HHS Public Access

Author manuscript

Contrib Tob Nicotine Res. Author manuscript; available in PMC 2024 August 14.

Published in final edited form as:

Contrib Tob Nicotine Res. 2021 November ; 30(4): 212–220. doi:10.2478/cttr-2021-0016.

Determination of Benzoic Acid and Benzyl Alcohol in E-Liquids (JUUL™ Pods) by Isotopic Dilution High-Performance Liquid Chromatography and Tandem Mass Spectrometry

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SUMMARY

The new pod devices like JUUL™, Vuse Alto™, myblu™, and other ?pod-mod" related products had a huge impact on the e-cigarette market, especially among teens and young adults due in particular to aggressive marketing on social media, wide availability, and discrete use due to their special design. These pod devices are designed to deliver nicotine levels per puff comparable to combustible cigarettes while producing smaller amounts of visible exhaled aerosol from the heating of e-liquids. Some of these liquids contain high concentrations of acids, such as benzoic acid, to allow higher nicotine deliveries with less harshness and throat irritation. Benzoic acid is a potential source of the human carcinogen benzene and a chemical of concern. Besides acids, flavoring agents such as benzyl alcohol, a local anesthetic that could facilitate tobacco smoke inhalation are also common in these devices. Both benzoic acid and benzyl alcohol in e-liquids might be of relevance for the health risk of vapers.

An isotope dilution high-performance liquid chromatography-tandem mass spectrometry (ID-HPLC-MS/MS) method has been developed for the detection of benzoic acid and benzyl alcohol in the JUUL™ brand e-liquids. The sample preparation consisted of a simple dilution followed by a mechanical stirring process. ID-HPLC-MS/MS was used to separate, identify, and quantify the benzoic acid and/or benzyl alcohol in diluted extracts. Detection limits were 0.11 and 9.05 ng/μL for benzyl alcohol and benzoic acid, respectively. Product variability estimated from the analysis of seven different e-liquids in triplicates (n = 21) yielded relative standard deviations ranging from 4.3% to 16.0% for benzyl alcohol and 6.3% to 11.1% for benzoic acid. The amount of benzoic acid (32.8 ± 2.8 mg/g; 3.3 ± 0.3%, w/w) and the nicotine-benzoic acid molar ratio (1.15

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DISCLAIMER

The findings and conclusions in this study are those of the author(s) and do not necessarily represent the official position of the U.S. Centers for Disease Control and Prevention or by the U.S. Department of Health and Human Services. Use of trade names and commercial sources is for identification only and does not constitute endorsement by the U.S. Department of Health and Human Services or the U.S. Centers for Disease Control and Prevention.

CONFLICTS OF INTEREST

None

± 0.02) remained relatively consistent among pod flavors. [Contrib. Tob. Nicotine Res. 30 (2021) 212–220]

ZUSAMMENFASSUNG

Neue Geräte mit Pod-System wie JUUL™, Vuse™ Alto™, myblu™ sowie andere mit den sogenannten ?Pod Mods" verwandte Produkte haben den Markt für E-Zigaretten enorm geprägt, insbesondere in der Gruppe der Teenager und jungen Erwachsenen. Dies ist vor allem auf das aggressive Marketing in den sozialen Netzwerken, die große Verfügbarkeit sowie die Möglichkeit der diskreten Nutzung aufgrund des besonderen Designs der Produkte zurückzuführen. Die Pod-Geräte sind so konstruiert, dass bei ihnen mit jedem Zug Nikotinkonzentrationen abgeben werden, die mit denen einer herkömmlichen Zigarette vergleichbar sind. Bei der Erwärmung der E-Liquids entstehen jedoch geringere Mengen an sichtbarem ausgeatmetem Aerosol. Einige Liquids weisen hohe Konzentrationen an Säuren wie z.B. Benzoesäure auf, die eine höhere Nikotinabgabe bei weniger Halsirritationen und einer geringeren Schärfe ermöglichen. Aus Benzoesäure kann potenziell Benzol entstehen, das als für den Menschen krebserregend und daher als bedenklich gilt. Neben den Säuren sind Aromastoffe wie das Lokalanästhetikum Benzylalkohol, das die Inhalation des Tabakrauchs erleichtern soll, in diesen Liquids gebräuchlich. Sowohl die Benzoesäure als auch der Benzylalkohol in den E-Liquids könnten für das gesundheitliche Risiko der Konsumenten von E-Zigaretten relevant sein.

Es wurde eine Methode zum Nachweis von Benzoesäure und Benzylalkohol in den E-Liquids der Marke JUUL™ mittels Isotopenverdünnung gekoppelt mit Hochleistungsflüssigchromatographie und Tandem-Massenspektrometrie (ID-HPLC-MS/MS) entwickelt. Die Vorbereitung der Probe erfolgte durch einfache Verdünnung gefolgt von einem mechanischen Rührprozess. Im Anschluss wurden Benzoesäure und/oder Benzylalkohol in den verdünnten Extrakten mit der ID-HPLC-MS/MS-Methode getrennt, identifiziert und quantifiziert. Die Nachweisgrenze lag für Benzylalkohol und Benzoesäure jeweils bei 0,11 bzw. 9,05 ng/μL. Die auf der Grundlage der Analyse von sieben verschiedenen E-Liquids als Triplikate (n = 21) geschätzte Produktvariabilität ergab relative Standardabweichungen von 4,3% bis 16,0% bei Benzylalkohol und 6,3% bis 11,1% bei Benzoesäure. Die Menge an Benzoesäure (32.8 ± 2.8 mg/g; 3,3 ± 0,3 %, w/w) und das Stoffmengenverhältnis Nikotin/Benzoesäure (1,15 ± 0,02) blieben bei den unterschiedlichen Pod-Aromen relativ konstant. [Contrib. Tob. Nicotine Res. 30 (2021) 212–220]

RESUME

Les nouveaux dispositifs à capsule tels que JUUL™, Vuse Alto™, myblu™ et autres produits proches des ?pod-mods" ont profondément transformé le marché des cigarettes électroniques, notamment sur le segment des adolescents et des jeunes adultes, en raison, notamment d'une campagne de marketing agressive sur les médias sociaux, d'une grande disponibilité et d'un usage discret rendu possible par un design spécifique. Ces dispositifs incorporant une capsule sont conçus pour administrer, à chaque bouffée, des niveaux de nicotine comparables aux cigarettes combustibles, tout en libérant une moindre quantité d'aérosols exhalés visibles lors de la chauffe des liquides à vapoter. Certains de ces liquides contiennent de hautes concentrations en acides tels que de l'acide benzoïque afin de permettre un apport plus important en nicotine tout en atténuant l'irritation de la gorge et l'âpreté. L'acide benzoïque constitue une source potentielle de benzène cancérigène pour les êtres humains et donc une substance chimique préoccupante. En plus de ces

acides, des agents aromatisants tels que l'alcool benzylique (un anesthésique local susceptible de faciliter l'inhalation de la fumée de tabac) sont communément utilisés dans ce type de dispositifs. Tant l'acide benzoïque que l'alcool benzylique présents dans les liquides à vapoter pourraient s'avérer pertinents en termes de risque de santé pour les vapoteurs.

Une méthodologie fondée sur une spectrométrie de masse en tandem à dilution isotopique couplée à une chromatographie liquide à haute performance (ID-HPLC-MS/MS) fut mise au point afin de détecter l'acide benzoïque et l'alcool benzylique dans les liquides à vapoter de la marque JUUL™. La préparation des échantillons consista en une simple dilution suivie d'un processus de brassage mécanique. La méthodologie par ID-HPLC-MS/MS fut utilisée pour séparer, identifier et quantifier l'acide benzoïque et/ou l'alcool benzylique dans les extraits dilués. Les seuils de détection furent respectivement observés à 0,11 ng/μL pour l'alcool benzylique et 9,05 ng/μL pour l'acide benzoïque. La variabilité des produits estimée sur la base d'une analyse de sept liquides à vapoter différents en triple exemplaires (n = 21) afficha des écarts types relatifs allant de 4,3% à 16,0% pour l'alcool benzylique et de 6,3% à 11,1% dans le cas de l'acide benzoïque. La quantité d'acide benzoïque (32,8 ± 2,8 (mg/g) (3,3 ± 0,3%, p/p) ainsi que le rapport molaire nicotine-acide benzoïque (1,15 ± 0,02) demeurèrent relativement constants indépendamment de la saveur des capsules. [Contrib. Tob. Nicotine Res. 30 (2021) 212–220]

INTRODUCTION

Although modern electronic cigarettes (e-cigarettes) were introduced over a decade ago (2006 in Europe and 2007 in the United States), the concept even then was not new (1–3). An earlier attempt in 1965 to develop e-cigarettes in the United States was not profitable despite being advertised as a safe and harmless way to smoke or quit smoking (4). The first product to show some success on the market was developed in China in 2003 by HON LIK (5) and marketed with the help of the China Inventors Program. HON's e-cigarette gained some favor among Chinese smokers as a potential cessation device or an alternative cigarette product.

The appearance of e-cigarettes has varied widely, and include those that look like regular cigarettes, to pens, personal vaporizers, tank systems, and computer memory sticks. The devices share similar design components, including a battery that powers an atomizer or heating element, a reservoir to hold a liquid which generally contains nicotine and flavoring agents solubilized in propylene glycol (PG) and/or glycerin (Gly), and a mouthpiece through which the user inhales the aerosol of the electronic juice. The two popular formats of e-cigarettes are open systems and closed ("pod") systems. Open systems allow the consumer to adjust the temperature and use different e-liquids individually. Closed pod-based systems such as JUUL™, Vuse Alto™, and myblu™ do not allow the user to make changes to the device and/or e-liquid. However, the distinction between open and closed systems for pod-based devices has become muddled as third parties offer refillable replacement carts (6).

Pod e-cigarettes have become extremely popular, especially the JUUL™ brand because they are easy and discreet to use, often resembling a computer memory stick, and generate less exhaled vapor (7–10). These products are also available in multiple flavors that go beyond the standard tobacco and menthol offerings. Pod e-cigarette companies target their

advertising to groups such as youth and military personnel (10–14), successfully using social media platforms such as YouTube, Facebook, and Instagram (15). According to the National Youth Tobacco Survey about 19.6% of high schoolers and 4.7% of middle schoolers reported e-cigarette use in 2020. Much higher nicotine concentrations can be made available in pod e-cigarettes due to the use of e-juices. The e-juices contain high concentrations of nicotine salts, which allow greater amounts of nicotine to be consumed per puff of aerosol produced. Traditional e-liquids, by contrast, are based on free nicotine, which is volatile and produces higher absorption rates in the body. Free nicotine, like the smoke of cigars that also have high free-nicotine levels, is associated with harshness that makes inhalation difficult due to irritation (16) and limits the nicotine concentration. The nicotine salt e-liquids used in pod e-cigarettes such as JUUL™ are prepared using weak acids, such as benzoic acid, which serves to reduce the pH of the e-liquid, allowing the administration of higher doses of protonated nicotine with reduced throat irritation. Some publications suggest that nicotine salt e-liquids can produce satisfaction levels similar to those of traditional cigarettes to quench nicotine desire (14, 17–20).

Although there is a global consensus that the use of e-cigarettes lowers exposure to many harmful compounds compared to combustible tobacco cigarettes (21), the use of e-cigarettes remains controversial due to the high concentrations of their e-liquid components. Gly, PG, flavoring additives and nicotine salts are present in high concentrations in some of these e-liquids and could contribute to health problems. High concentrations of nicotine, for example, have been reported to adversely affect brain development (22–24) and could exacerbate cardiovascular issues and atherogenesis via prolonged nicotine exposure especially in individuals with underlying disease such as chronic kidney disease, hypertension, cardiovascular diseases, etc. (25, 26). Gly, PG, and flavorings can lead to the formation of highly carcinogenic compounds such as carbonyl groups and benzene during the vaping process when the coil temperature is sufficiently high. Additionally, the use of benzoic acid in nicotine salt liquids, such as those used in JUUL™ devices, can increase benzene formation via decarboxylation of benzoic acid, which can occur during vaping as previously postulated by PANKOW *et al.* (19). The Benzene formation ($^{12}C_6$) was confirmed by NMR for e-cigarette aerosol. It was collected in DMSO-d_6 and generated with the EVOD™ tank-type atomizer (Kangertech, Shenzhen, China) device operated at 14 W (2 ohms resistance) using 50:50 PG:Gly (both $^{12}C_3$) containing benzoic acid ($^{12}C_6$) at 1% by weight. The expected strong singlet peak in DMSO-d_6 at 7.37 ppm (27) for benzene was detected which was supported by addition of standard benzene, causing the peak to increase without introduction of other peaks. Furthermore, the use of flavorings such as benzyl alcohol, considered a local anesthetic and harmful compound by inhalation: vapors may cause drowsiness, dizziness, respiratory irritation and irritation to eyes, nose and throat, could facilitate tobacco smoke inhalation (28). Most research on e-cigarettes has focused on analyzing the vapor phase produced by vaping. While a few publications have reported analysis of the liquid phase, most of these have focused on quantitative analysis of nicotine and qualitative analysis of flavorings, with only a few studies measuring benzoic acid and benzyl alcohol. In this manuscript, we describe the development and validation of an isotopic dilution high-performance liquid chromatography-tandem mass spectrometry

(ID-HPLC-MS/MS) method to measure benzoic acid and benzyl alcohol in e-liquids under our ISO 17025 accredited policies and procedures.

MATERIALS AND METHODS

Chemicals and materials

All solvents used were analytical and/or HPLC grade with purity greater than 99.5%. Methanol Plus (MeOH), benzoic acid, and benzyl alcohol certified reference materials (CRM) were purchased from Sigma-Aldrich (Milwaukee, MO, USA). Formic acid 0.1% in water and acetonitrile (Optima) were obtained from Fisher (Fair Lawn, NJ, USA). Benzoic acid (992 μg/mL) and benzyl alcohol (998 μg/mL) reference materials solutions were acquired from SPEX CertiPrep (Metuchen, NJ, USA). Benzoic acid (Ring-$^{13}C_6$, 99%) and benzyl alcohol (Ring-$^{13}C_6$, ≥ 98%) were procured from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA).

JUUL™ cartridges (5% nicotine, pods) (Pax Inc., San Francisco, CA, USA) were acquired in five different flavors (Fruit Medley, Mango, Cool Mint, Virginia Tobacco, and Creme Brulee) along with two U.S. e-liquid products through The Lab Depot, Inc. (Dawsonville, GA, USA) in June 2019. The U.S. e-liquid products selected are not necessarily representative of the U.S. market; they constitute a “convenience” sample, purchased solely to test validity of the method using commercial e-liquids. Samples were labeled and stored at room temperature until they were analyzed. JUUL™ refill cartridges were uncapped and used only one time for each analysis.

Standard preparation

Isotopically labeled internal standards—Separate isotopically labeled internal stock solutions were prepared by dissolving 1000.00 mg of benzoic acid-$^{13}C_6$ in 1 mL of methanol and 100.00 mg of benzyl alcohol-$^{13}C_6$ in 5 mL of methanol. Aliquots of 800 μL of both labeled internal standard stock solutions were mixed into a 10-mL volumetric flask and diluted with methanol. The resulting internal standard solution (ISTD) of 80 mg/mL benzoic acid-$^{13}C_6$ and 1.6 mg/mL benzyl alcohol-$^{13}C_6$ was divided into 2-mL aliquots and stored at −20 °C until used. An aliquot of ISTD was added to the dilution solution which is needed to dilute each sample (2.5 mL dilution solution containing ISTD/sample) to yield a final concentration of 0.63 μg/μL of benzoic acid-$^{13}C_6$ and 12,64 ng/μL of $^{13}C_6$-benzyl alcohol.

Native standards

Standards were prepared from CRMs and covered the full range of the analytes concentration observed during preliminary investigation of the e-liquids, specifically the JUUL™ samples. A native standard stock solution of benzoic acid was prepared by weighing 2.5 g into a 25-mL volumetric flask and dissolving it with methanol to yield a stock solution with a concentration of 100 μg/μL. A native stock solution of benzyl alcohol was prepared by weighing 25.00 mg into a 25-mL volumetric flask and dissolving it with methanol to generate a stock solution with a concentration of 1.00 μg/μL. Aliquots of the two analytes were diluted with MeOH to generate nine primary calibration standards with concentrations

ranging from 0.45 to 1.82 μg/μL for benzoic acid and from 0.50 to 27.20 ng/μL for benzyl alcohol.

Quality control (QC) materials

A 250-mL non-commercial matrix solution was prepared by mixing Gly and PG (1:1, v/v) and dividing it into three pools. The first pool (low concentration quality control, QCL) was prepared by spiking the corresponding amount of benzyl alcohol and benzoic acid (CRMs) to yield concentrations of 1.3 ng/μL and 0.50 μg/μL, respectively. The second pool (high concentration quality control, QCH) was spiked with a corresponding amount of benzyl alcohol and benzoic acid (CRMs) to yield concentrations of 13.1 ng/μL and 1.20 μg/μL, respectively. The third pool was not spiked. After being examined for the presence of possible endogenous and/or cross contamination of the two analytes, the third pool was used as matrix material for blank samples, accuracy, and matrix-effect validation steps. QCL and QCH pools were characterized to determine the mean, 95th, and 99th control limits by consecutive analysis of at least 2 samples/day from each QC pool for 20 days. After establishing the control limits of the pools, QC high and low samples were analyzed with each analytical run and evaluated for validity using a modification of the Westgard rules (29). These values were used to certify the performance and long-term analytical stability of the method. A solvent, blank, and QCs samples were analyzed to monitor background levels and guard against contamination from sample carryover.

Sample preparation

All e-liquid samples, QC materials, reagents, and calibration standards were brought to room temperature. To prepare the QCs and the unknown samples, a 100 μL aliquot was weighed on an analytical balance with a precision of ± 0.00001 g into an amber 16-mL vial.

Samples were diluted with 2.5 mL of MeOH/H_2O (1:1) containing the internal standards and agitated for 60 min at 160 rpm on a Barnstead Lab-line E-class orbital shaker (Dubuque, IA, USA).

Instrumental analysis

The diluted extract was analyzed using a Dionex Ultimate 3000 HPLC coupled to a Thermo Scientific TSQ Endura mass spectrometer® (ThermoScientific, San Jose, CA, USA). The HPLC separation was carried out on a Luna® PFP (2.0 mm × 150 mm, 3.0 μm particle size) column (Phenomenex, Torrance, CA, USA). The column temperature was kept at 45 °C and the injection volume was 10 μL. The mobile phase consisted of (A) 0.1% formic acetic acid in water and (B) methanol:acetonitrile (1:1). The HPLC gradient conditions applied are listed in Table 1. To prevent excessive buildup of sample residue inside the mass spectrometer ion source, a diverter to waste container valve was used for the first and last segment of the separation. The HPLC eluents were ionized by heated electro-spray ionization probe (H-ESI) in positive-ion mode using ultra-high purity nitrogen as the sheath and auxiliary gases at pressures of 30 psi and 10 psi, respectively.

The vaporizer and ion transfer tube temperatures were kept at 300 and 270 °C, respectively. Ultra-high purity argon (Airgas, Atlanta, GA, USA) was used as the collision gas at a

pressure of 1 mTorr. The mass spectrometer was operated in the selected reaction monitoring mode as shown in Table 2.

RESULTS AND DISCUSSION

The main objective in our group is to develop analytical techniques to monitor and measure chemical components that impact the release of nicotine in tobacco products. In this project, we focus on the analysis of benzoic acid and benzyl alcohol, chemicals that are considered potentially human harmful that are present in new and/or popular tobacco products. JUUL™ is a popular style of e-cigarettes in the United States, and at one point accounted for more than half of the market share in tracked retail channels and becoming so popular that its use has led to a new verb: “JUULing” (15). We developed and fully validated a method to ensure reliable results in detecting and quantifying benzyl alcohol and benzoic acid in e-liquids. Accuracy, dynamic range, linearity, detection limit, precision, and robustness (including stability) were assessed. In addition, matrix effects were also determined in order to confirm the use of solvent-based calibrators was appropriate.

A calibration curve was constructed for each analytical run using the response factors of ten calibrators covering the linear dynamic range (LDR) from 0.50 to 27.20 ng/μL and 0.45 to 1.82 μg/μL for benzyl alcohol and benzoic acid, respectively. The LDR was selected such that the lowest standard concentration was near the limit of detection (LOD) and the highest calibrator concentration was higher than the concentrations measured for some e-liquids in domestic products. Analysis of the calibration curves (n = 7) indicated that a linear regression with (1/×) weighting resulted in an optimal distribution of residuals, and calibration curves displayed a coefficient of determination (R^2) greater than 0.995, indicating appropriate linearity for the analysis of both compounds.

We estimated LODs by evaluating the signal-to-noise (S/N) ratio for a low concentration standard of 0.50 ng/μL and 0.45 μg/μL for benzyl alcohol and benzoic acid, respectively, injected into the instrument over the course of twenty days (n = 20). The LOD was extrapolated for an S/N value of 3 based on the mean (S/N) value of the 20 measurements. Table 3 shows the LODs calculated using this approach.

The absolute accuracy of the method was not determined because no reference e-liquids were available for evaluation. Accuracy was assessed by spiking a known amount of each analyte to the blank e-liquid samples at three different concentrations in triplicate (low, medium, and high) spanning the range of expected values in commercial samples. The averaged calculated percent at low, medium, and high recovery samples are reported in Table 3.

To guarantee the method’s long-term analytical stability and reproducibility of the results, a blank sample and two QC samples at low and high concentrations (QCL and QCH, respectively) were analyzed with each sample set consisting of twenty-five unknown samples and ten standards. Analysis of intermediate precision yielded relative standard deviations that are reported in Table 3. The mean value and limits of each QC pool remained constant throughout this study. No carryover was observed for any blank sample.

We needed to determine the effects of the matrix due to internal CDC guidelines. The matrix effects between e-liquid calibrators and solvent-based calibrators were evaluated by comparing the slopes of two sets of calibrators prepared in Gly and PG solution (1:1, *v*/*v*) (homemade matrix) and solution of methanol (without matrix). Ten point calibration curves were constructed on a laboratory fabricated matrix and only in methanol. Least squares slopes were calculated for five independent calibration curves, averaged for the matrix-based and solvent-based samples, and the averaged slopes were compared for both calibration sets. Both matrix-based and solvent-based calibrators demonstrated linearity with $R^2 > 0.99$ and matrix effects were minimal. The two analytes presented an enhancement in the slopes when the homemade matrix was used of 2.0% and 3.2% for benzyl alcohol and benzoic acid, respectively. The speciWcity of this method was ensured by the addition of two conWrmation ions for each analyte (Table 2). For quality control, the ratios of the quantification ion over confirmation ions of benzyl alcohol and benzoic acid were calculated and evaluated versus pre-established values. The use of the ion ratios allowed us to identify interferences in the chromatograms thus ensuring the reporting of high-quality data. In this way, we took advantage of the specificity offered by HPLC-MS/MS using ISTDs. Additionally, speciWcity was clearly demonstrated by the chromatographic resolution of real samples with retention times of 7.93 ± 0.03 and 10.61 ± 0.03 min for benzyl alcohol and benzoic acid, respectively. No chromatographic interferences were observed (Figure 1).

We assessed sample stability using two different e-liquid samples (low and high concentration) under two different conditions: room temperature (24 ± 2) °C and in a dark freezer at (−20 ± 2) °C.

To avoid confounding issues resulting from simultaneous degradation of the standard and internal standard, the concentrated internal standard stock solutions were kept separate at (−20 ± 2) °C. On days of analysis, a single vial of internal standard and vials corresponding to each sample from each environment were equilibrated to room temperature. Then, each vial was spiked with internal standard solution and vortexed. Results following 30 days under the speciWed conditions were determined as a percentage of the original response for the sample. After 30 days all samples exhibited less than a 10% change in apparent concentration. Samples were stored in the dark at −20 °C if deemed necessary. Samples were typically analyzed the day they were generated, but if storage was required, they were stored for no longer than 30 days.

The robustness of the method was assessed by changing Wve method parameters using three different conditions including the final conditions. All the analyses were performed in commercial samples at two different levels. The five method parameters evaluated were column temperature, sample extraction composition, injection volume, sample weight and extraction time. For each parameter tested, all other variables were set to their final method conditions. Column temperature, sample extraction composition, injection volume, sample weight and extraction time varied from ± 0.1% to ± 4.7% with respect to the final method conditions (Table 4), demonstrating that the technique is robust. Based on CDC internal parameters, a maximum variability in the robustness test of ± 5% with respect to the final method is acceptable for the intended purpose of the method.

The suitability of the method was evaluated in the analysis of five JUUL™ flavors (5% nicotine), two commercially available e-liquids, and two quality control samples (QCL and QCH) for the quantification of benzyl alcohol and benzoic acid. The two commercially available brands were randomly selected and are not representative of the U.S. market. The samples were analyzed in triplicate seven times (n = 21). Additionally, JUUL™ samples were analyzed for nicotine following a previously reported method (30, 31) to establish the ratio correlation between nicotine and benzoic acid.

Table 5 presents a summary of the measured results. Two of the five JUUL™ samples tested yielded detectable values for benzyl alcohol (BOH); these concentrations are low and agree with data previously reported (32). However, the concentration of BOH in the Cool Mint JUUL™ flavor was the highest of all the samples analyzed in the study, including the two-regular e-liquids and the QCH samples. The concentration of BOH in Cool Mint JUUL™ was 22 and 2 times higher than BOH concentration of the Mango JUUL™ flavor and QCH sample, respectively. It should be noted that JUUL™ Lab Corporate withdrew all flavors from the U.S. market (except for the menthol and tobacco flavors). This was in response to the January 2020 U.S. Food and Drug Administration regulation that prohibited the sale of flavors in non-disposable e-cigarettes except for menthol and tobacco flavored varieties (31). Of note, the amount of benzoic acid did not vary significantly between different flavors of the initial pods (32.8 ± 2.8 mg/g; 3.3 ± 0.3%, w/w). The average results for benzoic acid and nicotine in JUUL™ are lower than results previously reported in the literature (20).

The possible discrepancy in the results may be due to the fact that the previous results were expressed in mg/mL, so for comparison purposes, a weighted density of the e-liquid was used based on a theoretical composition of the JUUL™ e-liquids of 30% PG and 70% Gly (0.30 × 1.04 g/mL + 0.70 × 1.26 g/mL = 1.19 g/mL). The molar ratio of nicotine:benzoic acid (1 : 0.9) for all JUUL™ samples suggests that 90% of the nicotine present in JUUL™ samples is in protonated form as nicotine benzoate. The next step in our research will be to quantify benzoic acid, benzyl alcohol, benzene, total nicotine, and freebase nicotine captured in the aerosol particulate using a standard puffing regimen. Another interesting path could be to quantify the amount of benzene in the same phase due to the carcinogenic nature of this analyte (33) and see if it correlates with the benzoic acid content. We have also considered quantifying other carboxylic acids such as lactic, levulinic, salicylic, and tartaric acids in e-liquids that can be found in other commercial brands that use these types of acids.

CONCLUSIONS

We developed and validated an analytical method to determine the concentration of benzoic acid and benzyl alcohol in e-liquids using a dilution and mechanical extraction sample preparation step coupled with isotope-dilution HPLC/MS-MS. The new method was applied to the analysis of five JUUL™ flavors and two other U.S. commercial e-cigarette brands. This method is characterized by its straightforward sample preparation, sensitivity, selectivity, and precision. The stability and precision of the measurement system over several weeks demonstrated the robustness of the method. The method is suitable for the routine analysis of benzoic acid and benzyl alcohol in e-liquids.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Tameka S. Lawler for the nicotine analysis in e-liquids. Technical assistance provided by Juliana Giraldo Junco and Ngac Phuong for the benzoic acid/benzyl alcohol is gratefully appreciated.

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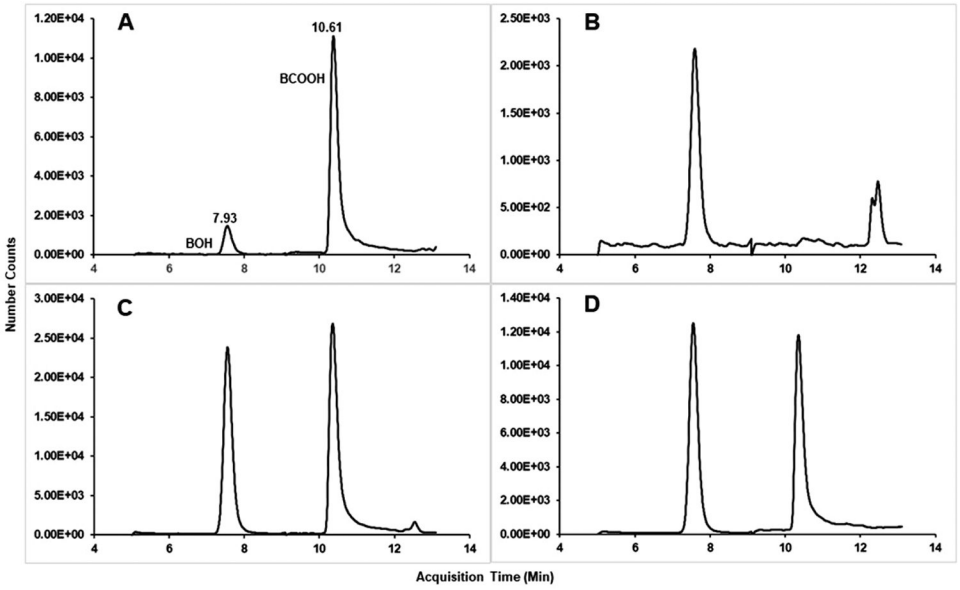

Figure 1.

Representative chromatograms of benzyl alcohol (BOH) and benzoic acid (BCOOH) quantification transitions for (A) quality control sample low concentration, (B) HAUS Cool Ice™, (C) JUUL Cool Mint™, (D) labeled internal standard.

Table 1.

HPLC gradient conditions for benzoic acid and benzyl alcohol analysis.

Run time (min)	Flow rate (mL/min)	Mobile phase A Composition (%)	Mobile phase B Composition (%)
0.0	0.225	80	20
2.0	0.225	30	70
11.0	0.225	30	70
12.0	0.250	80	20

Table 2.

Segments used in the mass spectrometer during analysis.

Segment	Segment time (min)	Compound	Scan type	Ions monitored (m/z)	Collision energy (eV)
1	0.00 – 6.00	NA	Full Scan		NA
2	6.00 – 9.50	BOH_Q	SRM	91.0 → 65.0	20.0
		BOH_C1	SRM	91.0 → 91.0	10.0
		BOH_C2	SRM	91.0 → 39.1	34.0
		BOH_L	SRM	97.0 → 69.0	20.5
3	9.55 – 13.50	BCOOH_Q	SRM	123.0 → 79.0	10.2
		BCOOH_C1	SRM	123.0 → 77.0	22.1
		BCOOH_C2	SRM	123.0 → 105.0	13.6
		BCOOH_L	SRM	129.0 → 85.0	10.0
4	13.55 – 20.0	NA	Full Scan		NA

BOH: benzyl alcohol; BCOOH: benzoic acid; _Q: quantitation; _C: confirmation; _L: Labeled; NA: Does not apply, SRM: selected reaction monitoring

Table 3.

Limits of detection (LOD), accuracy, and precision of the method.

Analyte	LOD (ng/μL)	Accuracy (%)			% RSD intermediate precision	
		Low	Medium	High	QCL	QCH
Benzyl alcohol	0.11	96.6 ± 1.9	95.4 ± 1.4	98.9 ± 2.3	5.3	4.4
Benzoic acid	9.05	92.2 ± 1.7	91.7 ± 4.1	93.8 ± 2.5	4.8	4.6

RSD: relative standard deviation; QCL: quality control samples at low concentration; QCH: quality control samples at high concentration

Table 4.

Results and conditions for the determination of method robustness.

Test parameters	Analyte	Method settings		Low level settings			High level settings		
		Method level	Amount (μg)[a]	Low level	Amount (μg)[a]	% Diff.	High level	Amount (μg)[a]	% Diff.
Column temperature	Benzyl alcohol	50 °C	3.76 ± 0.12	45 °C	3.69 ± 0.13	1.9	55 °C	3.66 ± 0.03	2.7
			61.65 ± 0.08		62.90 ± 1.34	2.0		61.54 ± 0.82	4.7
	Benzoic acid		3279 ± 50		3241 ± 40	1.2		3171 ± 35	3.3
			3158 ± 103		3038 ± 84	3.8		3053 ± 55	3.3
Sample dilution composition	Benzyl alcohol	MeOH:H_2O 50:50	2.95 ± 0.01	MeOH:H_2O 40:60	3.06 ± 0.06	3.7	MeOH:H_2O 60:40	2.96 ± 0.04	0.3
			52.12 ± 4.8		52.21 ± 0.59	0.2		52.42 ± 1.32	0.6
	Benzoic acid		3344 ± 72		3324 ± 90	0.6		3415 ± 77	2.1
			2711 ±201		2831 ±111	4.4		2813 ± 60	3.8
Injection volume	Benzyl alcohol	10 μL	3.00 ± 0.30	7 μL	3.11 ± 0.32	3.7	13 μL	3.01 ± 0.41	0.3
			61.26 ± 6.95		61.91 ± 7.09	1.1		61.17 ± 6.76	0.1
	Benzoic acid		3332 ± 396		3419 ± 437	2.6		3434 ± 386	3.1
			3077 ± 27		3137 ± 52	1.9		3147 ± 29	2.3
Sample weight	Benzyl alcohol	100 mg	3.03 ± 0.11	80 mg	3.09 ± 0.12	2.0	130 mg	3.05 ± 0.20	0.7
			53.54 ± 4.25		53.19 ± 3.05	0.7		54.15 ± 2.25	1.1
	Benzoic acid		3360 ± 68		3484 ± 58	3.7		3308 ± 78	1.5
			2806 ±210		2909 ± 90	3.7		2856 ±110	1.8
Extraction time	Benzyl alcohol	60 min	3.01 ± 0.07	42 min	2.98 ± 0.04	1.0	78 min	2.98 ± 0.06	1.0
			51.64 ± 4.04		51.75 ± 1.49	0.2		52.12 ± 0.96	0.9
	Benzoic acid		3345 ± 65		3293 ± 27	1.6		3327 ± 20	0.5
			2773 ± 235		2714 ± 21	2.1		2721 ± 23	1.9

% Diff.: Percentage of difference between the final method settings and the new settings used for the robustness study.

[a]Average amount in sample with standard deviation. All samples were prepared in triplicates.

Table 5.

Levels of benzyl alcohol (n = 21), benzoic acid (n = 21), and nicotine (n = 7) in JUUL™ liquids and two commercially available e-liquids.

Sample type	Benzyl alcohol Total (mg/g)			Benzoic acid (BA) Total (mg/g)			Benzoic acid Total (%,W/W)	Nicotine (N) Total (mg/g)			Molar ratio
	Average	STD	CV	Average	STD	CVs	Average	Average	STD	CV	N:BA
QCL	0.0340	0.0009	2.5	13.03	0.67	5.2	1.30	NP	—	—	NA
JUUL™ - Fruit Medley	ND	NA	NA	32.58	2.06	6.3	3.26	49.4	0.84	1.7	1.14
JUUL™ - Mango	0.0283	0.0045	16.0	33.64	3.74	11.1	3.36	50.6	0.92	1.8	1.13
JUUL™ - Cool Mint	0.6420	0.0485	7.6	33.18	2.84	8.5	3.32	49.3	0.75	1.5	1.12
JUUL™ - Virginia Tobacco	ND	NA	NA	32.52	2.54	7.8	3.25	50.3	0.88	1.7	1.16
JUUL™ - Creme Brulee	ND	NA	NA	32.20	2.62	8.2	3.22	50.2	1.08	2.2	1.17
HAUS™ - Cool Ice	0.0487	0.0021	4.3	ND	NA	NA	ND	NP	—	—	NA
Pacha Mama - Mango	0.0972	0.0065	6.7	ND	NA	NA	ND	NP	—	—	NA
QCH	0.3317	0.0118	3.6	29.87	1.27	4.3	2.99	NP	—	—	NA

ND: Non-detectable; NA: Does not apply; % CV: Coefficient of variation; STD: Standard deviation; NP: Non-performed; QCL: quality control sample-low concentration; QCH: quality control sample-high concentration; N: nicotine; BA: benzoic acid