

Article

Chemical Characterization of Electronic Cigarette (e-cigs) Refill Liquids Prior to EU Tobacco Product Directive Adoption: Evaluation of BTEX Contamination by HS-SPME-GC-MS and Identification of Flavoring Additives by GC-MS-O

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Abstract: The present study focused on the determination of benzene, toluene, ethylbenzene and xylenes (BTEX) concentration levels in 97 refill liquids for e-cigs selected by the Italian National Institute of Health as representative of the EU market between 2013 and 2015 prior to the implementation of the European Union (EU) Tobacco Product Directive (TPD). Most of the e-liquids investigated (85/97) were affected by BTEX contamination, with few exceptions observed (levels below the limit of quantification (LOQ) of headspace-solid phase micro extraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS) methodology). Across brands, concentration levels ranged from 2.7 to 30,200.0 µg/L for benzene, from 1.9 to 447.8 µg/L for ethylbenzene, from 1.9 to 1,648.4 µg/L for toluene and from 1.7 to 574.2 µg/L for m,p,o-xylenes. The variability observed in BTEX levels is likely to be related to the variability in contamination level of both propylene glycol and glycerol and flavoring additives included. No correlation was found with nicotine content. Moreover, on a limited number of e-liquids, gas chromatography-mass spectrometry-olfactometry (GC-MS-O) analysis was performed, allowing the identification of key flavoring additives responsible of specific flavor notes. Among them, diacetyl is a flavoring additive of concern for potential toxicity when directly inhaled into human airways. The data reported are eligible to be included in the pre-TPD database and may represent a reference for the ongoing evaluation on e-liquids safety and quality under the current EU Legislation.

Keywords: electronic cigarettes; flavoring additives; BTEX; contamination; headspace solid micro phase extraction; gas chromatography-olfactometry; human health; EU regulation

1. Introduction

Electronic cigarette (e-cig) use has increased extremely quickly worldwide over the last decade due to an intense marketing campaign aiming to advertise them as an aid to reducing and/or eliminating addiction to tobacco cigarette smoke [1]. Emerging in 2006 in China, e-cigs became widely available on the market throughout the world in 2008–2009. EU Commission public opinion surveys focused on the smoking attitudes of European citizens across 27 European Union (EU) member states highlighted that

e-cig consumption increased from 7.2% to 11.6% between 2011 and 2014 and is expected to increase further [2]. Despite the claims of manufacturers and retailers advertising e-cigs as a healthier way to smoke nicotine and other chemicals in public places, to date reliable sociological data confirming the effectiveness of e-cigs use in changing smokers' behavior (e.g., smoking cessation and/or reduction) are not exhaustive enough to draw certain conclusions [3–5]. On one hand, public opinion surveys have provided data suggesting a relationship between e-cig consumption and quitting and significant reduction of traditional tobacco smoking [6]. On the other hand, however, scientific research still raises doubts regarding the role of e-cigs in smoking cessation and highlights the interchangeable and simultaneous use of e-cigs with tobacco cigarettes [7,8]. Moreover, a controversial debate is still ongoing within the scientific community on potential adverse effects on the health of both users and bystanders. Concerns about e-cig consumption, specifically related to e-liquids composition, are: (a) the potential inhalation exposure to chemicals of concern present in e-liquid formulations as contaminants of the main ingredients (i.e., aromatic hydrocarbons, aldehydes, PAHs, heavy metals); (b) the potential exposure to harmful by-products formed during the vaporization process; and (c) the unknown and unpredictable long-term health effects due to flavoring additive and main ingredient (i.e., glycerol and propylene glycol) inhalation exposure [9–12]. In view of the health-related concerns raised by the international scientific community and EU member states' competent authorities, specific provisions concerning e-cigs manufacture, labelling, and advertising were included in the EU Tobacco Products Directive 2014/40/EU (TPD), entered into force on May 2014 and fully implemented in EU countries between 2016 and 2018 [13]. E-liquids, available on the market in bottles or in replaceable cartridges, are basically a mixture of propylene glycol, glycerol, and water (the latter generally in smaller quantities). The inclusion of propylene glycol and glycerol in e-liquid formulations is common due to humectant and solvent properties, although the use of other chemicals, such as ethanol (EtOH), has been recently reported in literature [14]. This basic formulation may be enriched with nicotine (in variable and allowed quantities) and a wide selection of flavoring additives, in order to provide users a satisfying and enhanced sensory perception while vaping.

1.1. Flavoring Additives

It is estimated that several hundred flavoring chemicals are currently used for e-liquid formulations, allowing consumers to choose on the market among several flavors belonging to menthol, tobacco, fruit (i.e., cherry, blueberry, strawberry, apple), sweets (i.e., caramel, vanilla, liquorice, chocolate) categories, to mention the most popular ones [15,16]. Scientific reports on addictive behaviors highlighted the key role of flavors in vaping initiation, especially among young adults, and the resulting addiction along to nicotine [17]. The inclusion of flavoring additives in e-liquids is one of the most debated issues. They are approved in foods, beverages, and cosmetics and included in the Generally Recognized As Safe (GRAS) list of the Flavors and Extracts Manufacturers' Association (FEMA); therefore their use is intended through ingestion and dermal contact routes, not for direct inhalation. As a result, both short- and long-term effects due to inhalation exposure cannot be predicted. Due to the lack of epidemiological data able to elucidate the issue and to be reliable foundations for human risk assessment, precautionary measures have to be taken. Moreover, besides this general precautionary principle, specific flavorings are worthy of further attention for their potential toxicity. For instance, 2,3-butanedione (usually named diacetyl) has been widely used in the past in microwave popcorn in the USA with the purpose to generate, depending on the concentration, buttery and caramel tastes. It is a chemical mentioned in the GRAS list and approved in certain limits for ingestion, therefore it is used as additive in foods [18]. Due to its flavoring properties it is also used in the manufacturing process of e-liquid formulations and its presence has been documented in previous investigations carried out in EU Member States, raising concerns in the scientific community regarding potential health implications [19,20]. In this regard, recently published scientific papers based on epidemiological data collected over recent decades have revealed that inhalation exposure to diacetyl is likely related to increased risk of a specific lung disease called bronchiolitis obliterans [21,22]. The use of flavoring

chemicals for e-liquid manufacture stimulated scientists to focus on safety and quality aspects of the formulations. As a result, the number of scientific publications on the chemical characterization of e-liquids in terms of flavoring additives has recently increasing. To cite the most recent studies, in 2017 Aszyk et al. carried out a comprehensive determination of flavoring additives on 25 e-liquid samples highlighting that limonene and benzyl acetate were the two most frequently detected [23]. In 2018 Girvalaki et al. reported findings from qualitative and quantitative analysis performed on 122 of the most commonly sold e-liquids in 9 EU member states. Among the 293 flavoring chemicals identified, menthol was the most frequently detected compound, regardless the overall e-liquid flavor [24]. Specific flavoring chemicals with known respiratory irritant properties or identified as inhalation toxicants were detected in other studies in relevant amounts, i.e., benzaldehyde by Kosmider et al., methyl cyclopentenolone and menthol by Vardavas et al., diacetyl and acetylpropionyl by Barhdadi et al. [19,25,26].

1.2. E-Liquids Contamination

The attention of scientists to the chemical composition of e-liquids has not only been aimed at the identification of flavoring chemicals, but also to address the issue concerning the potential presence of compounds of toxicological concern, such as volatile organic compounds (VOCs), due to main component contamination and low purity level of nicotine and flavors [11,27–30]. Among VOCs, aromatic hydrocarbons have attracted remarkable attention in view of a toxicity assessment of refill liquids due to the recognized carcinogenic properties of benzene, classified as carc. 1A according to EU CLP regulation [31]. Specific investigations were carried out to perform both qualitative and quantitative characterization in terms of VOCs of e-liquids commercially available on the EU market prior to the EU TPD implementation and after 2016, in order to verify the compliance of e-liquids distributed over EU countries with the TPD in force, in terms of both chemical composition and classification/labelling [24,32]. With specific regard to aromatic hydrocarbons, BTEX contamination has been detected in e-liquids available on extra-EU markets. Lim et al. highlighted the potential health hazards for e-cig users reporting the results of investigations made on 283 flavored liquids, 21 nicotine-content liquids, and 12 disposable cartridges [33]. BTEX coexisted in most of the investigated samples at relevant concentrations (e.g., benzene concentration ranging from 0.008 to 2.28 mg/L) and the contamination was hypothetically related to the use of petrogenic hydrocarbons in the extraction process of nicotine and flavors from natural plants. BTEX contamination of liquid formulations was also previously observed by Han et al. in a study aiming to assess VOCs levels in 55 refill liquids of 17 different brands available on the Chinese market [34]. Benzene and m,p-xylenes were found in all of the samples investigated, whilst ethylbenzene and toluene were detected with different frequencies. They all were present at comparable levels in the concentration range 1.10–17.31 µg/g. In view of the findings obtained to date on e-liquids composition in terms of a broad range of chemicals, reported above, it appears clear that the attention on the issue has to remain high to ensure that consumers' health is safeguarded and that compliance to safety and quality standards is guaranteed. On one hand there is the need for a comprehensive database referred to e-liquids both manufactured and imported in EU member states before the implementation of the TPD allowing us to define a pre-TPD baseline reference useful for comparison. On the other hand, ongoing investigations into e-liquids currently on the market are necessary to evaluate the effectiveness of TPD provisions in EU member states with regard to the manufacture and labelling of e-liquids, and to formulate further recommendations to policymakers.

1.3. Aim of the Present Study

The aim of the present study was to evaluate BTEX contamination across a representative group of refill liquids for e-cigs (n = 97) and to identify, in a selected sub-group (n = 5), the main flavoring additives responsible for the flavor/taste perceived. BTEX quantification was carried out applying headspace-solid phase micro extraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS) methodology.

The identification of flavoring additives was performed applying a hybrid analytical-sensory technique, the gas chromatography-mass spectrometry-olfactometry (GC-MS-O). This research activity has been carried out in the context of a more comprehensive national project supported by the Italian Ministry of Health and coordinated by the National Institute of Health aimed to evaluate in a comprehensive manner potential risks related to e-cig consumption. The refill liquids investigated were selected through a preliminary survey and were considered representative of the EU market between 2013 and 2015, prior to the implementation of TPD in most of EU member states. Therefore, the data here reported are eligible to be included in the pre-TPD database on e-liquids manufactured and/or imported in EU and may represent a useful reference for the ongoing evaluation on e-liquid safety and quality under the current EU Legislation.

2. Materials and Methods

2.1. E-Liquids Selection

In the framework of the national research project, the Italian National Institute of Health carried out a preliminary survey allowing to identify the most popular brands of e-liquids manufactured and imported in EU and representative of the EU market between 2013 and 2015. Ninety-seven e-liquids of 12 different brands, with and without Nicotine and characterized by different flavors, were purchased online from EU manufacturers and importers in 10–30 ml plastic bottles, as sold commercially. More specifically, the selected e-liquids were manufactured in Italy (n = 45), China (n = 28), France (n = 8), UK (n = 8), Germany (n = 4), and the USA (n = 4). E-liquid composition in terms of propylene glycol, glycerol, water content (expressed in %), nicotine content (expressed as mg/ml or mg/g) as well as characteristic flavor is reported in Table 1, as declared on product label. E-liquids belonging to different brands and within the same brand were classified with progressive letters and number, respectively (sample ID in Table 1). Moreover, three identical e-liquids in terms of brand, basic composition, flavor and nicotine content (e.g., samples 10, 11 and 12 C) belong to different production batches. Nicotine-containing e-liquids were 59 with variable content (11,14,16 and 18 mg/ml and 11, 18 mg/g), as reported on the product label. The remaining 38 e-liquids were declared nicotine-free. Most of the investigated e-liquids were flavored and may be included in the following typical flavor categories: tobacco (48), mint (17), sweets/candy (11), spicy (7), fruits (3), coffee (3), and alcohol (3). Before analysis, all e-liquids were properly stored at room temperature and kept away from direct sunlight, as recommended on the product label.

2.2. BTEX Determination by HS-SPME-GC-MS Analysis

2.2.1. Standards and Reagents

The reference standard benzene (99.96%), toluene (99.93%), ethylbenzene ($\geq 99.90\%$), p-xylene (99.90%), and benzene-d₆ (99.99%), the latter used as internal standard (IS), were purchased from Sigma Aldrich. The reagents methanol and propylene glycol used for the preparation of standard/calibration solutions, as well as blank and samples solutions of a purity grade of more than 99%, were purchased from Sigma Aldrich.

2.2.2. Standards and Calibration Solutions

For each compound, internal standard included, two standard stock solutions were preliminarily prepared. The first set of standard solutions (S1) was prepared diluting reference standards in methanol at a concentration of about 9×10^7 µg/L. The second set of standard solutions (S2) was prepared diluting S1 solutions in methanol (1:100 dilution) to obtain a concentration of about 9×10^5 µg/L.

Table 1. E-liquids composition and information: manufacturing country, % of the main components, characteristic flavor and nicotine content (expressed as mg/mL or mg/g).

Sample ID	Manufacturing Country	Propylen Glycol (%)	Glycerol (%)	Water (%)	Other (%) *	Flavor	Nicotine (mg/mL, ** mg/g)
1-A	China	-	-	-	-	coca cola	18
2-A	China	-	-	-	-	kiwi	18
3-A	China	-	-	-	-	Davidoff-tobacco	11
4-A	China	-	-	-	-	Green USA mix-tobacco	11
5-A	China	-	-	-	-	cigar	11
1-B	Italy	-	-	-	-	cuban cigar	18
2-B	Italy	-	-	-	-	natural	18
3-B	Italy	-	-	-	-	mint	0
4-B	Italy	-	-	-	-	tobacco USA	18
5-B	Italy	-	-	-	-	Virginia blend tobacco	0
6-B	Italy	-	-	-	-	natural	0
7-B	Italy	-	-	-	-	coffee	0
8-B	Italy	-	-	-	-	anise	0
9-B	Italy	-	-	-	-	cuban cigar	0
10-B	Italy	-	-	-	-	rum	0
11-B	Italy	-	-	-	-	biscuit	0
12-B	Italy	-	-	-	-	anise	18
13-B	Italy	-	-	-	-	liquirice	18
14-B	Italy	-	-	-	-	biscuit	18
15-B	Italy	-	-	-	-	tobacco USA	0
16-B	Italy	-	-	-	-	mint	18
17-B	Italy	-	-	-	-	Virginia blend tobacco	18
1-C	Italy	50	40	5-10	-	Virginia blend tobacco	0
2-C	Italy	50	40	5-10	-	Virginia blend tobacco	18
3-C	Italy	50	40	5-10	-	basic flavor	0
4-C	Italy	50	40	5-10	-	basic flavor	18
5-C	Italy	50	40	5-10	-	anise	0
6-C	Italy	50	40	5-10	-	anise	18
7-C	Italy	50	40	5-10	-	mint	0
8-C	Italy	50	40	5-10	-	mint	18
9-C	Italy	50	40	5-10	-	biscuit	0
10-C	Italy	50	40	5-10	-	biscuit	18
11-C	Italy	50	40	5-10	-	biscuit	18
12-C	Italy	50	40	5-10	-	biscuit	18
13-C	Italy	50	40	5-10	-	cuban cigar	0
14-C	Italy	50	40	5-10	-	cuban cigar	18
15-C	Italy	50	40	5-10	-	tobacco USA	0
16-C	Italy	50	40	5-10	-	tobacco USA	18
17-C	Italy	50	40	5-10	-	rum	0
18-C	Italy	50	40	5-10	-	cognac	0
19-C	Italy	50	40	5-10	-	coffee	0
20-C	Italy	50	40	5-10	-	liquirice	18
1-D	Italy	-	-	-	-	mint	0
2-D	Italy	-	-	-	-	mint	14
3-D	Italy	-	-	-	-	black tobacco	0
4-D	Italy	-	-	-	-	black tobacco	14
5-D	Italy	-	-	-	-	Virginia blend tobacco	0

Table 1. Cont.

Sample ID	Manufacturing Country	Propylen Glycol (%)	Glycerol (%)	Water (%)	Other (%) *	Flavor	Nicotine (mg/mL, ** mg/g)
6-D	Italy	-	-	-	-	Virginia blend tobacco	14
7-D	Italy	-	-	-	-	tobacco	0
8-D	Italy	-	-	-	-	tobacco	14
1-E	China	80	20	-	-	cuban cigar	0
2-E	China	80	20	-	-	cuban cigar	16
3-E	China	80	20	-	-	Davidoff-tobacco	0
4-E	China	80	20	-	-	Davidoff-tobacco	16
5-E	China	80	20	-	-	Virginia blend tobacco	0
6-E	China	80	20	-	-	Virginia blend tobacco	16
1-F	China	>45	<12	<1	<42	almond	11 **
2-F	China	>45	<12	<1	<42	bubble gum	11 **
3-F	China	>45	<12	<1	<42	cigar	11 **
4-F	China	>45	<12	<1	<42	cigar	18 **
5-F	China	>45	<12	<1	<42	cinnamon	11 **
6-F	China	>45	<12	<1	<42	coffee	11 **
7-F	China	>45	<12	<1	<42	Davidoff-tobacco	11 **
8-F	China	>45	<12	<1	<42	Davidoff-tobacco	18 **
9-F	China	>45	<12	<1	<42	lemon	11 **
10-F	China	>45	<12	<1	<42	Marlboro cigarettes	11 **
11-F	China	>45	<12	<1	<42	Marlboro cigarettes	18 **
12-F	China	>45	<12	<1	<42	mint	11 **
13-F	China	>45	<12	<1	<42	tobacco	11 **
14-F	China	>45	<12	<1	<42	tobacco	18 **
15-F	China	>45	<12	<1	<42	fruits	11 **
16-F	China	>45	<12	<1	<42	Virginia blend tobacco	11 **
17-F	China	>45	<12	<1	<42	Virginia blend tobacco	18 **
1-G	France	>80	<20	-	-	American blend-tobacco	0
2-G	France	>80	<20	-	-	American blend-tobacco	18
3-G	France	>80	<20	-	-	Virginia blend tobacco	0
4-G	France	>80	<20	-	-	Virginia blend tobacco	18
5-G	France	>80	<20	-	-	Habanos cigar-tobacco	0
6-G	France	>80	<20	-	-	Habanos cigar-tobacco	18
7-G	France	>80	<20	-	-	mint	0
8-G	France	>80	<20	-	-	mint	18
1-H	United Kingdom	50	50	-	-	tobacco	0
2-H	United Kingdom	50	50	-	-	tobacco	18
1-I	United Kingdom	>80	<20	-	-	Virginin Leaf - tobacco	0
2-I	United Kingdom	>80	<20	-	-	Virginin Leaf - tobacco	18
3-I	United Kingdom	>80	<20	-	-	mint	0
4-I	United Kingdom	>80	<20	-	-	mint	18
5-I	United Kingdom	>80	<20	-	-	cuban cigar	0
6-I	United Kingdom	>80	<20	-	-	cuban cigar	18
1-L	Germany	50	50	-	-	chocolate/vanille	0
2-L	Germany	50	50	-	-	chocolate/vanille	18
3-L	Germany	50	50	-	-	mint/herbs	0
4-L	Germany	50	50	-	-	mint/herbs	18
1-M	USA (California)	50	50	-	-	mint/vanilla/chocolate	0
2-M	USA (California)	50	50	-	-	mint/vanilla/chocolate	18
1-N	USA	20	80	-	-	thin mint	0
2-N	USA	20	80	-	-	thin mint	18

* Other components declared on the product label: tobacco essential and leaf oil, nicotine from tobacco leaf, plant extracts, trace level compounds; ** nicotine concentration expressed as mg/g; (-) means that information was not provided on product label.

Starting from S2 and with subsequent dilution with methanol, five solutions for each compound were prepared in the concentration range, approximately 20.0–450.0 µg/L (S3–S7). In order to simulate e-liquid basic composition, five matrix-matched calibration solutions for each compound were prepared by adding 100 µl of the corresponding S3–S7 solutions and 100 µl of benzene-d₆ solution (S2 set) in a headspace (HS)-vial containing 1 ml of laboratory-made liquid (90% propylene glycol, 10% water). Similarly, a blank solution was also prepared by adding 100 µl of benzene-d₆ solution (S2 set) and 100 µl of methanol in a HS-vial containing 1 ml of laboratory-made liquid (90% propylene glycol, 10% water). Both blank and matrix-matched calibration solutions were used for calibration, resulting in five concentration levels for each compound in the dynamic range between limit of quantification (LOQ) value and 45.0 µg/L.

2.2.3. Sample Preparation

Sample preparation prior analysis required the dilution of an aliquot of refill liquid (1 ml) with 100 µl of methanol and 100 µl of IS solution. The dilution with a proper solvent is fundamental to avoid inhomogeneous samples due to the difficulty in sampling exact volumes of high viscosity fluids [35].

2.2.4. HS-SPME-GC-MS Method Conditions and Performance Characteristics

The collection of BTEX in the volatile fraction of both calibration and sample solutions was carried out in 20-ml HS vials with magnetic screw caps provided with polytetrafluoroethylene (PTFE)/silicone septa (Agilent Technologies). BTEX were collected through adsorption onto the polydimethylsiloxane (PDMS) stationary phase-coated fused silica fiber (thickness 100 µm, length 1 cm) introduced into the sample vial. The PDMS fiber was left in the vial for 30 s at 50 °C. Mechanical stirring was performed for 5 s with a stirring speed of 500 rpm. Analyses were performed using a gas chromatograph (7890B Agilent Technologies, Santa Clara CA, USA) equipped with an automated sampler (Pal System, CTC Analytics AG, Zwingen, Switzerland), a split/splitless injector and a single-quad mass spectrometer (5977A Agilent Technologies, Santa Clara CA, USA). Once incubation was completed, the heated gas-tight syringe containing the fiber was automatically transferred into the GC injector via the automated sampler and BTEX were thermally desorbed at 250 °C for 300 s and injected into the GC column in split injection mode (split ratio 1:10). Separation was performed on capillary column semivolatiles, 30 m × 0.25 mm, i.d. 0.25 µm film thickness (Phenomenex). Helium (purity ≥ 99.999%) was applied as carrier gas at a constant flow rate of 1 ml/min. The GC oven temperature program used for optimal separation was: 40 °C for 2 min, ramped 8 °C/min up to 80 °C, then ramped 60 °C/min up to 250 °C. Transfer line and ion source temperatures were kept at 260 °C and 270 °C, respectively. The mass spectrometer was operated in electron impact (EI) ionization mode (70 eV). Identification of BTEX was based on comparison of the obtained mass spectra with those included in the National Institute of Standards and Technology (NIST) library (MassHunter software) and considered positive by library search match >800 for both forward and reverse matching. Further criteria for compounds identification were: (a) the matching of relative retention times (t_R) with those of the authentic standards within the allowed deviation of ± 0.05 min; and (b) the matching of ion ratios collected with those of the authentic standards within a tolerance of ± 20%. Quantification was performed in a selected ion monitoring mode (SIM). One quantifier ion and two qualifier ions were selected for each compound on the basis of their selectivity and abundance: 79 m/z as quantifier ion and 51 and 39 m/z as qualifier ions for benzene; 91 m/z as quantifier ion and 65 and 39 m/z as qualifier ions for toluene; and 91 m/z as quantifier ion and 106 and 51 m/z as qualifier ions for ethylbenzene and xylenes. Five point matrix-matched calibration curves were constructed for quantification ($r^2 > 0.995$) reporting compound/benzene-d₆ quantifier ion peak areas ratio vs amount ratio. Calibration curves were in the range 2.6–41.6 µg/L for benzene, 2.7–43.2 µg/L for toluene and xylenes isomers and 2.8–44.8 µg/L for ethylbenzene. The xylenes isomers were quantified on the basis of p-xylene response factor (e.g., p-xylene calibration curve) and reported as sum in Table 2. Chromatograms of a blank sample and a sample spiked with the BTEX standard solution (calibration level 3) were

compared in Figure S1 (Supplementary Material, Figure S1). The main performance characteristics of the HS-SPME-GC-MS method were also evaluated. Linearity was calculated on the basis of three sets of replicates for each calibration level on three different days. As for the results, all matrix-matched calibration curves were linear over the set concentration ranges: relative accuracy (%) for each point was within the $\pm 5\%$ of the expected concentrations, and all coefficients of determination (r^2) were >0.995 . Selectivity/specificity was assessed directly onto the chromatograms obtained from the blank and from spiked matrices. The occurrence of possible extra peaks was tested by monitoring in SIM mode qualifier and quantifier ions characteristic for each investigated compound onto the blank matrix chromatograms, within the retention time window expected for the analyte elution. Limit of detection (LOD) and LOQ values were assessed in the spiked matrix by determining the lowest concentration of the analytes that resulted in a signal-to-noise (S/N) ratio of ≥ 3 and ≥ 10 , respectively. LOD values were 1.4 $\mu\text{g/L}$ for benzene and toluene, 1.5 $\mu\text{g/L}$ for xylenes, and 1.6 $\mu\text{g/L}$ for ethylbenzene. LOQ values were 2.6 $\mu\text{g/L}$ for benzene, 2.7 $\mu\text{g/L}$ for toluene and xylenes and 2.8 $\mu\text{g/L}$ for ethylbenzene. Repeatability expressed as intra-day coefficients of variation (CV%) was evaluated on a set of results ($n = 6$ replicates) obtained for each analyte at three validation levels (i.e., LOQ values; 10.4 $\mu\text{g/L}$ for benzene, 10.8 $\mu\text{g/L}$ for toluene and xylenes, 11.2 $\mu\text{g/L}$ for ethylbenzene; 41.6 $\mu\text{g/L}$ for benzene, 43.2 $\mu\text{g/L}$ for toluene and xylenes and 44.8 $\mu\text{g/L}$ for ethylbenzene). Intra-day CV% values were 1.2–4.5% for benzene, 1.2–9.9% for toluene, 3.2–10.9% for ethylbenzene and 2.8–11.4% for xylenes. Intermediate precision (expressed as inter-day CV%) and recovery were calculated by analyzing the series within the three different days ($n = 18$ replicates). Inter-day CV% values were 5.1–15.3% for benzene, 6.6–10.0% for toluene, 8.8–14.6% for ethylbenzene and 9.4–15.4% for xylenes. Finally, recoveries were in the range of 96.6–113.0%.

2.3. Identification of Flavoring Additives by GC-MS-O Analysis

GC-MS-O methodology was revealed to be a powerful approach for accurate identification of volatile odor-active compounds in high-level complexity matrices through coupling traditional chromatographic analysis with human sensory perception [36–38]. For this reason, GC-MS-O methodology was applied in the present study, allowing us to accurately identify, on a limited number of e-liquids, the odor-active compounds responsible for the overall flavor perceived or of specific flavor notes.

2.3.1. Sample Selection and Preparation

The e-liquids subjected to the in-depth investigation were e-liquids with ID A 1-5 manufactured in China, with medium-high nicotine content and characterized by flavors covering different categories, from tobacco to fruits (Table 1). The aforementioned e-liquids were chosen for further study on the basis of collected data from BTEX investigation that highlighted high level of contamination. Moreover, during the preliminary survey and e-liquid selection made by the National Institute of Health, the brand A was already considered worthy of particular attention due to previous precautionary seizing actions made by Italian authorities and financial police. The preparation of the gaseous sample for GC-MS-O analysis starting from e-liquid formulation involved the use of the Adsorbent Tube Injector System device (ATISTM, Supelco). Before gaseous sample preparation, 250 μL of each e-liquid was preliminarily diluted, adding 250 μL of methanol, resulting in a solution with final volume of 500 μL . An aliquot (100 μL) of the obtained solution was injected by a syringe through the septum of the ATIS injection glassware and the volatile fraction was conveyed by ultrapure air flow (50 mL/min) into a collecting bag (Nalophan[®]), connected at the outlet of the injection glassware, resulting in a gaseous sample with a final volume of 2 L. The temperature, controlled by a thermometer inserted into the heating block, was set at 120°C. As a result, only the volatile fraction was collected into the bag, avoiding the vaporization of the high-boiling point fraction composed by propylene glycol and glycerol that would have resulted in two broad chromatographic peaks in the GC chromatogram.

2.3.2. GC-MS-O Analysis Conditions

The VOCs collected were analyzed using an air sampler-thermal desorber integrated system (UNITY 2™Markes International Ltd, Llantrisant, UK) connected to a gas chromatograph (7890 Agilent Technologies, Santa Clara CA, USA) equipped with an Olfactory Detection Port (ODP 3 Gerstel GmbH&Co, Mülheim an der Ruhr, Germany) and a single-quad mass spectrometer (5975 Agilent Technologies, Santa Clara CA, USA). The collection of VOCs onto the sorbent-pack focusing trap at -10°C of the desorption system UNITY2™ was performed by connecting the Nalophan bag to the inlet port of the automated air sampling device. The cold trap was flash heated to 300°C and the compounds were transferred via the heated transfer line (200°C) to the GC column and to the ODP port. The chromatographic separation was performed on a HP5-MS capillary column ($30\text{m} \times 250\mu\text{m} \times 0.25\mu\text{m}$). Carrier gas (Helium) flow was controlled by constant pressure and equal to 1.7 ml/min . The GC oven temperature program was set as follows: from 37°C up to 100°C at 3.5°C/min (ramp 1); and from 100°C up to 250°C at 15°C/min (ramp 2). After the GC separation, the column flow was split into two parts (ratio 1:1), one part was connected to the MS detector and the other one to ODP. The transfer line connecting the GC column and MS detector was kept at 250°C . The mass spectrometer was operated in electron impact (EI) ionization mode (70eV) in the mass range $20\text{--}250\text{ m/z}$. The effluent from the capillary column was connected to the ODP port through an uncoated transfer line (deactivated silica capillaries), constantly heated to prevent compounds condensation. Two trained panelists, one male and one female (24 years old), were asked to sniff in the conical ODP simultaneously with the GC run, indicating exactly when they start and stop perceiving the odor and providing a qualitative description of the odor (using suitable descriptors) [36] and odor intensity based on an intensity scale from 0 (no odor perceived) to 4 (strong odor). Auxiliary air (make-up gas) was added to the GC effluent to prevent the assessors' nose mucous membranes drying, which may potentially cause discomfort, especially in extended analysis sessions. The panelists involved in the present study had previously been selected according to a standardized procedure used for the panel selection in Dynamic Olfactometry, the official methodology for odor emissions assessment standardized by a European technical law (EN 13725/2003) [39]. The standardized procedure provides for individuals with average olfactory perception sensitivity that constitute a representative sample of the human population. The screening was performed evaluating the response to the most used reference gas, 1-butanol. Only assessors who fulfilled predetermined repeatability and accuracy criteria were selected as panelists. The identification of flavoring additives and other VOCs in e-liquid formulation was performed by comparing the mass spectra obtained with those listed in the NIST library (Agilent Technologies). It was considered valid when the confidence rating of mass spectra comparison was superior or equal to 95%. The attribution was further confirmed using the retention times of authentic compounds. Before GC-MS-O sessions, panelists were asked to carry out preliminary sensory tests by sniffing and vaping the liquid formulations. This preliminary approach revealed to be useful in appreciating discrepancies between the flavors reported on e-liquid labels and the overall flavor perceived by panelists' noses and mouths (see Section 3.2 in results section).

3. Results

3.1. Quantitative Analysis: BTEX Contamination of the Investigated E-Liquids

Single and total BTEX concentrations, expressed in $\mu\text{g/L}$, are reported in Table 2. As shown, most of the e-liquids investigated in the present study (85/97) were revealed to be affected, to a lesser or greater extent, by BTEX contamination. Only a few exceptions were observed with BTEX levels below the LOQ of the analytical methodology applied. Across all of the brands investigated (ID A-N, Table 1), concentration levels ranged from $2.7\text{ }\mu\text{g/L}$ to $30,200.0\text{ }\mu\text{g/L}$ for benzene, from $1.9\text{ }\mu\text{g/L}$ to $447.8\text{ }\mu\text{g/L}$ for ethylbenzene, from $1.9\text{ }\mu\text{g/L}$ to $1,648.4\text{ }\mu\text{g/L}$ for toluene and, finally, from $1.7\text{ }\mu\text{g/L}$ to $574.2\text{ }\mu\text{g/L}$ for m,p,o-xylenes. HS-SPME-GC-MS analysis of e-liquids with ID A (1-5), manufactured in China, highlighted a relevant contamination by BTEX with concentration levels up to four order of magnitude

higher than those determined in all the other investigated e-liquids, regardless of the manufacturing country and the chemical composition. More specifically, within brand A, benzene concentration levels ranged from 7,200.0 µg/L (sample 4-A) to 30,200.0 µg/L (sample 3-A), toluene concentration levels ranged from 764.4 µg/L (sample 1-A) to 1,648.4 µg/L (sample 4-A), ethylbenzene concentration levels ranged from 187.9 µg/L (sample 1-A) to 447.8 µg/L (sample 4-A) and, finally, m,p,o-xylenes concentration levels ranged from 201.8 µg/L (1-A) to 574.2 µg/L (sample 5-A). Moreover, making a comparison among samples ID A in terms of BTEX total concentration, it is possible to observe that 3-A shows the highest BTEX total concentration, equal to 32,151.1 µg/L. The comparison between samples ID A with all the other samples under investigation (ID B-N) revealed that benzene concentrations in 1-5 A samples were between one and four orders of magnitude higher than those determined in all the other e-liquids. Moreover, toluene concentrations in 1-5 A samples were up to three order of magnitude higher than those determined in all the other e-liquids, whilst ethylbenzene and m,p,o-xylenes were up to two order of magnitude higher. Benzene concentrations in 1–5 A samples were higher than toluene concentrations (from 4 to 22 times higher), a finding that was not observed for all the other samples characterized by toluene concentrations higher than benzene concentrations, with very few exceptions. To mention some examples, e-liquids with ID E and F manufactured in China showed toluene concentrations ranging from 20.7 µg/L to 96.2 µg/L and from 6.8 µg/L to 385.9 µg/L, respectively, in both cases one up to two order of magnitude higher than benzene concentrations. As already mentioned, some of the samples investigated were not affected by BTEX contamination. It is possible to observe that in most of the samples C (i.e., 1,2,3,8,10,12,14,15 and 17) and in samples 5D, G5, G6 the presence of BTEX was not detected at all with all concentration levels below the LOQ of the analytical methodology applied. Therefore the samples with ID C manufactured in Italy were revealed to be the highest quality e-liquids among all the tested samples. On the contrary, across samples with ID B-N, the highest BTEX total concentrations were associated with samples belonging to the batch with ID F (manufacture country China) with samples 12-F and 17-F showing the highest values, equal to 739.2 µg/L and 743.8 µg/L respectively. Therefore, it is possible to state that the highest BTEX contamination was observed in e-liquids belonging to two different brands (A and F), both of Chinese origin. Another important observation is that the highest BTEX total concentrations observed for most of the brands were associated with e-liquids characterized by mint flavor (brands B, F and L) and tobacco flavor (brands D, E, F and I).

Table 2. Benzene, toluene, ethylbenzene and xylenes (BTEX) concentration (expressed in µg/L) in the investigated e-liquids.

E-Liquid ID	Flavor	Benzene (µg/L)	Ethylbenzene (µg/L)	Toluene (µg/L)	m,p,o-Xylenes (µg/L)	BTEX Total (µg/L)
1-A	coca cola	11,000.0	187.9	764.4	201.8	12,154.1
2-A	kiwi	16,700.0	305.1	902.5	388.6	18,296.2
3-A	Davidoff-tobacco	30,200.0	295.8	1,331.7	323.6	32,151.1
4-A	Green USA mix-tobacco	7200.0	447.8	1,648.4	559.1	9,855.3
5-A	cigar	12,900.0	442.0	1,566.0	574.2	15,482.2
1-B	cuban cigar	<LOQ	<LOQ	5.9	<LOQ	5.9
2-B	natural	<LOQ	2.8	4.4	3.6	10.8
3-B	mint	2.7	39.0	42.8	77.3	161.8
4-B	tobacco USA	<LOQ	<LOQ	3.3	<LOQ	3.3
5-B	Virginia blend tobacco	<LOQ	<LOQ	3.6	<LOQ	3.6
6-B	natural	<LOQ	<LOQ	4.4	<LOQ	4.4
7-B	coffee	<LOQ	<LOQ	3.2	<LOQ	3.2
8-B	anise	<LOQ	<LOQ	3.7	<LOQ	3.7
9-B	cuban cigar	<LOQ	<LOQ	4.6	<LOQ	4.6
10-B	rhum	<LOQ	<LOQ	6.3	<LOQ	6.3
11-B	biscuit	<LOQ	<LOQ	4.0	<LOQ	4.0
12-B	anise	<LOQ	3.1	4.9	<LOQ	8.0
13-B	liquirice	<LOQ	<LOQ	4.3	4.6	8.9
14-B	biscuit	<LOQ	<LOQ	4.1	<LOQ	4.1
15-B	tobacco USA	<LOQ	<LOQ	3.3	<LOQ	3.3
16-B	mint	3.4	37.3	38.8	80.7	160.2
17-B	Virginia blend tobacco	<LOQ	2.8	3.3	<LOQ	6.1
1-C	Virginia blend tobacco	<LOQ	<LOQ	<LOQ	<LOQ	/
2-C	Virginia blend tobacco	<LOQ	<LOQ	<LOQ	<LOQ	/
3-C	basic flavor	<LOQ	<LOQ	<LOQ	<LOQ	/

Table 2. Cont.

E-Liquid ID	Flavor	Benzene (µg/L)	Ethylbenzene (µg/L)	Toluene (µg/L)	m,p,o-Xylenes (µg/L)	BTEX Total (µg/L)
4-C	basic flavor	<LOQ	4.0	7.3	18.8	30.1
5-C	anise	5.2	<LOQ	2.7	4.0	11.9
6-C	anise	<LOQ	<LOQ	8.3	<LOQ	8.3
7-C	mint	<LOQ	<LOQ	3.0	<LOQ	3.0
8-C	mint	<LOQ	<LOQ	<LOQ	<LOQ	/
9-C	biscuit	4.5	<LOQ	<LOQ	<LOQ	4.5
10-C	biscuit	<LOQ	<LOQ	<LOQ	<LOQ	/
11-C	biscuit	<LOQ	<LOQ	3.1	4.3	7.4
12-C	biscuit	<LOQ	<LOQ	<LOQ	<LOQ	/
13-C	cuban cigar	<LOQ	<LOQ	10.0	5.4	15.4
14-C	cuban cigar	<LOQ	<LOQ	<LOQ	<LOQ	/
15-C	tobacco USA	<LOQ	<LOQ	<LOQ	<LOQ	/
16-C	tobacco USA	<LOQ	<LOQ	7.9	<LOQ	7.9
17-C	rum	<LOQ	<LOQ	<LOQ	<LOQ	/
18-C	cognac	4.2	<LOQ	<LOQ	<LOQ	4.2
19-C	coffee	<LOQ	<LOQ	29.4	<LOQ	29.4
20-C	liquirice	<LOQ	<LOQ	5.0	<LOQ	5.0
1-D	mint	<LOQ	<LOQ	22.5	<LOQ	22.5
2-D	mint	<LOQ	<LOQ	29.1	<LOQ	29.1
3-D	black tobacco	<LOQ	<LOQ	11.4	<LOQ	11.4
4-D	black tobacco	<LOQ	<LOQ	10.9	<LOQ	10.9
5-D	Virginia blend tobacco	<LOQ	<LOQ	<LOQ	<LOQ	/
6-D	Virginia blend tobacco	2.7	<LOQ	5.2	26.4	34.3
7-D	tobacco	<LOQ	8.0	4.1	22.1	34.2
8-D	tobacco	<LOQ	<LOQ	7.9	19.9	27.8
1-E	cuban cigar	<LOQ	<LOQ	75.2	4.9	80.1
2-E	cuban cigar	<LOQ	7.3	96.2	9.8	113.3
3-E	Davidoff-tobacco	<LOQ	<LOQ	36.5	6.2	42.7
4-E	Davidoff-tobacco	<LOQ	4.9	73.3	9.2	87.4
5-E	Virginia blend tobacco	<LOQ	<LOQ	20.7	8.6	29.3
6-E	Virginia blend tobacco	6.7	6.9	25.9	15.1	54.6
1-F	almond	260.6	3.0	154.0	13.9	431.5
2-F	bubble gum	12.0	37.4	121.3	148.2	318.9
3-F	cigar	17.3	64.0	81.5	50.8	213.6
4-F	cigar	120.6	80.8	334.9	110.1	646.4
5-F	cinnamon	23.2	6.4	102.0	4.4	136.0
6-F	coffee	<LOQ	<LOQ	6.8	<LOQ	6.8
7-F	Davidoff-tobacco	113.6	19.5	212.0	85.4	430.5
8-F	Davidoff-tobacco	11.6	3.0	38.6	17.6	70.8
9-F	lemon	<LOQ	<LOQ	20.5	5.5	26.0
10-F	Marlboro cigarettes	13.8	<LOQ	35.4	11.4	60.6
11-F	Marlboro cigarettes	18.9	5.1	44.0	15.9	83.9
12-F	mint	7.8	111.9	326.5	293.0	739.2
13-F	tobacco	67.2	12.5	151.4	46.0	277.1
14-F	tobacco	9.1	2.8	31.8	12.6	56.3
15-F	fruits	<LOQ	<LOQ	14.6	5.9	20.5
16-F	Virginia blend tobacco	12.2	7.1	31.1	14.2	64.6
17-F	Virginia blend tobacco	176.6	40.8	385.9	140.5	743.8
1-G	American blend-tobacco	<LOQ	<LOQ	5.1	<LOQ	5.1
2-G	American blend-tobacco	<LOQ	<LOQ	4.3	<LOQ	4.3
3-G	Virginia blend tobacco	<LOQ	<LOQ	4.5	17.5	22.0
4-G	Virginia blend tobacco	<LOQ	<LOQ	11.9	3.9	15.8
5-G	Habanos cigar-tobacco	<LOQ	<LOQ	<LOQ	<LOQ	/
6-G	Habanos cigar-tobacco	<LOQ	<LOQ	<LOQ	<LOQ	/
7-G	mint	<LOQ	4.0	8.5	24.5	37.0
8-G	mint	<LOQ	3.0	9.4	18.4	30.8
1-H	tobacco	<LOQ	6.4	<LOQ	7.8	14.2
2-H	tobacco	<LOQ	<LOQ	<LOQ	2.8	2.8
1-I	Virginin Leaf - tobacco	<LOQ	<LOQ	3.5	4.3	7.8
2-I	Virginin Leaf - tobacco	<LOQ	<LOQ	4.6	<LOQ	4.6
3-I	mint	<LOQ	<LOQ	5.0	<LOQ	5.0
4-I	mint	<LOQ	<LOQ	3.2	<LOQ	3.2
5-I	cuban cigar	<LOQ	<LOQ	4.0	<LOQ	4.0
6-I	cuban cigar	<LOQ	<LOQ	2.7	<LOQ	2.7
1-L	chocolate/vanille	<LOQ	<LOQ	4.0	<LOQ	4.0
2-L	chocolate/vanille	<LOQ	<LOQ	4.0	2.8	6.8
3-L	mint/herbs	<LOQ	<LOQ	2.8	7.8	10.6
4-L	mint/herbs	<LOQ	<LOQ	4.8	4.5	9.3
1-M	mint/vanilla/chocolate	<LOQ	<LOQ	13.4	5.3	18.7
2-M	mint/vanilla/chocolate	<LOQ	4.1	27.3	15.4	46.8
1-N	thin mint	<LOQ	<LOQ	29.0	10.1	39.1
2-N	thin mint	<LOQ	<LOQ	10.0	<LOQ	10.0

3.2. GC-MS-O Qualitative Analysis: Identification of Flavoring Additives

The sensory evaluation report by GC-MS-O analysis of e-liquids ID A 1–5 is shown in Table 3. Molecular formula, CAS number and retention time (TR), expressed in minutes, of identified odor-active compounds, as well as the intensity of the odor perceived and the associated qualitative description provided by both trained panelists, are reported. GC/MS-O analysis of the sample 1-A with labelled flavor Coca cola allowed to distinctly identify 4 odor-active compounds: ethoxyethane, 2-ethoxybutane, camphene, and γ -terpinene. In more detail, the integration of chromatographic data with sensory perception revealed that the first odorous stimulus perceived by both assessors with intensity 3 (clear odor) and qualitatively described with the descriptor ‘sweet’ was associated with ethoxyethane eluted at 2.8 min. The odor-active compounds 2-ethoxybutane and γ -terpinene, eluted at 4.8 and 18.1 min respectively, were associated with the characteristic flavor of coca cola beverage and related to the overall flavor perceived during the preliminary odor test with the refill liquid. More specifically, 2-ethoxybutane was perceived by both panelists with intensity 3 and described as coca cola-like flavor while γ -terpinene was perceived by both the panelists with intensity 2, described with the descriptor ‘bitter’ and referred to the bitter aftertaste of coca cola. Another odor-active compound detected at the olfactory port and chromatographically identified was camphene, perceived by both assessors with intensity 2 and associated with citrus and fresh notes. The odor-active compounds 2-ethoxybutane, camphene and γ -terpinene are all classified by FEMA as flavoring agents with a specific flavor profile. 2-ethoxybutane is associated with the flavor profile ‘floral’ while camphene and γ -terpinene to the flavor profile ‘camphor/oil’ and ‘bitter/citrus’ respectively. Other sources e.g., The Good Scents Company (TGSC) Information System reports a more detailed flavor profile of camphene including minty, fresh, woody and citrus notes depending on the concentration confirming, in part, the assessors’ olfactory perception. Finally, as shown in Table 3, two odorous stimuli although distinctly perceived at the olfactory port approximately at 8.9 and 21.3 min were not identified due to chromatographic peaks not sufficiently intense to allow accurate identification. The lack of clear correspondence between sensory perception and chromatographic data highlights that, despite the potentialities of GC-MS-O technique, in certain cases the sensory perception of human nose is more sensitive than the analytical detection as reported by Plutowska et al., 2008 [40]. The GC-MS-O analysis of e-liquid 2-A with the characteristic kiwi flavor resulted in the identification of seven odor-active compounds. Most of the odorous stimuli were qualitatively described by assessors with the odor descriptors ‘sweet’ and ‘fruity’. The odor-active compounds identified, in order of chromatographic elution, were: ethoxy ethane (sweet, 2.8 min), ethyl acetate (aromatic/alcoholic, 3.6 min), 2-ethoxybutane (sweet/fruity, 4.8 min), methyl butanoate (fruity, 5.3 min), ethyl butanoate (fruity, 7.6 min), ethyl 2-methyl butanoate (fruity, 9.3 min), and methyl hexanoate (fruity, 12.3 min). Two were in common with e-liquid 1-A, i.e., ethoxy ethane and 2-ethoxy butane perceived by both assessors with intensity 3 and 2, respectively. The esters methyl butanoate, ethyl butanoate, ethyl 2-methylbutanoate, and methyl hexanoate are odor-active compounds with fruity attributes and represent a characteristic portion of the volatile aroma profile of fruits. They are also classified by FEMA as flavoring agents and are primarily used to impart fruity flavor in foods and beverages. Ethyl acetate is also included in the FEMA list of flavoring agents (with specification as food additive, carrier solvent) but its flavor profile is based on aromatic, brandy, and grape odor notes. Among the ‘sweet’ and ‘fruity’ odorous stimuli, both the assessors clearly indicated the one associated with the characteristic kiwi flavor, with odor intensity equal to 3. Comparing GC-MS results with the sensory response provided by both the panelists, ethyl 2-methyl butanoate was identified as the odor-active compound responsible of the kiwi flavor of the refill. This specific ester has been already identified in previous investigations by GC-MS and GC-MS-O as the key contributor of the aroma profile of several fruits such as pineapples [41], strawberries [42], cranberries [43] and melons [44]. The preliminary sensory tests (e.g., sniffing and vaping) on e-liquids 3-A, 4-A and 5-A, performed by both the panelists before GC-MS-O analytical sessions, allowed to appreciate a significant discrepancy between the flavor reported on the label and the overall flavor perceived. E-liquids 3-A and 4-A labels ‘Davidoff’ and ‘Green USA mix’ referred to tobacco brands

whilst e-liquid 5-A label reported 'cigar' flavor. In all three cases, the overall flavor coming from e-liquids vaporization should have simulated the characteristic notes of the tobacco leaves aroma (i.e., woody, leather). Instead, the qualitative description provided by both assessors highlighted that the overall e-liquids flavors were dominated by sweet and caramel-like notes with the only exception of e-liquid 5-A that in addition was characterized by distinct woody notes. GC/MS-O analysis of sample 3-A ('Davidoff' flavor) allowed to confirm the role of ethoxyethane in giving the formulation a characteristic sweet and pleasant flavor. Moreover, the odor-active compound found to be the key contributor to the caramel notes of the overall flavor was 2,3-butanedione (or diacetyl), whose relevance as a flavoring additive will be deeply discussed in Section 4. Similarly to samples 1-A and 2-A, other odorous stimuli perceived approximately at 17.6 and 20.4 min and resembling tobacco flavor were associated with low intensity chromatographic peaks and, as a result, the tentative attribution was not allowed. At this regard, it has been already highlighted in Tierney et al., 2015 that the majority of tobacco flavored liquids were found to contain confectionary flavor chemicals instead of tobacco extracts therefore it is likely that the flavor chemicals pattern (i.e., benzyl alcohol, vanillin, ethylacetate, maltol) included in the formulations for resembling tobacco flavor is not necessarily what is expected to be found in a tobacco extract [45]. Considerations made for sample 3-A are relevant also for sample 4-A ('Green USA mix' flavor). Ethoxyethane and diacetyl were also detected in sample 4-A and associated, similarly with sample 3-A, to sweet and caramel-like flavor notes respectively. In addition, ethoxybutane was identified and associated with sweet flavor notes. The attribution for other odorous stimuli perceived during the GC/MS run, approximately at 8.7, 11.6 and 17.6 min (the latter similarly with sample 3-A), was not successful due to low intensity chromatographic peaks. More specifically, in addition to tobacco-like flavor, herbaceous and grass/mint notes were perceived by assessors and this perception was considered reliable taking into account that, at least in principle, the formulation 'Green USA mix' should have simulated menthol-tobacco cigarettes and its characteristic menthol and herbaceous flavor notes. A comprehensive list of flavoring additives was obtained for sample 5-A ('cigar' flavor). Ethoxyethane and 2-ethoxybutane (both perceived with intensity 2) were confirmed as key contributors for sweet flavor notes while diacetyl (perceived with intensity 3) responsible for the caramel-like flavor. An interesting GC-MS-O outcome, allowing us to characterize the odor profile of the sample 5-A in a more distinctive way, was the identification of three odor-active compounds, perceived with odor intensity ranging from 1 to 2: α -terpinene (woody, 16.3 min), α -phellandrene (woody, 18.3 min), and α -terpinolene (woody/pine, 19.4 min). They all are classified as flavoring agents by FEMA: the associated flavor profile varies from woody, fresh, citrus, and spice notes in the case of α -phellandrene to pine flavor notes in the case of α -terpinolene. Their inclusion in the liquid formulation is therefore related to the intention of enriching the overall flavor profile of the product with woody and pine flavor notes with the purpose to simulate as closely as possible the cigar flavor. Finally, the integration of sensory perception and GC-MS chromatographic data failed in the identification of the odor-active compound perceived by both evaluators as responsible for the tobacco-like and burnt flavor, similar to what was previously observed for the sample 3-A.

Table 3. Gas chromatography-mass spectrometry-olfactometry (GC-MS-O) report: identified odor-active compounds with specification of molecular formula, CAS number, retention time (TR, min), odor description and intensity.

Sample ID	Compound Identified	Molecular Formula	CAS Number	Retention Time (TR, min)	Odor Description Panelist 1/Panelist 2	Odor Intensity Panelist 1/Panelist 2
1-A (Coca cola)	ethoxyethane	(C ₂ H ₅) ₂ O	60-29-7	2.8	sweet/sweet	3/3
	2-ethoxybutane	C ₆ H ₁₄ O	2679-87-0	4.8	Coca cola-like/sweet	3/3
	?			8.9	alcohol/sweet	2/1
	camphene	C ₁₀ H ₁₆	79-92-5	13.3	citrus/citrus,fresh	2/2
	γ-terpinene	C ₁₀ H ₁₆	99-85-4	18.1	bitter,citrus/coca cola-like,bitter	2/2
	?			21.3	pungent/no response	2/0
2-A (Kiwi)	ethoxyethane	(C ₂ H ₅) ₂ O	60-29-7	2.8	sweet/sweet	3/3
	ethyl acetate	C ₄ H ₈ O ₂	141-78-6	3.6	aromatic/alcoholic	1/1
	2-ethoxybutane	C ₆ H ₁₄ O	2679-87-0	4.8	sweet and fruity/sweet and fruity	2/2
	methylbutanoate	C ₅ H ₁₀ O ₂	623-42-7	5.3	fruity/fruity	2/2
	ethylbutanoate	C ₆ H ₁₂ O ₂	105-54-4	7.6	fruity/fruity	3/3
	ethyl 2-methylbutanoate	C ₇ H ₁₄ O ₂	7452-79-1	9.3	kiwi-like/kiwi-like	3/3
	methyl hexanoate	C ₇ H ₁₄ O ₂	106-70-7	12.3	fruity/fruity	2/2
3-A (Davidoff)	?			20.9	sweet/sweet	2/2
	ethoxyethane	(C ₂ H ₅) ₂ O	60-29-7	2.8	sweet/sweet	2/2
	2,3-butanedione	C ₄ H ₆ O ₂	431-03-8	3.3	caramel/caramel,sweet	3/3
	?			11.7	sweet/uncertain response	2/?
	?			17.6	tobacco-like/tobacco-like	1/1
4-A (Green USA Mix)	?			20.4	tobacco,burnt/tobacco, burnt	2/2
	ethoxyethane	(C ₂ H ₅) ₂ O	60-29-7	2.8	sweet/sweet	3/2
	2,3-butanedione	C ₄ H ₆ O ₂	431-03-8	3.3	caramel/caramel,sweet	2/2
	2-ethoxybutane	C ₆ H ₁₄ O	2679-87-0	4.8	sweet/sweet	2/2
	?			8.7	grass/mint	2/2
	?			11.6	herbaceous/herbaceous	2/2
	?			17.6	tobacco-like/tobacco-like	2/2
5-A (Cigar)	ethoxyethane	(C ₂ H ₅) ₂ O	60-29-7	2.8	sweet/sweet	2/2
	2,3-butanedione	C ₄ H ₆ O ₂	431-03-8	3.3	caramel/caramel,sweet	3/3
	2-ethoxybutane	C ₆ H ₁₄ O	2679-87-0	4.8	sweet/sweet	2/2
	α-terpinene	C ₁₀ H ₁₆	99-86-5	16.3	woody/woody	2/2
	α-phellandrene	C ₁₀ H ₁₆	99-83-2	18.2	woody/woody, spice	1/1
	terpinolene	C ₁₀ H ₁₆	586-62-9	19.4	woody/woody, pine	2/2
	?			20.4	tobacco-like,burnt/tobacco,burnt	2/2

4. Discussion

4.1. Discussion on BTEX Results

HS-SPME-GC-MS analysis of 97 e-liquids highlighted BTEX contamination. Experimental data obtained suggest that, during the period 2013–2015, contaminated e-liquids were commercially available on the EU market, particularly e-liquids imported into EU member states and manufactured in China. Taking into account all of the data obtained, no correlation was found between BTEX contamination levels and nicotine content, nor nicotine presence. The variability observed in BTEX contamination levels from one brand to another one is therefore likely to be related to the variability in contamination level of the basic components (i.e., propylene glycol and glycerol) and/or the flavoring additives included. In addition, the variability in BTEX contamination levels observed within the same brand is likely to be related to the flavoring additives used, and in the specific case of samples 10, 11 and 12 C, given the same flavor and nicotine content, to the contamination of basic components used in the production process of different batches. According to Regulation (EC) No 1272/2008 on Classification, Labelling and Packaging of substances and mixtures (CLP), benzene, toluene, ethylbenzene and m,o,p-xylenes are included in Annex VI, Table 3. Benzene is classified as carcinogenic for humans (Carc. 1A, H350: May cause cancer by inhalation), mutagenic (Muta. 1B, H340: May cause genetic defects), and represents a hazard when inhaled (Asp. Tox 1, H304: May be fatal if swallowed and enters airways; STOT RE 1, H372: causes damage to organs through prolonged and repeated exposure) [31]. Toluene is classified as reprotoxic (Repr. 2, H361d: Suspected of damaging the unborn child) and represents a hazard when inhaled (Asp.Tox 1, H304: May be fatal if swallowed and enters airways). Ethylbenzene and xylenes are both classified as follows: Acute tox. 4, H332: harmful if inhaled. Given all the information on toxicity classification reported above, more attention has necessarily to be paid to benzene, a human mutagenic and genotoxic carcinogen, detected in some e-liquids at high concentration levels. Therefore, an in-depth analysis of potential health effects due to inhalation exposure to benzene is due. Epidemiological studies over the years have provided evidence of a causal relationship between chronic inhalation exposure to benzene and serious adverse health effects and diseases, from non-cancer health effects (i.e., hematologic diseases and/or functional aberrations of immune, nervous, endocrine systems) to cancer (i.e., myeloid leukemia, non-Hodgkins lymphoma) [46]. Numerous studies have demonstrated that benzene metabolites, especially p-benzoquinone, are involved in the progression from cytotoxicity to carcinogenicity, as they activate oxygenated radical species able to cause DNA damage [47]. It has been estimated that approximately 50% of the quantity of inhaled benzene is adsorbed into the human body. Once introduced into the human body through the respiratory apparatus, benzene is preferentially adsorbed in fat-rich tissues (i.e., fat and bone marrow), owing to its lipophilic nature. Great concern about potential health hazards has been historically linked to occupational exposure (where higher benzene concentrations than in general environments are likely to be encountered) but knowledge on the issue, acquired over the years, has led the scientists and epidemiologists to be more and more focused on health effects induced by long term exposure of the general population to low concentrations of benzene. Although benzene is recognized as a ‘non-threshold carcinogen’ on the basis of the assumption that any exposure may result in some increase of risk, in the present study the carcinogenic risk related to the inhalation exposure to benzene resulting from the consumption of e-liquids affected by the highest contamination (brand A) has been estimated.

As reported in the results section, across all 97 e-liquids tested, benzene concentration levels ranged from 2.7 µg/L (in samples 3-B and 6-D, both produced in Italy) to 30,200.0 µg/L (sample 3-A produced in China). This means that, if we consider the daily average consumption of e-liquids by a regular vaper approximately equal to 3 ml per day [48], the total amount of benzene potentially inhaled by the vaper within one day would have ranged from 0.0081 µg to 90.6 µg. For the most contaminated Chinese brand (brand A) the total amount of daily inhaled benzene with 3 ml e-liquid consumption would have varied in the range 21.6–90.6 µg. Taking into account a regular vaper represented by an adult person with an average body weight of 60 kg, the daily consumption of brand A e-liquids would

result in benzene exposure of 0.00036–0.00151 mg/kg/day. A carcinogenic risk assessment for benzene may be performed comparing the estimated exposure with derived minimal effect level (DMEL) value, representing the level of exposure expressed as mg/kg/day below which the risk level of cancer is considered tolerable/acceptable (indicative tolerable risk level is 10^{-5} associated with a life-time risk for cancer of 1 per 100000 exposed individuals). The DMEL value for benzene, derived from reference values reported on Integrated Risk Information System (IRIS) website of United States Environmental Protection Agency (USEPA), is 0.0000182 mg/kg/day [49]. The comparison exposure-DMEL allows to point out that the daily consumption of Chinese e-liquids belonging to brand A would have resulted in a serious inhalation exposure scenario for active users with a risk level of cancer that is not acceptable. These results are of particular concern, also in light of the World Health Organization (WHO) guidelines for indoor air quality, published in 2010, where it is clearly stated that ‘no safe level of exposure to benzene can be recommended’ and that ‘from a practical standpoint, it is expedient to reduce exposure levels to as low as possible’ reducing or eliminating activities and materials that may release it [50].

4.2. Discussion of Flavoring Additives Results

Among the flavoring additives identified, diacetyl is certainly worthy of an in-depth analysis. Diacetyl is a volatile α -diketone and is a natural constituent of many regularly consumed foods (i.e., dairy products, fruits, coffee). Due to its flavor characteristics, it is widely used in the food manufacturing industry as a flavoring additive. It is added to a wide selection of foods and beverages to mainly impart butter and caramel taste and smell, depending on the concentration used. Its use in the food manufacturing industry is approved by competent governmental bodies such as U.S. Food and Drug Administration (U.S. FDA) and the National Institute for Occupational Safety and Health (NIOSH) and is currently authorized in EU member states according to EU Regulation No 872/2012. The potential risks for consumers health associated with the dietary exposure have been deeply evaluated over the years. As a result of safety evaluations, diacetyl has been determined to be ‘generally recognized as safe’ (e.g., GRAS) by the FEMA Expert Panel, and has been included in the FEMA GRAS list of authorized flavoring substances [51]. The European Food Safety Authority was also asked to take a position on the issue and the final opinion was that, on the basis of the safety evaluations carried out so far, the use of diacetyl in food is of no safety concern for humans. In this regard, however, it is important to point out that toxicological evaluations used to approve and support diacetyl as a flavoring additive in foods are related to ingestion, and therefore do not provide assurance of safety when other routes of exposure are involved, such as inhalation. In the early 2000s, concerns were raised with respect to potential toxicity for humans associated with inhalation exposure to diacetyl following the reported cases of a severe obstructive lung disease in diacetyl-exposed workers at microwave popcorn manufacturing plants in USA [52]. Preliminary evidence of an association between the occupational exposure to diacetyl and adverse effects on human respiratory apparatus has been reported by Kreiss et al., from a decline in respiratory function to development of a rare irreversible lung disease characterized by fixed airflow obstruction, called bronchiolitis obliterans [52]. Extensive scientific research on diacetyl has been carried out from then both confirming preliminary hypothesis on exposure-occurrence of lung disease association and adding new relevant scientific data [53]. Recently published papers have highlighted both neurotoxicity and impairment of cilia function in human airway epithelium [54,55]. Therefore, in light of the knowledge progressively acquired, the inclusion of diacetyl as flavoring additive in the manufacturing process of liquid formulations for e-cigs has rapidly become a much-debated issue in the scientific community due to foreseeable toxicological implications from direct inhalation exposure. In reaction to this, a prompt response came from e-liquids manufacturers with the replacement of diacetyl with 2,3-pentanedione (acetylpropionyl), an α -diketone showing similar flavor properties, but this option was soon revealed to be unsuccessful when scientific data on acetylpropionyl toxicity started to be published [56]. Our findings, although related to a limited number of samples, are in line with the results obtained in previous investigations highlighting the presence of diacetyl in e-liquids commercially available in EU member states in the

pre-TPD implementation period and with characteristic flavors appealing to teenagers and young adults [19,20,30]. Farsalinos et al., 2015 analyzed both liquid and aerosol matrices of a total number of 159 samples purchased from 36 manufacturers and retailers in 7 different countries. Diacetyl was found in 74% of the samples investigated and in a large proportion of sweet-flavored e-liquids, with similar concentrations in both liquid and aerosol. The simultaneous presence of acetylpropionyl also suggested that, instead of being used as a replacement, acetylpropionyl is often used in conjunction with diacetyl. Further, the authors highlighted that, for 47% of diacetyl-containing e-liquids, the daily exposure level ($\mu\text{g}/\text{day}$) for vapers could be higher than NIOSH-defined safety limits for occupational exposure. Barhdadi et al. investigated 12 flavored e-liquids by applying the HS/GC-MS method, properly developed for the screening and quantification of diacetyl and acetylpropionyl in e-liquids. The samples were provided by the Belgium Federal Agency for Medicinal and Health Products and collected either upon inspections in vaping shops or through seizure activity by Belgian authorities in the period 2013–2015, similar to the present study. The authors reported that only two sweet-flavored e-liquids contained measurable amounts of diacetyl and the determined concentrations were $6.04 \mu\text{g}/\text{g}$ and $98.84 \mu\text{g}/\text{g}$. Finally, 42 e-liquids selected from among the 14 most popular brands dominating both the USA and EU markets in 2013 were investigated by Varlet et al. in terms of chemical and biological constituents. Diacetyl was detected in three e-liquids, two of them characterized by tobacco flavors and one by candy flavor. Similarly to Farsalinos et al., comparison with the NIOSH safety limit was made, revealing that one tobacco flavored e-liquid that resulted diacetyl-positive could lead to exposure higher the recommended limit. Although approximate for estimating risk for e-cig users, the use of occupational exposure limits is affected by several limitations [19,57]. This approach has raised some resistance, mainly because occupational safety limits for toxicants, for instance for diacetyl, have been set for workers not for the general population and are related to inhalation exposure scenarios not applicable to e-cigs users. According to the authors' knowledge, other two studies carried out by Allen et al. in 2017 and Omayie et al. in 2019 have raised concerns about diacetyl, confirming its inclusion as flavoring additive in refill liquids for e-cigs (diacetyl detected in 39 of 51 tested refills and in 150 of 277 samples, respectively), but in both cases the investigated samples were considered dominating the current extra-EU market and therefore are not representative of the EU market before the implementation of TPD. To summarize, our findings on diacetyl, although related to a limited number of e-liquids manufactured in China and commercially available in the EU during the period 2013–2015, are in line with the results obtained in other investigations made on larger sets of samples representative of the EU market at that time. The only discrepancy on diacetyl presence detectable among the studies performed before the TPD implementation was reported by Girvalaki et al. in 2018. The authors evaluated the chemical composition of 122 e-liquids selected among the most commonly sold brands in 9 EU member states in mid-2016 before the TPD implementation. The result of this comprehensive investigation was a list of 177 compounds detected (e.g., flavoring additives and other VOCs), the majority with associated Globally Harmonized System of Classification and Labeling of Chemicals (GHS) health hazard statements. Diacetyl, however, was not detected in the samples tested, and therefore not included in the list. This discrepancy between Girvalaki et al. and the other abovementioned studies may be related or to the different period of e-liquids selection (2013–2015 versus 2016), although both periods were before TP -implementation, when the first actions aimed to the progressive replacement/elimination of diacetyl started to be made on a voluntary basis by some EU manufacturers and importers, or it simply reflects the potential heterogeneity due to the multitude of samples commercially available on the EU market in the period of reference. To date, following the implementation of TPD in most EU member states in 2016, both manufacturers and importers are obliged to submit a notification to competent authorities reporting detailed information on refill liquids (Article 20) [13]. The notification must report the list of all the ingredients (including flavoring additives) contained in e-liquid formulations for e-cigs available on the market and indication of related quantities as well. It must be noted, however, that according to TPD, the use of diacetyl is neither explicitly prohibited nor subjected to restriction. In addition, due to difficulty in defining a

typical inhalation exposure scenario fitting all vapers habits (high variability in daily e-liquid amount consumed), there is no scientific consensus on the maximum allowed level of diacetyl in e-liquids. Therefore, to date, diacetyl use as a flavoring additive in e-liquids remains an open issue, suggesting not only that quality controls remain necessary, even in e-liquids labelled as diacetyl-free, but also that the potential solution at the EU level to ensure that e-liquids supplied to consumers are safe is to follow the direction of some EU member states that proposed the ban of diacetyl and other flavoring additives of concern [58].

5. Conclusions

In the present paper, results from a study on the chemical characterization of levels of BTEX in 97 e-liquids, representative of the EU market between 2013 and 2015 prior the implementation of TPD in most EU member states, are reported. To our knowledge, there have been very few studies focused on BTEX analysis in refill fluids and cartridges for e-cigs commercially available on the EU market in the pre-TPD implementation period. Therefore, although the e-liquids investigated may not be representative of the current EU market, our findings may represent a useful reference for the ongoing evaluation on the effectiveness of e-liquid safety and quality requirements under the current legislative framework. Most of the e-liquids investigated were revealed to be affected, to a lesser or greater extent, by BTEX contamination. Few exceptions were observed (12 of 97 samples). High variability in BTEX total concentration level was observed from one brand to another, ranging from 2.7 µg/L to 32,151.1 µg/L. The contamination is likely to be related to the contamination of propylene glycol and glycerol, and/or the flavoring additives used. No correlation was found between BTEX concentration levels and nicotine content/presence. Moreover, it was estimated that an inhalation exposure of very high concern would have occurred for active users vaping the most contaminated e-liquids (brand A), characterized by high concentration levels (7,200–30,200 µg/L) of benzene, a known human carcinogen. Our findings, therefore, point out that higher quality ingredients should have been used and that quality control on the formulations should have been applied prior their introduction on the EU market in 2013–2015 period. Further investigations carried out on a limited number of e-liquids aimed at the identification of flavoring additives through GC-MS-O application confirmed, in the reference period of the present study, the use of diacetyl, a flavoring additive approved for foods but associated with the onset of a severe lung disease when inhaled. This finding is in line with results obtained by other investigations made in the same period on a larger number of e-liquids sold in EU, highlighting the use of diacetyl in the e-liquid manufacturing industry due to poor awareness of the potential harm to humans. There are now sufficient toxicological data on the potential adverse effects of diacetyl and other flavoring chemicals when directly inhaled into the human airways, and therefore harmonized regulation at EU level on flavoring additives use in e-liquids, resulting in ban or restriction, should be fully addressed, in order to ensure health protection.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4433/11/4/374/s1>, Figure S1: Comparison of the Chromatograms of a blank sample and a sample spiked with the BTEX standard solution (calibration level 3).

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