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Rapid Development and Validation of a Liquid Chromatography-Tandem Mass Spectrometry Method to Measure Cannabinoids in Bronchoalveolar-Lavage Fluid of Patients with e-Cigarette, or Vaping, Product Use-Associated Lung Injury

Christina R. Brosius,[†] Kevin T. Caron,[†] Connie S. Sosnoff,* Benjamin C. Blount, and Lanqing Wang

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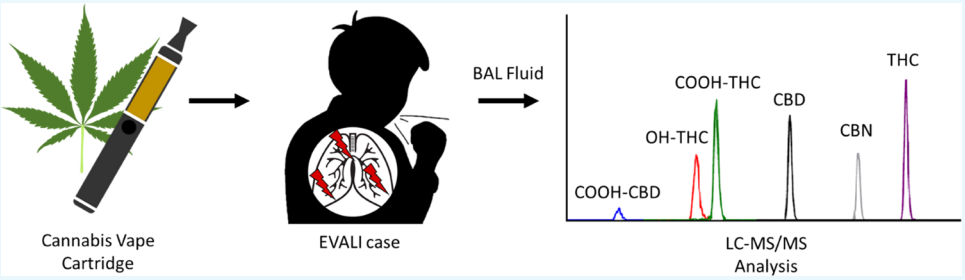

ABSTRACT: In 2019, the Centers for Disease Control and Prevention responded to an outbreak of e-cigarette, or vaping, product use-associated lung injury (EVALI). Bronchoalveolar-lavage (BAL) fluid from EVALI patients was available for analysis to investigate a range of potential toxicants that might be present at the presumed site of lung injury. Our laboratory developed and validated a novel method to measure cannabinoids and their metabolites in BAL fluid to aid in the investigation of the toxicants that might be the cause of EVALI. In this paper, we describe a sensitive liquid chromatography-tandem mass spectrometry method to measure the following six cannabinoids: Δ^9-tetrahydrocannabinol (THC), THC metabolites 11-nor-9-carboxy-THC and 11-hydroxy-THC, cannabinol, cannabidiol (CBD), and CBD metabolite 7-nor-7-carboxycannabidiol. Cannabinoids were extracted from BAL fluid using solid-phase extraction. Accuracy, precision, stability, and limits of detection were determined from replicate analyses of spiked BAL pools. The lower limits of detection ranged from 0.019 to 0.153 ng/mL for a sample volume of 150 μL. Overall accuracy ranged from 71.0 to 100.8%. Within-run imprecision (measured by the coefficient of variation) was below 8%, and between-run imprecision was below 21% for all analytes and concentrations tested. The method was applied to samples from 59 EVALI case patients. We identified THC, CBD, or their metabolites in 76% of EVALI patient samples. These findings support previous evidence that THC-containing products played a major role in the EVALI outbreak and help to inform public health recommendations.

INTRODUCTION

In August 2019, the Centers for Disease Control and Prevention (CDC) began collecting data from states regarding cases of severe pulmonary diseases associated with the use of electronic cigarette (e-cigarette) products from state health departments across the United States.[1] Eventually, more than 2800 similar cases resulted in hospitalization for what was eventually termed e-cigarette, or vaping, product use-associated lung injury (EVALI).[2] As the outbreak progressed, public health officials investigating possible sources of chemical exposure needed further information about whether patients were using only nicotine-containing products, only vaping products containing cannabis, or both nicotine and cannabis.[3−5] Reports of EVALI patients having recently used cannabis-containing vape products[6] as well as accounts that a number of chemical diluents were being added to these products[7] suggested an urgent need to test EVALI patients for exposure to toxicants, either inadvertent contaminants or intentional additives, that could be a cause of the lung injury. Testing patients for exposure to cannabis and nicotine[8] could help associate lung injury with the products that were used and thus help determine the source of toxicant exposure. To detect harmful substances at the presumed site of injury, CDC

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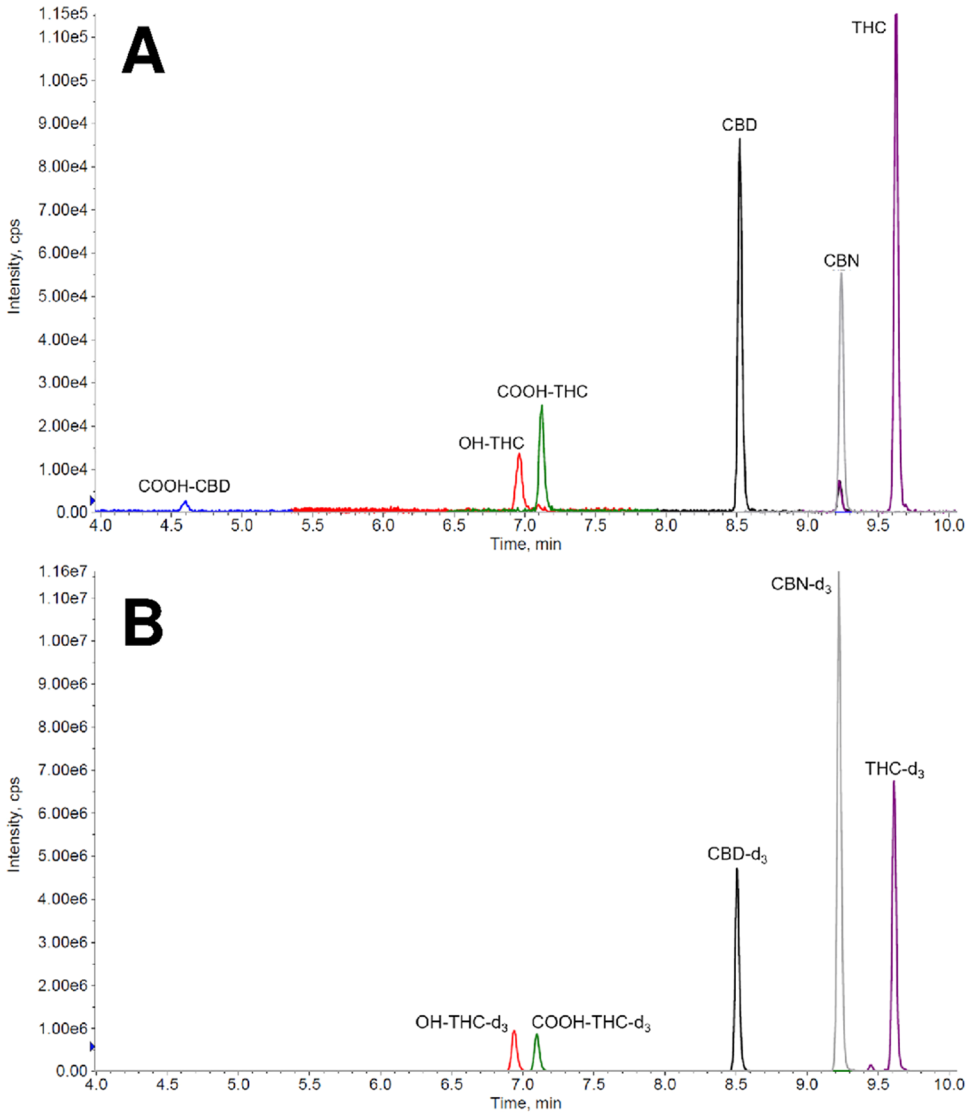

Figure 1. (A) Extracted ion chromatogram of 7-nor-7-carboxycannabidiol (COOH-CBD), 11-hydroxy-THC (OH-THC), 11-nor-9-carboxy-THC (COOH-THC), cannabidiol (CBD), cannabinol (CBN), and Δ^9-tetrahydrocannabinol (THC) quantitation ions, by retention time, in Calibrator 06 (2.5 ng/mL). (B) Extracted ion chromatogram of isotopically labeled internal standards, OH-THC-d_3, COOH-THC-d_3, CBD-d_3, CBN-d_3, and THC-d_3 in the same calibrator.

collaborated with state health departments to test residual bronchoalveolar-lavage (BAL) fluid, obtained via bronchoscopy from EVALI patients, for a number of possible chemicals that could be associated with the source of exposure.[9]

To establish the presence or absence of cannabinoids in the BAL fluid of EVALI patients, CDC rapidly developed and validated a method to detect six cannabinoids of interest: Δ^9-tetrahydrocannabinol (THC), 11-nor-9-carboxy-THC (COOH-THC), 11-hydroxy-THC (OH-THC), cannabidiol (CBD), 7-nor-7-carboxycannabidiol (COOH-CBD), and cannabinol (CBN). THC is the principal psychoactive constituent in cannabis and is metabolized to COOH-THC as well as OH-THC in the human body. CBD is a major nonpsychoactive cannabinoid found in a variety of cannabis-containing products and is metabolized to COOH-CBD. CBN is not biosynthesized by cannabis plants but is a primary product of THC degradation with exposure to light and air.[10]

In February 2020, CDC laboratories reported detecting vitamin E acetate, a thickening agent found in some cannabis-containing vape products,[11] in 48 of 51 BAL samples from EVALI cases and cannabinoids in 40 of 47.[12] The following report describes the BAL fluid method CDC used to evaluate EVALI patient exposure to cannabinoids.

RESULTS

We developed this assay rapidly during the initial weeks of the EVALI public health emergency, so the method validation process was abbreviated. The current method is based on our existing validated urinary cannabinoid assay, with modifications from preliminary research for a serum cannabinoid assay.[13] We used the urinary cannabinoid calibrators and quality control (QC) pools and the same sample extraction procedure that we had already validated for urinary cannabinoids. The high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) analysis was also the same as our urinary method; however, we added COOH-CBD as an analyte to the method. Our urinary assay is based on a 0.5 mL sample size. We did not know how much BAL fluid would be available for cannabinoid analysis, so we

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developed and validated the method based on only 50 μL of BAL fluid. After analyzing the first batch of EVALI BAL fluid samples using 50 μL, we subsequently used 150 μL of BAL fluid when adequate volume was available.

We did not have enough blank BAL fluid to use for calibrators, so we used phosphate-buffered saline as the matrix for the calibrators, QCs, and blanks. THC, CBD, and CBN are lipophilic and will stick to plastic, glass, and other materials used for sample preparation. To minimize losses due to adsorption, we used silanized glassware whenever possible and prepared all cannabinoid solutions including internal standards (ISTDs), calibrators, and QCs in methanol and water (v/v 60:40).

Calibration Curve. The extracted ion chromatograms of the quantitation ions (panel A) and the ISTD ions (panel B) for a calibrator (CAL-06, 2.5 ng/mL) are shown in Figure 1.

Calibration curves showed good linearity with coefficients of determination, r^2, greater than 0.995 for all analytes. The instrumental linear range varied by analyte: for THC, COOH-THC, and CBN, it ranged from 0.01 to 500 ng/mL; for CBD, it ranged from 0.1 to 500 ng/mL; and for OH-THC and COOH-CBD, the linear range was from 0.2 to 500 ng/mL. We observed an interference in the two lowest OH-THC calibrators (CAL-01 and CAL-02) that prevented accurate peak integrations, and it sometimes prevented accurate integration of CAL-03 (0.2 ng/mL) as well. Because of this, the linear range for OH-THC was sometimes shortened at the low-concentration end. Representative regression equations are shown in Table 1.

Table 1. Representative Regression Equations for Cannabinoids in Phosphate-Buffered Saline[a]

analyte	regression equation	r^2
THC	$y = 0.1327x - 0.00013$	0.9996
COOH-THC	$y = 0.2173x + 0.00052$	0.9997
OH-THC	$y = 0.1060x + 0.00240$	0.9998
CBD	$y = 0.1620x - 0.00060$	0.9997
COOH-CBD	$y = 0.0187x + 0.00040$	0.9980
CBN	$y = 0.0374x + 0.00013$	0.9997

[a]THC, Δ^9-tetrahydrocannabinol; COOH-THC, 11-nor-9-carboxy-THC; OH-THC, 11-hydroxy-THC; CBD, cannabidiol; COOH-CBD, 7-nor-7-carboxycannabidiol; and CBN, cannabinol.

Accuracy. Accuracy was assessed by replicate analyses of two unique BAL pools spiked at three concentration levels. We analyzed them in triplicate along with the two unspiked BAL pools on each of 2 days. Overall accuracy for each concentration level was the mean of the accuracy results obtained from each spiked pool and ranged from 71.0 to 100.8%. Overall accuracy for the lowest spiked pool (0.5 ng/mL) ranged from 85.5 to 100.8%. All accuracy results are presented in Table 2.

Precision. Within-run and between-run precisions were determined from repeat analyses of two unique BAL pools spiked at two concentration levels. We analyzed them in duplicate in two separate runs each day for 3 days. The coefficient of variation (CV) did not exceed 9.5% for any analyte at either concentration level except for COOH-CBD in the low-concentration pool where the between-run CV was 20.7%. The overall method CV ranged from 5.7 to 22.2% for the low pool and from 4.2 to 11.4% for the high pool. Long-term interday precision was determined from the analysis of data from two QC pools assayed over 5 months. All long-term precision CVs were less than 12% for QCL except for OH-THC and COOH-CBD, which had CVs of 17.0 and 29.6%, respectively. Long-term CVs for QCH ranged from 3.3 to 8.4%. All precision results are presented in Table 3.

Limit of Detection (LOD) and Linear Range. The method LOD was evaluated as 3 times S_0, the extrapolated standard deviation at zero concentration.[14] Five BAL pools spiked with cannabinoids at five low concentrations were assayed repeatedly over the course of several days. Regression lines were obtained after plotting the standard deviations vs the mean concentrations of the five pools, and S_0 was determined for each analyte. We analyzed the BAL LOD pools using a sample size of 50 μL. For sample volumes different from 50 μL, the LOD can be calculated as follows: LOD at sample volume 2 = (LOD at 50 μL) × (50 μL)/(sample volume 2 in μL). For a sample volume of 150 μL, the LODs range from 0.019 ng/mL for COOH-THC to 0.153 ng/mL for OH-THC. All method LODs are given in Table 4.

The linear range of this method is from the method LOD, or the lowest valid calibrator concentration (whichever is higher), to the highest valid calibrator concentration. Sample results were reportable if they were within the linear range of the calibration curves for that day.

Specificity. The initial specificity of the assay was established by analyzing 10 commercial BAL samples. No interfering peaks were seen in the quantitation chromatograms for any analyte in any of the samples. Confirmation ion ratios and relative retention times were checked, and no sample results were reported for samples if these were outside of the established limits. Figures 2 and 3 show sample chromatograms from EVALI cases with detectable analytes and with no detectable analytes, respectively.

We investigated carryover effects by injecting a blank sample after a high QC sample four times and found no carryover in any analyte.

Stability. We evaluated the stability of cannabinoids in BAL fluid by comparing the initial measurements of the spiked accuracy and precision pools obtained on day 1 to the final measurements obtained on day 3. After they were spiked, the BAL pools were kept at 4 °C for the duration of the time period except when they were brought to room temperature for sampling each day. The difference between mean measurements obtained on day 1 compared to that on day 3 showed no consistent trend by analyte or by pool.

EVALI Case Results. We found detectable levels of cannabinoids in 45 out of 59 (76%) samples from EVALI cases. We detected all analytes except for CBD. COOH-THC was the most prevalent cannabinoid detected (43 positive, 0.021−127 ng/mL), followed by THC (18 positive, 0.046−1.48 ng/mL), CBN (7 positive, 0.036−0.311 ng/mL), COOH-CBD (4 positive, 0.134−0.156 ng/mL), and OH-THC (3 positive, 0.325−2.76 ng/mL).

DISCUSSION

We developed and validated an HPLC-MS/MS method to measure six cannabinoids in BAL fluid with good sensitivity, specificity, and precision. Our precision results met the recommendations in FDA's Bioanalytical Method Validation Guidance for Industry[15] (CVs < 15%) for all pools and analytes except for COOH-CBD at the low-concentration level. Long-term precision as measured by the QC pools was good for all analytes for both pools except for COOH-CBD

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Table 2. Accuracy Results for BAL Pools Spiked with Analytes at Three Concentration Levels[d]

analyte	spiked amount (ng/mL)	pool 1 measured mean[a] (ng/mL)	pool 1 accuracy (percent of spike)	pool 2 measured mean[a] (ng/mL)	pool 2 accuracy (percent of spike)	overall accuracy[b]
THC	0	0.040		0.000		
	0.500	0.535	99.1	0.512	102.4	100.8
	2.00	1.80	88.2	1.72	85.9	87.1
	100	84.8	84.7	82.7	82.7	83.7
COOH-THC	0	0.005		0.000		
	0.500	0.434	85.6	0.433	86.6	86.1
	2.00	1.66	82.5	1.64	81.8	82.1
	100	84.1	84.1	84.5	84.5	84.3
OH-THC	0	0.000		0.000		
	0.500	0.490	97.9	see note[c]		97.9
	2.00	1.38	69.1	1.64	82.1	75.6
	100	77.4	77.4	80.5	80.5	79.0
CBD	0	0.010		0.000		
	0.500	0.444	88.8	0.471	94.2	91.5
	2.00	1.40	70.0	1.45	72.5	71.3
	100	69.5	69.5	82.0	82.0	75.8
COOH-CBD	0	0.042		0.010		
	0.500	0.537	98.9	0.486	95.2	97.1
	2.00	1.76	85.9	1.44	71.7	78.8
	100	87.0	86.9	91.7	91.7	89.3
CBN	0	0.005		0.002		
	0.500	0.445	88.2	0.416	82.8	85.5
	2.00	1.43	71.1	1.65	82.2	76.6
	100	80.9	80.9	82.2	82.2	81.6

[a]Average of six replicates. [b]Average of the accuracy measured in two pools. [c]OH-THC could not be measured due to a contaminant. [d]BAL, bronchoalveolar-lavage; THC, Δ^9-tetrahydrocannabinol; COOH-THC, 11-nor-9-carboxy-THC; OH-THC, 11-hydroxy-THC; CBD, cannabidiol; COOH-CBD, 7-nor-7-carboxycannabidiol; and CBN, cannabinol.

Table 3. Precision Results for Repeat Analyses of Two BAL Pools Spiked with Analytes[a]

analyte	low pool (N = 12) mean (ng/mL)	within-run (CV%)	between-run (CV%)	method (CV%)	high pool (N = 12) mean (ng/mL)	within-run (CV%)	between-run (CV%)	method (CV%)	QCL mean (ng/mL)	QCL long-term (CV%)	QCH mean (ng/mL)	QCH long-term (CV%)
THC	1.39	5.1	9.2	10.5	72.3	4.3	9.1	10.1	0.409	8.6	200	4.8
COOH-THC	1.56	3.1	4.7	5.7	82.2	1.0	4.1	4.2	0.453	9.3	218	5.3
OH-THC	1.52	7.9	9.5	12.4	77.6	1.9	4.6	5.0	0.465	17.0	218	5.3
CBD	1.44	4.6	9.5	10.5	73.2	6.5	9.3	11.4	0.452	11.7	209	3.3
COOH-CBD	1.63	7.9	20.7	22.2	90.2	4.9	2.8	5.6	0.557	29.6	237	8.4
CBN	1.48	4.7	8.7	9.9	75.1	4.5	8.3	9.4	0.436	6.9	207	5.4

[a]BAL, bronchoalveolar-lavage; QCL, low-concentration quality control pool; QCH, high-concentration quality control pool; THC, Δ^9-tetrahydrocannabinol; COOH-THC, 11-nor-9-carboxy-THC; OH-THC, 11-hydroxy-THC; CBD, cannabidiol; COOH-CBD, 7-nor-7-carboxycannabidiol; and CBN, cannabinol.

Table 4. Limits of Detection (LOD) for Six Analytes in 150 μL BAL Fluid[a]

analyte	LOD (ng/mL)
THC	0.035
COOH-THC	0.019
OH-THC	0.153
CBD	0.078
COOH-CBD	0.094
CBN	0.030

[a]BAL, bronchoalveolar-lavage; THC, Δ^9-tetrahydrocannabinol; COOH-THC, 11-nor-9-carboxy-THC; OH-THC, 11-hydroxy-THC; CBD, cannabidiol; COOH-CBD, 7-nor-7-carboxycannabidiol; and CBN, cannabinol.

and OH-THC for the low QC pool. The most likely reason that COOH-CBD CVs were high is that we did not have an isotopically labeled analogue to use as ISTD for that analyte. As mentioned before, the OH-THC chromatograms had a small interference in the quantitation ion that prevented accurate quantitation at low concentrations, and this probably contributed to the higher CV at low concentration. The interfering peak can be seen in the OH-THC quantitation ion chromatogram of Figure 2.

The method accuracy results overall were within 29% of expected. This is less accurate than FDA method validation guidelines. However, it should be noted that accuracy at the lowest concentration (0.5 ng/mL) was good and within 15% of expected for all analytes, which follows the FDA guidelines.

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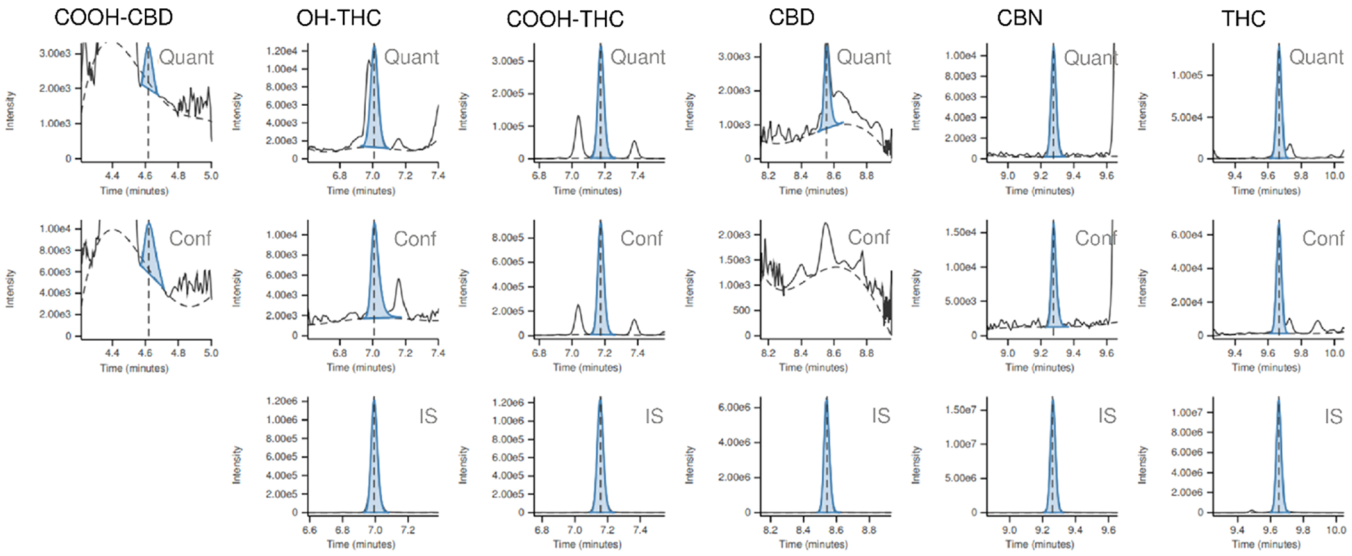

Figure 2. Chromatograms of 7-nor-7-carboxycannabidiol (COOH-CBD), 11-hydroxy-THC (OH-THC), 11-nor-9-carboxy-THC (COOH-THC), cannabidiol (CBD), cannabinol (CBN), and Δ^9-tetrahydrocannabinol (THC) quantitation (Quant), confirmation (Conf), and internal standard (IS) ions in an EVALI case BAL sample by retention time. COOH-THC (4.46 ng/mL), OH-THC (0.325 ng/mL), THC (0.311 ng/mL), CBN (0.045 ng/mL), and COOH-CBD (0.155 ng/mL) were detected. CBD was below the limit of detection.

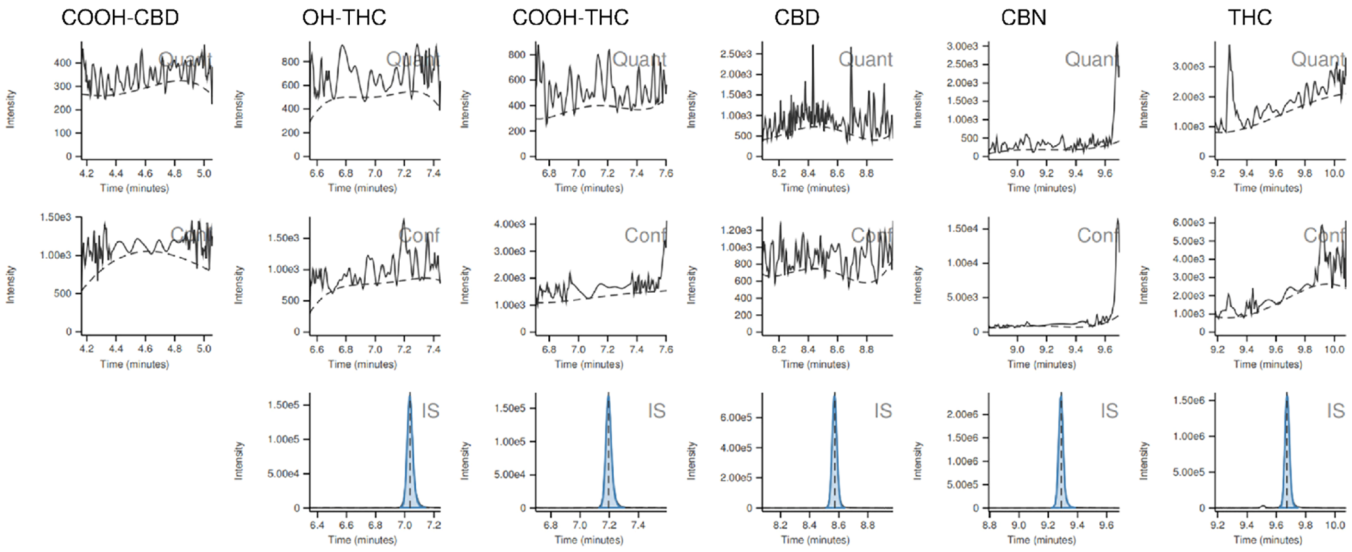

Figure 3. Chromatograms of 7-nor-7-carboxycannabidiol (COOH-CBD), 11-hydroxy-THC (OH-THC), 11-nor-9-carboxy-THC (COOH-THC), cannabidiol (CBD), cannabinol (CBN), and Δ^9-tetrahydrocannabinol (THC) quantitation (Quant), confirmation (Conf), and internal standard (IS) ions in an EVALI case BAL fluid sample by retention time. No cannabinoids were detected.

Among the patient samples with detectable cannabinoids, 69% were measured at 1 ng/mL or less, which is where our assay produced the best accuracy.

We found only one published method that quantifies cannabinoids in BAL fluid. Rotolo et al. used GC/MS to measure five cannabinoids (THC, COOH-THC, OH-THC, CBD, and CBN) in BAL fluid from cannabis smokers suffering from lung disease.[16] They detected THC, CBD, and CBN in 6 out of 15 BAL samples and OH-THC in 2. They did not detect COOH-THC in any samples. They had information about the last time the patients smoked cannabis, which ranged from 2 to 14 days for the patients with detectable cannabinoids and from 16 to 35 days for those whose BAL samples did not have detectable cannabinoids. In contrast to Rotolo et al., we did not detect CBD in any EVALI samples, and COOH-THC was the most prevalent cannabinoid that we detected. We did not have information on time since the last use of cannabis products for our samples. Most EVALI patients reported the use of THC-containing products,[17] with some patients additionally reporting smoking cannabis. Another difference in our methods is the volume of BAL fluid used for analysis; we used 150 μL for most samples and Rotolo et al. used 1 mL.

Concentrations of substances in BAL fluid are difficult to interpret. The volume of saline instilled into the lung and the lavage fluid volume recovered from the lung varies widely, especially when lavage is not performed using a standardized process. The BAL fluid samples we analyzed came from EVALI cases from 20 states. While BAL collection protocols were standardized within individual institutions, protocols vary among institutions, adding to the variation of soluble component concentration levels.[18,19] The purpose of our assay was to determine whether BAL samples from EVALI

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cases were positive for cannabinoids to provide how commonly we found evidence of the use of THC-containing products in EVALI patients. Our method proved adequate for this purpose.

Our method has several limitations. Of primary importance is the shortened method validation process. If time had not been of such importance, we would have performed more experiments to understand the reasons behind the lower-than-ideal accuracy results at the higher concentrations of the accuracy pools we used. We would have investigated more thoroughly the stability of our analytes at different conditions. We did not have enough BAL fluid to create a calibration curve in the BAL matrix, and we were not able to determine matrix effects on analyte recoveries. We believe our precision for COOH-CBD would have been better if we had had access to an isotopically labeled ISTD, which is available now. And finally, our assay may have achieved higher sensitivity and/or better selectivity if we had had the time to explore other LC columns with smaller diameter particles (such as 1.8 μm or less), instead of using two 2.6 μm columns in series. The major strength of our method is its excellent sensitivity, which gave us the ability to detect analytes using the very limited sample size that was available to us from the EVALI cases.

In conclusion, we developed and validated a sensitive HPLC-MS/MS method to detect six cannabinoids in BAL fluid with good precision and selectivity and decent accuracy. It was fit for the purpose to investigate cannabinoids as a potential co-exposure with toxicants that cause vaping-associated lung injury.

MATERIALS AND METHODS

Standards and Reagents. Certified reference material stock solutions of native THC, COOH-THC, OH-THC, CBD, and CBN and their isotopically labeled counterparts, THC-d_3, COOH-THC-d_3, OH-THC-d_3, CBD-d_3, and CBN-d_3, were purchased from Cerilliant Corporation (Round Rock, TX). Native COOH-CBD was purchased from Toronto Research Chemicals (North York, ON, Canada). Acetonitrile (HPLC grade), methanol (HPLC grade), formic acid (≥99.5%), ammonium formate (≥99%), and phosphate-buffered saline were purchased from Fisher Scientific (Pittsburgh, PA). Water (HPLC grade) was purchased from JT Baker (Phillipsburg, NJ).

Calibrator Solutions, Internal Standard Spiking Solution, and Calibration Curve. Calibrator spiking solutions were prepared from THC-, COOH-THC-, OH-THC-, CBD-, and CBN-certified reference materials by serial dilution with methanol and water (v/v 60:40). A high-concentration working solution, WS-A, was first prepared by transferring 1.0 mL of each certified solution (1 mg/mL) to a volumetric flask and diluting to 50 mL with methanol and water (v/v 60:40) to make a mixed stock of the five analytes at 20 μg/mL. A second working solution, WS-B, was prepared by diluting 5.0 mL of WS-A to 50 mL in a volumetric flask with methanol and water (v/v 60:40) to produce a solution with analytes at 2.0 μg/mL. Calibrator spiking solutions were prepared at 13 concentration levels from 0.01 to 500 ng/mL by three overlapping serial dilutions of WS-A and WS-B with methanol and water (v/v 60:40). The COOH-CBD material became available only after we made the first set of calibrators, so a second set of calibrator spiking solutions was prepared from the COOH-CBD-certified reference stock solution in the same manner and at the same concentrations as the mixed calibrators. Calibrator spiking solution concentrations are listed in Table 5.

Table 5. Calibrator Spiking Solution Concentrations for All Analytes

calibrator spiking solution	analyte concentration (ng/mL)
CAL-01	0.01
CAL-02	0.1
CAL-03	0.2
CAL-04	0.5
CAL-05	1.25
CAL-06	2.5
CAL-07	6.25
CAL-08	12.5
CAL-09	25
CAL-10	62.5
CAL-11	125
CAL-12	250
CAL-13	500

The ISTD spiking solution was prepared by mixing together isotopically labeled certified reference materials at appropriate volumes and diluting with methanol and water (v/v 60:40) to achieve final concentrations of 60 ng/mL for COOH-THC-d_3, CBD-d_3, and CBN-d_3 and 100 ng/mL for THC-d_3 and OH-THC-d_3. A labeled standard for COOH-CBD was not available, so COOH-THC-d_3 was used as the internal standard for COOH-CBD. All calibrator solutions and the ISTD spiking solution were stored in Teflon-capped amber glass vials at <−20 °C.

Thirteen calibrators (CAL-01−CAL-13) were created during sample preparation by spiking 50 μL of the calibrator spiking solutions (both the multianalyte spiking solutions and the COOH-CBD-only spiking solutions) to 13 calibrator vials along with 50 μL of ISTD spiking solution. Calibrators were prepared each day in the same manner as unknown samples and analyzed in order from low to high concentration to create the calibration curves for samples analyzed that day.

BAL Pools. BAL pools were created using anonymous BAL samples acquired commercially from Discovery Life Sciences (Huntsville, AL). They were shipped frozen on dry ice and then stored in −70 °C freezers until analyzed. Ten individual BAL fluids were screened for cannabinoid levels, and five samples with nondetectable results for each analyte were combined to create two unique blank BAL pools. The blank pools were used for accuracy, precision, stability, and LOD testing.

BAL pools were spiked with solutions of THC, COOH-THC, OH-THC, CBD, and CBN from different stock solutions than those used to make calibrators. Only one stock solution of COOH-CBD was available at the time, so it was used for both calibrators and BAL pools. Pools to test accuracy were prepared in duplicate by spiking both BAL pools at three concentration levels: 0.5, 2.0, and 100 ng/mL. Two of the accuracy pools were used to test precision (2.0 and 100 ng/mL). Two of the accuracy pools were also used to evaluate the LOD (0.5 and 2.0 ng/mL) along with three additional BAL pools spiked at 0.15, 0.25, and 1.0 ng/mL.

Quality Control Spiking Solutions. Spiking solutions for QC samples were prepared at low- (QCL) and high (QCH)-concentration levels from dilutions of the stock solutions WS-A and WS-B (described above) with methanol and water (v/v

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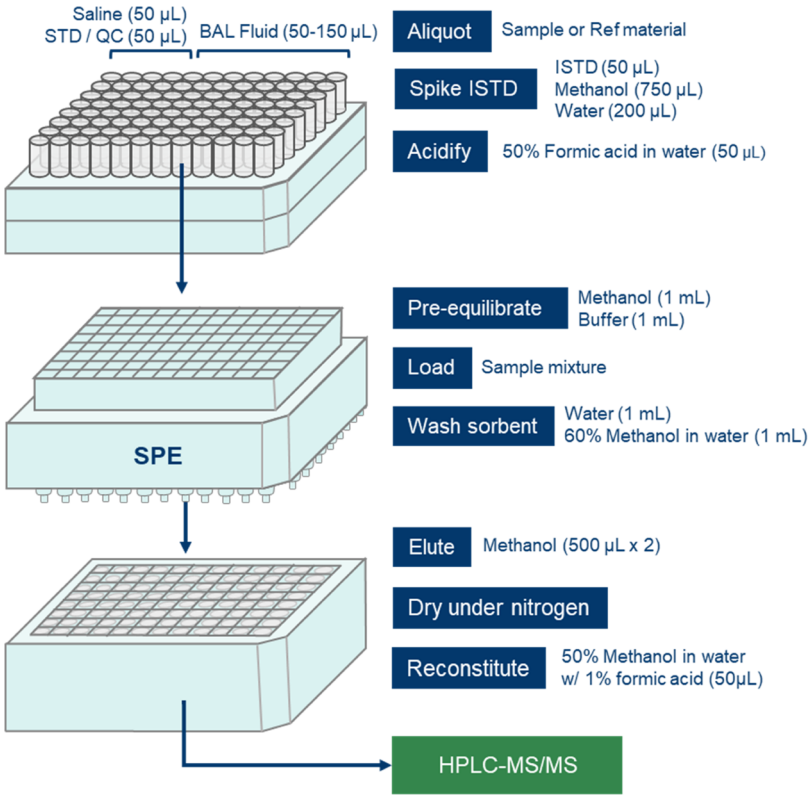

Figure 4. Sample preparation process for cannabinoids in BAL fluid.

60:40). Both sets of stock solutions (multianalyte and COOH-CBD-only) were used to create the two QC spiking solutions; QCL spiking solution (0.5 ng/mL) was made by diluting WS-B at 1:4000, and QCH spiking solution (250 ng/mL) was made by diluting WS-A at 1:80. The QC spiking solutions were stored in Teflon-capped amber glass vials at <−20 °C and reprepared as needed.

Sample Preparation. Each analytical run consisted of two QCs (low and high), two blanks, and up to 22 unknown samples. Blank samples were 50 µL of phosphate-buffered saline. QC samples were 50 µL of phosphate-buffered saline into which 50 µL of a QC spiking solution was added. Thirteen calibrators and up to three analytical runs were prepared on one 96-well plate.

BAL fluid was thawed and centrifuged at 1500 rpm for 12 min at 4 °C. The supernatant was transferred to 2 mL cryogenic vials. Each BAL sample was vortexed for approximately 10 s to homogenize the sample prior to aliquoting. A Hamilton Microlab STAR system (Reno, NV) was used to transfer ISTD, calibrator and QC spikes, phosphate-buffered saline, water, methanol, and formic acid. Automated liquid transfers were performed using Hamilton CORE black conductive filter pipette tips, 50 and 1000 µL. Water (200 µL) and methanol (750 µL) were transferred to silanized flat-bottom clear glass vials (1.5 mL capacity) arranged in a 96-well plate format. Each vial received 50 µL ISTD spiking solution (5 ng THC-d_3 and OH-THC-d_3, 3 ng COOH-THC-d_3, CBD-d_3, and CBN-d_3). Calibrator, QC, and blank vials received 50 µL of phosphate-buffered saline. The multianalyte calibrator spiking solutions (50 µL) and the COOH-CBD-only calibrator spiking solutions (50 µL) were transferred to each calibrator vial. QC vials were spiked with 50 µL QC spiking solutions. BAL samples were hand-pipetted. The BAL samples were first analyzed at 50 µL, and then the sample volume was increased to 150 µL for all samples that had enough BAL fluid. Lower sample volumes were used if there was not enough BAL fluid. Each sample vial was then acidified by the addition of 50 µL of 50% formic acid in water.

The contents of each vial were loaded onto a 96-well C18 SPE fixed well plate (ISOLUTE-96, 100 mg, Biotage, Charlotte, NC), pre-equilibrated with 1.0 mL of methanol and 1.0 mL of buffer (5 mM ammonium formate with 0.05% formic acid). The sample mixture was soaked on the SPE sorbent for 10 min and then gently pushed through the sorbent with approximately 1.0 psi pressure using in-house nitrogen (NM20ZA Peak Generator, Peak Scientific Instruments, Billerica, MA) on a Biotage Pressure+ 96 manifold. Samples were washed with 1.0 mL water and 1.0 mL methanol and water (v/v 60:40) and then dried under nitrogen (25 psi) for 5 min. After drying, samples were eluted with 0.5 mL methanol into 0.7 mL silanized clear glass flat-bottom tapered inserts in a polypropylene 96-well deep square well collection plate. Samples were evaporated to near-dryness under nitrogen using a Biotage TurboVap evaporator (35 °C, 38 psi), and then the plate was eluted a second time with 0.5 mL methanol and evaporated to dryness. The residuals were reconstituted with 50 µL methanol and water (v/v 50:50) with 1% formic acid. Ten microliters of each reconstituted sample were injected into the LC-MS system. The overall sample preparation arrangement is depicted in Figure 4.

Instrumental Analysis. Instrumental analysis was performed using a Shimadzu Nexera HPLC system (Columbia, MD) coupled to a Sciex API 6500 triple quadrupole tandem mass spectrometer with a TurboIonSpray source (Framingham, MA). The Shimadzu Nexera system consisted of a CBM-20A controller, a DGU-20A3 degassing unit, two LC30AC

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Table 6. Multiple Reaction Monitoring (MRM) Ion Transitions, Mass Spectrometric Voltage Settings, and Scheduled MRM Time Periods for Cannabinoids in BAL Fluid[a]

			mass spectrometer setting (V)				
analyte	ion	precursor ion/product ion (m/z)	DP	EP	CE	CXP	scheduled MRM window (min)
THC	quantitation	315.2/193.1	65	10	32	12	8.95−10.45
THC	confirmation	315.2/123.1	65	10	41	11	8.95−10.45
THC-d_3	internal standard	318.2/196.1	60	8	32	12	8.95−10.45
COOH-THC	quantitation	345.1/193.1	50	10	37	15	6.45−7.95
COOH-THC	confirmation	345.1/299.2	50	10	28	16	6.45−7.95
COOH-THC-d_3	internal standard	348.1/196.1	60	8	37	13	6.45−7.95
OH-THC	quantitation	331.1/193.1	40	10	34	11	6.25−7.75
OH-THC	confirmation	331.1/201.0	40	10	33	11	6.25−7.75
OH-THC-d_3	internal standard	334.0/196.1	50	8	35	11	6.25−7.75
CBD	quantitation	315.2/193.1	65	10	32	12	7.55−9.05
CBD	confirmation	315.2/123.1	65	10	41	11	7.55−9.05
CBD-d_3	internal standard	318.2/196.2	50	8	31	13	7.55−9.05
COOH-CBD	quantitation	345.1/193.1	50	10	37	15	3.85−5.35
COOH-CBD	confirmation	345.1/299.2	50	10	27	16	3.85−5.35
CBN	quantitation	311.1/208.0	55	10	40	11	8.55−10.05
CBN	confirmation	311.1/241.0	55	10	26	12	8.55−10.05
CBN-d_3	internal standard	314.2/223.1	55	7	30	13	8.55−10.05

[a]BAL, bronchoalveolar-lavage; THC, Δ^9-tetrahydrocannabinol; COOH-THC, 11-nor-9-carboxy-THC; OH-THC, 11-hydroxy-THC; CBD, cannabidiol; COOH-CBD, 7-nor-7-carboxycannabidiol; CBN, cannabinol; DP, declustering potential; EP, entrance potential; CE, collision energy; and CXP, cell exiting potential.

pumps, a SIL-30ACMP autosampler (held at 4 °C), and a CTO-20A column oven (at 40 °C). Chromatographic separation was conducted using two reversed-phase columns connected in series (Kinetex C18 2.6 μm 2.1 × 100 mm^2, Phenomenex, Torrance, CA) with a precolumn filter at a flow rate of 0.45 mL/min. Samples were eluted through the column using a binary gradient of 0.05% formic acid in water (mobile phase A) and acetonitrile (mobile phase B). Briefly, the percentage of mobile phase B was increased from 45% at 0 min to 60% over 5 min, and then to 98% until 10 min and held at 98% until 13 min. At 13 min, it was decreased to 45% and held there until the end of the cycle time at 15 min.

Mass spectrometric analysis was performed in a positive-ion mode with the following parameters: source temperature, 650 °C; ionspray voltage, 5500; ion source gas 1 (zero grade air), 80 psi; ion source gas 2 (zero grade air), 80 psi; curtain gas (nitrogen), 35 psi; collision gas (nitrogen), 9 psi. Scheduled multiple reaction monitoring (MRM) was used to monitor all quantitation and confirmation ion mass transitions for each native analyte, along with the corresponding ISTD mass transition. The MRM ion transitions, mass spectrometric voltage settings, and scheduled MRM time periods are detailed in Table 6.

BAL Specimens from EVALI Cases. Biospecimen collection from EVALI cases has been previously described.[12] Briefly, the state health departments from 20 states assisted the CDC in obtaining 64 BAL specimens collected by clinical care teams in the course of treating individual patients. EVALI case specimens came from patients who qualified as having a probable or confirmed case of EVALI, some of which arrived at our lab after our previous report.[12] Of the 64 EVALI case specimens, 59 had enough BAL fluid for cannabinoid analysis. A human-subject research review was conducted by CDC, which determined that this work did not meet the regulatory definition of research under 45 CFR 46.102(d) and was determined to be a nonresearch public health response activity.

Data Analysis. ASCENT software (Indigo BioAutomation, Carmel, IN) was used to integrate chromatogram peaks, generate calibration curves, and calculate analyte concentrations. Calibration curves were constructed by plotting peak area ratios of native to labeled compounds in the calibrators against the expected concentrations using $1/x$ weighted linear least-squares regression with the origin ignored. Sample results were calculated using calibration curves generated from calibrators analyzed the same day.

A blank and two QC pools were analyzed in each analytical run with unknown samples. Blanks were usually zero, but when they were nonzero, their value was subtracted from the calculated sample values in the run. All accepted QC data met the requirements of the multirule QC program of the Division of Laboratory Sciences, CDC.[20] Statistical analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC).

Method Validation. We followed an abbreviated process to validate the method, which included assessments of accuracy, precision, sensitivity, selectivity, and stability.

We assessed accuracy by spiking two blank BAL pools with known amounts of cannabinoids at three concentrations (0.5, 2.0, and 100 ng/mL) for a total of six spiked pools. We analyzed all six spiked pools and the unspiked BAL pools in triplicate on two separate days for a total of 48 results. The mean background concentrations in the unspiked pools were determined and then subtracted from the mean sample results for that day and pool. Accuracy was calculated as (measured mean concentration − mean concentration of unspiked pool)/(added concentration).

Within-run and between-run precisions were determined from repeat analyses of two BAL pools spiked with known amounts of cannabinoids at two concentrations (2.0 and 100 ng/mL). The pools were analyzed in duplicate in two analytical runs on three separate days for a total of 12 results per pool. Long-term precision was determined from the analysis of data from two QC pools (QCL at 0.5 ng/mL and

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QCH at 250 ng/mL) assayed in 28 runs from October 2019 to February 2020.

We evaluated the method LOD based on the extrapolated standard deviation at zero concentration, as specified by Taylor.[14] We prepared BAL pools at five concentrations (0.15, 0.25, 0.5, 1.0, and 2.0 ng/mL) of the six cannabinoids and analyzed them repeatedly over the course of several days. For each analyte, we plotted the standard deviation of each pool against the mean concentration of the pool. The *y*-intercept of the extrapolated regression line is an estimate of S_0, where S_0 is the standard deviation at zero analyte concentration, and the method LOD is defined as 3 times S_0.

We established the initial specificity of the assay by analyzing 10 individual BAL samples to look for chromatographic interferences. To confirm specificity in study samples, we monitored the confirmation ion ratios (confirmation ion peak area/quantitation ion peak area) and relative retention times between the quantitation and ISTD peaks. Confirmation ion ratio limits were calculated from the mean of the confirmation ion ratio of all calibrators greater than 0.5 ng/mL analyzed that day. The acceptable confirmation ion ratio range was set at ±25% of the mean. Relative retention times between the quantitation and ISTD peaks were acceptable if they were within 3 s. Relative retention times were checked between the quantitation and confirmation ions for each analyte as well to make sure correct peaks were chosen. In addition, all chromatographic peaks were inspected for interferences and acceptable peak shapes.

The stability of cannabinoids in BAL fluid was evaluated in spiked BAL pools kept at 4 °C over 3 days. Carryover effects were investigated by injecting blank samples after high QC samples.

AUTHOR INFORMATION

Corresponding Author

Connie S. Sosnoff − *Tobacco and Volatiles Branch, Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, Georgia 30341, United States;* orcid.org/0000-0001-9162-633X; Email: css3@cdc.gov

Authors

Christina R. Brosius − *Tobacco and Volatiles Branch, Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, Georgia 30341, United States*

Kevin T. Caron − *Tobacco and Volatiles Branch, Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, Georgia 30341, United States*

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

Lanqing Wang − *Tobacco and Volatiles Branch, Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, Georgia 30341, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.1c04815

Author Contributions

†C.R.B. and K.T.C. contributed equally to this work and share the first authorship.

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 Health and Human Services or the Centers for Disease Control and Prevention.

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