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Novel Methods for the Analysis of Toxicants in Bronchoalveolar Lavage Fluid Samples from E-cigarette, or Vaping, Product Use-Associated Lung Injury (EVALI) Cases: Terpenes.

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Abstract

Rationale: Over 2,800 e-cigarette, or vaping, product use-associated lung injury (EVALI) cases have been reported to the Centers for Disease Control and Prevention (CDC) during August 2019-February 2020. Bronchoalveolar lavage (BAL) fluid samples from 51 EVALI and 99 non-EVALI cases were analyzed for toxicants including terpenes. We describe a novel method to measure selected terpenes in BAL fluid by gas chromatography-tandem mass spectrometry (GC/MS/MS).

Methods: α-Pinene, ß-pinene, ß-myrcene, 3-carene, and limonene were measured in BAL fluid specimens by headspace solid-phase microextraction/gas chromatography/tandem mass spectrometry. We created and characterized BAL fluid pools from non-EVALI individuals to determine assay accuracy, precision, linearity, limits of detection, and analytical specificity. All measurements were conducted in accordance with the CDC's Division of Laboratory Sciences rigorous method validation procedures.

Results: Matrix validation experiments showed that calibration curves in BAL fluid and saline had similar slopes, with differences less than 7%. Assay precision ranged from 2.52% - 5.30%. In addition, the limits of detection for the five analytes ranged from 1.80 – 16.8 ng/L, and their linearity was confirmed with R^2 values >0.99.

Conclusions: We developed and validated a method to quantify selected terpenes in BAL fluid specimens using GC/MS/MS. The assay provided accurate and precise analyses of EVALI and non-EVALI BAL fluid specimens in support of CDC's EVALI response. This method is applicable to the determination of a broad range of terpenes in BAL fluid specimens.

Keywords

Bronchoalveolar lavage fluid; GC/MS/MS; EVALI; terpenes

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Rationale

A total of 2,807 hospitalized e-cigarette, or vaping, product use-associated lung injury (EVALI) cases or deaths have been reported to the Centers for Disease Control and Prevention (CDC) from 50 states, the District of Columbia, and two U.S. territories (Puerto Rico and U.S. Virgin Islands) as of February 18, 2020[1]. Additionally, 68 deaths have been reported in 29 states, and the District of Columbia[1]. EVALI cases have continued to decline through February 2020, after sharply increasing in August 2019 and peaking in September 2019. Blount et al showed that vitamin E acetate, an additive in some THC-containing e-cigarette, or vaping, products, is strongly linked to EVALI[2,3]. In that study, bronchoalveolar lavage (BAL) fluid samples from 51 EVALI cases from 16 states and a comparison group of samples from 99 individuals without EVALI were analyzed for toxicants, including vitamin E acetate, plant oils, medium chain triglyceride (MCT) oil, coconut oil, petroleum distillates, and terpenes. BAL fluid specimens are obtained by injecting normal saline into the lung and applying mild suction to retrieve a fraction of that saline. Terpenes are volatile organic compounds (VOCs), and can be analyzed using gas chromatography-tandem mass spectrometry (GC/MS/MS).

Our laboratory developed a method to quantify terpenes in serum in support of the National Centers for Health Statistics' National Health and Nutrition Examination Survey (NHANES)[4]. Specifically, we provide measurements of terpenes in serum to obtain nationally representative estimates of the U.S. population's exposure to VOCs[5]. To support CDC's response to the EVALI crisis, we modified and validated our existing terpenes in serum method to accurately detect selected terpenes in BAL fluid samples. In this report, we describe the method performance parameters (e.g., accuracy, linearity, accuracy) for measuring α-pinene, ß-pinene, ß-myrcene, 3-carene, and limonene in BAL fluid.

Methods

Five terpenes were measured in BAL fluid specimens by headspace solid-phase microextraction (SPME)/gas chromatography/ tandem mass spectrometry. All glassware, headspace vial septa, vials, and reagent water were cleaned and verified to be free of those VOCs being analyzed[6]. After cleaning, glassware and septa were stored in a vacuum oven prevent recontamination. All method parameters were examined in accordance with the CDC's Division of Laboratory Sciences rigorous method validation procedures, which are based on standard practices[7]. Table 1 shows a summary of optimized transitions (*m/z*), collision energies (eV), and retention times (min) of selected terpenes and the stable isotope-labeled internal standards

Standards preparation

Primary stock solutions were prepared from neat materials diluted with either purge and trap grade methanol or low-VOC water (Table 2)[8]. The primary stock solution concentrations were based on the gravimetric measure of mass transferred to the volumetric flasks. Working standards (standards 1–9) were prepared in low-VOC water. Aqueous working standards were formulated in volumes of 25-mL quantities with low-VOC water and added internal standard. Each of the aqueous working standards was transferred into cleaned 10-mL

headspace vials using a positive displacement pipetter. The vials were immediately sealed with recently cleaned caps and grouped by concentration in separate wide mouth sample jars to prevent cross contamination. The standard set was stored in a dedicated refrigerator at 2–6°C and analyzed as part of an analytical run within one week.

Preparation of isotopically labeled internal standard solutions

Primary isotopically labeled internal standard stock solutions were made by dilution of the neat compound into purge and trap grade methanol (Table 2). Isotopically labeled internal standards (ISTD) were of adequate chemical and isotopic purity (>95%) to produce levels needed for accurate quantitation. The isotopic purity of internal standards was at least 97%. Isotopically labeled internal standards were checked for any spectral overlap with the corresponding native analogs. Concentrations of the primary labeled internal standard stock solutions ranged from 2- to 10-mg/mL. The primary isotopically labeled internal standard stock solutions were stored in a freezer below −70 °C until use. Secondary isotopically labeled internal standard stock solutions were made by combining primary stock solutions and diluting to pre-established concentrations. Working isotopically labeled internal standard solutions were prepared weekly from the secondary stock solutions. The secondary stock solutions were added to the standard formulations, water blanks, quality control samples, and specimen samples.

Preparation of quality control materials

Quality control (QC) materials were prepared at two concentration levels in pooled human serum. The concentration homogeneity across the lot was evaluated by comparing samples prepared at the beginning, middle, and end of the batch. Any variability of more than 25% for any analyte resulted in reformulation of the affected lot.

Instrumentation and operation

Sample analysis was performed using a PAL system (CTC Analytics AG Zwingen, Switzerland) autosampler, coupled to an Agilent 7890B gas chromatograph and a 7010 triple quadrupole mass spectrometer (Agilent, Santa Clara CA). Samples were queued on an autosampler tray and maintained at 15 ± 0.5 °C until they were analyzed. During analysis the samples were transferred to an agitating incubator set to 350 rpm and 40 ± 1 °C as the headspace is sampled with a 80-μm Carboxen-PDMS coated SPME fiber (Supelco, Bellefonte PA) for 10 min. The SPME fiber was then immediately transferred into the injection port of an Agilent fitted with a glass liner with an i.d. of 0.75 mm and held at 250 ± 0.5 °C. The sample was introduced into an Agilent DB-624 column (25 m x 0.2 mm x 1.12 μm film) via splitless injection set to a 70 mL/min purge flow and maintained a constant flow of 1 ± 0.05 mL/min of helium throughout the run. In-line, after the injection port, is a cryogenic trap. At the start of the GC run the cryotrap is set to −100 °C for 2 min, then ballistically heated to approximately 220 °C. The GC oven temperature profile is programmed to ramp starting from 50°C (3 min hold) at 5°C/min to 125°C. Then, it ramps at 50°C/min to a final temperature of 180°C and holds at this temperature for 7.3 minutes. Analysis was performed using electron ionization in positive mode. We used nitrogen as collision gas for multiple reaction mode and optimized MS/MS parameters (Table 1). Of note, all transitions were selected by choosing the strongest responses in a full scan (single

quad GC/MS run) and collision energies were optimized for each transition of each analyte (Table 1). During the analytical run, the SPME fiber remained in the GC injection port until ready to collect the next sample and was not exposed to the laboratory air for more than 1 min to reduce ambient air contamination.

Calibration and calibration verification

All calibration standards were prepared in phosphate buffered saline or low-VOC water because it was difficult to consistently obtain reduced VOC background levels in BAL matrixes below detectable levels. Matrix spike experiments (matrix validation) were performed to verify that calibration curves in low-VOC water and saline had the same slope (Table 3). Characterization was performed once at nine different concentrations distributed across the analytical range.

Accuracy, precision, linearity, limits of detection, analytical specificity

Accuracy was evaluated by comparing three different spiked levels in pooled BAL fluid specimen matrix with freshly prepared standards at analyte concentrations ranging from 0.484 to 11.8 μg/L (Table 4). We prepared three standard solutions (low, medium, and high) by spiking 200-μL of standards 4, 6, and 7 into three 10-mL flasks of methanol, respectively. Six SPME vials containing 500-μL pooled BAL fluid samples were spiked with 40-μL of each standard solution (n=18) and ISTD solution.

Precision was evaluated by analysis of QC samples. Six repetitive injections of a blank, a low QC, and a high QC were analyzed. We calculated the mean and the coefficient of variation (CV) for the results (Table 5). We determined the assay's linearity by creating individual analyte calibration curves of at least six calibrators and blank buffered saline. These solutions were analyzed six times. Limits of detection (LOD, Table 6) were calculated as three times the standard deviation at zero concentration ($3S_0$)[9, 10]. In addition, analytical specificity was established by confirming similarity (within 25%) of quantitation ion response ratios (native peak area/ISTD peak area) to those of the confirmation ion.

Analysis of BAL fluid specimens

Prior to analysis, all BAL fluid specimens and QCs, were mixed by a rotating mixer for at least 15 min. before 500-μL of sample was aliquoted into a 10-mL SPME vial. All samples were spiked with 40-μL of the working ISTD solution (except for standards which were spiked with ISTD upstream) before the SPME vials were hermetically sealed.

Results

Chromatographic separation

We spiked pooled BAL fluid samples with the target terpenes to examine their chromatographic separation in matrix (Figure 1). This was done to ascertain that there were no overlapping peaks, and to optimize sample injection volume and temperature gradient. Of note, Figure 1 is truncated to start at 14 minutes since there were no peak of interest prior to a retention time of 14 minutes. All target analytes eluted from 14 – 18 minutes. In addition,

we show the chromatogram obtained from an actual case (Figure 2) that had a detectable level of limonene. This result is described by Blount et al[3].

Matrix validation

We performed matrix validation experiments to verify that calibration curves in saline (BAL fluid's matrix) and low-VOC water had the same slope (Table 3). Characterization was performed once at nine different concentrations distributed across the analytical range. Our terpenes in serum method uses QC pools prepared in serum, and that method's matrix validation also showed that the slopes were comparable (all <5% differences).

Accuracy, precision, assay linearity

Accuracy was evaluated by comparing three different spiked levels in pooled BAL fluid specimen matrix with water standards, in which both samples were prepared similarly (Table 4). We obtained a pooled BAL fluid sample from non-EVALI patients, which was used to establish the assay's accuracy by fortifying it with known target analyte concentrations. We obtained coefficients of variation less than 6% for all analytes. In addition, six repetitive injections of a blank, a low QC, and a high QC were analyzed (Table 5). The QC samples were prepared in serum; our matrix validation study confirmed the suitability of serum QC pools for this purpose, given the limited availability of pooled BAL fluid sample. All QC targets met CDC's established precision acceptance criteria (imprecision within 20%).

We created individual analyte calibration curves of at least six calibrators and a blank (phosphate buffered saline) and analyzed them six times to examine the linearity of the method. All target analytes demonstrated linearity in the calibration range, with R^2 values >0.990.

Limits of detection, analytical specificity

Limits of detection are presented in Table 6. In addition, analytical specificity was established by confirming similarity (within 25%) of quantitation ion response ratios (native peak area/ISTD peak area) to those of the confirmation ion. Moreover, we checked for interferences in at least six BAL fluid specimens. All analytes passed the interference check.

Discussion

We successfully developed and validated a method to quantify five terpenes in BAL fluid specimens using GC/MS/MS. The assay provided accurate and robust analyses of controls and case study BAL fluid specimen in support of CDC's EVALI response. Case study results are available in a published report by Blount et al[3].

There are important limitations to the analysis of terpenes (and other volatile toxicants) in BAL fluid specimens. Interpreting measurements of chemicals in BAL fluid presents several challenges. The volume of BAL fluid obtained from a patient can vary according to the technique used to obtain it, thus expressing concentrations per volume of BAL fluid that may not be acceptable to quantitatively compare results among individuals. Other than normal saline, BAL fluid should be sampling the epithelial lining fluid, and anything present

in it. Interpretation of BAL fluid concentrations must consider blood as a source for analytes which are present in blood if the BAL specimen shows evidence of red blood cells in it (i.e., it shows a red tinge). In addition, BAL fluid specimen collection and handling prior to analysis must address the volatile nature of some of the target analytes. Since BAL fluid specimens are not typically assayed for the presence of terpenes and other VOCs, the specimens may not have been hermetically sealed upon collection. We showed, however, that terpenes in BAL fluid can be detected when handled appropriately.

This analytical approach is applicable to the determination of a broad range of VOCs in BAL fluid specimens with detection limits in the parts-per-trillion range. Of note, the five terpenes tested only represent a fraction of the most volatile terpenes that may be present in BAL fluid. However, they are ubiquitous in the environment and in consumer products. Less volatile terpenes (e.g., squalene) are not suitable for quantitation by this method. The analysis of volatile terpenes in BAL fluid at parts-per-trillion levels is an extremely complex measurement. However, there are no alternative analytical methods that achieve the sensitivity and specificity described in this method capable of quantifying selected terpenes in BAL fluid in a high-throughput manner.

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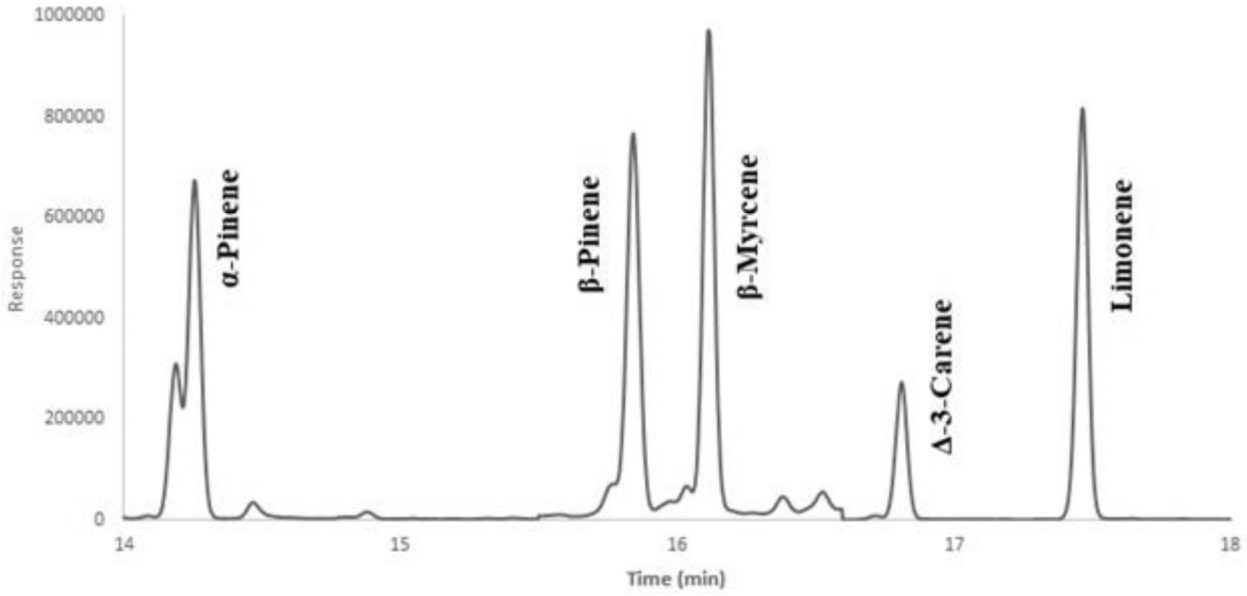

Figure 1.
Spiked pooled BAL fluid sample chromatogram.

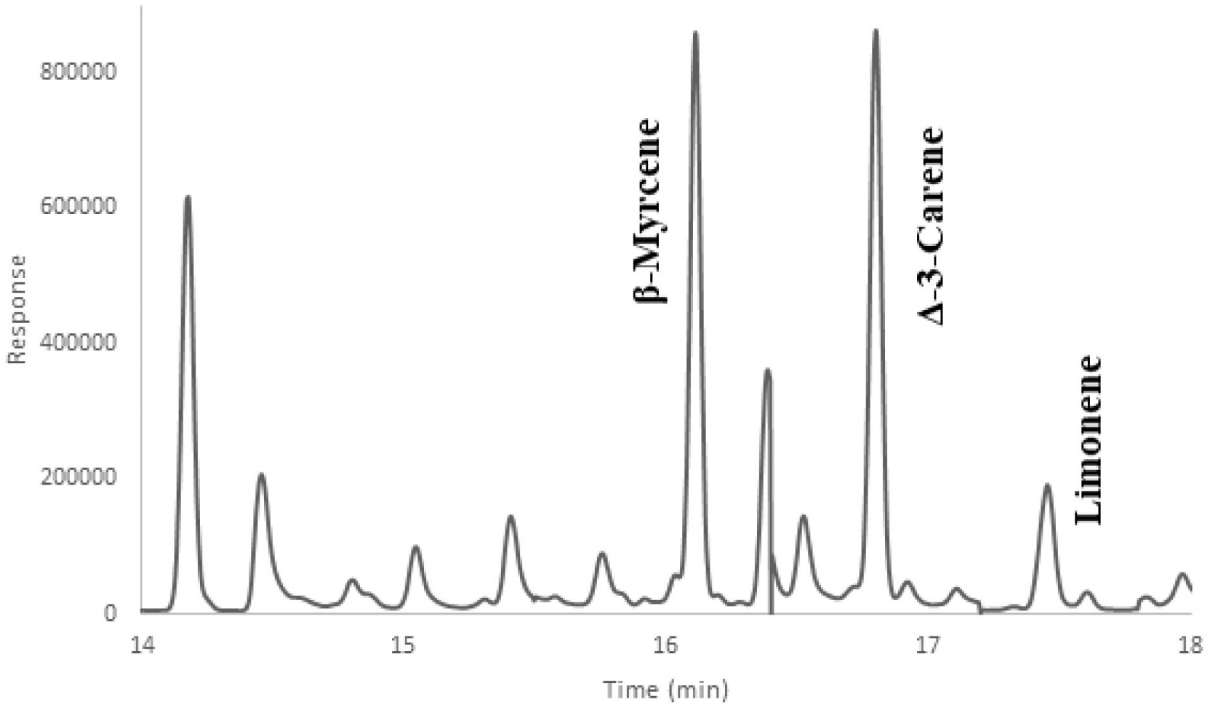

Figure 2.
EVALI case study BAL fluid specimen chromatogram.

Table 1.

Optimized transitions (*m/z*), collision energies (eV), and retention times (min) of selected terpenes with isotopically labeled internal standards.

Analyte	Mass (g/mol)	Internal Standard	Retention Time (min)	1° Transition	2° Transition	ISTD Transition
α-Pinene	136.23	α-Pinene-$^{2}H_3$	14.23	93→77 15 eV	93→91 9 eV	96→77 16 eV
β-Pinene	136.23	β-Pinene-$^{2}H_4$	15.82	93→77 14 eV	93→91 9 eV	97→79 15 eV
β-Myrcene	136.23	β-Myrcene-$^{13}C_3$	16.11	93→77 6 eV	93→91 9 eV	72→43 8 eV
3-Carene	136.23	3-Carene-$^{13}C_3$	16.80	136→93 8 eV	136→121 6 eV	139→93 8 eV
Limonene	136.24	Limonene-^{13}C-$^{2}H_2$	17.45	136→94 2 eV	136→93 13 eV	139→96 3 eV

Table 2.

Analyte and internal standard solution concentrations.

Analyte Stock Solutions (μg/L)		Standard Solution Concentrations (μg/L)									ISTD Stock Solutions
Analyte	**ISTD**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**Concentration (μg/L)**
α-Pinene	α-Pinene-$^{2}H_{3}$	16.3	32.5	81.3	182.5	325.0	405.6	676.3	1353.1	3525.6	8
β-Pinene	β-Pinene-$^{2}H_{4}$	16.3	32.5	81.3	183.1	326.3	407.5	679.4	1358.1	3567.5	8
β-Myrcene	β-Myrcene-$^{13}C_{3}$	15.0	30.0	75.0	168.8	300.0	375.0	624.4	1248.8	3245.0	10
Δ−3-Carene	3-Carene-$^{13}C_{3}$	16.9	33.1	83.1	187.5	333.1	416.9	694.4	1388.8	3598.1	10
Limonene	Limonene-^{13}C-$^{2}H_{2}$	80.0	160.6	401.3	902.5	1603.8	2005.0	3341.9	6685.0	17368.1	8

Table 3.

Matrix comparison (matrix validation) for selected terpenes.

Analyte	Water Matrix			Saline Matrix			Slope Difference (%)
	Slope	Intercept	R^2	Slope	Intercept	R^2	
α-Pinene	1.57	0.004	0.993	1.6	0.012	0.995	0.64
β-Pinene	20.4	−0.127	0.995	19.0	−0.039	0.993	6.86
β-Myrcene	1.84	2.318	0.998	1.8	2.318	0.999	2.17
3-Carene	3.41	0.033	0.997	3.3	0.032	0.997	4.4
Limonene	2.48	0.103	0.999	2.3	0.154	1.000	6.45

Table 4.

Assay accuracy for the measurement of selected terpenes in BAL fluid.

Analyte	Coefficient of Variation (%)		
	Low Level	Med Level	High Level
α-Pinene	5.42	4.39	4.60
β-Pinene	4.18	4.81	3.84
β-Myrcene	3.78	3.84	2.37
3-Carene	4.08	3.21	3.45
Limonene	4.33	3.08	4.84

Table 5.

Precision using quality control samples.

Analyte	QC Low (μg/L)			QC High (μg/L)		
	Target Concentration	Mean Concentration	% CV	Target Concentration	Mean Concentration	% CV
α-Pinene	0.754	0.57	3.54	1.64	1.19	2.84
β-Pinene	0.542	0.45	4.78	1.44	1.13	4.99
β-Myrcene	0.563	0.54	5.30	1.38	1.15	4.39
3-Carene	0.874	0.90	2.52	1.78	1.63	1.70
Limonene	3.24	2.65	2.64	7.61	6.23	2.59

Table 6.

Limits of detection and quantitation range for selected terpenes in BAL fluid.

Analyte	Limits of Detection (μg/L)	Quantitation Range (μg/L)
α-Pinene	0.006	0.026 – 5.64
β-Pinene	0.002	0.026 – 5.71
β-Myrcene	0.004	0.024 – 5.19
3-Carene	0.002	0.027 – 5.76
Limonene	0.017	0.128 – 27.8