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Article

Sensitive Quantification of Nicotine in Bronchoalveolar Lavage Fluid by Acetone Precipitation Combined With Isotope-Dilution Liquid Chromatography-Tandem Mass Spectrometry

Baoyun Xia,* Benjamin C. Blount, and Lanqing Wang*

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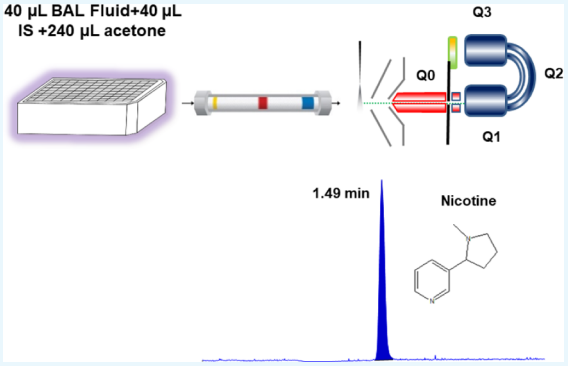
ABSTRACT: The United States experienced an outbreak of e-cigarette, or vaping, product use-associated lung injury (EVALI) that began in August 2019. Patient diagnosis and treatment sometimes involved bronchoscopy and collection of the bronchoalveolar lavage (BAL) fluid. Although this matrix has been useful for understanding some chemical exposures in the lungs, no methods existed for measuring the nicotine content. Therefore, we developed a simple and sensitive method for measuring nicotine in the BAL fluid. Nicotine was extracted from the BAL fluid using acetone precipitation in a 96-well plate format to increase the sample throughput (200 samples/day). We optimized liquid chromatography column conditions (e.g., mobile phase, column temperature) and mass spectrometry parameters to improve the signal-to-noise ratio and lower limits of detection (LOD) for measuring nicotine in the BAL fluid. The LOD for nicotine in the BAL fluid was 0.050 ng/mL at a sample volume of 40 μL of the BAL fluid. The within-day and between-day imprecision and bias were less than 10%. This method detected nicotine in 15 of 43 BAL fluids from EVALI case patients. This method is useful for understanding recent inhalational exposure to nicotine as part of characterizing EVALI or similar illnesses.



INTRODUCTION

As of February 18, 2020, the national outbreak of e-cigarette, or vaping, product use-associated lung injury (EVALI) had affected 2807 hospitalized patients across all 50 states, the District of Columbia, the U.S. Virgin Islands, and Puerto Rico, and 68 deaths have been confirmed in 29 states and the District of Columbia.[1] The National Emergency Department (ED) data and the active case reporting from state health departments for ED visits related to e-cigarette, or vaping, products show a sharp rise in symptoms or cases of EVALI in August 2019, a peak in September 2019, and a gradual but persistent decline thereafter.[1] Analysis of bronchoalveolar lavage (BAL) fluid samples found vitamin E acetate—an additive used in some tetrahydrocannabinol (THC)-containing e-cigarette, or vaping, products—to be present in BAL fluids from 48 of 51 case patients (94%) but not in the study participants without EVALI.[2−4] However, evidence is not sufficient to rule out the contribution of other chemicals of concern, including chemicals in either THC or non-THC products, in some of the reported EVALI cases.[5,6]

Nicotine is the primary tobacco-specific alkaloid in tobacco products (e.g., cigarettes, e-cigarettes) and their emissions.[7,8] Nicotine is highly addictive, which can lead to routine tobacco product use and chronic exposure to the carcinogens and bioactive compounds in these products and their emissions. The presence of nicotine in biological specimens indicates exposure to tobacco products or products containing nicotine, either through the active use of tobacco products or other products containing nicotine, or from secondhand smoke (SHS) exposure.[9,10] Many publications describe nicotine measurements using liquid chromatography-tandem mass spectrometry (LC-MS/MS) in different matrices including urine,[11−22] plasma or serum,[11,23−27] saliva,[15,20,25] and hair.[28−32] The lowest nicotine LODs for these methods are in the range of 1.00−10.0 ng/mL. We previously developed a sensitive LC-MS/MS method for measuring serum nicotine with LOD at about 0.050 ng/mL.[33] To support the emergency response to EVALI by the U.S. Centers for Disease Control and Prevention (CDC), the existing serum method was modified and validated to quantify nicotine in 43 BAL fluid samples obtained from EVALI case patients.

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MATERIALS AND METHODS

Standards and Reagents. Standards obtained from Toronto Research Chemicals, Ontario, Canada, were nicotine salicylate (Cat. #N428010), nicotine (Cat. #N412450), and nicotine-$^{13}CD_3$ (Cat. #N412424). Nicotine solution in 1.0 mg/mL methanol (Cat. #N-008) was obtained from Cerillient (Round Rock, TX). Other chemicals used and their sources include the following: acetone (Optima, A.C.S., Fisher Scientific, Waltham, MA, Cat. #A929SK-4), acetonitrile (A.C.S./HPLC Certified Solvent, Honeywell, Charlotte, NC, Cat. #BJAH015-4), ammonium hydroxide (Certified A.C.S. PLUS, Fisher Scientific, Cat. #A669S-500), hydrochloric acid (HCl) (Certified A.C.S. PLUS, Fisher Scientific, Cat. #A144-500), and high-performance liquid chromatography (HPLC) water (Tedia Company, Inc., Fairfield, OH, Cat. #WS2211001). Acidified HPLC water (pH about 3) was prepared by adding 8 drops of concentrated HCl into 4 L of HPLC water.

Instrumentation. LC-MS/MS analysis was performed using a Shimadzu Nexera ultra-high-performance liquid chromatography (UHPLC)/HPLC mixed module (Columbia, MD) in tandem with a Sciex API 6500 triple quadrupole system (Framingham, MA). The Shimadzu Nexera system consisted of a DGU-20A3 degasser, two LC30AC and one LC20AD pumps, a SIL-30ACMP autosampler, a CTO-20A column oven, and a CBM-20A controller. Chromatographic separation was conducted on a reversed-phase column (Agilent Poroshell, HPH-C18, 2.1 × 100 mm^2, 2.7 μm; CA).

The sample was eluted by a linear gradient of mobile phase A (0.05% ammonium hydroxide in H_2O, v/v) and mobile phase B [100% acetonitrile (ACN)] at a flow rate of 0.4 mL/min. Details for the LC gradient are shown in Table 1. We used post-column infusion of ACN at 0.1 mL/min to increase the signal response of nicotine in the mass spectrometer. Temperatures of the autosampler and LC column oven were held at 8 and 55 °C, respectively.

Table 1. LC Gradient Program for Chromatographic Separation of Nicotine[a]

time	module	event	% mobile phase B by volume
0.01	controller	start	
0.02	pumps	pump B conc	23
4.00	pumps	pump B conc	88
4.01	pumps	pump B conc	100
5.00	pumps	pump B conc	100
5.01	pumps	pump B conc	23
6.00	controller	stop	

[a]Mobile phase A is 0.05% ammonium hydroxide in water and mobile phase B is 100% acetonitrile.

Mass spectrometric analysis was carried out in the positive-ion mode with the following parameters: ionspray voltage at 3000 V, source heater temperature at 600 °C, curtain gas at 40 psi, ion source gas 1 at 90 psi, ion source gas 2 at 88 psi, and collision gas at 9 psi. All LC-MS/MS data were recorded at unit mass resolution in multiple reaction monitoring (MRM) mode. The MS/MS parameters for nicotine and its isotopically labeled internal standard (ISTD) are provided in Table 2. Analyst software 1.62 (Framingham, MA) was used to operate the LC-MS/MS system. We generated a formatted output file containing the final calculated concentration data and directly uploaded it to the laboratory information management system. Unknown samples were evaluated individually according to a set of quality assurance (QA) rules, including the difference in retention times of ISTD and native-ion transition peaks, confirmation ion ratio, ISTD peak area, and concentration exceeding calibration dynamic range. Batch quality controls (QCs) were evaluated according to modified Westgard QC rules.[34]

Table 2. Tandem Mass Spectrometer Parameters for NIC_QUAN, NIC_CONF, and NIC_ ISTD[a]

analyte	Q1 mass	Q3 mass	DP	EP	CE	CXP
NIC_QUAN	163.1	130.0	51	10	29	14
NIC_CONF	163.1	117.0	51	10	35	6
NIC_ISTD	167.1	130.0	51	10	29	16

[a]NIC_QUAN, nicotine quantitation transition; NIC_CONF, nicotine confirmation transition; NIC_ISTD, nicotine isotope internal standard (Nicotine_$^{13}CD_3$) transition; DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, cell exiting potential.

Preparation of Standard Solutions and ISTD Spiking Solution. Stock solutions were prepared using nicotine or nicotine salicylate and isotopically labeled nicotine_$^{13}CD_3$. Equivalent sources may be used for standards as well. To calculate the free nicotine concentration, we multiplied the ratio of the formula weight (FW) of nicotine by the FW of nicotine salicylate (162.23/300.35) when the latter was used as the standard. The initial stock solution was prepared by weighing the standard in a 100 mL polypropylene volumetric flask and then adding acidified HPLC water. The calibration curve standards at 12 concentration levels (from 0.00 to 5.00 ng/mL) were prepared by serial dilution and mixing of stock solutions in acidified HPLC water (pH about 3) (Table S2). Aliquots of 1.0 μL of each calibration standard were injected in the LC-MS/MS for analysis. We constructed calibration curves by plotting the peak area ratios of standards and ISTD against the concentration of standards using weighted linear regression (weight = $1/X$). Only values within the linear range of the assay were reported. The reportable range was 0.050−50.0 ng/mL.

The labeled ISTD solution was prepared by diluting the stock solution into the final working concentration to 12.5 ng/mL.

Sample Preparation. Aliquots of 40 μL of solution containing isotopically labeled nicotine-$^{13}CD_3$ (12.5 ng/mL) were spiked into 40 μL of acidified HPLC water in a polypropylene 96-well plate. We then added 40 μL of blank, low quality control (QCL), high quality control (QCH), or BAL fluid samples to the plate and mixed them. Finally, 240 μL of cold acetone was added to each well and the plate was sealed. Samples were refrigerated at 4 °C for at least 45 min to precipitate the salts and protein, and the precipitate was removed by centrifugation (30 min at 3200g and 4 °C). Immediately following centrifugation, we transferred a portion (100 μL) of the top BAL fluid/acetone solution to a new 96-well plate. An additional 100 μL of acidified HPLC water was added and mixed well. We directly injected 1 μL of the residual supernatant onto the LC-MS/MS.

BAL fluid pools were created for method validation experiments of the method from 12 anonymous BAL fluids purchased commercially from Discovery Life Sciences (Huntsville, AL). The individual BAL fluid was screened for the nicotine level to reduce the nicotine background level in the pooled BAL fluid below detectable levels.

EVALI Case Patients. The BAL fluid was collected as part of the clinical care of hospitalized EVALI cases. If residual BAL fluid was retained for an EVALI case, then the BAL fluid was

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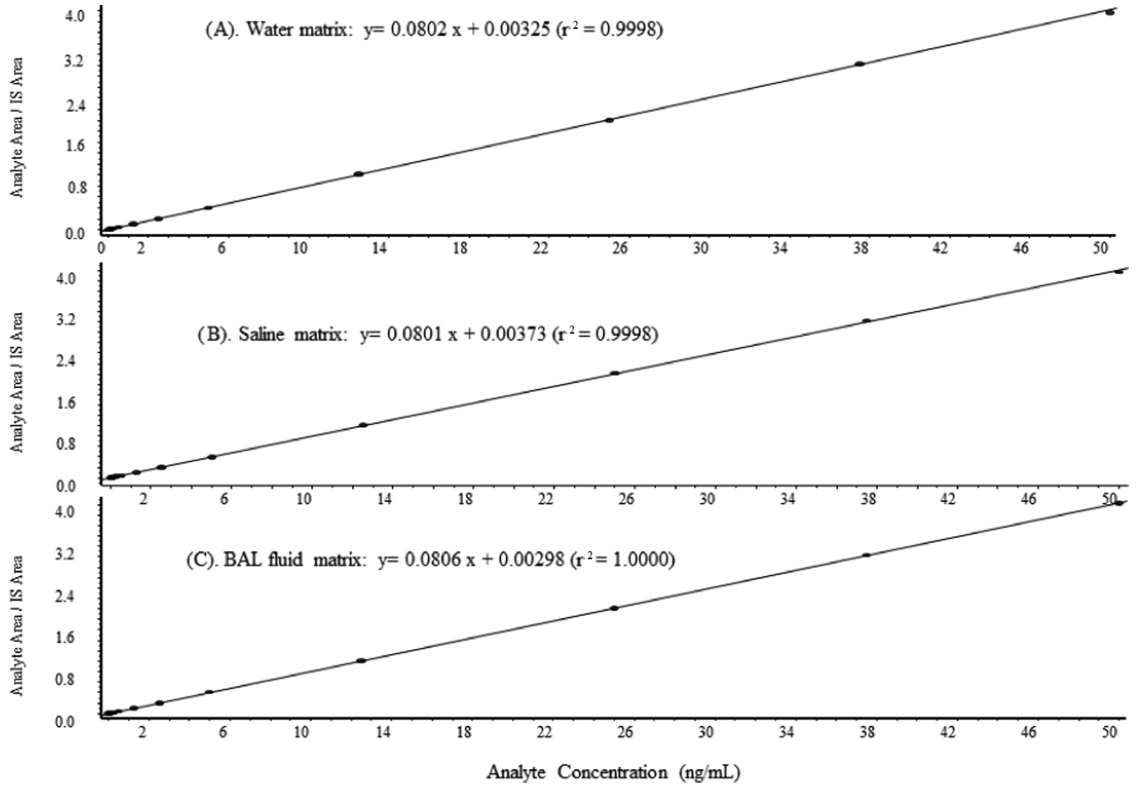

Figure 1. Nicotine calibration curves prepared in different sample matrices. (A) Water; (B) aqueous saline; and (C) BAL fluid.

Table 3. Accuracy and Recovery for the Measurement of Nicotine in BAL Fluid Pools

		BALF1					BALF2						
			measured concentration					measured concentration					
	replicate	spike concentration (ng/mL)	day 1	day 2	mean	recovery (%)	spike concentration (ng/mL)	day 1	day 2	mean	recovery (%)	mean recovery (%)	SD (%)
BALF	1	0.000	0.000	0.000	0.000		0.000	0.004	0.000	0.001		93.7	1.0
	2		0.000	0.000				0.000	0.000				
	3		0.000	0.000				0.003	0.000				
spike 1	1	1.00	0.963	0.937	0.943	94.3	1.00	0.934	0.903	0.928	92.7		
	2		0.941	0.910				0.951	0.948				
	3		0.954	0.953				0.936	0.894				
spike 2	1	5.00	4.78	4.81	4.73	94.7	5.00	4.74	4.60	4.66	93.1		
	2		4.72	4.69				4.69	4.58				
	3		4.74	4.66				4.61	4.71				
spike 3	1	50.0	47.9	47.5	47.4	94.9	50.0	46.3	46.4	46.4	92.8		
	2		47.0	47.7				46.1	47.0				
	3		47.5	47.1				45.7	47.0				

accepted by CDC for analysis (no other inclusion/exclusion criteria). Samples were refrigerated or frozen after collection and shipped to CDC on dry ice. Samples with limited volume were prioritized for toxicant assays and were analyzed for nicotine only if an adequate volume was available (N = 43). The CDC human subjects research review concluded that this information collection did not meet the regulatory definition of research under 45 CFR 46.102(d) and was therefore determined to be a nonresearch public health response activity.

RESULTS AND DISCUSSION

System Cleanup and Sample Preparation. Cleaning up the LC-MS/MS system to reduce the baseline background is a key step for acquiring a low LOD in nicotine measurements. The following procedures were used to decrease the noise resulting from trace amounts of nicotine in water or different container surfaces. We flushed the LC system for 2 h with acidified HPLC water and washed containers and pipette tips with acetone or ACN. We used high-quality HPLC-grade water and ACN to prepare mobile-phase solution and prepared mobile phase A fresh before every run. After optimizing the percentage of the mobile phase B (100% ACN) percentage, we found that starting at 23% B for LC gradient gave the best result for nicotine measurements.[33] Table S1 shows different factors optimized, which gave the nicotine the best sensitivity and lowest baseline noise for nicotine. Figure S1 shows the LC-MS/MS profile before and after cleanup procedures. The sample preparation procedure in this method is one-step acetone precipitation

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Table 4. Within-Run, Between-Run, and Total Precision of Nicotine in BAL Quality Control Pools

	QCH (n = 20)				QCL (n = 20)			
analyte	mean (ng/mL)	within-run (CV %)	between-run (CV %)	method (CV %)	mean (ng/mL)	within-run (CV %)	between-run (CV %)	method (CV %)
nicotine	4.71	1.3%	0.4%	1.4%	0.936	1.7%	1.5%	2.3%

Table 5. Stability of Nicotine in BAL QC Pools

	initial measurement	three freeze−thaw cycles	initial measurement	benchtop stability	initial measurement	processed-sample stability
			QCL			
replicate 1	0.951	0.923	0.951	0.892	0.951	0.944
replicate 2	0.960	0.940	0.960	0.966	0.960	0.980
replicate 3	1.00	0.930	1.00	0.911	1.00	0.979
mean	0.970	0.931	0.970	0.923	0.970	0.968
% difference from initial measurement		−4.05%		−4.88%		−0.28%
			QCH			
replicate 1	4.80	4.59	4.80	4.59	4.80	4.77
replicate 2	4.76	4.46	4.76	4.66	4.76	4.79
replicate 3	4.58	4.53	4.58	4.73	4.58	4.86
mean	4.71	4.53	4.71	4.66	4.71	4.80
% difference from initial measurement		−3.96%		−1.13%		1.98%

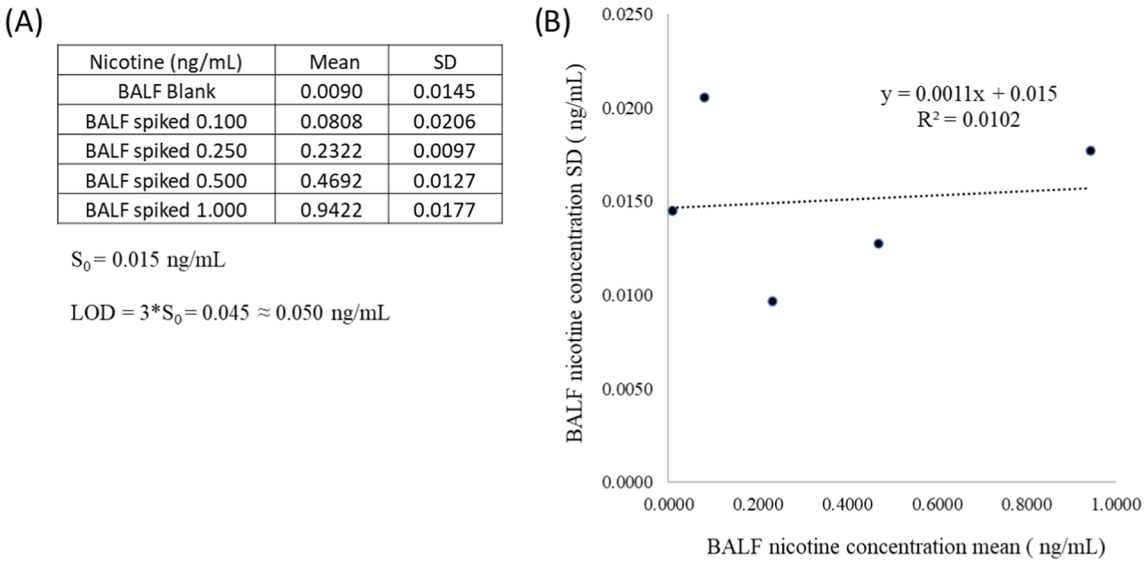

Figure 2. LOD calculations for nicotine in BAL fluids. (A) Mean and standard deviation (SD) of nicotine concentration was measured in the BAL fluid blank pool and BAL fluid pools spiked at different concentrations. (B) Standard deviation (SD) was plotted against the concentration means. The S_0 is the Y-intercept.

within a 96-well plate format. The samples were centrifuged and injected into LC-MS/MS without evaporation. In addition, the total run time including column equilibration and autosampler movement was 6 min. This high throughput method can measure 200 samples per day.

Matrix Matched Calibration and Linearity. Calibration curves were constructed using water, saline, and the BAL fluid as matrices. The saline and BAL fluid calibration standards were prepared using the same procedure as for the unknowns. The detailed standard preparation is shown in Tables S2−S4. The resulting calibration curves are shown in Figure 1. Strong linearity was observed in water (r^2 = 0.9998, panel A), aqueous saline (r^2 = 0.9998, panel B), and BAL fluid (r^2 = 1.0000, panel C). The concentrations measured in each matrix displayed a deviation of less than 5%. The influence of the BAL fluid matrix on the calibration curves was estimated by comparing the curve slopes built using BAL fluid matrix, saline, and water matrix. The slopes differed by <5%, indicating that a matrix has minimal impact on the quantification of nicotine based on calibration curves prepared in water (Figure 1).

Accuracy. We assessed accuracy through recovery analyses of BAL fluids after known amounts of nicotine are added (spiked in). We screened the commercial BAL fluid samples and selected the samples with nicotine levels below LOD for the blank pool. We made two pools: BALF1 and BALF2. For each blank pool, we spiked it with nicotine at zero concentration and 1.00, 5.00, and 50.0 ng/mL. For each of these concentrations, spiking was done in triplicate, resulting in a total of 12 samples. We analyzed the 12 samples in 2 analytical runs on 2 separate days, resulting in a total of 24 results. The recovery of the added analyte was

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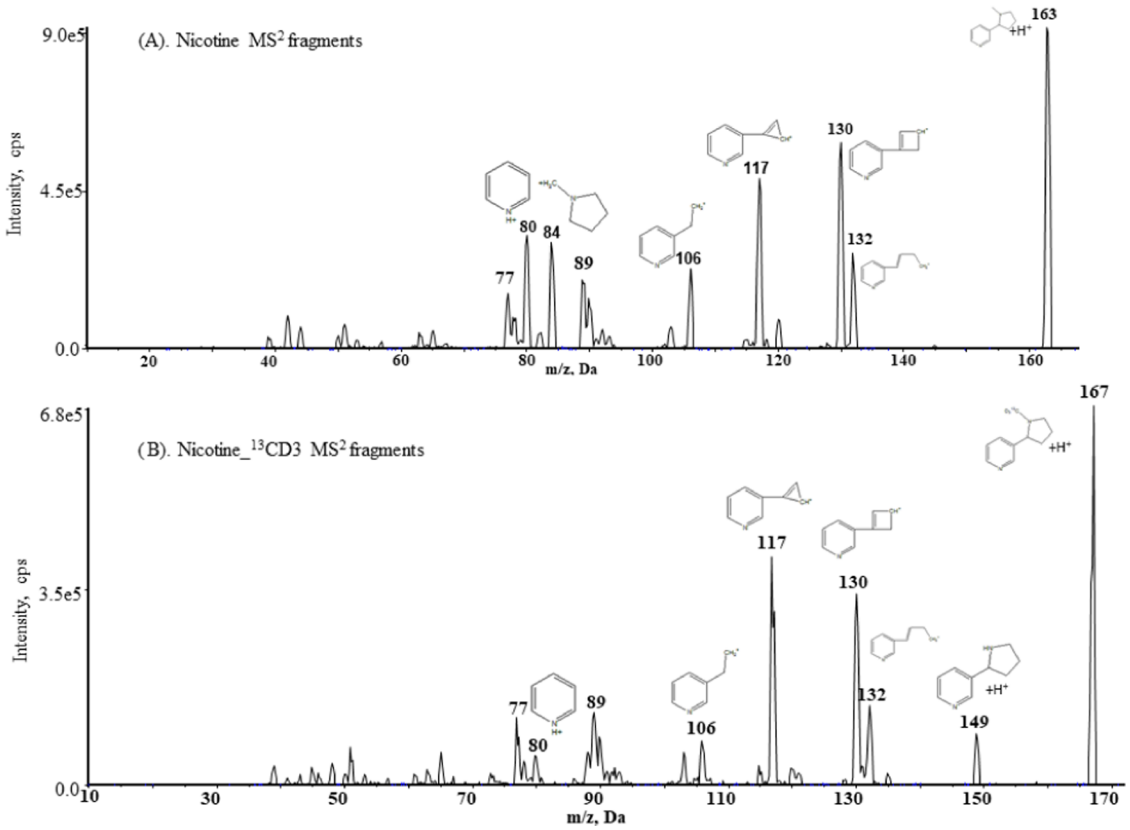

Figure 3. MS/MS (MS^2) product ion profiles. (A) Nicotine and (B) nicotine-$^{13}CD_3$.

calculated as [(final concentration − initial concentration)/added concentration] and ranged from 92.7 to 94.9% with a mean recovery of 93.7%, as shown in Table 3.

Precision. We used two concentrations of QC materials (QCL and QCH) to determine precision. For each QC material, we acquired 20 measurements in 10 different analytical runs (2 analyses per run) to calculate the coefficient of variation (CV) in within-run, between-run, and overall precision. The QCL had a within-run CV of 1.7%, a between-run CV of 1.5%, and a total CV of 2.3%. The QCH had a within-run CV of 1.3%, a between-run CV of 0.4%, and a total CV of 1.4%. The total CV for QCL or QCH was less than 10%. The precision of within-runs and between-runs is shown in Table 4.

Stability. We evaluated nicotine stability with the QCL and QCH and data are shown in Table 5. Three replicates of the QCL and QCH were freshly prepared for the evaluation. We tested the nicotine stabilities in BAL fluid by conditions that a sample is likely to encounter during analysis, including freeze−thaw cycles, benchtop stability, and processed-sample stability. We performed 3 freeze−thaw cycles as follows: removing the QCL and QCH from the −70°C freezer, allowing them to stand at room temperature for 4 h, and refreezing them. Nicotine concentration was analyzed before the initial freeze and after the final thaw. The percentage difference from the initial measurement and after the 3 freeze−thaw cycles was 4.0% of decrease for QCL and QCH. We tested benchtop stability by allowing samples to stand at room temperature for 24 h before being processed. The calculated percentage of decrease from initial measurement of QCL and QCH was 4.9 and 1.1%, respectively. We evaluated the processed-sample stability by allowing the processed QCL and QCH in the autosampler to stand at 8 °C for 24 h. Compared to the initial measurements, the calculated percentage difference from the initial measurement of QCL and QCH was −0.3 and 2.0%, respectively. Nicotine was stable in the BAL fluid during these sample processing procedures.

LOD. The LOD was calculated based on the extrapolated standard deviation at zero concentration.[35] Five BAL fluid pools (blank, 0.100, 0.250, 0.500, and 1.00 ng/mL) were spiked and analyzed repeatedly on different days ($N > 20$) (Figure 2). We plotted the standard deviation of each pool against the concentration means. We obtained the Y-intercept S_0, which was the standard deviation at zero-analyte concentration. Nicotine LOD was calculated as 3 times the S_0. The LOD was 0.050 ng/mL. We also investigated the carryover effect: following an injection of the highest calibrator, no nicotine peak was observed on the next injection of a blank solvent.

Analytical Specificity. We verified analytical specificity to ensure that only the correct component was measured and also examined the effects of potentially interfering substances. We scanned the nicotine product ions to selectively monitor ion transitions (Figure 3) and checked retention times for nicotine and nicotine-$^{13}CD_3$ to ensure consistency within runs. To confirm the right analyte in unknown samples, we also calculated the quantitation ion/confirmation ion ratio. Potential interferences with nicotine were investigated in human BAL fluid samples. Figure 4 shows representative chromatograms of a patient's BAL fluid profile compared with a water bank and a saline profile.

Application to BAL Fluid Samples. BAL fluids were obtained and analyzed from 43 EVALI cases. All of the detected results were within the linear range of the assay. Nicotine was detected in 35% of the 43 BAL fluid samples, indicative of recent use of an inhalation nicotine product such as a nicotine-containing e-cigarette or a conventional cigarette.

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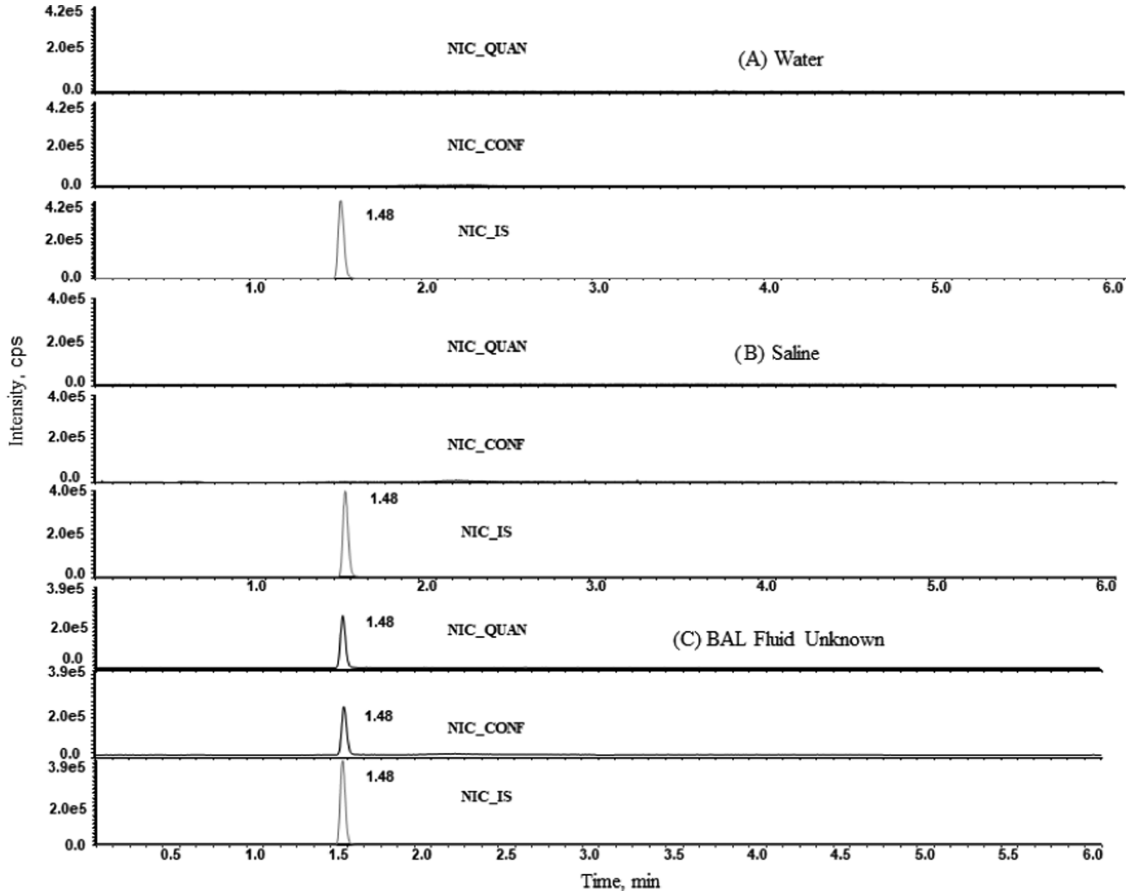

Figure 4. Nicotine LC-MS/MS profiles in (A) water, (B) saline, and (C) BAL fluid unknown. NIC_QUAN: nicotine quantitation transition; NIC_CONF: nicotine confirmation transition; and NIC_IS: nicotine isotope internal standard (Nicotine_$^{13}CD_3$).

CONCLUSIONS

We developed a sensitive method for quantifying nicotine in BAL fluids using acetone precipitation combined with isotope-dilution LC-MS/MS. Because of its high sensitivity, precision, accuracy, and throughput, this assay required only 40 μL of samples for detecting nicotine exposure in the BAL fluid matrix. The method has an LOD of 0.050 ng/mL, which is approximately 10 times more sensitive than the LODs of previously reported methods for urine, serum, or plasma in the literature. This method is useful for understanding the recent inhaled-nicotine exposure as it relates to nicotine uptake as well as forensic investigations to link inhaled product use with acute lung injuries such as those observed in EVALI case patients.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c05696.

Factors that affect the nicotine sensitivity and baseline noise; standard curve prepared in water; standard curve with the saline matrix and process as unknowns; standard curve with the BAL fluid matrix and process as unknowns; comparison of the nicotine LC-MS/MS profile of (A) before system cleanup and (B) after system cleanup (PDF)

AUTHOR INFORMATION

Corresponding Authors

Baoyun Xia − *Tobacco and Volatiles Branch, Division of Laboratory Sciences, National Center for Environmental Health, U.S. Centers for Disease Control and Prevention (CDC), Atlanta, Georgia 30341, United States;* orcid.org/0000-0001-5081-7447; Phone: 770-488-0148; Email: vvq2@cdc.gov

Lanqing Wang − *Tobacco and Volatiles Branch, Division of Laboratory Sciences, National Center for Environmental Health, U.S. Centers for Disease Control and Prevention (CDC), Atlanta, Georgia 30341, United States*; Phone: 770-488-7914; Email: lfw3@cdc.gov

Author

Benjamin C. Blount − *Tobacco and Volatiles Branch, Division of Laboratory Sciences, National Center for Environmental Health, U.S. Centers for Disease Control and Prevention (CDC), Atlanta, Georgia 30341, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.0c05696

Notes

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention. Use of trade names and commercial sources is for identification only and does not constitute an endorsement by the U.S. Department of

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■ REFERENCES

(1) Outbreak of Lung Injury Associated with the Use of E-Cigarette, or Vaping, Products, 2019. https://www.cdc.gov/tobacco/basic_information/e-cigarettes/severe-lung-disease.html?s_cid=osh-stuhome-spotlight-006.

(2) Jiang, H.; Ahmed, C. M. S.; Martin, T. J.; Canchola, A.; Oswald, I. W. H.; Garcia, J. A.; Chen, J. Y.; Koby, K. A.; Buchanan, A. J.; Zhao, Z.; Zhang, H.; Chen, K.; Lin, Y. H. Chemical and Toxicological Characterization of Vaping Emission Products from Commonly Used Vape Juice Diluents. *Chem. Res. Toxicol.* **2020**, *33*, 2157−2163.

(3) Blount, B. C.; Karwowski, M. P.; Shields, P. G.; Morel-Espinosa, M.; Valentin-Blasini, L.; Gardner, M.; Braselton, M.; Brosius, C. R.; Caron, K. T.; Chambers, D.; Corstvet, J.; Cowan, E.; De Jesús, V. R.; Espinosa, P.; Fernandez, C.; Holder, C.; Kuklenyik, Z.; Kusovschi, J. D.; Newman, C.; Reis, G. B.; Rees, J.; Reese, C.; Silva, L.; Seyler, T.; Song, M. A.; Sosnoff, C.; Spitzer, C. R.; Tevis, D.; Wang, L.; Watson, C.; Wewers, M. D.; Xia, B.; Heitkemper, D. T.; Ghinai, I.; Layden, J.; Briss, P.; King, B. A.; Delaney, L. J.; Jones, C. M.; Baldwin, G. T.; Patel, A.; Meaney-Delman, D.; Rose, D.; Krishnasamy, V.; Barr, J. R.; Thomas, J.; Pirkle, J. L. Vitamin E Acetate in Bronchoalveolar-Lavage Fluid Associated with EVALI. *N. Engl. J. Med.* **2020**, *382*, 697−705.

(4) Blount, B. C.; Karwowski, M. P.; Morel-Espinosa, M.; Rees, J.; Sosnoff, C.; Cowan, E.; Gardner, M.; Wang, L.; Valentin-Blasini, L.; Silva, L.; De Jesus, V. R.; Kuklenyik, Z.; Watson, C.; Seyler, T.; Xia, B.; Chambers, D.; Briss, P.; King, B. A.; Delaney, L.; Jones, C. M.; Baldwin, G. T.; Barr, J. R.; Thomas, J.; Pirkle, J. L. Evaluation of Bronchoalveolar Lavage Fluid from Patients in an Outbreak of E-cigarette, or Vaping, Product Use-Associated Lung Injury − 10 States, August-October 2019. *Morb. Mortal. Wkly. Rep.* **2019**, *68*, 1040−1041.

(5) Kleinman, M. T.; Arechavala, R. J.; Herman, D.; Shi, J.; Hasen, I.; Ting, A.; Dai, W.; Carreno, J.; Chavez, J.; Zhao, L.; Kloner, R. A. E-cigarette or Vaping Product Use-Associated Lung Injury Produced in an Animal Model From Electronic Cigarette Vapor Exposure Without Tetrahydrocannabinol or Vitamin E Oil. *J. Am. Heart Assoc.* **2020**, *9*, No. e017368.

(6) Ghinai, I.; Navon, L.; Gunn, J. K. L.; Duca, L. M.; Brister, S.; Love, S.; Brink, R.; Fajardo, G.; Johnson, J.; Saathoff-Huber, L.; King, B. A.; Jones, C. M.; Krishnasamy, V. P.; Layden, J. E. Characteristics of Persons Who Report Using Only Nicotine-Containing Products Among Interviewed Patients with E-cigarette, or Vaping, Product Use-Associated Lung Injury - Illinois, August-December 2019. *Morb. Mortal. Wkly. Rep.* **2020**, *69*, 84−89.

(7) Hukkanen, J.; Jacob, P., 3rd; Benowitz, N. L. Metabolism and disposition kinetics of nicotine. *Pharmacol. Rev.* **2005**, *57*, 79−115.

(8) Benowitz, N. L. Nicotine addiction. *N. Engl. J. Med.* **2010**, *362*, 2295−2303.

(9) Pirkle, J. L.; Flegal, K. M.; Bernert, J. T.; Brody, D. J.; Etzel, R. A.; Maurer, K. R. Exposure of the US population to environmental tobacco smoke: the Third National Health and Nutrition Examination Survey, 1988 to 1991. *JAMA* **1996**, *275*, 1233−1240.

(10) Pirkle, J. L.; Bernert, J. T.; Caudill, S. P.; Sosnoff, C. S.; Pechacek, T. F. Trends in the exposure of nonsmokers in the U.S. population to secondhand smoke: 1988-2002. *Environ. Health Perspect.* **2006**, *114*, 853−858.

(11) Yue, B.; Kushnir, M. M.; Urry, F. M.; Rockwood, A. L. Quantitation of nicotine, its metabolites, and other related alkaloids in urine, serum, and plasma using LC-MS-MS. *Methods Mol. Biol.* **2010**, *603*, 389−398.

(12) Xu, X.; Iba, M. M.; Weisel, C. P. Simultaneous and sensitive measurement of anabasine, nicotine, and nicotine metabolites in human urine by liquid chromatography-tandem mass spectrometry. *Clin. Chem.* **2004**, *50*, 2323−2330.

(13) von Weymarn, L. B.; Thomson, N. M.; Donny, E. C.; Hatsukami, D. K.; Murphy, S. E. Quantitation of the Minor Tobacco Alkaloids Nornicotine, Anatabine, and Anabasine in Smokers' Urine by High Throughput Liquid Chromatography-Mass Spectrometry. *Chem. Res. Toxicol.* **2016**, *29*, 390−397.

(14) Taghavi, T.; Novalen, M.; Lerman, C.; George, T. P.; Tyndale, R. F. A Comparison of Direct and Indirect Analytical Approaches to Measuring Total Nicotine Equivalents in Urine. *Cancer Epidemiol., Biomarkers Prev.* **2018**, *27*, 882−891.

(15) Shaik, F. B.; Nagajothi, G.; Swarnalatha, K.; Kumar, C. S.; Maddu, N. Quantification of Nicotine and Cotinine in Plasma, Saliva, and Urine by HPLC Method in Chewing Tobacco Users. *Asian Pac. J. Cancer Prev.* **2019**, *20*, 3617−3623.

(16) Miller, E. I.; Norris, H. R.; Rollins, D. E.; Tiffany, S. T.; Wilkins, D. G. A novel validated procedure for the determination of nicotine, eight nicotine metabolites and two minor tobacco alkaloids in human plasma or urine by solid-phase extraction coupled with liquid chromatography-electrospray ionization-tandem mass spectrometry. *J. Chromatogr. B* **2010**, *878*, 725−737.

(17) Meger, M.; Meger-Kossien, I.; Schuler-Metz, A.; Janket, D.; Scherer, G. Simultaneous determination of nicotine and eight nicotine metabolites in urine of smokers using liquid chromatography-tandem mass spectrometry. *J. Chromatogr. B* **2002**, *778*, 251−261.

(18) McGuffey, J. E.; Wei, B.; Bernert, J. T.; Morrow, J. C.; Xia, B.; Wang, L.; Blount, B. C. Validation of a LC-MS/MS method for quantifying urinary nicotine, six nicotine metabolites and the minor tobacco alkaloids–anatabine and anabasine–in smokers' urine. *PLoS One* **2014**, *9*, No. e101816.

(19) Marclay, F.; Saugy, M. Determination of nicotine and nicotine metabolites in urine by hydrophilic interaction chromatography-tandem mass spectrometry: Potential use of smokeless tobacco products by ice hockey players. *J. Chromatogr. A* **2010**, *1217*, 7528−7538.

(20) Kataoka, H.; Inoue, R.; Yagi, K.; Saito, K. Determination of nicotine, cotinine, and related alkaloids in human urine and saliva by automated in-tube solid-phase microextraction coupled with liquid chromatography-mass spectrometry. *J. Pharm. Biomed. Anal.* **2009**, *49*, 108−114.

(21) Heavner, D. L.; Richardson, J. D.; Morgan, W. T.; Ogden, M. W. Validation and application of a method for the determination of nicotine and five major metabolites in smokers' urine by solid-phase extraction and liquid chromatography-tandem mass spectrometry. *Biomed. Chromatogr.* **2005**, *19*, 312−328.

(22) Wei, B.; Feng, J.; Rehmani, I. J.; Miller, S.; McGuffey, J. E.; Blount, B. C.; Wang, L. A high-throughput robotic sample preparation system and HPLC-MS/MS for measuring urinary anatabine, anabasine, nicotine and major nicotine metabolites. *Clin. Chim. Acta* **2014**, *436*, 290−297.

(23) Yuan, C.; Kosewick, J.; Wang, S. A simple, fast, and sensitive method for the measurement of serum nicotine, cotinine, and nornicotine by LC-MS/MS. *J. Sep. Sci.* **2013**, *36*, 2394−2400.

(24) Shu, I.; Wang, P. Simultaneous serum nicotine, cotinine, and trans-3′-hydroxycotinine quantitation with minimal sample volume for tobacco exposure status of solid organ transplant patients. *J. Chromatogr. B* **2013**, *928*, 139−145.

(25) Byrd, G. D.; Davis, R. A.; Ogden, M. W. A rapid LC-MS-MS method for the determination of nicotine and cotinine in serum and saliva samples from smokers: validation and comparison with a radioimmunoassay method. *J. Chromatogr. Sci.* **2005**, *43*, 133−140.

https://doi.org/10.1021/acsomega.0c05696
ACS Omega 2021, 6, 13962−13969

(26) Baumann, F.; Regenthal, R.; Burgos-Guerrero, I. L.; Hegerl, U.; Preiss, R. Determination of nicotine and cotinine in human serum by means of LC/MS. *J. Chromatogr. B* **2010**, *878*, 107−111.
(27) Abdallah, I. A.; Hammell, D. C.; Stinchcomb, A. L.; Hassan, H. E. A fully validated LC-MS/MS method for simultaneous determination of nicotine and its metabolite cotinine in human serum and its application to a pharmacokinetic study after using nicotine transdermal delivery systems with standard heat application in adult smokers. *J. Chromatogr. B* **2016**, *1020*, 67−77.
(28) Tzatzarakis, M. N.; Vardavas, C. I.; Terzi, I.; Kavalakis, M.; Kokkinakis, M.; Liesivuori, J.; Tsatsakis, A. M. Hair nicotine/cotinine concentrations as a method of monitoring exposure to tobacco smoke among infants and adults. *Hum. Exp. Toxicol.* **2012**, *31*, 258−265.
(29) Miller, E. I.; Murray, G. J.; Rollins, D. E.; Tiffany, S. T.; Wilkins, D. G. Validation of a liquid chromatography-tandem mass spectrometry method for the detection of nicotine biomarkers in hair and an evaluation of wash procedures for removal of environmental nicotine. *J. Anal. Toxicol.* **2011**, *35*, 321−332.
(30) Kim, J.; Cho, H. D.; Suh, J. H.; Lee, J. Y.; Lee, E.; Jin, C. H.; Wang, Y.; Cha, S.; Im, H.; Han, S. B. Analysis of Nicotine Metabolites in Hair and Nails Using QuEChERS Method Followed by Liquid Chromatography-Tandem Mass Spectrometry. *Molecules* **2020**, *25*, No. 1763.
(31) Inukai, T.; Kaji, S.; Kataoka, H. Analysis of nicotine and cotinine in hair by on-line in-tube solid-phase microextraction coupled with liquid chromatography-tandem mass spectrometry as biomarkers of exposure to tobacco smoke. *J. Pharm. Biomed. Anal.* **2018**, *156*, 272−277.
(32) Chetiyanukornkul, T.; Toriba, A.; Kizu, R.; Kimura, K.; Hayakawa, K. Hair analysis of nicotine and cotinine for evaluating tobacco smoke exposure by liquid chromatography-mass spectrometry. *Biomed. Chromatogr.* **2004**, *18*, 655−661.
(33) Xia, B.; McGuffey, J.; Xia, Y.; Guillot, T.; McGahee, E.; Wang, L.; Blount, B. *Sensitive, Rapid and High Throughput Measurement of Nicotine in Human Serum by Automation and Liquid Chromatography-Atmospheric Pressure Ionization Tandem Mass Spectrometry*; American Chemical Society: Washington, DC, 2017.
(34) Caudill, S. P.; Schleicher, R. L.; Pirkle, J. L. Multi-rule quality control for the age-related eye disease study. *Stat. Med.* **2008**, *27*, 4094−4106.
(35) Taylor, J. K. Quality assurance of chemical measurements. *Anal. Chem.* **1981**, *53*, 1588A−1596A.

https://doi.org/10.1021/acsomega.0c05696
ACS Omega 2021, 6, 13962−13969