Title

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Journal

Chemical research in toxicology, 34(6)

ISSN

1520-5010

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Publication Date

2021-05-17

Peer reviewed

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

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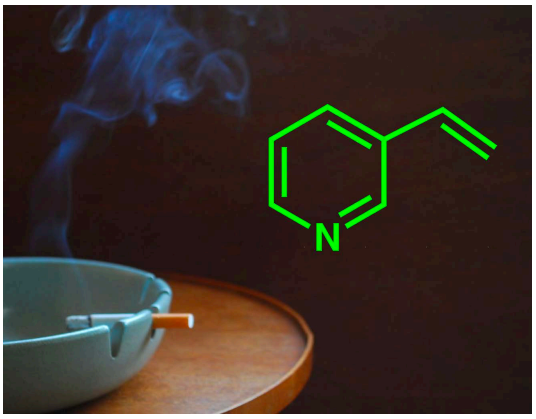
KEYWORDS: Tobacco products, e-cigarettes, biomarkers of tobacco smoke, tobacco alkaloids, secondhand smoke, thirdhand smoke.



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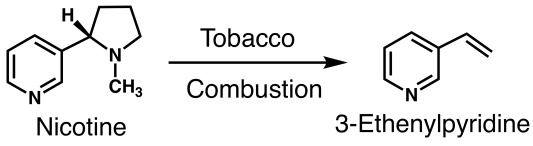
ABSTRACT : In studies of tobacco toxicology, including comparisons of different tobacco products and exposure to secondhand or thirdhand smoke, exposure assessment using biomarkers is often useful. Some studies have indicated that most of the toxicity of tobacco smoke is due to gas-phase compounds. 3-Ethenylpyridine (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 urine of smokers and in 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] in studies of tobacco smoke exposure and toxicity specific biomarkers and environmental tracers for both particulate matter and the gas phase are desirable.[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) range from about 4 – 30 µg/cigarette. Concentrations in sidestream (SS) cigarette smoke (emitted by the smoldering cigarette) are much higher, about 200 – 600 µg/cigarette (Table 1).[1,4,18-21] Since 3-EP has high specificity for tobacco smoke, and due to the 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



Scheme 1. Formation of 3-EP by pyrolysis of nicotine

well, and therefore it has been proposed as an environmental tracer for VOCs derived from tobacco smoke.[4]

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]	US, Non-Filter[b]	23	640	28
Brunnemann *et al*. 1978[20]	US Cigar[b]	30	2550	85
Kulshreshtha *et al*. 2003[21]	Full Flavor	4.57		
Hoffmann et al 2001[1]	Non-Filter[c]	7 – 30		
Hodgeson *et al*. 1996[6]	Reference 1R4F[a]		680	
Hodgeson *et al*. 1996[6]	6 Commercial Brands[a]		450 - 890	

a. Both gas and particle phases
b. Measured as a mixture with 3,4-lutidine
c. Vapor-Phase

A number of tobacco smoke VOCs have been measured as metabolites in urine of smokers[30-32] and in people exposed to secondhand smoke (SHS),[33] 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 urine using headspace solid-phase microextraction (SPME) gas chromatography – tandem mass spectrometry (GC-MS/MS); (2) present data on concentrations of 3-EP in urine of non-smokers and in urine of people using various combusted (cigarettes, cigars) and non-combusted (smokeless, 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 synthesis of standards were of analytical reagent grade or 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 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, USA) were used in the injection port in when SPME arrows were used. The liner used for SPME arrow is splitless single taper gooseneck w/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[34] and by Alacid and Nájera[35]. 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[36] (0.5 mL, 820 mg, 5 mmol) in an argon-flushed 100 mL flask equipped with a reflux condenser, a septum inlet and attached to a mineral oil bubbler. Potassium carbonate (1.3 g ,10 mmol) dissolved in 2 mL argon-flushed water was added. The mixture was refluxed with stirring for 2 hr 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 dichloromethane, and 10 mg 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 about 50 mmHg to remove the solvent. Subsequently, the remaining liquid was distilled bulb-to-bulb (Kugelrohr) at 30 mmHg, air bath temperature 115° C to give 241 mg of colorless liquid, 44% yield. A considerable amount of yellow viscous liquid was left undistilled, presumably 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 ether, the product was filtered and washed with 2 mL ether. The product was dried under suction giving 200 mg of white solid. This was recrystallized from 1.4 mL of ethanol, washed with 2 mL ethanol followed by 2 mL 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 above for 3-EP d_4. The free base was obtained in 66% yield. 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[37]: 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).

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, correcting 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 QC working solutions ranging from 20 ng/ml to 2000 ng/ml. 1 μg/mL 3-EP-d_4 in water was used as the internal standard working solution. Nonsmokers urine found to be free of 3-EP and 3-EP-d_4 was used to prepare the standards and QCs. 20 μL aliquots of standard working solution were spiked into 2 mL 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, 20 ng/ml and the QCs were 0.2, 0.4, 2 and 8 ng/ml. The final concentration for internal standard is 5 ng/ml. Standards and controls were freshly prepared before each use.

Sample Preparation. 10 μL internal standard working solution (10 ng of 3-EP-d_4) was spiked into 2 mL urine sample, standard, or QC sample. 1 tablespoon of sodium chloride (approx. 700 mg) and 75 μL 50% potassium phosphate tribasic were added. The amount of sodium chloride added was an excess of what was necessary for reaching saturation, so the ionic strength of all samples was essentially the same. The final pH of the urine samples was about 8. Since 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. After the first 5 min of preconditioning, the SPME tool including the fiber/arrow transfers the sample from the autosampler tray to the incubation station. Then the SPME fiber/arrow is returned to the conditioning station to complete the remaining 5 min of conditioning. In the incubation station, samples were continuously agitated 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 stirred 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. Once extraction was completed, 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.

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 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 thickness of 30 μm and 100 μm, and two PDMS SPME arrows with a sorbent length of 20 mm and sorbent film thickness of 100 μm and 250 μm were used. 2 mL of 100 ng/mL 3-EP and 100 ng/mL 3-EP-d_4 solution saturated with K_2HPO_4 in water was used for this evaluation. 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 headspace vapor. Consequently, 120 μm PDMS/DVB SPME arrow was tested as a comparison to 250 μm PDMS SPME arrow. 2 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 120 μm PDMS/DVB SPME arrow extracted three times more 3-EP compared to PDMS 250μm 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 non-volatile salt, then evaporated to dryness, reconstituted with 2 ml 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.

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 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 was 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 ion source was 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 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 following injection of the study samples. QC samples were run through the sequence among study samples. An equation and correlation coefficient for a representative standard curve is $Y = 0.003340 + 0.3318*X$, $r^2 = 0.9997$

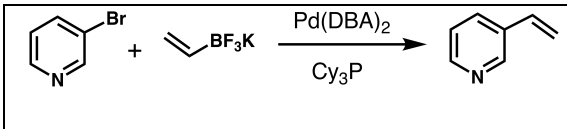
Human Urine Samples. Urine samples were available from previous studies.[32,38-41] All studies received approval of the appropriate institutional review boards. Sixteen urine samples were obtained from cigarette smokers in a multi-site, randomized clinical trial.[38] Participants were 18 years or older, smoked of 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.[39] 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 on the Clinical Research Center at Zuckerberg San Francisco General Hospital.[32] These samples were 24-hour collections during ad libitum water pipe smoking, but participants were asked to smoke a minimum of twice per day. Eight urine samples were 24 hr 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.[40] This study was also carried out on the Clinical Research Center at Zuckerberg San Francisco General Hospital. The smokeless tobacco users (N = 11) were using their usual brand

smokeless tobacco products. These samples were obtained from the University of Minnesota Biorepository, that contains biological samples from users of various tobacco products. Urine samples from 9 non-smokers exposed to SHS in a discotheque were from nonsmoking adolescents, taken at baseline and after at least 4 hours in a smoking-allowed disco.[41] The 23 urine samples from non-smokers not exposed to SHS were obtained in San Francisco. Smoking status and SHS exposure was by self-report and/or the nicotine metabolite cotinine concentration 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 urine of people exposed to tobacco smoke, and (2) measure concentrations of 3-EP in 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, that on distillation yielded a colorless distillate and a considerable amount of tarry residue. One sample received as a solid had apparently polymerized. Due to obvious stability issues, and to make available reasonable quantities of both unlabeled 3-EP and a stable isotope-labeled analog 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.[34,35] (Scheme 2) The modifications included the reaction solvent, reaction



Scheme 2. Synthesis of 3-EP

conditions, and catalyst that resulted in a good yield in a short period of time (2 hr), obviating the need to carry out the reaction for 22 hrs while 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 on storage, we prepared known salts of 3-EP (hydrochloride, chloroplatinate, and chloroaurate)[37] and a new one, the oxalate, as 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.

Method development.

SPME fiber selection. A PAL SPME arrow was used for the solid phase microextraction. Polydimethylsiloxane (PDMS) and polydimethylsiloxane/divinylbenzene (PDMS/DVB) coated SPME were selected for testing for 3-EP because they are commonly used sorption phase materials and suitable for relatively nonpolar compound and bipolar compounds, respectively. Moreover, the molecular weight of 105 is within the application range of these two SPME coatings. Finally, PDMS coated SPME was selected over PDMS/DVB coated SPME because it provided a much cleaner background while maintaining a similar sensitivity. PDMS SPME fibers and SPME arrows were tested under the same instrument parameters to compare their capability and achieve the best sensitivity. Two PDMS SPME fibers with a sorbent length of 10 mm and sorbent film thickness of 30 μm and 100 μm, and two PDMS SPME arrows with a sorbent length of 20 mm and sorbent film thickness of 100 μm and 250 μm were used. 2 mL of 100 ng/mL 3-EP and 100 ng/mL 3-EP-d_4 solution saturated with K_2HPO_4 in water was used for this evaluation. 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 adsorb the analyte from

headspace vapors. Therefore, 250 μm PDMS SPME arrows were selected for further method development.

Extraction conditions. The effect of extraction temperature was studied from 60°C 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 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 min, 2 min 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 3-EP peak area, demonstrating that a longer extraction time than 2 min did not improve sensitivity. Therefore, a 2 min extraction time was used.

The effect of stirring. Sample stirring is generally used in SPME to shorten the equilibrium time needed for extraction.[42] Sample stirring was tested in this study with two different stirring rates of 600 rpm and 1200 rpm with an extraction time of 2 min. 2 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 that 1200 rpm produced a peak that was two times higher than 600 rpm.

The effect of salt and base. Salting-out is generally used in SPME in order to decrease the analyte solubility and to keep the ionic strength in real samples similar to standards.[43] Base addition was also applied in our study since 3-EP is a weak base with a pKa value around 5. Keeping the sample basic with a pH value greater than 7 ensures that 3-EP stays in free-base form and can be released from the solution. Saturated potassium phosphate dibasic and saturated sodium chloride with 50% K_3PO_4 addition were compared in these experiments. The result showed that 75 μL 50% K_3PO_4 solution is needed to adjust typical urine samples to a basic range, and 150 μL 50% K_3PO_4 solution is needed for 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 μL or 150 μL 50% K_3PO_4 addition for our sample preparation.

Desorption conditions. Desorption time in the injector was set to 1 min since 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.

Method Validation. (Table 2) The analytical method was validated according to protocols for bioanalytical method validation generally applicable for pharmacokinetic studies and biomarkers

Table 2. Intra-assay and inter-assay 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	Inter-assay precision (CV%), n=18, 3-5 different runs	Inter-assay accuracy (CV%), 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

in drug development.[44-46] Standard curves were constructed at six levels ranging from 0.2 to 20 ng/mL by spiking 3-EP into blank urine matrix. Precision, accuracy, and lower limit of quantitation (LLOQ) were evaluated by analyzing six different nonsmoker urines spiked with 3-

EP, who had no known secondhand smoke exposure, in the same run and over three to five different runs. Intra-assay and inter-assay 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. 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 nonsmoker urine, which were further divided into

Table 3. Stability of 3-EP in acidified and non-acidified urine samples.

Concentration (ng/ml)	pH Status	r.t. (24h)	4° C (30 days)	−20° C (30 days)
0	Non-acidified	<0.2	NA[a]	<0.2
	Acidified	<0.2	<0.2	<0.2
2	Non-acidified	2.22	NA[a]	2.04
	Acidified	2.12	2.130	2.10

[a]NA means not applied

two groups with three samples in the acidified group (pH 2-3, adjusted with sodium bisulfate) and two samples in the non-acidified group. The acidified group samples were stored at three temperatures with different periods, which were room temperature for 24 hours, and 4° C and −20° C for 30 days. The non-acidified group samples were stored at room temperature for 24 hours and −20° C for 30 days, respectively. After the storage period, the stability samples were processed as unknown samples and stability was evaluated. The results (Table 3) showed that 3-EP is stable under all examined pH and temperatures, with the accuracy between 102.0% and 111.2%.

Concentrations of 3-EP in Urine of People Using Various Tobacco Products (Table 4).

Since cigarette smoking remains the most prevalent form of tobacco use, the first groups of samples analyzed were cigarette smokers, and non-smokers who reported no significant

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

Product Use or Exposure	3-EP Urine Concentration, ng/mL				
	Mean (N)	Median	SD (Range)	Detection Frequency	p[b]
Cigarette Smokers	1.08 (16)	0.61	1.31 (BLQ-5.12)	88%	**0.01**
Cigarette Smokers[c]	0.45 (16)	0.27	0.63 (BLQ-2.56)	63%	0.07
Cigar Smokers[c]	0.66 (16)	0.42	0.87 (BLQ-3.74)	88%	**0.03**
Water Pipe Smokers	BLQ (10)	BLQ	0.09 (BLQ-0.42)	10%	0.34
e-Cigarette Users	BLQ (8)	BLQ	All BLQ	0%	0.99
Smokeless Tobacco Users	BLQ (11)	BLQ	0.02 (BLQ-0.22)	9%	0.34
Non-Smokers Pre Disco[d]	BLQ (9)	BLQ	All BLQ	0%	0.99
Non-Smokers Post Disco[d]	0.20 (9)	0.14	0.08 (BLQ-0.38)	44%	0.05
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/square root 2 was used.
[b]P-values are for differences between tobacco product use and non-tobacco users by t test.
[c]These participants were dual users of cigarettes and small cigars in a crossover study in which participants either smoked cigarettes or small cigars in different study blocks.[38]
[d]These participants were-non smokers who spent several hours in smoking-allowed discotheques.[36]

exposure to SHS. The goals were to determine whether measurable amounts of 3-EP are excreted in urine of smokers, and whether 3-EP is present in urine of non-smokers.

Two groups of cigarette smokers were studied. The first, N = 16, were daily cigarette smokers in a multi-site, randomized clinical trial.[38] 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%.

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.[39] 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 0.66 (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 0.45 (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 1.

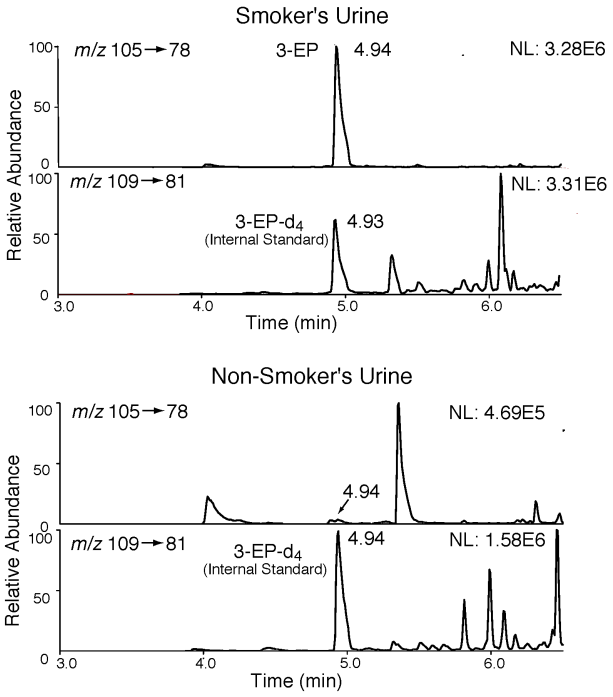


Figure 1. GC-MS/MS chromatograms of 3-EP in a smoker's urine and a non-smoker's urine

We also analyzed urine from electronic cigarette users,[40] water pipe (hookah) smokers,[32] and daily smokeless tobacco users. 3-EP concentrations were BLQ in urine of all of the 8 e-cigarette users. 3-EP concentrations were BLQ in urine of all but one of the 10 water pipe smokers. 3-EP concentrations were BLQ in urine of all but one of the 11 smokeless tobacco users (Table 4).

Concentrations of 3-EP in sidestream smoke are substantial (Table 1) and exposure in non-smokers 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.[41] For 9 non-smoking

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 urine of 4 subjects, with a mean for all 9 of 0.21 ng/mL, 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 homes of smokers and non-smokers, mean 3-EP concentrations of 0.08 $\mu g/m^3$ (N = 24, median undetectable) were found in non-smokers' 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 cigarettes smoked.[47] In our studies, concentrations of 3-EP in urine of non-smokers (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 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 is 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, 47]

Since 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 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 urine of 8 electronic cigarettes users. For 11 users smokeless tobacco, 3-EP was detected in 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 water pipe on a research ward, 3-EP was detected in urine of just one participant at a concentration of 0.42 ng/mL. Since these participants excreted substantial concentrations of nicotine metabolites,[32] we conclude that the temperatures achieved during water pipe 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 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. Distinguishing combusted tobacco use from 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 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, react with other substances in the environment, and substances that can be re-emitted from surfaces. Exposure to toxic substances in THS can be by transdermal absorption, ingestion of dust and hand-to-mouth behavior by young children, as well as by 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, that have a strong affinity for surfaces and can persist for long periods indoors after smoking ceases,[3] 3-EP has 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.

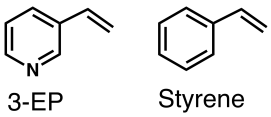


Figure 2. Structures of 3-EP and styrene

Concentrations of 3-EP in tobacco smoke are substantial (Table 1). Because of its structural similarity to styrene, a probable human carcinogen (Figure 2), consideration of the potential toxicity of 3-EP is warranted.[48] Surprisingly, very little has been published on its potential toxicity. In a study published in 1992,[49] 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).[50] We are unaware of any studies of 3-EP toxicity other than the 1992 study, which suggests that further studies are warranted.

CONCLUSIONS AND FUTURE STUDIES

We have demonstrated, we believe for the first time, that 3-EP is present and measurable in 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. Since 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.[48] 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 non-smokers exposed to tobacco smoke. Our study also calls attention to the significant exposure in both smokers and in non-smokers exposed to SHS, and that the paucity of toxicity data on 3-EP suggests the need for further toxicological studies.

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Author Contributions

Peyton Jacob and Jia Liu conceived and designed the study, and carried out data analysis. Jia Liu developed the analytical method and performed the urine analyses. Peyton Jacob synthesized the standards. Christopher Havel assisted with method development and data acquisition. Neal Benowitz, Dorothy Hatsukami, Eduardo Lazcano-Ponce, and Andrew Strasser 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.

Funding Sources

Financial support from the California Tobacco-Related Disease Research Program (TRDRP), 28PT- 0077 (P. Jacob) and 28PT-0013 (N. Benowitz), and from the National Institutes of Health, P30 DA012393 (P. Jacob), R01 CA141531 (D. Hatsukami), U54 CA229973 (A. Strasser), is gratefully acknowledged. The content is solely the responsibility of the authors and does not necessarily represent the official views of the TRDRP, the National Institutes of Health, or the US Food and Drug Administration that provided funding for U54 CA229973.

ACKNOWLEDGEMENTS

We thank Lisa Yu, Kristina Bello, Ti Won, and Marlene Gerodias for their assistance the analysis of samples for this study.

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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