Formation of Diacetyl and Other α-Dicarbonyl Compounds during the Generation of E-Vapor Product Aerosols

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ABSTRACT: Exposure to diacetyl (DA) has been linked to the respiratory condition bronchiolitis obliterans. Previous research has demonstrated that DA and other α-dicarbonyl compounds can be detected in both the e-liquids and aerosols of e-vapor products (EVPs). While some EVP manufacturers may add these compounds as flavor ingredients, the primary objective of this work was to determine the potential for the formation of α-dicarbonyl compounds during the generation of aerosols from EVPs where no DA or other α-dicarbonyl compounds are added to the e-liquid. A novel ultraperformance liquid chromatography-mass spectrometry-based analytical method for the determination of DA, acetyl propionyl, glyoxal, and methylglyoxal was developed and validated. Next, eight commercially available cig-a-like-type EVPs were evaluated for α-dicarbonyl formation. Increased levels of α-dicarbonyls were observed in the aerosols of all evaluated EVPs compared to their respective e-liquids. Mechanistic studies were conducted using a model microwave reaction system to identify key reaction precursors for DA generated from propylene glycol (PG) and carbon-13-labeled glycerin (GLY). These studies, along with the corresponding retrosynthetic analysis, resulted in the proposed formation pathway where hydroxyacetone is generated from PG and/or GLY. Hydroxyacetone then participates in an aldol condensation with formaldehyde where formaldehyde can also be generated from PG and/or GLY; the resultant product then dehydrates to form DA. This proposed pathway was further investigated through in situ synthetic organic experiments within the model microwave reaction system. This work establishes that DA is formed in the aerosol generation process of the EVPs tested though at levels below toxicological concern.

INTRODUCTION

E-cigarettes, also known as e-vapor products (EVPs) and electronic nicotine delivery systems (ENDS), are a growing tobacco product category. On June 22, 2009, the Family Smoking Prevention and Tobacco Control Act1 was signed into law granting the United States Food and Drug Administration (FDA) regulatory authority over tobacco products, such that oversight of cigarettes, smokeless tobacco, and roll-your-own tobacco was effective immediately. In 2016, the FDA published the final rule establishing oversight of cigars, EVPs, and other tobacco products effective on August 8, 2016.2 As a result, EVPs ultimately require authorization from the FDA through the premarket tobacco product application (PMTA) pathway to either remain on the market or to enter interstate commerce.3 The FDA published guidance for the industry to assist in the submission of PMTAs for ENDS.3 Within this guidance, FDA suggests studies that could be conducted to demonstrate that the product is “appropriate for the protection of public health” and includes a list of select constituents that should be considered for analysis in both e-liquids and aerosols.3 These constituents, often referred to as harmful and potentially harmful constituents (HPHCs), included the flavorings diacetyl (DA) and acetyl propionyl (AP).

The Food and Extract Manufacturers’ Association (FEMA) has deemed the flavor compounds DA (FEMA #2370) and AP (FEMA #2841) as generally recognized as safe (GRAS) for use in consumer products intended for ingestion.11 However, GRAS status does not apply to consumer products intended for inhalation. Many, if not all, GRAS compounds, including DA and AP, have not been evaluated for inhalation safety.11 The use of DA in microwave popcorn manufacturing led to the recognition of its occupational exposure risk. In 2000, the cases of eight former operators from a popcorn manufacturing site diagnosed with bronchiolitis obliterans, a chronic and often fatal respiratory/pulmonary disease, were reported to the Missouri Department of Health, as summarized by Kreiss et al.12 Elevated indoor air concentrations resulted when DA-containing flavor systems were heated prior to application, which resulted in elevated exposure for the operators. Epidemiology studies ultimately linked the respiratory disease to DA exposure.13 Additionally, Hubbs et al.15 demonstrated
changes in protein homeostasis in the airways of DA-exposed mice. These researchers made the association between protein damage in airway epithelium as a mechanism of action for airway injury induced by DA exposure. Hubbs et al. also determined that acute inhalation exposure to AP caused airway epithelial damage similar to what was observed in the DA studies when rats were dosed at 112, 241, 318, and 354 ppm for 6 h, and Morgan et al. demonstrated the chemical reactivity and respiratory toxicity of DA.

There are many different e-liquid flavors currently available in the commercial marketplace where varying combinations of propylene glycol (PG) and glycerin (GLY) are used as the base solvents. This wide range of commercial flavors can include sweet, fruity, mint, citrus, and tobacco flavors. Farsalinos et al. and Sleiman et al. reported that two HPHCs (DA and acetyl propionyl (AP); also known as 2,3-butanedione and 2,3-pentanedione, respectively) have been identified in some commercially available e-liquids. A recent infographic from the Centers for Disease Control refers to DA as a flavoring and also indicates that it can be contained in some e-cigarette aerosols. These compounds contain the structural similarity of an α-dicarbonyl functionality and specifically an α-diketone substructure for DA and AP. These α-dicarbonyls are naturally occurring compounds found in many types of fruits, vegetables, and nuts and can also be found in many products of fermentation such as butter and cheese. They are used in the commercial food industry as artificial flavorings to impart a characteristic butter flavor note and have been used in microwave popcorn manufacturing.

Several researchers have demonstrated that thermal degradation of PG and GLY can occur to produce compounds such as carbonyls (e.g., formaldehyde, acetaldehyde, and acrolein) that can be detected in EVP aerosols at levels higher than those found in the corresponding e-liquid. Data (unpublished) indicated it was possible that α-dicarbonyl compounds could form under certain thermal conditions experienced during EVP aerosol formation. Data (unpublished) indicated it was possible that α-dicarbonyl compounds could form under certain thermal conditions experienced during EVP aerosol formation. Measurable levels of DA and AP in EVP liquids and aerosols were also reported by Allen et al. and Moldoveanu et al. Consequently, the primary objective of this work was to confirm the formation of α-dicarbonyls during the thermal process of EVP aerosol formation and investigate the mechanism of their formation.

## MATERIALS AND METHODS

**Chemicals, Standards, and Reagents.** Acetic acid, acetonitrile (ACN), GLY, and PG were obtained from Fisher Scientific (Pittsburgh, PA). o-Phenylenediamine (OPD), hydroxyacetone (HA), and formaldehyde (33% solution in water, v/v) were obtained from Sigma-Aldrich (St. Louis, MO). 13C2-labeled GLY was obtained from AptoChem Inc. (Montreal, Canada). G, MG, DA, AP, quinoxaline (product #Q1603; 99% purity), 2-methylquinoxaline (product #M80202, 97% purity), and 2,3-dimethylquinoxaline (product #D184977, 97% purity) were purchased from Sigma-Aldrich. Quinoxaline-d6 (product #D-1033, 98% purity) was purchased from CDN Isotopes (Pointe-Claire, Canada).

The analytical method employs o-phenylenediamine (OPD) as the derivatizing agent that generates the corresponding quinoxalines from the respective α-dicarbonyls. The compounds 2-methyl,3-ethylquinoxaline, 2,3-dimethylquinoxaline-d6, and 2-methyl,3-ethylquinoxaline-d6 were synthesized from the corresponding diketone or deuterated diketone and OPD according to the method of Delpivo et al. with minor modification, as depicted in Scheme 1. The same preparation of the internal standards was used for preparing both the standards and the samples. Two additional α-dicarbonyls, glyoxal (G) and methylyglyoxal (MG), were added to the method, as they have been reported in the literature to be potential precursors to DA in certain thermal processes with sugars and in Maillard reactions.

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**2,3-Dimethylquinoxaline-d6.** o-Phenylenediamine (176.1 mg, 1.6 mmol) was transferred to a 100 mL volumetric flask and dissolved in 50 mL of acetonitrile. Thirty milligrams (0.33 mmol) of 2,3-butanedione-d6 was added and the solution was mixed on a wrist-action shaker for 20 min. The solution was brought to volume with acetonitrile and used without further purification.

**2-Methyl,3-ethylquinoxaline-d6.** o-Phenylenediamine (135.8 mg, 1.26 mmol) was transferred to a 100 mL volumetric flask and dissolved in 50 mL of acetonitrile. 2,3-Pentanedione-d6 (26.4 mg, 0.25 mmol) was added and the solution was mixed on a wrist-action shaker for 20 min. The solution was brought to volume with acetonitrile and used without further purification.

**2-Methyl,3-ethylquinoxaline.** o-Phenylenediamine (3.28 g, 30.3 mmol) was transferred to a 100 mL volumetric flask and dissolved in 50 mL of acetonitrile. 2,3-Pentanedione (0.61 g, 6.1 mmol) was added and the solution was mixed on a wrist-action shaker for 20 min. The solution was brought to volume with acetonitrile and used without any purification.

**Analytical Method.** An ultraperformance liquid chromatography-mass spectrometry (UPLC-MS) method was developed, which utilizes an Agilent Poroshell Bonus-RP (2.1 x 100 mm2, 2.7 μm pore size) (Santa Clara, CA) analytical column. The instrument used for these studies was a Waters Acquity UPLC with a Xevo TQD mass spectrometer. A gradient elution was used, where mobile phase A (MP-A) was 100% Type-1 water and mobile phase B (MP-B) was 100% ACN. The flow rate was 0.6 mL/min. The initial gradient conditions were 100% MP-A, which was held for 1 min followed by a linear gradient to 70:30 MP-A/MP-B over 4 min and held for 1 min. The runtime was 6 min with positive electrospray ionization MS detection in single-ion monitoring mode. The collected masses were 131, 145, 159, and 173 m/z, which correspond to the quinoxaline derivatives of G, MG, DA, and AP. The masses of the deuterated internal standards were 135, 165, and 178 for quinoxaline-d6, dimethylquinoxaline-d6, and methylthiylquinoxaline-d6, respectively.

Standard solutions were prepared from a commercial or synthetic material as stated above by performing a serial dilution of stock solutions with hexane as the diluent (see the Supporting Information for experiments performed to determine the accuracy of the synthesized 2-methyl,3-ethylquinoxaline calibration standards). Calibration was carried out using the relative responses of the
matched internal standards. For MG, quinoxaline-$d_4$ was used as the internal standard. An example chromatogram is shown in Figure 1.

**Sample Preparation.** E-liquid samples were prepared by weighing 500 mg of sample into 5 mL of 50 mM OPD/0.2% acetic acid (v/v), adding internal standard, and extracting into
Table 1. Validation Summary

| parameter          | glyoxal (μg/g) | methylglyoxal (μg/g) | diacetyl (μg/g) | acetyl propionyl (μg/g) |
|--------------------|---------------|---------------------|----------------|------------------------|
| linearity          | R² ≥0.995     | R² ≥0.995           | R² ≥0.995      | R² ≥0.995              |
| accuracy           | e-liquid (%)  | aerosol (%)         | aerosol (%)    | aerosol (%)            |
| e-liquid (%)       | 82–114        | 78–111              | 80–112         | 82–117                 |
| aerosol (%)        | 40–2400       | 12–800              | 24–2400        | 36–3600                |
| interday precision | e-liquid (%)  | <5                  | <5             | <7                     |
| aerosol (%)        | <16           | <13                 | <8             | <11                    |
| LOQ                | e-liquid (ng/g) | 40                  | 12             | 24                     |
| aerosol (ng/g)     | 40            | 12                  | 24             | 36                     |

“Transfer efficiency not evaluated. Recovery samples used (n = 5). *Commercial product used (n = 15). **Assuming 0.5 g of generated aerosol.

Table 2. Analysis of E-Liquids and Aerosols from Eight Commercially Available EVPs (N = 4)

| product ID | aerosol mass (g) | e-liquid aerosol increase | diacetyl (μg/g) | acetyl propionyl (μg/g) |
|------------|------------------|--------------------------|----------------|------------------------|
| A          | 0.124            | 0.24                     | <LOQ           | ND                     |
| B          | 0.124            | 0.24                     | <LOQ           | <LOQ                   |
| C          | 0.120            | 3.50                     | 0.12           | 0.30                   |
| D          | 0.128            | 1.60                     | <LOQ           | ND                     |
| E          | 0.125            | 1.70                     | <LOQ           | ND                     |
| F          | 0.121            | 0.29                     | <LOQ           | ND                     |
| G          | 0.107            | 0.24                     | <LOQ           | ND                     |
| H          | 0.099            | 0.06                     | <LOQ           | ND                     |

“ND, none detected. *LOQ of diacetyl = 24 ng/g. **LOQ of acetyl propionyl = 36 ng/g; LOQ of glyoxal = 40 ng/g; LOQ of methylglyoxal = 12 ng/g.

2 mL of hexane by mechanical shaking for 10 min. A 2 μL aliquot of the hexane layer was analyzed by UPLC-MS. Lower amounts of samples (100 mg instead of 500 mg) were used if the analyte values were above the calibration curve. The large amount of PG and GLY that makes up the EVP matrix is detrimental to instrument performance and induces matrix effects and interferences, both of which need to be avoided for a robust routine analysis method. In the current implementation, the PG and GLY are retained in the acidified aqueous layer along with other basic compounds. The unprotonated quinoxalines are transferred into the hexane layer due to their pKₐ of approximately 0.6. This technique eliminates the need for performing sample clean-up by solid-phase extraction. Hexane is typically not injected into a reversed-phase chromatography system due to solvent miscibility; however, no deleterious effects were observed in the chromatography when the injection volume was 2 μL or less.

EVP aerosol samples were generated from the designated devices using an Analytical Vaping Machine LX20E2 (Borgwaldt; Hamburg, Germany) and a puff regimen of 55 mL puff volume, 5 s puff duration, and 30 s puff interval under a square wave puff profile. The aerosol collection system consisted of a Cambridge filter pad (CFP) followed by one impinger containing 20 mL of 50 mM OPD/0.2% acetic acid (v/v). After aerosol collection, the CFP and the impinger solution were combined in a 40 mL vial, treated with an internal standard solution and 2 mL of hexane, and extracted by mechanical shaking for 10 min. A 2 μL aliquot of the hexane layer was analyzed by UPLC-MS. To limit the possibility of cross contamination, the equipment used for the EVP sample collections had only been used for the collection of e-vapor and not for any aerosol collections from conventional cigarettes.

Method Validation. The method was then validated for fitness-for-purpose to analyze trace levels of the analytes of interest following the International Conference on Harmonization (ICH) guidelines. The validation reference sample consisted of e-liquid prepared in-house with a composition of 50:50 PG/GLY, 2.5% nicotine (w/w), and 15% water (w/w). The validation figures of merit are summarized in Table 1. Briefly, the method gave a linear response with coefficients of determination greater than 0.995 across the working range of the calibration standards. Recoveries for e-liquid and aerosol samples ranged from 76 to 116% and 80 to 110%, respectively (see the Supporting Information for experimental details). The method interday precision was less than 16% relative standard deviation (RSD) for both e-liquid and aerosol samples over 3 days. The limit of quantification (LOQ) for e-liquid and aerosol samples was set to the lowest calibration standard for each analyte, 40, 12, 24, and 36 ng/g for G, MG, DA, and AP, respectively.

Analysis of Commercial Products. Commercial EVPs were acquired in 2017 at local convenience stores in the Richmond, VA area, and all contained rechargeable batteries with disposable e-liquid cartridges. MarkTen XL Classic and MarkTen XL Menthol e-cigarettes were obtained internally (products A and B, respectively, in Table 2). The e-liquids were removed from the cartridges by centrifugation at 4000 rpm for 10 min in a 50 mL conical tube. The aerosol was collected using the standard conditions for aerosol collection that were stated above, and 25 puffs were collected from each sample. The samples were prepared according to the method and analyzed by UPLC-MS. All results were corrected against a blank method sample. The blank method sample was generated by collecting an empty smoke machine port along with the aerosol sample. This sample was then prepared according to the method. The correction was performed by subtracting the value of the blank method sample from the value of the sample for each analyte.
Model Reaction System: Development and Verification. To improve the efficiency of subsequent mechanistic studies, a model reaction system was developed, wherein a multitude of reactant combinations were evaluated. The same reference e-liquid [50:50 PG/GLY + 2.5% (w/w) nicotine + 15% water (w/w)] from the method validation study was used for the subsequent evaluations. Traditional heating using an oven, heat block, as well as a Rapid-Oxy oxygen stability system (Anton Paar, Ashland, VA), were evaluated without the generation of acceptable results. Traditional heating resulted in sample decomposition, while the Rapid-Oxy system generated only oxidized products where MG was produced from PG; both of which produced products that were not representative of those formed within the aerosol generated by the EVP system that was being modeled. The use of a Discovery SP Hybrid microwave reactor (CEM Corp, Matthews, NC) was screened with encouraging results and selected for further optimization. The microwave conditions were then optimized for DA formation by varying the temperature from 80 to 220 °C under a fixed power of 200 W and over time from 1 to 15 min at 160 and 180 °C. The samples were then prepared and analyzed as described above. To verify the model reaction system, these product distributions were then compared to the product distribution of the aerosol generated by a MarkTen XL e-cigarette using the same reference e-liquid mentioned above. The reference e-liquid was loaded into empty MarkTen XL cartridges (NuMark LLC, Richmond, VA; 3.5 Ω). The samples consisted of the aerosol generated from 50 puffs using the standard puffing regimen and collection system described above. Samples were prepared in the same manner as above and analyzed by UPLC-MS. The optimum conditions were determined to occur after heating 500 mg of sample to 180 °C for 3 min under a fixed power of 200 W where the decomposition/reaction product distributions were similar for DA and AP. This was accomplished by measuring the levels of the four analytes of interest created under the microwave conditions and comparing those to the product distribution in the aerosol generated by an EVP device. It is to be emphasized that the microwave reaction system is a model system that under select reaction conditions produces a reaction environment that is representative of the reaction environment encountered within a specific EVP under normal operating conditions; in this case, the model system was developed with the MarkTen XL e-cigarette. As has been discussed in other publications,57−40 microwave heating phenomena differ from conventional heating phenomena, and hence, the time and temperature conditions at which the reactions occur in the microwave system are not similar to the conditions under which the same reactions happen in an EVP heating element. Due to the limitations with the infrared temperature control of the microwave model systems, the actual temperatures in the reaction media may be elevated above 180 °C.41−43

Studies with Stable Isotope-Labeled GLY. Isotopic labeling studies were performed using GLY where all three carbons are isotopically labeled (13C3-GLY). For these studies, an e-liquid sample composed of 50:50 PG/13C3-GLY containing 2.5% nicotine (w/w) and 15% water (w/w) was prepared. The sample was heated under the standard microwave conditions and taken through the sample preparation procedure. The samples were analyzed by the analytical method using a selected ion monitoring MS method that incorporated all of the relevant isotope masses for DA. These data were used to determine the relative isotopic abundance of DA with natural isotopic abundance and mass bias corrections being applied, according to Paine et al.44

Experiments to Evaluate the Mechanistic Pathway for DA Formation. The data obtained from the isotopically labeled GLY studies were used to perform a retrosynthetic analysis to identify key precursors required for the formation of DA. This analysis leads to the proposed reaction pathway and is discussed below.

The proposed pathway was further investigated by applying synthetic organic chemistry reactions. The influence of added HA and formaldehyde on the generation of DA by the reference e-liquid was evaluated under the standard microwave conditions. Experiments were performed where either HA or formaldehyde was added to individual reactions at amounts of 12.5, 25, or 50 ng. The samples were prepared and analyzed according to the analytical method.

Reactions containing stoichiometric amounts of both HA and formaldehyde (100 μM each) in PG as the reaction media were then performed under acid- and base-catalyzed conditions to further probe the aldol reaction. Varying concentrations of nicotine were used for the base-catalyzed conditions, while varying concentrations of acetic acid were used for the acid-catalyzed conditions. Nicotine was chosen for the base due to its presence in EVP liquids. The reactions were performed under the standard microwave conditions and analyzed per the method.

Toxicological Evaluation−Risk Assessment. The toxicological evaluation was conducted using the methodology similar to the one described by Flora et al.52 Quantitative risk assessment calculations were performed for DA using published exposure limits, as established by the American Conference of Governmental Industrial Hygienists (ACGIH) and the National Institute of Occupational Safety and Health (NIOSH). The ACGIH threshold limit value (TLV) for DA is 0.04 mg/m3 (0.01 ppm) and the NIOSH recommended exposure limit (REL) is 0.02 mg/m3 (0.005 ppm). In general, TLVs and maximum allowable concentrations provided by ACGIH and NIOSH specify the limiting exposure concentrations of chemicals for daily inhalation during an 8 h workday over the course of a working lifetime. These levels are based upon an expert review of the published scientific literature in the areas of medicine, toxicology, industrial hygiene, and epidemiology and represent a level of exposure that a typical healthy worker can experience repeatedly without adverse health effects. Using TLVs, the daily exposure to a chemical without expected adverse health effects can be calculated by assuming a daily air inhalation rate of 28.8 m3/day or 1.2 m3/h.

RESULTS AND DISCUSSION

E-liquids from eight commercially available EVPs (Table 2, product IDs A−H), and their corresponding aerosols, were analyzed for the production of G, MG, DA, and AP using the validated method described herein. Samples were generated according to the procedures for e-liquid and aerosol outlined above. The increase in the analyte yield from e-liquid to aerosol was calculated by subtracting the e-liquid value from the aerosol value. This increase is indicative of analyte formation during aerosol generation.

As shown in Table 2, all commercial EVPs tested contained measurable amounts of G and MG in the e-liquids. Only two of the eight EVPs (products C and F) contained both DA and AP in the e-liquids. DA was also found to be present in the e-
liquid for product D, while AP was also detected in the e-liquid for product H. The aerosol yields were then acquired and compared to the e-liquid analysis. G and MG were elevated in all of the products relative to their respective e-liquid (with the exception of G in product A). G increases ranged from 0.26 to 37.81 μg/g and MG increases ranged from 0.39 to 17.74 μg/g. DA also showed elevated levels in aerosol for the products that contained DA in their respective e-liquid (products C and F), and measurable levels of DA were detected in all of the aerosols of the commercial products ranging from 0.08 to 4.88 μg/g of generated aerosol or 3.2 to 195.2 ng/puff. Moldoveanu et al. detected DA levels in commercial EVP aerosols in the range of 0.34 to 23.8 ng/puff for the first 50 puffs of the device similar to our findings. AP levels were largely unaffected, with products C and F showing a slight increase in AP when comparing yields in the e-liquid to the corresponding yields in the generated aerosol.

The increase in DA content between the native e-liquid and the aerosol was indicative of the formation of DA during aerosol generation potentially through a thermal degradation pathway. AP generation was not as prevalent as DA formation in the devices tested. This could indicate that AP formation follows a different pathway of formation than DA or that the kinetics for its formation are slower when compared to the rate of DA formation.

Mechanistic investigations were then conducted to understand the DA formation pathway within the aerosol. As previously described, a model reaction system was developed based on microwave heating, which was used for the subsequent investigations. Initial studies were conducted to optimize the conditions for DA formation in this model reaction system where time and temperature were varied. The results of these initial time—temperature studies are presented in Figure 2. The results from the microwave reaction system indicated a linear dependence over time for DA formation at each of the two temperatures measured (160 and 180 °C). The condition of 180 °C for 3 min under the fixed power mode was selected for further investigations as it produced the high measurable DA yields in a short amount of time.

The yields of DA generated from the aerosol were similar to the yields generated by the microwave system, indicating that the specific conditions used for the microwave model system are not well suited for studying G and MG generation in the particular EVP device used (MarkTen XL). However, the elevated G and MG formation could be rationalized by their formation occurring primarily through oxidation processes as observed in their formation from sugars. This is further supported by the Rapid-Oxy results, wherein a portion of the PG was converted to MG. It could be assumed that the reaction environment that is created during aerosol generation is highly aerobic due to the nature of the puff being 21% oxygen, the composition of ambient air. The same levels of molecular oxygen would not be achievable in the closed vessel of the microwave system. These differences in molecular oxygen availability could be a potential explanation for oxidation pathways predominating in the aerosol generation process. The formation of G and MG is the subject of future work.

The yields of DA generated from the aerosol were similar to the yields generated by the microwave reaction system, indicating that the microwave reaction system and conditions were a suitable model system for studying DA formation. AP was not observed at detectable levels in the e-liquid, aerosol, or in the microwave reaction system.

Once the equivalency of the model reaction system was verified, isotope labeling studies were performed with fully 13C-labeled GLY to aid in elucidating the DA formation pathway. The results of these studies are shown in Figure 4, and they indicate that PG alone was capable of producing all of the required precursors for DA formation based on the 24% relative abundance of the DA lacking incorporated 13C. The major pathway, representing 47% of the DA yield, produced DA with a single 13C incorporated. This provided key precursor information, indicating that the predominant pathway for DA formation involved a three-carbon compound originating from PG and a one-carbon compound originating from sugars. This is further supported by the Rapid-Oxy results, wherein a portion of the PG was converted to MG.
from GLY. The least favorable pathway, 7%, was observed for the three $^{13}$C-incorporated DA, which indicated that the three-carbon precursor could originate from GLY as well, and that the one-carbon precursor was also capable of being formed from PG. The relative abundance of the four $^{13}$C-incorporated DA (17%) was similar in preference to the non-$^{13}$C-incorporated pathway and indicated that GLY can produce both precursors; the one-carbon and three-carbon precursors. To summarize, DA appears to be formed from the reaction of a three-carbon and a one-carbon compound and both of these precursors can originate either from PG or from GLY.

Applying the technique of retrosynthetic analysis where formaldehyde is known to be a common one-carbon synthon, the following reaction scheme was proposed and is detailed in Scheme 2. This proposal relies on the production of HA, the three-carbon precursor being formed from either PG or GLY and has been identified previously in EVP aerosols. This compound can be formed in one step from GLY via a dehydration reaction or in one step from PG via an autoxidation reaction. Formaldehyde has been shown to form from GLY and PG by several common mechanisms. Once these precursors are present, HA (1) equilibrates to the enol form (2), which subsequently participates in an aldol condensation with formaldehyde (3) to produce the dihydroxy ketone (4) as well as the other potential regioisomer. This aldol product (4) then dehydrates to form the enol form of DA (5), which in turn converts to DA (6) via tautomerization. As classical aldol and mixed aldol condensations are not primarily driven by the keto-enol equilibrium constant, especially when secondary dehydration reactions are possible, the enol nucleophile concentration could be considered infinite. This is due to the secondary dehydration reaction and Le Chatelier’s Principle driving the keto-enol equilibrium to the enol form. An exhaustive physical organic mechanistic study is out of the scope of this report. However, basic structure-reactivity principles of the classical aldol condensation can prove useful in supporting this proposed mechanism.

The presence of formaldehyde and HA in the microwave reaction products was confirmed by analyzing the samples using a previously published analytical method, which relied on the standard 2,4-dinitrophenylhydrazine derivatization chemistry followed by UPLC-MS analysis. These results indicated that formaldehyde was being produced at a level of 25.6 μg/g in the microwave system, which is comparable to the corresponding aerosol value of 20.1 μg/g. HA was also present in the reaction mixture at 3.6 μg/g of e-liquid. The results of experiments performed to confirm the proposed mechanism of DA production are depicted in Figure 5. Base levels of DA formation were determined with the reference e-liquid (50:50 PG/GLY + 2.5% nicotine (w/w) + 15% water (w/w)), as previously described. This served as the control reaction to determine the influence of added HA to the reaction mixture on total DA yield. These experiments showed a significant increase in DA yield with increasing HA concentration, which supports the proposed mechanism such that increasing the concentration of HA in the reaction mixture shifts the equilibrium toward the formation of the nucleophilic enol. A subsequent condensation with formaldehyde followed by a secondary dehydration reaction would drive the reaction pathway toward the DA product. The analogous experiment, where formaldehyde was added to the reaction mixture, did not show any appreciable effect on DA formation compared to the control. This could be attributed to the concentration of
formaldehyde being in excess from native thermal degradation of PG and GLY. The model reaction system produced a formaldehyde yield of 25.6 μg/g of e-liquid or 852 nmole of formaldehyde per gram of e-liquid. The HA level of 3.6 μg/g of e-liquid converts to 48.6 nmole of HA per gram of e-liquid being produced by the microwave system. When comparing these results in a stoichiometric manner, the formaldehyde is present in approximately 17-fold excess as compared to the HA amount. As observed in our addition experiments, this natively produced large excess could prevent the addition of more formaldehyde from having an increasing effect on diacetyl production.

The influence of acid and base catalysis, a well-documented characteristic of classic aldol and mixed aldol condensations, was also investigated. In this case, samples containing 100 μM HA and 100 μM formaldehyde in 5 mL of PG were investigated where varying amounts of base (nicotine) and acid (acetic acid) were titrated into the solutions. These results are shown in Figure 6. Consistent with the proposed mechanism, increasing concentrations of base, which is nicotine in this example, resulted in increased DA yields. The analogous experiments using acetic acid as the acid catalyst showed similar results with increasing DA yields as the concentration of acid increased except at the 100 μL level. This could be attributed to the volume of aqueous acetic acid used increasing the concentration of water in the samples to a point that the rate of the dehydration step is substantially decreased. This was also consistent with the reactivity of aldol condensations.

A toxicological risk assessment was also performed on the measured DA levels of the commercial products from above using occupational exposure limits established by ACGIH and NIOSH. These exposure limits are based on toxicological data using decreased lung function as the adverse outcome and do not account for exposure from the recreational use of tobacco products. Using calculations previously detailed by Flora et al. and the assumptions listed in the methods section, the ACGIH TLV of 0.04 mg/m³ is equivalent to 384 μg DA/day and the NIOSH REL of 0.02 mg/m³ is equivalent to 192 μg DA/day. Internal data (unpublished) show that the average consumer of cig-a-like EVPs uses two e-liquid-filled cartridges per day similar to published data for cig-like EVPs where the reported use was one to two cartridges per day. Given that the maximum measured DA from one cartridge was 4.88 μg (Table 2, product F), the total daily average exposure can be estimated at 9.76 μg/day—about 40 times lower than the ACGIH TLV and about 20 times lower than the NIOSH REL.

In addition, given the elevated levels of G and MG in product H (Table 2), a toxicological risk assessment of combined G and MG aerosol levels in product H was conducted to compare potential daily exposure to an acceptable occupational exposure limit. The ACGIH TLV for G of 0.1 mg/m³ is equivalent to 960 μg/day. In the absence of an established regulatory value for MG, the ACGIH TLV for G was used as a comparison for both. Both G and MG are structurally similar, and although their metabolic pathways may differ slightly, both react with proteins and ultimately generate advanced glycation end products (AGEs). Using the same exposure assumptions as above, G (37.87 μg/g) and MG (17.74 μg/g) aerosol levels for product H were combined for a total of 55.61 μg/g, which was then compared to the ACGIH TLV for G (980 μg/day). The estimated total daily exposure of G and MG combined, assuming two cartridges per day, is approximately nine times lower than the ACGIH TLV for G.

## CONCLUSIONS

While the presence of DA has been reported in e-vapor liquids as a flavor additive, the observation of DA formation during aerosol generation from e-liquids has not been previously reported. To add to the scientific understanding of EVPs, this research provides a potential explanation for DA formation in EVP aerosols generated from e-liquids, including those that contained no added or measurable DA. A unique and selective method of analysis for α-dicarbonyl compounds was developed and validated following ICH guidelines. A market survey of
eight commercial EVPs with rechargeable batteries and disposable e-liquid cartridges was conducted for four \( \alpha \)-dicarbonyl compounds of interest. A model microwave reaction system that models the reactive environment of an electronic heating element-based system was developed and verified by comparing the yields of \( \alpha \)-dicarbonyl compounds within the aerosol generated from a commercial e-cigarette to the yields generated within the model microwave reaction system. This microwave system, in combination with the analytical method, was utilized in \( ^{13}\text{C} \) labeling studies to elucidate the reaction pathways that produced elevated levels of DA, comparable to those observed in the aerosol of e-cigarettes. These studies resulted in the proposed pathway for the formation of DA as shown in Scheme 1, which was further supported through the concerted synthetic organic reactions. These results were consistent with the proposed aldol/mixed aldol condensation reactions.

This research demonstrates that both PG and GLY, which are the major components of e-liquids, can undergo thermal degradation to form formaldehyde, a well-described thermal degradation product of EVP systems, as well as hydroxyacetone (HA). In this environment, HA is in equilibrium with its enol/enolate form to an appreciable amount to participate in an aldol condensation with formaldehyde. This aldol product is yet to be identified and should be considered as a reactive intermediate, which rapidly dehydrates to the enol form of DA.

As discussed, DA concentrations in the aerosols of the eight commercial EVPs tested were determined to be about 20–40 times lower than established occupational exposure limits. However, this research was limited to cig-a-like EVPs and the results might differ for other EVP device types. Additionally, the DA levels measured in these commercial EVPs were two orders of magnitude lower than those observed in the smoke generated from machine smoking of conventional tobacco cigarettes. Based on the data from Pierce et al., the overall mean DA in cigarette smoke was 250 \( \text{µg} \)/cigarette. Assuming an average usage of 14.1 cigarettes/day, the average daily exposure for DA is approximately 360 times lower in the tested EVPs than that from smoking conventional cigarettes.

**ASSOCIATED CONTENT**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c02018.

Accuracy study of synthetic 2-methyl,3-ethylquinoxaline; example chromatograms of synthetic and commercial materials; additional linearity figures of merit information; experimental details of accuracy studies (PDF)

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**ABBREVIATIONS USED**

ACGIH, American Conference of Governmental Industrial Hygienists; ACN, acetonitrile; AP, acetyl propanoyl (2,3-pentanedione); CFP, Cambridge filter pad; DA, diacetyl (2,3-butanedione); EVP, electronic vapor product; ENDS, electronic nicotine delivery systems; FDA, United States Food and Drug Administration; FEMA, Food and Export Manufacturers Association; G, glyoxal; GLY, glycerin; GRAS, Generally Recognized As Safe; HA, hydroxyacetone; HPHC, harmful and potentially harmful constituent; ICH, International Conference on Harmonization; LOQ, limit of quantification; MG, methylglyoxal; MP-A, mobile phase A; MP-B, mobile phase B; NIOSH, National Institute of Occupational Safety and Health; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, N-nitrosornicotine; OPD, o-phenylenediamine; PG, propylene glycol; PMTA, premarket tobacco product application; REL, recommended exposure limit; RSD, relative standard deviation; TLV, threshold limit value; UPLC-MS, ultraperformance liquid chromatography-mass spectrometry

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