS/MS Determination of Nicotelline, Other Alkaloids and TSNA in PM, in Tobacco Extracts, and in House Dust.”

Extraction of PM Collected on Filters

Cambridge filters or Pallflex Emfab membrane filters from chamber experiments were extracted in 50 mL polypropylene centrifuge tubes by sonicating in 25 mL of 0.1 N HCl in 50% methanol at 60 °C for 60 min followed by vortexing for 5 min.

LC-MS/MS Determination of Nicotelline, Other Alkaloids and TSNA in PM, in Tobacco Extracts, and in House Dust

Aliquots (0.1 – 0.25 mL) of the extracts described above, or 30 to 100 mg samples of house dust, were spiked with internal standards, 50 ng each of NNN-d_4, NNK-d_4, nicotelline-d_8 and 500 ng of bipyridine-d_4. To these were added 2 mL of aqueous 45% potassium carbonate/5% tetrasodium EDTA (w/v) and 5 mL of 45:45:10 dichloromethane/pentane/ ethyl acetate. The tubes containing the dust samples were sonicated at 60 °C for 60 min. All samples, dust and extracts, were vortexed for 5 min. The tubes were centrifuged, frozen in a dry ice- acetone bath, and the extracts were transferred to 13 × 100 mm tubes containing 0.5 mL of 1 M sulfuric acid. The tubes were vortexed, centrifuged, frozen, and the organic layers were poured off and discarded. The aqueous phases were made alkaline with 0.5 mL 45% potassium carbonate/5% tetrasodium EDTA and extracted with 4 mL of 45:45:10 dichloromethane/pentane/ethyl acetate as above. After centrifugation and freezing of the aqueous layers, the solvent was transferred new tubes and evaporated (SpeedVac). The residues were reconstituted in 0.2 mL of 10% methanol 100 mM ammonium formate for LC-MS/MS analysis. The samples were analyzed on the Thermo Vantage system (see "Instrumentation," above). A Phenomenex 15 cm × 3mm Kinetex 2.6 μm PFP column was used, using a gradient starting with 20% aqueous methanol/10 mM ammonium formate changing to 80% methanol/10 mM ammonium formate over 6 min. The flow rate was 0.6 mL per min, and column temperature was 50°C. The mass spectrometer was operated in the heated electrospray ionization (HESI) mode, the spray voltage was 4000, the vaporizer temperature was 400 °C, and the heated capillary temperature was 325 °C. The resolution of Q1 was set at 0.5 amu FWHM; the resolution of Q3 was 0.7 amu FWHM. Optimum MS/MS transition, collision energies and source parameters were determined by infusion solutions of the analytes into the ion source. The SRM transitions used for quantitation, collision energies, and analyte retention times are in the Supporting Information, Table S3. Standard concentrations ranged from 400 ng per tube down to 10 pg depending upon the expected concentration range. The calibration curves were linear with 1/X weighting. Lower limits of quantitation (LLOQs) were 10 pg per sample for NNN, NNK and nicotelline. The LLOQ for anatalline was 30 pg/sample.

As a test for the specificity of the method, unburned tobacco and PM extracts were analyzed for nicotelline and anatalline using two additional SRM transitions as qualifier ions. Results calculated using three transitions for each analyte were in good agreement (Table S4, Supporting Information). We also analyzed tobacco and PM extracts by GC-MS and compared the results to those obtained using LC-MS/MS. The extracts were further extracted as described above under "Characterization of Alkaloid Standards," "*GC-EI-MS Analysis of Anatalline Standard and Tobacco Extract.*" Instrument parameters were the same except that data was collected in the SIM mode, *m/z* 239 for anatalline, and *m/z* 164 for the internal standard, anabasine-d_4. Likewise, the results of analyses of PM and unburned tobacco for anatalline and nicotelline by LC-MS/MS SRM and GC-EI-MS SIM were in good agreement, although anatalline concentrations by GC-MS were somewhat higher (Table S5, Supporting Information). Recovery data for nicotelline spiked into clay soil are in the Supporting Information, Table S6.

GC Determination of Nicotine

The extracts described above were diluted, and concentrations of nicotine were determined by gas chromatography with nitrogen-phosphorus detection using 5-methylnicotine as the internal standard. The original method[50] has been modified for capillary GC.[51] The limit of quantitation is 1 ng/ml.

Identification of Nicotelline *N*-Oxides in Human Urine

A Bakerbond SPE Octadecyl (C_{18}) column, 6 mL/ 500 mg (Mallinckrodt Baker, Inc., Phillipsburg, NJ) was conditioned by passing through in succession, with gentle vacuum, 2 mL each of dichloromethane, methanol, and water. To a 50 mL urine sample from a smoker was added 2.5 mL of saturated aqueous sodium phosphate dibasic (pH 8.8), the sample was applied to the column under gentle vacuum over ~ 5 min, and sucked dry. The column was washed with 2 mL water followed by 2 mL dichloromethane, and then eluted with 2 mL methanol. The methanol eluate in a glass culture tube was dried with a stream of nitrogen at 60 °C. The residue was dissolved in 1 mL water (vortexing), then 2 mL of 50% (w/v) tripotassium phosphate and 4 mL dichloromethane was added. The tube was vortexed for 3 min, centrifuged, and then placed in a dry ice-acetone bath to freeze the aqueous (lower) layer. The extract was poured into glass culture tubes and evaporated to dryness using a current of nitrogen at 60 °C. The residue after evaporation of the dichloromethane extract was dissolved in 150 μL Millipore purified water and 15 μL were injected into the Vantage LC-MS system (see “Instrumentation,” above) operated using heated electrospray ionization (HESI) in the product ion scan mode. The resolution of Q1 was set at 0.1 amu FWHM; the resolution of Q3 was 0.7 amu FWHM. The column was a Phenomenex Kinetex PFP 100 A, 10 cm × 3 mm, 2.6 μm particle size. The column temperature was 50 °C, and the flow rate was 0.5 mL/min. The initial solvent composition was 10% aqueous methanol containing 10 mM ammonium formate, changing to 100% methanol containing 10 mM ammonium formate over 6 min and maintained for 1.5 min before returning to the initial composition. The parent mass was 150.12, the collision energy was 25 eV, the argon collision gas pressure was 1.5 mT, and the product ion mass range was 50–260 amu. Chromatograms and spectra are presented in Figure 4.

Three 10 mL smokers’ urine samples were extracted as described above, with the following changes: The volume of sat aq sodium phosphate dibasic was 0.5 mL, and Fisher Prep-Sep C18 SPE 1 mL columns (Fisher Scientific, Fairlawn, NJ) instead of Bakerbond columns were used. The evaporation residues were dissolved in 200 μL Millipore purified water (vortexing) and 10 μL were injected into the Vantage LC-MS system. The same LC column, mobile phase and gradient, and MS parameters as described above were used, except that the data was acquired in the SRM mode. Two transitions were monitored: *m/z* 250 → 233 and *m/z* 250 → 207; for both the collision energy was 25 eV and argon collision gas pressure was 1.5 mT. Chromatograms are presented in Figure 5.

Determination of Nicotelline *N*-Oxides in Human Urine

The method is based on the reduction of the nicotelline-N-oxides in urine to nicotelline using titanium trichloride. Internal standard, 100 μL of 10 ng/mL nicotelline-d_8 or nicotelline-*N*-oxides-d_8, was added to 1 mL urine samples, and standards prepared in non-smoker’s urine, in 13 × 100 mm glass culture tubes. Standards were prepared from both nicotelline and nicotelline-*N*-oxides. $TiCl_3$ (100 μL of 20% w/v) was added, and the tubes were allowed to stand for 30 min at room temperature, after which they were made basic by the addition of 0.5 mL of saturated tetrasodium EDTA. The samples were then extracted with 4 mL of 2:1 toluene/ethyl acetate, and then back extracted into 0.5 mL of 1 M sulfuric acid. The acid layer was separated and made basic with 0.5 mL of 50% potassium carbonate, and then extracted with 4 mL of 1:1 dichloromethane/pentane. The extracts were

evaporated, reconstituted in mobile phase and analyzed for nicotelline by LC-MS/MS in HESI mode on the Thermo Quantum as described above under "Nicotelline in PM of Mainstream (MS) and Sidestream (SS) Cigarette Smoke." The vaporizer and capillary temperatures were 450 and 350 °C respectively and the sheath and auxiliary gasses were 40 and 5 L/min. The ion spray voltage was set at 4000 volts. Standard concentrations of nicotelline and nicotelline-*N*-oxide(s) ranged from 1.37 pg/mL to 3000 pg/mL. Standard curves were generated using both nicotelline and the *N*-oxides. The limit of quantitation was 4.12 pg/mL for both the nicotelline and the nicotelline-*N*-oxide(s) standard curves. The standard curves were nearly identical, confirming the purity of the *N*-oxides standard. Precision and accuracy are in Table S7 in the Supporting Information.

Estimation of the Elimination Half-Life of Nicotelline in Urine of Abstaining Smokers

Urine samples were collected from 3 research subjects over time following smoking cessation, as described previously in a study for determination of the half-life of NNAL.[52] Nicotelline-*N*-oxides were measured as described above under "Determination of Nicotelline N-Oxides in Human Urine." Samples were collected after the last cigarette was smoked, and at 2 time points during the 15-h period following cessation. Half-lives were calculated from semilog plots of concentration vs. time (midpoint of sample collection period), where $t_{1/2} = \ln(2)/k$ and k is the slope of the concentration-time curve. This study was approved by the institutional review boards of the University of California, San Francisco, and the University of Silesia, Poland.

RESULTS

The goal of our studies was to investigate the possibility that nicotelline could be used as a biomarker and an environmental tracer for PM derived from tobacco smoke. Our studies included (1) developing methods for quantitation of nicotelline, and measuring the amounts in MS and SS cigarette smoke; (2) measuring the amounts of nicotelline in the PM and gas phase of aged cigarette smoke; (3) determining the relationships between nicotelline mass and PM mass in aged cigarette smoke to evaluate the potential use of nicotelline for measuring environmental contamination by tobacco smoke; (4) developing a method for determination of nicotelline and metabolites in human urine to evaluate its use as a biomarker, and (5) developing a method for determination of nicotelline in settled house dust, an easily obtained and commonly used matrix for assessing indoor contamination by toxic substances.

Synthesis and Characterization of Analytical Standards

To develop a robust quantitative mass spectrometric method for nicotelline, a stable isotope-labeled internal standard was required. We developed a synthesis of nicotelline-d_8 to use as the internal standard. This method was also used to synthesize unlabeled nicotelline for use as an analytical standard. Synthesis of nicotelline has been reported previously,[43,44] and it is commercially available. Both nicotelline isotopomers were characterized by physical and spectral properties. Melting points were within a few degrees of the published value for natural nicotelline. Analyses for carbon, hydrogen, and nitrogen were in good agreement with theoretical values. The spectrum obtained from an extract of cigarette butts (Figure 1) was virtually identical to the MS/MS product ion spectrum of synthesized nicotelline. Electron ionization mass spectra are in the Supporting Information section (Figure S1). A mixture of mono-*N*-oxides of nicotelline was synthesized by reaction of nicotelline with *m*-chloroperoxybenzoic acid. These were characterized by LC-MS/MS and comparison of spectra to nicotelline *N*-oxides extracted from smokers' urine as described below. Anatalline from a commercial source was characterized by LC-MS/MS and GC-MS analyses and

comparison of spectra with those obtained from tobacco extracts (Figures 1 and 2). The EI spectra (Figure 2) were similar to those reported in the literature for anatalline.[45,53]

Analytical Methods for Nicotelline, Metabolites, and Other Tobacco Alkaloids

A LC-MS/MS method was developed for determination of nicotelline in PM derived from cigarette smoke and in unburned tobacco. The method was extended to include determination of other tobacco alkaloids in aged cigarette smoke collected in chamber studies, and TSNA in house dust. TSNAs were measured because they are largely associated with the particulate matter of tobacco smoke,[4] and are carcinogens of major concern. Extracts from various matrices were analyzed for nicotine using an established GC method.[50,51] An LC-MS/MS method was developed to determine nicotelline metabolites in human urine.

Nicotelline in MS and SS Cigarette Smoke

PM was collected from MS and SS smoke using a smoking machine. Nicotelline and PM in cigarette smoke of 5 US, 5 Polish, and 10 Chinese brands are presented in Table 1. In accord with some but not all previous studies, PM yields per cigarette were higher in SS smoke than in MS smoke.[54–57] PM yields across brands and between different countries did not vary greatly, ranging from 8.8 to 19.8 mg/cigarette in MS smoke, and from 22.5 to 43.8 mg/cigarette in SS smoke, for this selection of mainly “Full Flavor” cigarettes. As with PM, nicotelline yields per cigarette were higher in SS smoke than in MS smoke, ranging from about 600 to 2800 ng/cigarette in MS and about 5500 to 22,000 ng/cigarette in SS smoke. Nicotelline yields in both MS and SS smoke were lower for this selection of Chinese cigarettes than those from the US and Poland.

Partitioning of Nicotelline between the PM and Gas Phase

Two sets of experiments were performed to investigate the partitioning of nicotelline between the PM and gas phases. The first was a simple experiment in which SS smoke was passed through a Cambridge filter, followed by traps containing dilute aqueous acid to capture nicotelline, which is a weak base. The filters were extracted and analyzed for nicotelline in the PM, and the traps were analyzed for gas phase nicotelline. In addition, we measured nicotine, also a basic substance, in filters and traps. Nicotine has been reported to be mainly in the PM of MS cigarette smoke,[57,58] but in SHS/ETS nicotine is present in the both the PM and gas phase.[1,3,59] The results from analyses of two American cigarettes are presented in Table 2. Nicotelline was found only in the PM collected on the Cambridge filters, whereas varying amounts of nicotine passed through the filters and were collected in the acid traps.

A second series of experiments was carried out with a custom-built system for generation, aging, and delivery of cigarette smoke for human exposure studies.[49] Smoke generated using a smoking machine was collected at three points, Sites 2, 4, and 5, Figure S1. The aging time at Site 2 was 3 min, at Sites 4 and 5 the aging time was 30 min. Smoke was collected on an untreated membrane filter connected in series to a second filter treated with sodium bisulfate to collect basic substances present in the gas phase.[1] The experimental design is similar to that used by Hammond *et al*.[1] to investigate partitioning of nicotine between PM and the gas phase of cigarette smoke in a chamber, using tandem filters in which the second was coated with sodium bisulfate. Both filters were analyzed for nicotelline, as well as three other tobacco alkaloids, nicotine, myosmine, and 2,3′-bipyridine. Nicotelline was found almost entirely in the material collected on the first filter. In accord with previous studies reporting that nicotine is in both the PM and in the gas phase of SHS/ETS,[1,3,59] nicotine was found on both filters, mainly on the second filter treated with bisulfate. Myosmine and 2,3′-bipyridine were found mainly on the first filter, but with

significant amounts on the second. The results are presented in Table 3. The data are expressed as mass/m^3 to conform with previous studies of tobacco smoke chemistry.[1,3,60]

Correlation of Nicotelline with PM in Aged Cigarette Smoke

To investigate the possibility that nicotelline could be used as a quantitative marker for measuring environmental contamination by tobacco smoke, cigarette smoke from 5 popular US brands was generated, aged, and collected on membrane filters in the system described above. The smoke was generated for time periods ranging from 2 to 6 h, and collected at sites in the system that provided different elapsed times between smoke generation and collection, and different stages of transport through the system (Sites 2, 4, and 5 in Figure S2, Supporting Information). Thus, the PM samples collected had been exposed to different conditions that have been demonstrated to affect partitioning of substances in the smoke between PM and gas phase and between PM and surfaces. The objective was to determine whether or not the mass of nicotelline correlated with the mass of PM, because changes in PM composition, such as loss of volatiles and/or loss of nicotelline during aging and transport, could limit the utility of nicotelline as a quantitative marker, and would be reflected in a lower correlation coefficient. Smoke was collected from each brand on a separate day, providing 4 to 8 samples per brand, with PM masses ranging from about 0.8 to 8 mg. Linear regression was used to determine correlations between nicotelline (ng) and PM (mg). The data, presented in Table 4, Figure 3, and Figure S3 in the Supporting Information demonstrate an excellent correlation between PM and nicotelline. The age of the smoke (3 min or 30 min) had no effect on the correlation between PM and nicotelline mass.

Origin of Nicotelline: Conversion of Anatalline to Nicotelline during Combustion

Since we could not find published quantitative data on nicotelline in tobacco or in tobacco smoke, we analyzed unburned cigarette tobacco as well as smoke. The results were initially puzzling: amounts of nicotelline were much less, per cigarette, in unburned tobacco than in the smoke. Since incomplete extraction was a possibility, several different extraction procedures for unburned tobacco were evaluated, including acid (aq HCl or acetic acid), pH 4.5 buffer, strong base, sonication, and mechanical mixing. But the result was the same: much less nicotelline was found in the unburned tobacco than in smoke (Table 5). Except for the procedure using pH 4.5 buffer, all procedures gave comparable nicotelline recovery. Extraction data are in Table S2 of the Supporting Information.

Anatalline [*cis*-2,4-di(3-pyridyl)piperidine],[45,53] another little-studied tobacco alkaloid, has structural similarities to nicotelline (Chart 1). In anatalline, the central ring is a piperidine instead of a pyridine ring. It occurred to us that anatalline might be converted to nicotelline by oxidative processes during combustion, and if anatalline were present in higher concentrations than those of nicotelline in unburned tobacco, this could explain the higher levels of nicotelline found in smoke. This was indeed found to be the case. We measured anatalline and nicotelline in unburned tobacco and in PM of cigarette smoke. Anatalline concentrations in cigarette tobacco were about 100 times higher than nicotelline concentrations, but nicotelline yields were about 2–3 times higher than anatalline yields in the PM of cigarette smoke (Table 5, Table S2, Supporting Information). Conversion of anatalline to nicotelline during combustion was demonstrated by spiking anatalline into a “cigarette” prepared from oregano, and measuring nicotelline in the smoke generated from its burning (Table 5). From 30 μg anatalline added to the oregano, 2.2 μg nicotelline were recovered from the PM. Nicotelline was not detected in the PM from an oregano “cigarette” that was not spiked with anatalline.

Nicotelline Metabolites in Urine of Smokers

Because nicotelline is a tripyridine, we surmised that it would be metabolized mainly to *N*-oxides, and this appears to be the case. Little if any unchanged nicotelline was detected in urine of smokers. However, treatment of smokers' urine with titanium trichloride, a reagent that converts *N*-oxides to the parent amines,[61] resulted in readily measurable concentrations of nicotelline (Figure 6). Furthermore, we synthesized a mixture containing at least two of the three possible mono-*N*-oxide regioisomers, and showed that at least two of these isomers are excreted in urine of smokers. LC retention times and MS/MS product ion spectra of the standard and those from a smoker's urine extract were in good agreement (Figures 4 and 5). Interestingly, the isomeric composition in smokers' urine was different from the synthesized standards and individual differences were apparent, as shown in Figure 5. As *N*-glucuronides are possible metabolites as well, we incubated smokers' urine samples with beta-glucuronidase, but nicotelline was not detected in the post-deconjugation incubates. Concentrations of *N*-oxide metabolites were measured in urine of smokers before and after smoking cessation, and used to make an estimate of the half-life. Prior to cessation, concentrations in 3 subjects ranged from about 350 to 600 pg/mL, declining with a half-life about 2 hours (Figure 7).

Nicotelline, NNN, NNK, and Nicotine in House Dust of Smokers and Non-Smokers

To further evaluate the applicability of nicotelline as an environmental tracer, we analyzed house dust from homes of smokers and non-smokers (Figure 8). Nicotine was detected in dust from all homes. Nicotine in house dust from smokers' homes has been previously reported.[62,63] Nicotelline was detected in all homes except one sample from a non-smoker's home. Levels were much higher in homes of smokers than of non-smokers. The TSNAs, NNN and NNK, which are carcinogens of concern and have been reported to be mainly in the PM of tobacco smoke[4] were found in smokers' homes and in some non-smokers' homes. House Dust Standard Reference Material® 2585 from the National Institute of Standards & Technology (NIST) contained nicotine, nicotelline, and TSNA levels that indicated some of the sources originated from venues where smoking was allowed. (Table 6).

DISCUSSION

In this report we describe experiments supporting the utility of nicotelline as an environmental tracer and biomarker for the PM derived from tobacco smoke.

Nicotelline yields did not vary greatly among several popular brands of US and Polish cigarettes for both MS and SS smoke, but yields were somewhat lower in the Chinese cigarettes that we studied, which were herbal sub-brands of a brand popular in southern China (Table 1). Nicotelline appears to have high specificity for tobacco. To our knowledge, other sources of nicotelline have not been reported. *Duboisia hopwoodii*, a plant found in Australia that contains nicotine in sufficient quantities that Aborigines have used it for its stimulant effects,[64] also contains anatalline,[65] which could oxidize to nicotelline. Nicotine and other tobacco alkaloids have been found in various plants, and other sources of nicotelline cannot be ruled out.[66,67]

To study nicotelline partitioning between the PM and gas phase after cigarette smoke is released into the environment, we used a custom built system for generating and aging cigarette smoke to collect PM and basic substances in the gas phase of smoke derived from 5 popular US brands. Nicotelline was found almost entirely in the PM (Table 3). In Table 3 we present data for nicotine, 2,3′-bipyridine, and myosmine as well as for nicotelline. All are basic substances, and we measured them in order to validate our method for separating PM from the gas phase and for collecting nicotelline present in the two phases. Our method is

based on that of Hammond *et al.*, in which tandem filters, a particle filter followed by a bisulfate treated filter, were used to collect nicotine in the PM and gas phases.[1] A concern with collecting gas phase components downstream from a particle filter is that adsorption of gas phase components on the particle filter might occur. Studies of nicotine partitioning have addressed this issue by the use of denuders or base-treated filters to minimize vapor-phase nicotine adsorption.[59,68,69] However, we believe that our data for the other alkaloids argue against the possibility that significant amounts of gas phase nicotelline were adsorbed on the particle filter. Although very little nicotelline was collected on the second, bisulfate treated filter, substantial amounts of the other three alkaloids were trapped on the second filter. If gas phase nicotelline were being quantitatively adsorbed on the first filter, one would expect the same for 2,3′-bipyridine, for which a substantial amount was found on the second filter. The two alkaloids, one a bipyridine and the other a tripyridine, would be expected to have similar adsorptive properties. The capture of 2,3′-bipyridine by the second, bisulfate-treated filter argues against the possibility that the bisulfate was not acidic enough to capture vapor-phase nicotelline (calculated pKa 3.69), as the two alkaloids have similar calculated pKa values (Table 7).

Nicotelline mass and PM mass were highly correlated (Table 4, Figure 3, and Figure S3 in the Supporting Information). This supports our proposal that nicotelline can be used to estimate the extent of environmental contamination by tobacco smoke PM. The regression equation for the line presented in Figure 3, which represents data from 5 brands of US cigarettes, for PM collected at various stages of transport through the smoke generation/aging system, and different times (3 or 30 min) between generation and collection, provides a relationship between nicotelline mass and PM mass that might be used for such an estimate:

$$\text{Tobacco Smoke PM (mg)} = (0.00064)(\text{ng Nicotelline}) + 0.048 \quad (1)$$

However, we should caution that further studies would be desirable to confirm our findings under conditions more typical of real-world environmental conditions.

We also used the data in Table 3 to calculate gas/particle partitioning constants K_p (m^3 μg^{-1}) as described by Pankow *et al.*

$$K_p = c_p / c_g \quad (2)$$

where c_p (ng μg^{-1}) is the concentration in the particle phase and c_g (ng m^{-3}) is the concentration in the gas phase (equation 2).[3,60] Theory predicts that volatility is the major factor in determining gas/particle partitioning of substances in tobacco smoke. Compounds with relatively low volatility have higher K_p values than volatile substances, and exist predominantly in the particle phase (PM) of the smoke.[60] The logs of our calculated K_p values for nicotelline, nicotine, myosmine, and 2,3′-bipyridine are in Table 7. The K_p calculated for nicotelline was about 4 orders of magnitude greater than that of nicotine. Our log K_p for nicotine, −4.18, compares favorably to the range of log K_p values (−2.83 to −4.93) reported by Pankow *et al*. for pH-adjusted SHS/ETS.[3] The log K_p value of −2.83 was for SHS/ETS collected in a large public hall. Samples were treated with ammonia at partial pressures ranging from 0 to 127×10^{-6} atmosphere, which resulted in a minimum log K_p value of −4.93. The pH of the smoke, which can change during aging, has a large influence on the K_p of nicotine.[3] Loss of volatile bases during aging causes the pH to decrease, and K_p to increase, because a greater percentage of nicotine is in the less volatile protonated form. Our log K_p value of −4.18 is consistent with some loss of basic substances during aging, but less so than in Paknow *et al.*'s "real world" SHS/ETS (log Kp = −2.83) that was presumably

more extensively aged smoke that had been exposed to other environmental conditions. We should also point out that K_p values may differ between brands due to differences in composition of the smoke, and that smoke dilution may affect composition and the K_p.

The K_p values and PM (TPM) concentrations in μg m^{-3} can be used to calculate the fraction in the particle phase (f_p) (equation 3).[60]

$$f_p = (K_p\,\mathrm{TPM})/(1+K_p\,\mathrm{TPM}) \quad (3)$$

We calculated f_p for nicotelline, nicotine, myosmine, and 2,3′-bipyridine (Table 7), which indicated that nicotelline is almost entirely in the PM, with f_p = 0.998. We should caution, however, that the concentrations of nicotelline in the gas phase were so low that the amounts collected on the bisulfate treated filters were near the detection limit of the analytical method, potentially compromising the precision and accuracy of the determination of K_p. But the amounts of nicotelline collected on the particle filters were sufficiently high (100 to 500 times the lower limit of quantitation) that there is little doubt that nicotelline is almost entirely in the PM (Table 3). We should also point out that, strictly speaking, calculations of K_p and f_p are for equilibrium measurements,[60] and our PM and gas phase collections made under conditions of constant flow might not have been under equilibrium conditions. Nevertheless, the good agreement for the nicotine K_p that we calculated compared to the values determined by Pankow *et al*.[3] suggests that our method provided reasonable estimates of K_p and f_p. We should also point out that f_p is dependent on the concentration of PM (equation 3). Our K_p and f_p values are means of 6 determinations calculated using the data in Table 3. PM concentrations were relatively constant, mean 2112 μg m^{-3}, RSD = 27%.

The utility of nicotelline as a biomarker of exposure to tobacco smoke PM is supported by our demonstration that nicotelline is predominantly in the PM phase, and that its metabolites can be measured in urine of smokers. Nicotelline has other attributes that make it an attractive biomarker. Its chemical structure, a tripyridine, suggests that it should not undergo extensive metabolism. This led us to predict, and the results of our experiments support our prediction that nicotelline is metabolized predominately to *N*-oxide metabolites. We found that a mixture of at least 2 of the 3 possible regioisomeric *N*-oxides is excreted in smokers' urine. These metabolites can be reduced and analyzed as nicotelline, an advantage for exposure assessment. Substances with complex biotransformation pathways leading to several metabolites are not ideal biomarkers. Individual differences in metabolism would likely produce different ratios of metabolites, and measuring a single metabolite may not provide a good estimate of exposure. Measuring a total of the most plentiful metabolites, if known, may avoid this problem, but would complicate the analytical chemistry. Demonstrating that the mixture of *N*-oxides, that likely represent a high percentage of absorbed nicotelline, can be determined by reduction with titanium trichloride supports the utility of nicotelline as a biomarker of exposure.

We also present evidence that nicotelline can be generated from anatalline, another little-studied tobacco alkaloid, by dehydrogenation during combustion. Dehydrogenation of anatalline with a palladium catalyst at high temperature has been reported.[53] Formation of nicotelline from anatalline suggests the possibility that the nicotelline present in unburned tobacco could be formed by oxidation of anatalline during curing and aging. Leete *et al.* proposed that nicotelline is not a biosynthetic product in the tobacco plant, suggesting that its formation involves trimerization of dihydro- and tetrahydro-pyridines.[70] To our knowledge, the complete biosynthetic pathway leading to anatalline has not been elucidated. It has been suggested that anabasine or anatabine is a biosynthetic precursor of anatalline.[45]

Anatalline is produced in cell cultures of *Nicotiana tobacum*,[45,71] at concentrations higher than those of nicotine, and a geometric isomer has been detected as well.[45] In our extracts of tobacco analyzed by GC-MS or LC-MS/MS we did not find evidence of an isomeric substance, although it is possible that an isomer, if present, was not separated by our chromatographic systems.

There are some limitations to our study. We did not have the resources to generate and analyze smoke from a large number of cigarette brands that would be representative of those consumed by smokers. However, the slopes of the regression lines obtained in the correlations of PM with nicotelline, for 5 popular American cigarette brands ranged from 0.000581 to 0.000831, which suggests that PM/nicotelline ratios are not highly variable (Table 4). Future studies of a wider range of cigarette brands, with comparison to other smoke constituents determined using FTC, Health Canada, ISO, or CORESTA protocols would be desirable. Studies of the conversion of anatalline to nicotelline, and reactions of anatalline with other tobacco constituents that might occur during tobacco curing and processing or during combustion, including quantitative studies using isotope-labeled anatalline would also be desirable.

The data in Table 1 were obtained from MS and SS smoke generated using a smoking machine. In the environment, tobacco smoke undergoes physical and chemical changes, such as loss of volatiles and changes in particle size distribution.[72] Therefore, it is not surprising that the PM/nicotelline ratio for MS and SS smoke was higher than the ratio for smoke from the generation/aging system that had been diluted with air and collected 3 or 30 min after generation. For fresh MS and SS smoke, using data in Table 1 for US cigarettes, the PM/nicotelline ratios (mg/ng) were 0.0086 and 0.0021, respectively. From the data used to prepare Figure 3, for PM collected 3 or 30 min after generation, the ratio was 0.00065. The latter value is probably a better model for real-world environmental conditions, and it was gratifying to find such a good linear relationship between nicotelline and particulate matter (Figure 3) when the points represented different aging times, different collection points, different smoke generation times, and five brands of US cigarettes.

We should also point out that rigorous determination of the partitioning (K_p) of nicotelline between the PM and gas phases may require experiments carried out under equilibrium conditions,[60] and the use of denuders or other techniques to minimize the possibility that vapor-phase nicotelline is adsorbed on the filters.[59,68,69] Additional studies of partitioning between the phases would be desirable, including studies of partitioning in particles of different sizes. Studies of the environmental stability of nicotelline would also be desirable, as well as structural characterization of the *N*-oxide metabolites.

Due to the well-documented hazards of SHS/ETS exposure,[73] and current interest in the potential hazards of exposure to aged tobacco smoke residues, termed “thirdhand smoke” (THS),[8] there is continuing interest in improving methodology for measuring tobacco smoke contamination of the environment. We found that nicotelline can be measured in settled house dust from homes of smokers, and in some homes of non-smokers at levels much lower than those found in homes of smokers. As PAHs of low volatility can persist in carpets for years or decades,[74,75] the low volatility of nicotelline suggests that it also may persist for long periods, and would be suitable for assessment of long-term tobacco smoke contamination. But even though the vapor pressure is low (Table 7) nicotelline could nevertheless volatilize over time.

In summary, we present data supporting the utility of nicotelline as a biomarker and tracer for tobacco smoke derived PM, and experiments demonstrating that nicotelline can be formed from anatalline during combustion. Other than our recent report of nicotelline in

extracts of cellulose strips that had been exposed to cigarette smoke,[35] data on concentrations of nicotelline and anatalline in tobacco smoke do not appear to have been published. We are unaware any studies of the pharmacology and toxicology of these two alkaloids, except for a study of nicotelline as a potential cytochrome P450 inhibitor.[76] Our data on the presence of TSNAs in house dust is also significant. It has been shown that settled house dust can be a source of exposure to toxic substances,[74,75,77] but until very recently the presence of TSNAs in house dust had not been reported.[78] Future studies will include applications of nicotelline as a tracer for tobacco smoke in the environment, and efforts to improve analytical sensitivity for measuring urine concentrations in non-smokers, in order to utilize it as a biomarker in studies of SHS and THS exposure. We will also explore the applicability of anatalline and/or its metabolites as biomarkers of tobacco use and tobacco smoke exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

APCI	Atmospheric Pressure Chemical Ionization
BLQ	Below Limit of Quantitation
ISO	International Organization for Standardization
ISTD	Internal Standard
LLOQ	Lower Limit of Quantitation
MS	Mainstream
NNK	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN	N′-nitrosonornicotine
PICI	Positive Ion Chemical Ionization
PM	Particulate Matter
SHS	Secondhand Smoke
SRM	Selected Reaction Monitoring
SS	Sidestream
TPM	Total Particulate Matter

TSNA Tobacco-Specific Nitrosamine

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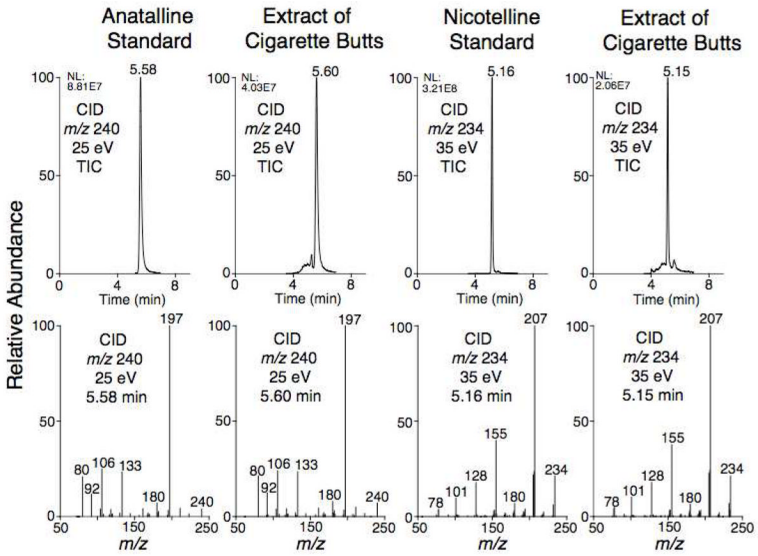


Figure 1.

LC-MS/MS chromatograms (upper panels) and product ion spectra (lower panels) of nicotelline and anatalline extracted from cigarette butts, and corresponding chromatograms and spectra of reference standards.

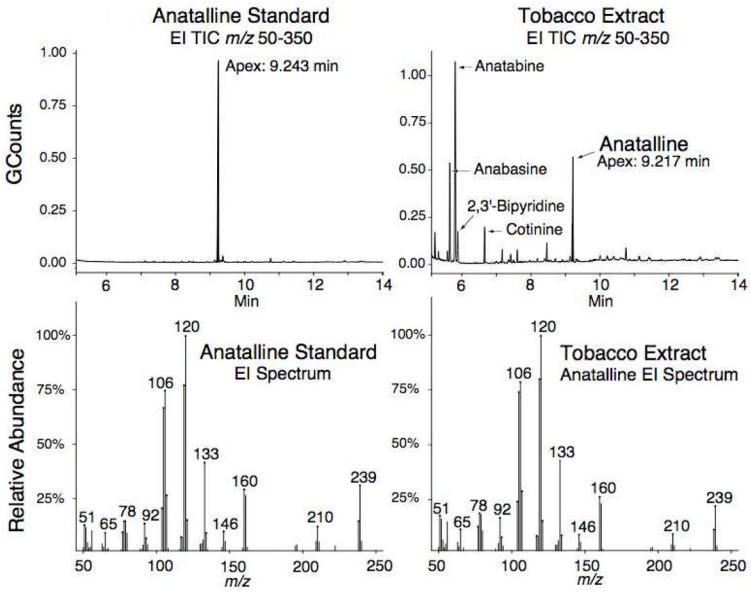


Figure 2.
GC-EI-MS chromatograms and spectra of anatalline standard and tobacco extract.

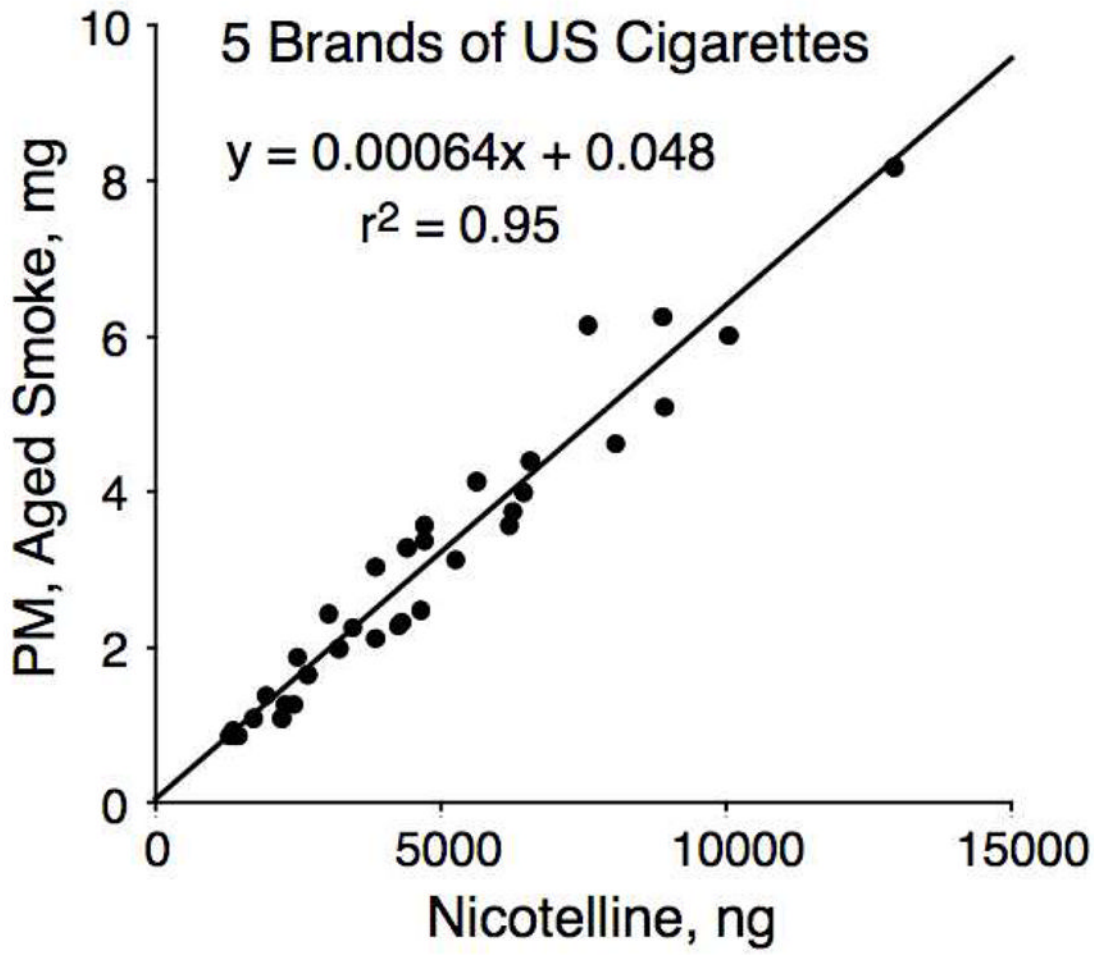


Figure 3.
Correlation of nicotelline mass with PM mass in aged cigarette smoke from 5 US Brands. Smoke was generated from multiple cigarettes for each brand for times ranging from 2 to 6 h, and collected at two sampling ports in the smoke generation/aging system, corresponding to 3 or 30 min after generation. See Experimental Procedures section for details.

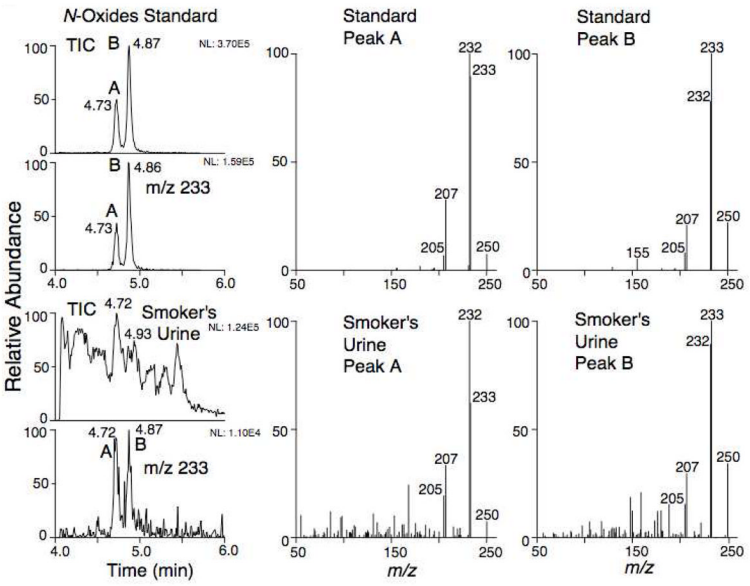


Figure 4.

LC-MS/MS chromatograms and product ion spectra of nicotelline *N*-oxides extracted from a smoker's urine sample, and corresponding chromatograms and spectra from synthesized nicotelline *N*-oxides.

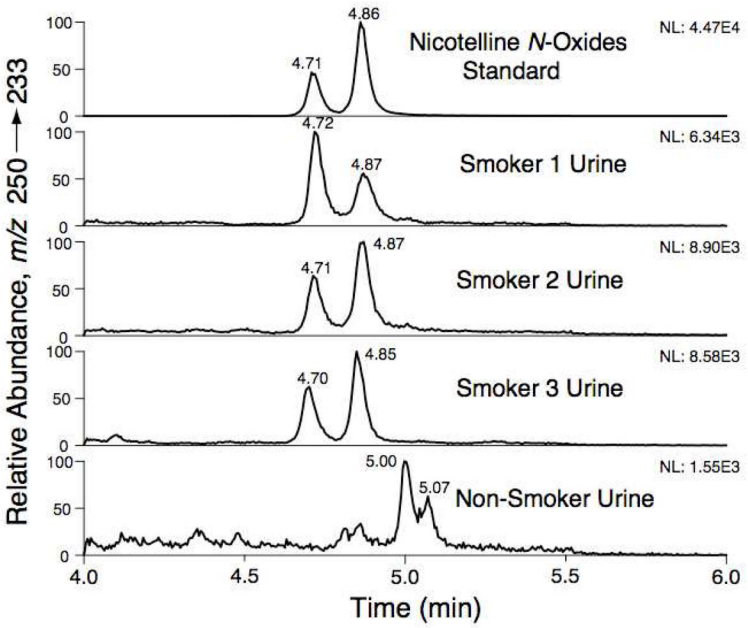


Figure 5.
LC-MS/MS SRM chromatograms of nicotelline *N*-oxides extracted from smokers' urine, and chromatogram of synthesized nicotelline *N*-oxides.

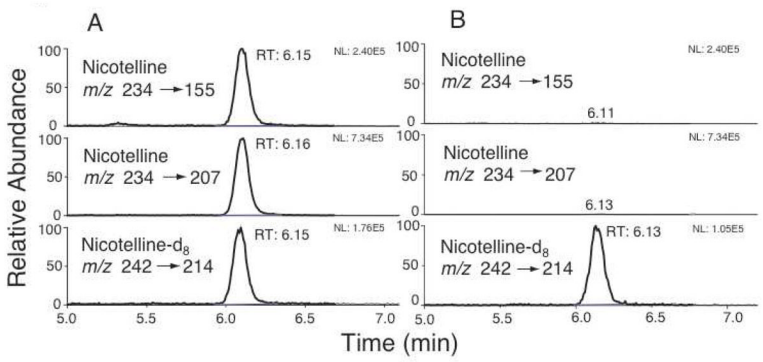


Figure 6.

LC-MS/MS chromatograms of a smoker's urine extracts treated with titanium trichloride before extraction. A, during smoking; B, following cessation. The respective Y-axis maxima are the same on both A and B for the two respective SRM chromatograms of natural nicotelline.

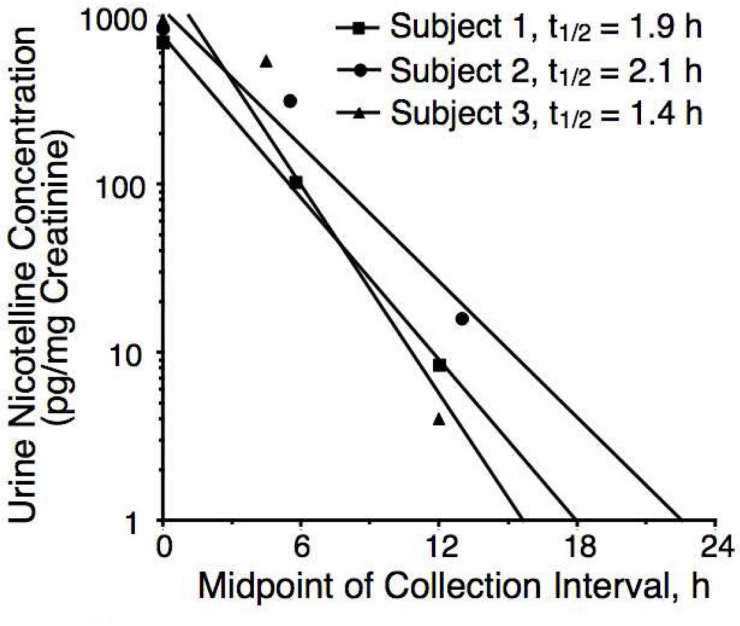


Figure 7.
Concentrations of nicotelline in titanium trichloride treated urine samples from three human subjects before and after smoking cessation.

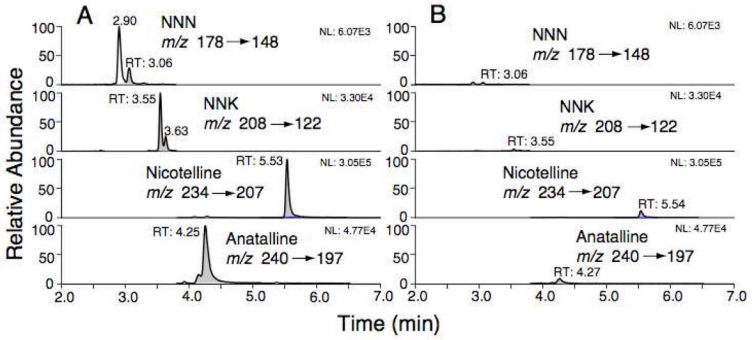
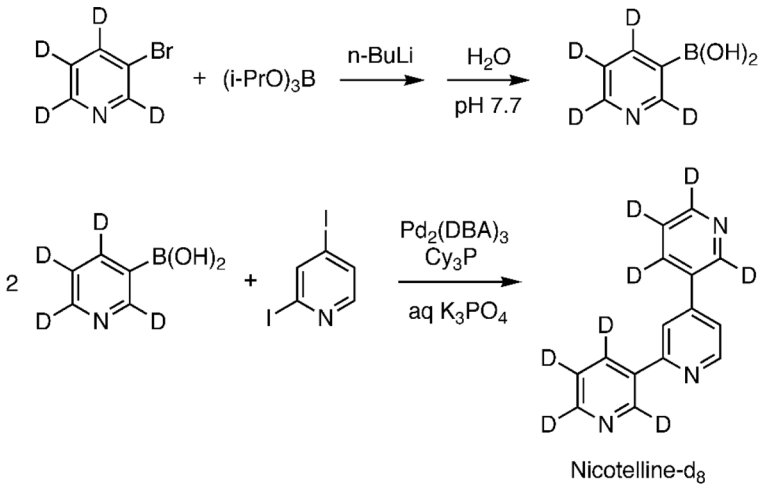


Figure 8.

LC-MS/MS chromatograms of house dust extracts containing nicotelline, anatalline, NNN, and NNK. A, from a smoker's home; B, from a non-smoker's home. The respective Y-Axis maxima are the same on both A and B to illustrate the difference between the smoker's home and the non-smoker's home. Concentrations are in Table 6, Smoker's Home #1, and Non-Smoker's Home #2. NNK consists of two peaks, because it is a mixture of *syn*- and *anti*-isomers. The isomers equilibrate and cannot be isolated, but the rate of equilibration at room temperature is slow on the time scale of the HPLC separations, resulting in two peaks. NNN likewise consists of two isomers, but these were not separated under our HPLC conditions.



Scheme 1.
Synthesis of Nicotelline-d_8

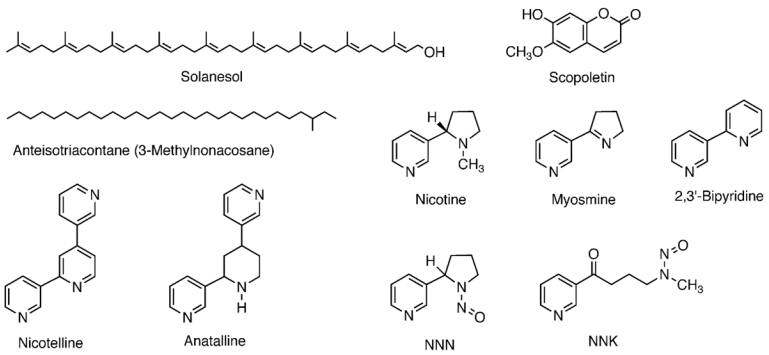


Chart 1.
Structures of Tobacco Alkaloids, TSNA, and Tracers of Tobacco Smoke Particulate Matter.

Table 1

Nicotelline and Particulate Matter (PM) in Mainstream and Sidestream Cigarette Smoke[a]

Cigarette Brands	Mainstream PM, mg/cig	Mainstream Nicotelline, ng/cig	Sidestream PM, mg/cig	Sidestream Nicotelline, ng/cig
US Cigarettes[b]				
B&H 100s	16.7 (1.5)	1375 (145)	40.5 (2.4)	12980 (629)
GPC Full Flavor 100s	16.2 (1.7)	1139 (60)	31.1 (1.7)	9323 (360)
Kool 100s Menthol	19.8 (1.1)	1081 (65)	43.8 (2.7)	10960 (658)
Marlboro Red Flip-Top hard Box	15.6 (2.0)	2833 (400)	27.2 (2.2)	20560 (1870)
Marlboro Lights King Size	12.2 (1.4)	2379 (216)	29.1 (4.1)	22440 (1320)
Mean	**16.1 (2.7)**	**1761 (795)**	**34.3 (7.4)**	**15250 (5890)**
Polish Cigarettes[b]				
Marlboro Red Hard Box PL	13.5 (3.0)	2209 (332)	28.8 (2.1)	22260 (697)
Marlboro Light Hard Box PL	8.8 (1.0)	1540 (140)	27.1 (3.5)	19760 (1100)
LM Red Hard Box PL	11.7 (0.9)	1571 (99)	29.1 (2.4)	14160 (1140)
LM Blue Hard Box PL	10.4 (1.1)	767 (84)	34.3 (4.3)	8229 (929)
LM Green Menthol Hard Box PL	9.4 (0.1)	626 (64)	32.3 (2.1)	7154 (788)
Mean	**10.8 (1.9)**	**1342 (649)**	**30.3 (2.9)**	**14310 (6730)**
Chinese Cigarettes[c]				
A	14.0 (3.0)	877 (48)	23.7 (1.9)	6753 (449)
B	15.2 (1.9)	928 (38)	24.4 (2.1)	7597 (975)
C	15.8 (2.5)	975 (69)	24.5 (0.7)	8103 (436)
D	16.3 (1.7)	838 (91)	26.2 (2.1)	6675 (748)
E	14.6 (2.4)	832 (91)	26.2 (2.1)	5648 (1750)
F	15.4 (1.6)	899 (70)	24.8 (2.2)	7134 (632)
G	19.3 (3.4)	1116 (155)	27.6 (3.6)	9062 (926)
H	10.4 (1.2)	634 (80)	22.5 (4.5)	7171 (888)
I	15.3 (1.3)	795 (72)	22.8 (3.2)	5520 (158)
J	17.5 (3.5)	939 (56)	23.9 (1.3)	6590 (385)
Mean	**15.4 (2.3)**	**883 (125)**	**24.4 (1.5)**	**7025 (1070)**
Kentucky Reference 3R4F[d]	**10.6 (0.8)**	**1070 (156)**	**26.7 (2.7)**	**14860 (1620)**

[a] Smoke was generated using a smoking machine, with puff parameters and smoke collection similar to ISO 3308 and ISO 4387for mainstream smoke and ISO 20773 for sidestream smoke. See Experimental Procedures section.

[b] For US and Polish brands, the values are a mean (SD) for 5 individual cigarette analyses.

[c] Chinese cigarettes were 10 herbal sub-brands of a brand popular in southern China. Chinese herbal cigarettes are prepared from tobacco, with powdered herbal medicines or their extracts added to the tobacco filler. Values are a mean (SD) for 10 cigarettes.

[d] The PM value for the 3R4F Kentucky Reference Cigarettes is a mean (SD) of 4 cigarettes for mainstream and a mean of 5 (SD) for sidestream smoke. The nicotelline values are a mean (SD) for 15 cigarettes. University of Kentucky product specification for TPM is 10.9 (0.2).

Table 2

Nicotelline and Nicotine in the Particulate Matter and in the Gas Phase of Sidestream Smoke[a]

Cigarette, Fraction	Nicotelline, μg/cig	Nicotine, mg/cig
Marlboro		
PM (Filter)	16.7	2.98
Gas Phase (Acid Traps)	ND	1.32
Benson & Hedges		
PM (Filter)	16.6	2.73
Gas Phase (Acid Traps)	ND	0.14

[a] Sidestream smoke was collected by passing through a Cambridge filter, which was connected in series to four traps, with fritted glass dispersion tubes immersed in 50 mL 0.1 M HCl in 50% aqueous methanol. Extracts of the filters and aliquots of the aqueous methanol were analyzed. The LLOQs for nicotelline and nicotine were 0.05 μg/cig and 0.00005 mg/cig, respectively.

Table 3

Nicotelline, Nicotine, Myosmine, and 2,3′-Bipyridine in the Particulate Matter and the Gas Phase of Aged Cigarette Smoke[a]

Sampling Time	Smoke Aging Time[b]	Particulate Matter μg/m^3	Nicotelline Particle Filter (1st) μg/m^3	Nicotelline Treated Filter (2nd) μg/m^3	Nicotine Particle Filter (1st) μg/m^3	Nicotine Treated Filter (2nd) μg/m^3	Myosmine Particle Filter (1st) μg/m^3	Myosmine Treated Filter (2nd) μg/m^3	Bipyridine Particle Filter (1st) μg/m^3	Bipyridine Treated Filter (2nd) μg/m^3
2 h	3 min	2980	3.50	0.0152	8.14	99.9	0.80	0.64	0.62	0.26
2 h	30 min	1717	2.87	0.0057	3.68	24.2	0.39	0.09	0.08	0.02
4 h	3 min	2781	3.86	0.0066	9.15	73.2	0.81	0.32	0.53	0.10
4 h	30 min	1852	3.16	0.0024	3.65	25.6	0.36	0.08	0.08	0.03
6 h	3 min	2372	3.82	0.0059	15.0	84.6	1.10	0.43	0.65	0.12
6 h	30 min	1567	2.62	0.0026	4.48	21.9	0.32	0.10	0.08	0.03
Mean		**2212**	**3.31**	**0.0064**	**7.35**	**54.9**	**0.63**	**0.28**	**0.34**	**0.09**
RSD, %		**27**	**15**	**73**	**60**	**64**	**51**	**82**	**84**	**100**

[a] Cigarette (Marlboro Red) smoke was generated from multiple cigarettes with a smoking machine, mixed, and aged in the smoke generation/aging system (see Experimental Procedures section and diagram in Supporting Information, Figure S2). Smoke was generated and collected over times ranging from 2 to 6 hours using tandem filters. The first was a PTFE coated glass fiber filter to collect > 0.3 μm particles, the second was a similar filter impregnated with sodium bisulfate to trap basic compounds not retained by the particle filter (gas phase). The 3 min aging collection point was from Site 2 “pre-aging,” meaning before the aging chamber, collected after mixing from the ducting upstream from the aging chamber. The 30 min collection point was from Site 4 “pre-subject,” collected from the ducting downstream from the aging chamber.

[b] Aging time refers to the time elapsed from generation by the smoking machine and sample collection.

Table 4

Pearson Correlations and Slopes of Regression Lines for Nicotelline (ng) and Particulate Matter (mg) in Aged Cigarette Smoke from 5 US Brands[a]

Brand	r^2	Slope
Camel	0.971	0.000719
Camel Blue	0.957	0.000831
Marlboro Gold	0.995	0.000582
Marlboro Red	0.972	0.000581
Newport	0.989	0.000616
All Brands	**0.948**	**0.000634**

[a]Smoke was generated from multiple cigarettes for each brand for times ranging from 2 to 6 h, and collected at two sampling ports in the smoke generation/aging system, corresponding to 3 or 30 min after generation. See Experimental Procedures section for details. Correlation coefficients were not different by paired t test

Table 5

Conversion of Anatalline to Nicotelline: Concentrations and Yields of the Two Alkaloids in Tobacco and in Sidestream Smoke

Products	Anatalline Tobacco	Nicotelline Tobacco	Anatalline Smoke	Nicotelline Smoke
Normalized on Weight Basis	**μg/g**	**μg/g**	**ng/mg**	**ng/mg**
Marlboro-27	132	0.87	198	745
Benson and Hedges	78	0.76	189	602
Amounts per Cigarette	**μg/cig**	**μg/cig**	**μg/cig**	**μg/cig**
Marlboro-27	79.8	0.53	4.63	17.4
Benson and Hedges	53.6	0.53	4.81	15.3
Oregano Cigarette[a]	n.a.	n.a.	n.a.	ND
Oregano Cigarette Spiked with 30 μg Anatalline[a]	n.a.	n.a.	n.a.	2.2

[a]Dried oregano from a grocery store was used to prepare the oregano “cigarettes.” A solution of anatalline in methanol was applied to oregano before preparing one of the oregano cigarettes.

Abbreviations: n.a., not analyzed; ND, none detected.

Table 6

Nicotine, Nicotelline, and TSNA Concentrations in House Dust, ng/g (ppb)[a]

Sample	Nicotine	Nicotelline	NNK	NNN
Smokers' Homes				
Smoker's Home #1	174,000	297	131	64.9
Smoker's Home #2	22,400	48.4	36.9	5.97
Mean	**98,200**	**173**	**84.0**	**35.4**
Non-Smokers' Homes[b]				
Non-Smoker's Home #1	3,230	8.13	1.93	1.25
Non-Smoker's Home #2	4,730	2.01	1.55	1.12
Non-Smoker's Home #3, Sample 1	729	1.1	4.98	1.83
Non-Smoker's Home #3, Sample 2	335	0.76	1.29	BLQ
Non-Smoker's Home #3, Sample 3	909	1.25	6.14	1.75
Mean	**1,990**	**2.65**	**3.18**	**1.27**
NIST SRM #2585, House Dust	**18,500**	**78.4**	**29.4**	**12.7**
Clay Soil	**11**	**BLQ**	**BLQ**	**BLQ**

[a]Values are the average of 3 to 4 replicate determinations. Lower limits of quantitation (LLOQs) for nicotine, nicotelline, NNK, and NNN were 10 ppb, 0.15 ppb, 0.3 ppb, and 0.6 ppb, respectively. BLQ = below lower limit of quantitation.

[b]Non-Smoker's home #3 samples are from 3 different vacuum cleaners used for different parts of the house.

Table 7

Calculated Values of Gas/Particle Partitioning Constants (K_p)[a] and Fraction in the Particulate Matter (f_p)[a] of Aged Cigarette Smoke, and Literature Values for other Properties[b,c] of Nicotelline, Nicotine, Myosmine, and 2,3′-Bipyridine

Alkaloid	Log K_p	f_p	Log *p*	MW	pKa
Nicotine	−4.18	0.13	−1.52	162	8.00
Myosmine	−2.88	0.73	−1.33	146	6.66
2,3′-Bipyridine	−2.77	0.78	−2.57	156	3.25
Nicotelline	−0.54	0.998	−6.05	233	3.69

[a] K_p and f_p calculated using the data in Table 3, arithmetic mean of the 6 determinations, 2 sites and 3 smoke generation times

[b] From Chemical Abstracts, calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994–2012 ACD/Labs)

[c] *p* = calculated vapor pressure