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Article

3-Ethenylpyridine Measured in Urine of Active and Passive Smokers: A Promising Biomarker and Toxicological Implications

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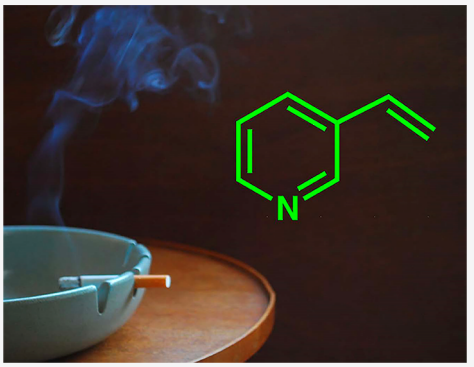
ABSTRACT: In studies of tobacco toxicology, including comparisons of different tobacco products and exposure to secondhand or thirdhand smoke, exposure assessment using biomarkers is often useful. Some studies have indicated that most of the toxicity of tobacco smoke is due to gas-phase compounds. 3-Ethenylpyridine (3-EP) is a major nicotine pyrolysis product occurring in the gas phase of tobacco smoke. It has been used extensively as an environmental tracer for tobacco smoke. 3-EP would be expected to be a useful tobacco smoke biomarker as well, but nothing has been published about its metabolism and excretion in humans. In this Article we describe a solid-phase microextraction (SPME) GC-MS/MS method for determination of 3-EP in human urine and its application to the determination of 3-EP in the urine of smokers and people exposed to secondhand smoke. We conclude that 3-EP is a promising biomarker that could be useful in studies of tobacco smoke exposure and toxicology. We also point out the paucity of data on 3-EP toxicity and suggest that additional studies are needed.



INTRODUCTION

Numerous toxic substances are present in tobacco smoke.[1,2] Tobacco smoke is an aerosol, a mixture of particles and gases that include vapors of volatile organic compounds (VOCs). Both the gas phase and the particulate matter contain harmful substances that may be causative agents for the major diseases associated with smoking, including lung and heart disease and cancer.[3−5] Some modeling studies suggest that most of the toxicity of tobacco smoke is due to gas-phase substances.[6−9] Because gas-phase compounds may distribute differently in the environment[10] than particle-phase compounds, and because they may have different modes of absorption into the body,[11] specific biomarkers and environmental tracers for both particulate matter and the gas phase are desirable in studies of tobacco smoke exposure and toxicity.[12]

3-Ethenylpyridine (3-EP) is a pyrolysis product of nicotine and possibly other tobacco alkaloids (Scheme 1).[13,14] The presence of 3-ethenylpyridine in tobacco smoke has been known for many years.[15−17] Reported concentrations in mainstream (MS) cigarette smoke (inhaled by the smoker) are in the range ∼4−30 μg/cigarette. Concentrations in sidestream (SS) cigarette smoke (emitted by the smoldering cigarette) are much higher, ∼200−600 μg/cigarette (Table 1).[1,4,18−21] Because 3-EP has high specificity for tobacco smoke and is present in high concentrations in SS smoke, it has been widely used as an environmental tracer for secondhand smoke (SHS).[4,22−29] It is mainly in the gas (vapor) phase of tobacco smoke.[1,14] 3-EP has emission rates and distribution characteristics that are similar to VOCs in tobacco smoke that have other sources as well, and therefore it has been proposed as an environmental tracer for VOCs derived from tobacco smoke.[4]

A number of tobacco smoke VOCs have been measured as metabolites in the urine of smokers[31−33] and people exposed to secondhand smoke (SHS),[34] but none are tobacco-specific. 3-EP is tobacco-specific, and if 3-EP or its metabolites could be measured in human biofluids, they would be promising candidates for gas-phase biomarkers. In this article, we (1) describe an analytical method for measuring 3-EP in human

Scheme 1. Formation of 3-EP by Pyrolysis of Nicotine

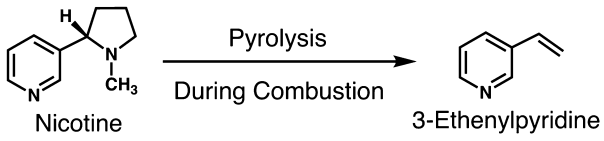


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Table 1. Concentrations of 3-EP in Mainstream and Sidestream Cigarette Smoke (μg/cig)

study	cigarette brand/ type	mainstream	sidestream	SS/ MS
Koszowski et al. 2009[18]	3R4F reference cigarette[a]	4.1	219	53
Koszowski et al. 2009[18]	full flavor[a]	9.5	531	56
Sakuma et al. 1984[19]	burley tobacco[a]	13.2	451	34.2
Brunnemann et al. 1978[20]	U.S., unfiltered[b]	23	640	28
Brunnemann et al. 1978[20]	U.S. cigar[b]	30	2550	85
Kulshreshtha et al. 2003[21]	full flavor	4.57		
Hoffmann et al. 2001[1]	unfiltered[c]	7−30		
Hodgson et al. 1996[4]	reference 1R4F[a]		680	
Hodgson et al. 1996[4]	6 commercial brands[a]		450−890	
Cancelada et al. 2019[30]	IQOS[d]	2−3	0.03−0.05	

[a]Both gas and particle phases. [b]Measured as a mixture with 3,4-lutidine. [c]Vapor phase. [d]Heat not burn product, μg/heatstick.

urine using headspace solid-phase microextraction (SPME) gas chromatography−tandem mass spectrometry (GC-MS/MS); (2) present data on the concentrations of 3-EP in the urine of nonsmokers and of people using various combusted (cigarettes and cigars) and noncombusted (smokeless and e-cigarettes) tobacco products and secondhand-smoke exposure that support its potential as a biomarker of tobacco smoke exposure; and (3) propose that toxicological studies of 3-EP are needed.

EXPERIMENTAL PROCEDURES

Chemicals. 3-Ethenylpyridine and 3-ethenylpyridine-d_4 were synthesized as described below. Both unlabeled 3-ethenylpyridine and 3-ethenylpyridine-d_4 can be purchased from Toronto Research Chemicals (TRC), North York, ON, Canada, and other suppliers. Reagents and solvents used for sample extractions and syntheses of standards were of analytical reagent grade or high-performance liquid chromatography (HPLC) grade. Unless otherwise specified, chemicals used in the synthesis of 3-ethenylpyridine and 3-ethenylpyridine-d_4 were from commercial vendors.

Instrumentation. For characterization of the standards, 3-EP and 3-EP-d_4, GC-MS was carried out using an Agilent 6890 GC interfaced with an Agilent 5973 MSD operated in the positive ion chemical ionization mode using isobutene as the reagent gas. ^{1}H NMR spectra were recorded using a Bruker Avance III HD 400 instrument at 400 MHz, and ^{13}C NMR spectra were recorded at 100 MHz. Chemical shifts were reported in parts per million (ppm, δ). Proton-coupling patterns are described as singlet (s), doublet (d), broad doublet (br d), and doublet of doublets (dd). For the determination of 3-EP in urine, GC-MS/MS analyses were carried out with a Trace1310 GC coupled to a TSQ 8000 Evo triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA). Solid-phase microextraction was performed on a Thermo CTC TriPlus RSH autosampler equipped with an accessory for SPME fiber/arrow conditioning and for sample incubation and extraction. Conventional SPME fibers were desorbed in splitless mode with a 0.75 mm i.d. SPME liner through a standard inlet septum. A Merlin Microseal septum replacement and a Merlin Microseal nut (Merlin Instrument Company, Half Moon Bay, U.S.A.) were used in the injection port when SPME arrows were used. The liner used for the SPME arrow is a splitless single taper gooseneck with a wool Topaz liner (Restek Corporation, Bellefonte, PA), which has a wider inner diameter of 2 mm. A PAL 250 μm PDMS SPME arrow (CTC Analytics AG, Zwingen, Switzerland) was used for sample microextraction.

Synthesis of 3-EP-d_4 and Oxalate Salt. The method is based on the Suzuki−Miyaura cross-coupling reactions of potassium vinyltrifluoroborate with 3-bromopyridine described by Molander and Brown[35] and Alacid and Nájera.[36] Argon-flushed isopropyl alcohol (5 mL) was added to a mixture of potassium vinyltrifluoroborate (805 mg, 6 mmol), tricyclohexylphosphine (100 mg 0.36 mmol), palladium(0) bis(dibenzylideneacetone) ($Pd(DBA)_2$) (100 mg 0.17 mmol), and 3-bromopyridine-d_4, which was synthesized by the method of Englert and McElvain[37] (0.5 mL, 820 mg, 5 mmol) in an argon-flushed 100 mL flask equipped with a reflux condenser and a septum inlet and attached to a mineral oil bubbler. Potassium carbonate (1.3 g,10 mmol) dissolved in 2 mL of argon-flushed water was added. The mixture was refluxed with stirring for 2 h under a static pressure of argon. Analysis of an aliquot by GC-MS indicated that all of the 3-bromopyridine-d_4 had reacted. Dichloromethane (5 mL) was added to the reaction mixture, which was filtered through Celite that had been prewashed with isopropyl alcohol. The filter cake was washed with 5 mL of dichloromethane, and 10 mg of butylated hydroxytoluene (BHT) was added to the filtrate to inhibit possible polymerization of the product. The lower aqueous layer was removed and discarded, and the organic layer was distilled using a water bath that was gradually heated to 50 °C under vacuum, increasing to ∼50 mmHg to remove the solvent. Subsequently, the remaining liquid was distilled bulb-to-bulb (Kugelrohr) at 30 mmHg, and an air bath temperature 115 °C to give 241 mg of colorless liquid, a 44% yield. A considerable amount of yellow viscous liquid was left undistilled, which was presumably a polymeric material. A 1 M solution of oxalic acid dihydrate in isopropyl alcohol was prepared. To 201 mg of 3-EP-d_4 (1.84 mmol) was added 1.84 mL of the 1 M oxalic acid in a 20 mL vial. The vial was vortexed, and the salt precipitated. This was diluted with 2 mL of ether, and the product was filtered and washed with 2 mL of ether. The product was dried under suction, giving 200 mg of white solid. This was recrystallized from 1.4 mL of ethanol and washed with 2 mL of ethanol followed by 2 mL of ether. Air-drying under suction provided 85 mg of white needles (mp 123−124 °C). ^{1}H NMR (D_2O): δ 6.81 (dd, J = 17.6, 10.8 Hz, 1H), 6.08 (d, J = 17.6 Hz, 1H), 5.65 (d, J = 10.8 Hz, 1H). ^{13}C NMR (D_2O): δ 164.7 (carbon in the oxalate group), 137.3, 129.8, 121.7. From GC-MS analysis (isobutane CI), extracting the ion chromatogram corresponding to 3-EP-d_0 (m/z 106), no m/z 106 was detected, verifying its suitability as a mass spectrometric internal standard for 3-EP.

Synthesis of 3-EP. Unlabeled 3-EP and the oxalate salt were prepared from 3-bromopyridine (10 mmol) as described earlier for 3-EP-d_4. The free base was obtained in 66% yield. Only trace impurities were detected by GC-MS. The oxalate salt had mp 124−125 °C. ^{1}H NMR (D_2O): δ 8.73 (br d, 1H), 8.59 (br d, J = 8.4 Hz, 1H), 8.56 (br d, J = 6.0, 1H), 7.94 (dd, J = 8.4, 6.0 Hz, 1H), 6.81 (dd, J = 17.6, 10.8 Hz, 1H), 6.08 (d, J = 17.6 Hz, 1H), 5.65 (d, J = 10.8 Hz, 1H). ^{13}C NMR (D_2O): δ 164.7 (carbon in the oxalate group), 143.2, 139.3, 138.8, 137.5, 129.9, 127.1, 121.7. The composition of the salt was verified as being 1:1 by GC-MS comparison with freshly distilled 3-EP free-base. For 3 aliquots of a solution extracted and analyzed, the amount determined was within 5−10% of the specified amount, when correcting the salt for the oxalic acid content, as being 53.8% base. Small amounts of 3-EP base were converted to salts that have been previously reported.[38] Hydrochloride, white powder, mp 115−116.5 °C (lit 114−115°); chloroaurate, yellow solid, mp 137−140° (lit 138−140°); chloroplatinate, orange solid, mp 155−157° dec (lit 158−160).

Analytical Method Development: SPME Phase Selection and Optimization. Polydimethylsiloxane (PDMS)- and polydimethylsiloxane/divinylbenzene (PDMS/DVB)-coated SPME arrows were compared for the coating selection. There are two types of SPME coatings: polymeric films for absorption of analytes and particles embedded in polymeric films for adsorption of analytes. PDMS belongs to the first type and is suitable for relatively nonpolar compounds. PDMS/DVB belongs to the second type and has a

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bipolar feature. Traditional SPME fibers were also evaluated to compare their performance to SPME arrows and to optimize the sensitivity. Two PDMS SPME fibers with a sorbent length of 10 mm and sorbent film thicknesses of 30 and 100 μm, and two PDMS SPME arrows with a sorbent length of 20 mm and sorbent film thicknesses of 100 and 250 μm, were used. Two mL of 100 ng/mL 3-EP and 100 ng/mL 3-EP-d_4 solution saturated with K_2HPO_4 (pH ≈ 9) in water was used for this evaluation. 3-EP is a weak base with a calculated pK_a of 4.86 at 25 °C.[39] Adjusting the pH to >7 ensures that 3-EP is in the free-base form and can be released from the solution. We found that the 250 μm PDMS SPME arrow provided the highest peak response for 3-EP and 3-EP-d_4, which indicated its superior ability to absorb the analyte from the headspace vapor. Consequently, a 120 μm PDMS/DVB SPME arrow was tested as a comparison to the 250 μm PDMS SPME arrow. Two mL of 0.2 ng/mL 3-EP and 5 ng/mL 3-EP-d_4 solution saturated with K_2HPO_4 in water was used for this evaluation. Although a 120 μm PDMS/DVB SPME arrow extracted three times more 3-EP compared to a PDMS 250 μm one under the same conditions, it extracted more impurities as well, resulting in no improvement in the S/N ratio. In an attempt to obtain a cleaner extract and improve the S/N ratio resulting in better sensitivity, a liquid extraction step was tried before SPME. Urine was extracted with methyl *tert*-butyl ether after making it basic with sodium hydroxide. The organic layer was acidified to convert 3-EP to a nonvolatile salt, then evaporated to dryness, reconstituted with 2 mL of water, and then analyzed by the SPME method. This resulted in good recovery for 3-EP and 3-EP-d_4 (50−100%), but the background of the blank urine sample did not show a significant improvement compared to the direct SPME method. Therefore, 250 μm PDMS SPME arrows were selected for further method development by the direct headspace SPME method.

Optimization of Extraction Conditions. The effect of extraction temperature was studied from 60 to 80 °C. In these experiments, 2 mL of 1 ng/mL 3-EP and 100 ng/mL 3-EP-d_4 solution saturated with K_2HPO_4 (pH ≈ 9) was used. The results showed that heating at 80 °C resulted in the highest partitioning of the analytes into the headspace, and thus this temperature was used. For extraction time, three extraction times of 1, 2, and 5 min were compared. We found that 2 min resulted in greater peak areas than 1 min, but 5 min provided negligible improvement in the 3-EP peak area, demonstrating that an extraction time longer than 2 min did not improve the sensitivity. Therefore, a 2 min extraction time was used. Sample mixing is generally used in SPME to shorten the equilibrium time needed for extraction.[40] Sample mixing was tested in this study with two different rates, 600 and 1200 rpm, with an extraction time of 2 min. Two mL of 1 ng/mL 3-EP and 100 ng/mL 3-EP-d_4 solution saturated with K_2HPO_4 was used. We found that 1200 rpm produced a peak that was 2 times higher than that for 600 rpm. Salting-out is generally used in SPME to decrease the analyte solubility and to keep the ionic strength in real samples similar to standards.[41] Base addition was also applied to keep the pH well above the calculated pK_a of 3-EP (4.86),[39] as discussed earlier. At a sample pH of ∼8, 3-EP should be 99.9% in the free-base form. Saturated K_2HPO_4 and saturated sodium chloride with 50% K_3PO_4 aqueous solution (w/w) addition were compared in these experiments. The result showed that 75 μL of 50% K_3PO_4 solution is needed to adjust typical urine samples to a basic range, and 150 μL of 50% K_3PO_4 solution is needed for the acidified urine samples available in some of our studies. There was essentially no difference in recovery between the two procedures. Therefore, we chose saturated sodium chloride with 75 or 150 μL of 50% K_3PO_4 addition for our sample preparation.

Optimization of Desorption and Injection Conditions. The desorption time in the injector was set to 1 min because any longer desorption time did not significantly increase the peak area. A splitless injection mode was used so that all of the vaporized sample could be applied to the column. After a splitless time of 1 min, the split ratio was set to 50:1 for the purpose of septum and injector purge. The column was kept at a low temperature of 40 °C for 1 min to focus all 3-EP on the head of the column. A longer desorption time requires a longer splitless time and longer initial column temperature time, which may cause a tailing peak as the desorption time increases. Under the condition of 1 min desorption, good sensitivity was achieved with a peak width of 0.06 min.

Working Standards and Controls. A 1.00 mg/mL stock standard solution of 3-EP, as the free base, was prepared in water from 3-EP oxalate, corrected for the composition of 1:1 3-EP/oxalic acid. The stock solution was then diluted successively with water to form a set of 9 standards and quality control (QC) working solutions ranging from 20 to 2000 ng/mL. One μg/mL of 3-EP-d_4 in water was used as the internal standard working solution. Nonsmokers' urine that was found to be free of 3-EP and 3-EP-d_4 was used to prepare the standards and QCs. Twenty μL aliquots of standard working solution were spiked into 2 mL of urine to prepare the analytical run/calibration standards and controls in the range of 0.2−20 ng/mL. The final concentrations for the standards were 0.2, 0.5, 1, 5, 10, and 20 ng/mL, and the QCs were 0.2, 0.4, 2, and 8 ng/mL. The final concentration for the internal standard is 5 ng/mL. Standards and controls were freshly prepared before each use.

Sample Preparation. Ten μL of internal standard working solution (10 ng of 3-EP-d_4) was spiked into 2 mL of urine sample, standard, or QC sample. One tablespoon of sodium chloride (∼700 mg) and 75 μL of 50% w/w potassium phosphate tribasic aqueous solution were added. The amount of sodium chloride added was an excess of what was necessary for reaching saturation, so the ionic strengths of all samples were essentially the same. The final pH of the urine samples was ∼8. Because some urine samples had been acidified for stability purposes, a larger volume of 50% potassium phosphate tribasic, 150 μL, was added to those samples.

Extraction Procedure. Samples were stored in the autosampler tray at room temperature (23 °C). Prior to extraction, the SPME fiber/arrow was preconditioned in the conditioning station at 250 °C for 10 min under a stream of nitrogen at 5.0 mL/min. Before the preconditioning began, the SPME tool including the fiber/arrow transferred the sample from the autosampler tray to the incubation station, where the autosampler mixed the samples with a rotating motion at 600 rpm for 10 min at 80 °C. After the sample incubation time, the sample was transferred to a stirring station where the sample vials' septa were pierced by the fiber/arrow and the sorption phase was immersed into the sample headspace while the vial was continuously mixed for 2 min at 1200 rpm to adsorb the analyte. The sample vial penetration depth was set to 55 mm in order to ensure constant and complete immersion of the sorption phase. After the 2 min extraction period, the fiber/arrow was transferred into the GC injector for thermal desorption. Subsequently, the fiber/arrow was cleaned for 15 min in the corresponding conditioning station at 250 °C prior to adsorbing and injecting the following sample.

GC Chromatography. The analyte was desorbed from the fiber/arrow in the injection port at 250 °C for 1 min. Analyte separation was accomplished using a 30 m × 0.25 mm fused silica column, 0.25 μm HP-5MS stationary phase (Agilent Technologies, Palo Alto, CA). Helium (99.995%, Airgas, Radnor, PA) was used as the carrier gas with a flow rate of 1.2 mL/min. Nitrogen (99.999%, Airgas) acted as the split and septum purge gas and also the gas for cleaning the SPME fiber/arrow at the time of conditioning. A splitless injection mode was used for the first 1 min. After a splitless time of 1 min, the split ratio was set to 50:1. The oven temperature program was as follows: the initial temperature was set at 40 °C and held for 1 min, followed by a first temperature ramp of 20 °C min^{-1} to 150 °C, and a second ramp of 80 °C min^{-1} to 280 °C, with a final time of 3 min.

Mass Spectrometry. Electron ionization at 70 eV was used. Data were acquired in the selected reaction monitoring (SRM) mode. The transitions 105 to 78 and 109 to 81 at a collision energy of 12 eV were used for 3-EP and the internal standard 3-EP-d_4, respectively, with argon (99.998%, Airgas) as the collision gas. The transfer-line temperature was set to 280 °C, and the ion source was set to 275 °C.

Instrument Calibration and Data Analysis. The XCalibur software was used to generate calibration curves (linear regression, 1/*X* weighting, ignore origin) and calculate concentrations using peak area ratios of analyte/internal standard. Standard curves were linear from 0.2 to 20 ng/mL for six concentrations spanning this range. Two

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sets of standards and QCs were included in each run of 20−30 clinical samples. Typically, one set of standards was injected at the beginning of the run, and one set was injected following the injection of the study samples. QC samples were run through the sequence among the study samples. The equations and correlation coefficients for the standard curves run with the study samples are as follows by date:

01/27/2020: $Y = 0.0104749 + 0.329096X$, $r^2 = 0.9985$
02/06/2020: $Y = 0.00915605 + 0.30328X$, $r^2 = 0.9994$
02/18/2020: $Y = 0.00334046 + 0.331841X$, $r^2 = 0.9997$
06/17/2020: $Y = 0.0661857 + 0.309408X$, $r^2 = 0.9992$
07/08/2020: $Y = 0.0102504 + 0.288638X$, $r^2 = 0.9992$
09/06/2020: $Y = 0.000327393 + 0.274545X$, $r^2 = 0.9982$
09/10/2020: $Y = -0.00433334 + 0.279534X$, $r^2 = 0.9991$

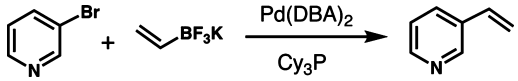
Human Urine Samples. Urine samples were available from previous studies.[33,42−45] All studies received approval of the appropriate institutional review boards. Sixteen urine samples were obtained from cigarette smokers in a multisite, randomized clinical trial.[42] Participants were 18 years or older, smoked five or more cigarettes per day, and had no current interest in quitting smoking. A second set of 16 samples from cigarette smokers were from a crossover study of dual users of small cigars and cigarettes in Philadelphia (December 2012−December 2015) collected during the cigarette-smoking arm.[43] Samples from these same subjects during the small cigar-smoking arm were also analyzed. Ten samples from water pipe smokers were from a crossover study of water pipe and cigarette smoking carried out by the Clinical Research Center at Zuckerberg San Francisco General Hospital.[33] These samples were 24-h collections during ad libitum water pipe smoking, but participants were asked to smoke a minimum of twice per day. Eight urine samples were 24 h collections from e-cigarette users in San Francisco in a crossover study of use of e-cigarettes, and combusted cigarettes were collected during the vaping arm.[44] This study was also carried out by the Clinical Research Center at Zuckerberg San Francisco General Hospital. The smokeless tobacco users ($N = 11$) were using their usual brand of smokeless tobacco products. These samples were obtained from the University of Minnesota Biorepository, which contains biological samples from users of various tobacco products. Urine samples from 9 nonsmokers exposed to SHS in a discotheque were from nonsmoking adolescents, taken at baseline and after at least 4 h in a smoking-allowed disco.[45] The 23 urine samples from nonsmokers not exposed to SHS were obtained in San Francisco. Smoking status and SHS exposure were by self-report and/or the nicotine metabolite cotinine concentration being below the established cutpoint for determining smoking status.

RESULTS

The goals of our study were (1) to develop an analytical method with adequate sensitivity and specificity to measure 3-EP in the urine of people exposed to tobacco smoke and (2) to measure concentrations of 3-EP in the urine of people who used various tobacco products to evaluate its utility as a biomarker.

Synthesis of Standards. The foundation of any analytical method is the availability of a standard with documented identity and purity. 3-EP is commercially available as the free base, and purities of 95% or greater may be specified. However, on receipt from more than one vendor, it was a black liquid, which on distillation yielded a colorless distillate and a considerable amount of tarry residue. One sample received as a solid had apparently polymerized. Because of obvious stability issues, and to make available reasonable quantities of both unlabeled 3-EP and a stable isotope-labeled analogue needed as a mass spectrometric internal standard, we modified and optimized published methods for the synthesis of 3-EP via Suzuki−Miyaura cross-coupling reactions of potassium vinyltrifluoroborate with 3-bromopyridine.[35,36] (Scheme 2) The

Scheme 2. Synthesis of 3-EP



modifications included the reaction solvent, reaction conditions, and catalyst that resulted in a good yield in a short period of time (2 h), obviating the need to carry out the reaction for 22 h while being heated in a sealed tube, and facilitated isolation of the product by a simple distillation rather than column or flash chromatography. Because of the instability of 3-EP during storage, we prepared known salts of 3-EP (hydrochloride, chloroplatinate, and chloroaurate),[38] as well as a new one, the oxalate, because amine salts are generally more stable than the free bases. The oxalate was chosen as the primary standard because the hydrochloride appeared to be hygroscopic and because the expense of the precious metals discourages their use. We should point out that 3-EP obtained from commercial sources would be satisfactory if purified before making working standards. A simple distillation should suffice.

Method Development. Because of the volatility of 3-EP and potential interference from extraction solvents, we reasoned that headspace SPME would be more satisfactory than methods involving liquid injection. A number of parameters were evaluated, as described in the Experimental Procedures section. These included the SPME fiber sorbent types and film thicknesses, sorbent lengths, extraction times and temperatures, and sample mixing rate. We found that 250 μm polydimethylsiloxane (PDMS) SPME arrows and an extraction temperature of 80 °C provided the best results in terms of sensitivity and chromatograms free from interfering substances.

Method Validation. The analytical method was validated according to protocols for bioanalytical method validation generally applicable for pharmacokinetic studies and biomarkers in drug development (Table 2).[46−48] Standard curves were constructed at six levels ranging from 0.2 to 20 ng/mL by spiking 3-EP into a blank urine matrix. Precision, accuracy, and lower limit of quantitation (LLOQ) were evaluated by analyzing six different nonsmoker urines, from people who had no known secondhand smoke exposure, spiked with 3-EP in the same run and over 3−5 different runs. Intra-assay and interassay precision (CV%) ranged from 0.6 to 8.7% for all four concentration levels, and accuracy (percent of expected) ranged from 92.1 to 104.5% (Table 2). The LLOQ was determined to be 0.2 ng/mL on the basis of a CV < 20% and an accuracy bias within ±20%.

Specificity was determined by analyzing urine samples from 23 people who do not use tobacco products. All of them had 3-EP concentrations below the limit of quantitation of 0.2 ng/mL. Precision was also evaluated on a pooled smokers' urine sample, which was prepared by mixing equal amounts of urines from three different smokers. Eighteen duplicate analysis were carried out over three runs on three consecutive days with six samples on each day. Intra-assay precision (CV%) ranged from 1.7 to 4.0%, and the interassay precision (CV%) is 6.1% (Table 2).

The stability of 3-EP in urine was evaluated by testing two pH conditions and three storage temperatures (Table 3). In these experiments, two concentrations of 3-EP (0 and 2 ng/mL) were utilized. Five samples for each concentration level were prepared by spiking desired amounts of 3-EP into

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Table 2. Intra-assay and Interassay Precision and Accuracy for Determination of 3-EP in Urine

expected amount (ng/mL)	intra-assay precision (CV%), n = 6	intra-assay accuracy (% of expected), n = 6	interassay precision (CV%), n = 18, 3−5 different runs	interassay accuracy (% of expected), n = 18, 3−5 different runs
0.2	4.1	104.5	6.8	100.5
0.4	3.4	92.1	8.7	98.8
2	2.9	97.6	4.4	97.5
8	0.6	92.4	3.7	92.8
day 1, pool	4.0	NA[a]; mean = 4.94 ng/mL		
day 2, pool	3.7	NA[a]; mean = 5.48 ng/mL		
day 3, pool	1.7	NA[a]; mean = 4.91 ng/mL		
3 days pool			6.1	NA[a]; mean = 5.11 ng/mL

[a]NA, accuracy not applicable for pooled smokers' urine.

Table 3. Stability of 3-EP in Acidified and Nonacidified Urine Samples (Concentrations in ng/mL over Time)

condition	initial[a]	24 h	48 h	7 days	30 days
RT, no acid	0	<0.2			
	2	2.22			
	4.90	4.42	4.74		
RT, acid[b]	0	<0.2			
	2	2.12			
	4.14	3.86	4.10		
4 °C, no acid	0				
	2				
	4.90		4.97	4.74	
4 °C, acid[b]	0				<0.2
	2				2.13
	4.14		4.61	4.54	
−20 °C, no acid	0				<0.2
	2				2.04
	4.90			4.72	
−20 °C, acid[b]	0				<0.2
	2				2.10
	4.14			4.47	

[a]Concentrations of 4.90 and 4.14 are pooled smokers' urine. The others are concentrations spiked into nonsmokers' urine. [b]With solid sodium bisulfate.

nonsmoker's urine, which were further divided into two groups with three samples in the acidified group (pH 2−3, adjusted with solid sodium bisulfate) and two samples in the nonacidified group. The acidified group samples were stored at three temperatures with different time periods, which were room temperature for 24 h and 4 and −20 °C for 30 days. The nonacidified group samples were stored at room temperature for 24 h and −20 °C for 30 days, respectively. We also analyzed pooled smokers' urine samples, acidified and nonacidified, over the course of 7 days. We did not find significant increases or decreases in concentrations under any conditions for the spiked nonsmoker's urine or the pooled smokers' urine. The percent differences from initial concentrations ranged from −10% to +11%, which is within acceptable precision for this type of analysis[46] (Table 3).

Concentrations of 3-EP in Urine of People Using Various Tobacco Products. Because cigarette smoking remains the most prevalent form of tobacco use, the first groups of samples analyzed were cigarette smokers and nonsmokers who reported no significant exposure to SHS. The goals were to determine whether measurable amounts of 3-EP are excreted in the urine of smokers and whether 3-EP is present in the urine of nonsmokers. Two groups of cigarette smokers were studied. The first, N = 16, were daily cigarette smokers in a multisite, randomized clinical trial.[42] The concentrations of 3-EP in urine ranged from below the limit of quantitation (BLQ) to 5.12 ng/mL, with of mean of 1.08 (SD = 1.31) and a detection frequency of 88% (Figure 1). The second group was from a study of dual users of cigarettes and small cigars in Philadelphia, in a crossover study in which the participants either smoked cigarettes or small cigars in different study blocks.[43] This allowed us to compare 3-EP excretion in the same people for the two different products. During the cigarette-smoking arm, the concentrations of 3-EP in urine ranged from BLQ to 2.56 ng/mL, with of mean of 0.45 (SD = 0.63) and a detection frequency of 63%. During the cigar-smoking arm, the concentrations of 3-EP in urine ranged from BLQ to 3.74 ng/mL, with of mean of 0.66 (SD = 0.87) and a detection frequency of 88%. Concentrations in urine of 23 adults who did not use tobacco products were all below the limit of quantitation (Table 4). Representative GC-MS/MS chromatograms are presented in Figure 2.

We also analyzed urine from electronic cigarette users,[44] water pipe (hookah) smokers,[33] and daily smokeless tobacco users. 3-EP concentrations were BLQ in the urine of all of the 8 e-cigarette users. 3-EP concentrations were BLQ in the urine of all but 1 of the 10 water pipe smokers. 3-EP concentrations were BLQ in the urine of all but 1 of the 11 smokeless tobacco users (Table 4).

Concentrations of 3-EP in sidestream smoke are substantial (Table 1), and exposure in nonsmokers exposed to SHS could be significant. We measured concentrations of 3-EP in urine from a study of SHS exposure in smoking-allowed Mexican discotheques.[45] For 9 nonsmoking participants prior to entering the discos, 3-EP concentrations were below the limit of quantitation. Following spending several hours in the discos, 3-EP was detectable in the urine of 4 subjects, with a mean for all 9 subjects of 0.21 ng/mL, with a range BLQ−0.38 ng/mL (Table 4).

DISCUSSION

In this article we provide data supporting 3-EP as a promising biomarker for the gas phase of tobacco smoke. Previous studies demonstrated that 3-EP has high specificity for tobacco smoke.[23,24] Undetectable amounts or low concentrations of 3-EP have been reported in venues where smoking has not occurred as compared to venues where smoking takes place. For example, in a study comparing VOC concentrations in the homes of smokers and nonsmokers, mean 3-EP concentrations of 0.08 $\mu g/m^3$ (N = 24, median undetectable) were found in nonsmokers' homes compared to a mean of 1.28 $\mu g/m^3$ (N = 25) in smokers' homes. The authors also found a significant correlation between 3-EP concentrations and the number of

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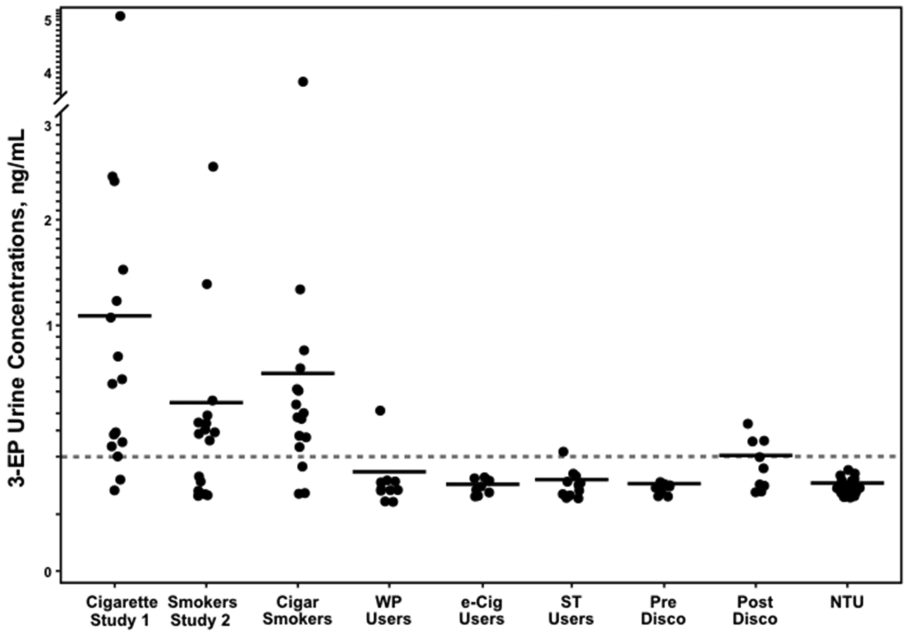

Figure 1. Concentrations of 3-EP in the urine of people using tobacco products and of non-tobacco users. Horizontal lines in columns are mean concentrations, and the dashed line is the limit of quantitation. WP = water pipe, ST = smokeless tobacco, and NTU = non-tobacco user. Participants in the disco study were non-tobacco users. If below the limit of quantitation (BLQ), LLOQ/$\sqrt{2}$ was used.

Table 4. 3-Ethenylpyridine Concentrations[a] in Urine of People Using Tobacco Products, People Exposed To Secondhand Smoke (SHS), and People Without SHS Exposure Who Did Not Use Tobacco Products

	3-EP urine concentration, ng/mL					cotinine,[b] ng/mL
product use or exposure	mean (*N*)	median	SD (range)	detection frequency	*p*[c]	mean
cigarette smokers	1.08 (16)	0.61	1.31 (BLQ-5.12)	88%	0.01	5400 (total)
cigarette smokers[d]	0.45 (16)	0.27	0.63 (BLQ-2.56)	63%	0.07	3800 (total)
cigar smokers[d]	0.66 (16)	0.42	0.87 (BLQ-3.74)	88%	0.03	3500 (total)
water pipe smokers	BLQ (10)	BLQ	0.09 (BLQ-0.42)	10%	0.34	920 (total)
e-cigarette users[e]	BLQ (8)	BLQ	all BLQ	0%	0.99	1400 (total)
smokeless tobacco users	BLQ (11)	BLQ	0.02 (BLQ-0.22)	9%	0.34	5100 (total)
nonsmokers pre-disco[f]	BLQ (9)	BLQ	all BLQ	0%	0.99	0.38 (free)
nonsmokers post-disco[f]	0.21 (9)	0.14	0.08 (BLQ-0.38)	44%	0.05	20 (free)
non-tobacco users	BLQ (23)	BLQ	all BLQ	0%		

[a]Lower limit of quantitation (LLOQ) = 0.2 ng/mL. If below the limit of quantitation (BLQ), LLOQ/$\sqrt{2}$ was used. [b]Total cotinine is the sum of cotinine glucuronide and unconjugated cotinine. Free cotinine is unconjugated cotinine. About equal amounts of free cotinine and cotinine glucuronide are excreted by most smokers.[49] [c]*p*-values are for differences between tobacco product use and non-tobacco users by *t* test. [d]These participants were dual users of cigarettes and small cigars in a crossover study in which participants smoked either cigarettes or small cigars in different study blocks.[43] [e]These samples were analyzed in duplicate. [f]These participants were nonsmokers who spent several hours in smoking-allowed discotheques.[45]

cigarettes smoked.[50] In our studies, the concentrations of 3-EP in the urine of nonsmokers (N = 23) were below the limit of quantitation. 3-EP concentrations were measurable in most urine samples from combustible tobacco users, confirming its specificity for tobacco smoke (Table 4).

Specific biomarkers for both the particle phase and the gas phase are desirable because the two phases distribute differently in the environment,[10] and compounds in them may have different modes of absorption in the respiratory tract.[11] 3-EP exists primarily in the gas phase of cigarette smoke, a requisite for it to be a useful marker for gas-phase compounds.[1] The validity of 3-EP as a biomarker for gas-phase components is supported by studies showing that 3-EP is a useful tracer for VOCs derived from tobacco smoke. 3-EP has been utilized for source apportionment to estimate the contribution of SHS (ETS) to concentrations of VOCs in indoor air.[4,50]

Because 3-EP is produced by pyrolysis at high temperature[13,14] and does not appear to be naturally occurring in tobacco, it would not be expected to be present in the urine of people using e-cigarettes or smokeless tobacco. That indeed was found to be the case (Table 4). 3-EP was not detected in the urine of 8 electronic cigarettes users. For 11 users of smokeless tobacco, 3-EP was detected in the urine of only one study participant, and the concentration (0.22 ng/mL) was just above the LLOQ, possibly the result of exposure to SHS. For 10 study participants who smoked a water pipe on a research ward, 3-EP was detected in the urine of just one participant at a concentration of 0.42 ng/mL. Because these participants excreted substantial concentrations of nicotine metabolites,[33] we conclude that the temperatures achieved during water pipe

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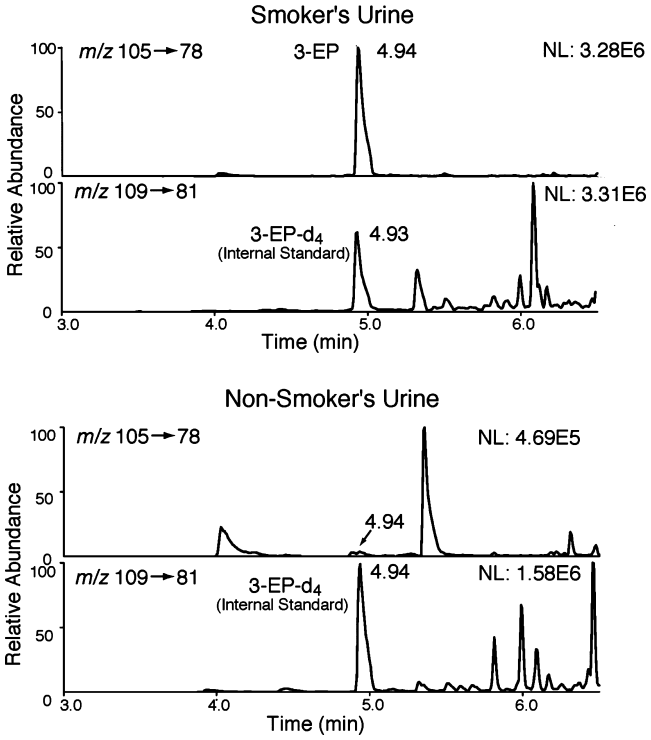

Figure 2. GC-MS/MS selected reaction monitoring (SRM) chromatograms of 3-EP in a smoker's urine and a nonsmoker's urine. Parent-to-product ion transitions are indicated in the upper left corners of the chromatogram panels.

smoking generally are not high enough to convert nicotine to 3-EP. Therefore, a potential application of 3-EP is a biomarker to distinguish combusted tobacco use from the use of other tobacco products. We should note that typical water pipe use does not involve tobacco combustion per se. It involves placing a piece of burning charcoal on top of a moist mixture of fruit and tobacco, and the smoker inhales the resulting aerosol without actual combustion of the smoking product. Our results are consistent with those of a study of water pipe smoke contamination indoors, in which increased concentrations of nicotine and benzene were measured, but 3-EP concentrations were below the detection limit before and after water pipe smoking.[51] Distinguishing combusted tobacco use from the use of other products is of interest in studies comparing exposure and health effects in people using different or multiple products.

Another possible application of 3-EP is as a biomarker for secondhand smoke (SHS) exposure and to distinguish secondhand smoke exposure form thirdhand smoke (THS) exposure. From a toxicological standpoint, this is important because modes of exposure are different and strategies for reducing exposure are different. SHS consists of airborne particles and gases, exposure is primarily by inhalation, and strategies for reducing exposure include room ventilation and avoiding venues where people smoke. THS consists of the residues that remain and react with other substances in the environment and substances that can be reemitted from surfaces. Exposure to toxic substances in THS can be by transdermal absorption, ingestion of dust and hand-to-mouth behavior by young children, and inhalation. Strategies for reducing THS exposure include remediation of the venue by thorough cleaning or even replacing carpets, furniture, and wallboard in extreme cases, as well as avoiding venues where THS is present.[10] Unlike nicotine and other less-volatile substances in tobacco smoke, which have a strong affinity for surfaces and can persist for long periods indoors after smoking ceases,[3] 3-EP has a relatively low affinity for surfaces[29] and due to its volatility is removed fairly rapidly by ventilation. Therefore, 3-EP should be a selective marker for SHS exposure, and its concentrations in biofluids could potentially be used to distinguish SHS exposure from THS exposure.

Concentrations of 3-EP in tobacco smoke are substantial (Table 1). Because of its structural similarity to styrene, a probable human carcinogen (Figure 3), consideration of the potential toxicity of 3-EP is warranted.[52] Surprisingly, very little has been published on its potential toxicity. In a study published in 1992,[53] it was reported that 3-EP was not mutagenic in *Salmonella typhimurium* strains TA 1535, TA 1538, TA 98, and TA 100, nor was it genotoxic in the rat hepatocyte DNA-repair test. No significant incidence of lung adenoma or of any other type of tumors was found after intraperitoneal injection in A/J mice. However, in this same study, styrene was not found to be mutagenic or genotoxic and likewise did not lead to a significant incidence of any type of tumor. Subsequent studies have determined that styrene is carcinogenic, and it is considered a probable human carcinogen by the International Agency for Research on Cancer (IARC).[54] We are unaware of any studies of 3-EP toxicity other than the 1992 study, which suggests that further studies are warranted.

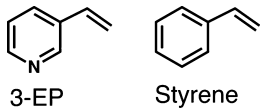

Figure 3. Structures of 3-EP and styrene.

CONCLUSIONS AND FUTURE STUDIES

We have demonstrated, we believe for the first time, that 3-EP is present and measurable in the urine of smokers and that it may have utility as a biomarker of gas-phase toxicants in studies of tobacco smoke exposure and toxicity. There are some limitations to our studies. Larger numbers of samples from people using various product types and samples from people of different demographics will be needed to properly determine 3-EP concentrations on a population basis. Because 3-EP was not detected in all smoking participants, improvements in the sensitivity of the analytical method would be desirable. Nothing is known about the half-life of 3-EP, and if it is short 3-EP might not be detectable if sufficient time had elapsed between smoking and the time of sampling for concentrations to fall below the LLOQ. It is possible that metabolites of 3-EP may be present in higher concentrations than 3-EP and might be amenable to more sensitive detection. Studies on the metabolism of 3-EP are underway in our laboratory.[52] Our study provides proof of concept that, with improvements in method sensitivity and/or identification of a more abundant metabolite, 3-EP could become a generally useful gas-phase biomarker in smokers and in nonsmokers exposed to tobacco smoke. Our study also calls attention to the significant exposure in both smokers and nonsmokers exposed to SHS and the fact that the paucity of toxicity data on 3-EP suggests the need for further toxicological studies.

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Author Contributions

P.J. and J.L. conceived and designed the study and carried out data analysis. J.L. developed the analytical method and performed the urine analyses. P.J. synthesized the standards. C.M.H. assisted with method development and data acquisition. N.L.B., D.K.H., E.L.-P., and A.A.S. designed and directed the studies that generated the urine samples from tobacco product users. All authors contributed to writing the manuscript and approved the final version.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

3-EP, 3-ethenylpyridine; SPME, solid-phase microextraction; VOC, volatile organic compound; MS cigarette smoke, mainstream cigarette smoke; SS cigarette smoke, sidestream cigarette smoke; PDMS, polydimethylsiloxane; Cy_3P, tricyclohexylphosphine; DBA, dibenzylideneacetone; LLOQ, lower limit of quantitation; BLQ, below the limit of quantitation; SHS, secondhand smoke; THS, thirdhand smoke

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This paper was published on May 17, 2021, with an incorrect column heading in Table 2. The corrected version was reposted on May 17, 2021.

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