**“Toxicological and Analytical assessment of e-cigarette refill components on airway epithelia”**

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**Abstract:** There are over 2.6 million users of e-cigarettes in the United Kingdom alone. E-cigarettes have been promoted as safer alternative to traditional cigarettes. The addition of flavours and aromas have also proven to be popular with younger generations. In this communication we investigated the composition of the e-cigarette refills and assessed the biological effect of e-cigarettes refills on Beas2B (epithelium cells). We established that e-cigarette refills are complex mixtures of solvent vehicle, flavours with or without nicotine and their components are toxic towards the cells.

**Keywords:** e-cigarettes, flavours, Beas2B, toxic.

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## Background

Electronic cigarettes (e-cigarettes, e-cig or personal vaporisers (PV) are battery-powered devices that deliver vaporised chemicals to the user, current sales are over $1.7 billion for 2013 (Orellana), while there are over 7500 flavour variations at the moment (Sherwood). They may contain nicotine alongside other chemicals, such as flavourings and enhancers, while some variants may contain tobacco extracts[1]. The key differences between conventional and e-cigarettes are that e-cigarettes do not usually contain tobacco[2]. Smoking conventional cigarettes leads to the combustion of tobacco products. The process of heating in e-cigarettes is gentler than in conventional cigarettes[2]. Several studies clearly show that e-cigarettes vapours have less combustion products than the ones produced by regular cigarettes, many of which originating from regular cigarettes are carcinogenic[2], New manufacturers are increasing the heating temperature in e-cigarettes to allow for a more “real” effect. As 30% of the cancer deaths in USA are caused by tobacco, more precisely from the tar component which is the killer (Hartung short), it is understandable that e-cigarettes (with no tar) are being branded a safer alternative to tobacco.

E-cigarettes are composed of a cartridge or tank which is used to store liquid material containing the “e-liquid”, “e-juice” or “nicotine solution”[11]. The cartridge serves as a reservoir of storage for the liquid and also acts as the mouthpiece of the e-cigarette. A heating element is used as an atomiser to turn the liquid into a vapour[10], and a power source such as a battery, which can be either manual or automatic, make up the rest of the device. The vapour is only produced while the heating element is activated and not between puffs. The vaporised liquid condenses into an aerosol, later inhaled to deliver nicotine and flavourings[11,12]. The vapour is generated by heating the solution to temperatures ranging from 65°C to 120°C, with a reported maximum atomiser

temperature of approximately 250°C[11]. This can increase the chances of carbonyl formation. Different models are available with some more manual to control the delivery and temperature (Breland). Propylene glycol and glycerine are used as carriers with the first one being the more widely employed, even though glycerine has been used in traditional cigarettes (Carmines). The vapour can contain carbonyl compounds like formaldehyde, acetaldehyde, and acrolein, which have been shown in numerous studies to be toxic. Formaldehyde and acetaldehyde are classified as carcinogens[13,14] and acrolein as an irritant[15].

E-cigarettes are sold as a healthier option to tobacco smoke and physicians are currently asked about their opinions in this area (Arnold). Furthermore, around 95% of the general population believe them to be healthier than conventional cigarettes (Kaisar). So far the research has proven that the e-cigarette vapour is not benign, but less hazardous than traditional cigarettes (Arnold). E-cigarette users commented on open forums on the internet about side effects such as headaches, respiratory tract irritations and digestive problems (Arnold). Clinical studies has shown that only 10% of traditional smokers quit smoking after switching to e-cigarettes. The biggest change observed was in the reduction of traditional cigarettes per day in favour of e-cigarette puffing (Kaisar). On the pro e-cigarette side, the absence of the tar products, pyrolysis and lower plasma nicotine content (around 10% that of the tobacco cigarette) would make it a healthier option for traditional cigarette smokers. A clinical study has also shown that cell blood counts and markers are statistically not affected by exposure to e-cigarette users and passive user. On the contrary, the exposure to tobacco cigarettes (users and passive users) showed an indication of inflammation after 3 hours (Flouris).

However, there is conflicting information regarding the risks posed to public health and the health benefit from e-cigarettes. The Consumer Advocates for Smoke-free

Alternatives Association (CASAA), has reported a significant risk reduction when assessed against regular cigarettes[4]. However, various studies indicate that e-cigarettes may produce long-term and short-term side effects, such as airway resistance, irritation of the airways, redness of the eyes and drying out the throat[6-8]. Research has been focused on the toxicological effects of e-cigarettes on lung, heart and cancer (Orellana). While some reports might have inconsistencies or conflict of interest. The general view indicates a toxic effect (McKee). The World Health Organisation (WHO) and the Food and Drug Administration (FDA), have indicated that the safety and the potential health damage of e-cigarettes and its constituents have not been fully studied and so remain undetermined[9,10]. Guidelines form the FDA indicate an inclination towards the enforcement if the same rules applying to traditional cigarettes for the term of sales and marketing strategies of electronic cigarettes (Orellana).

The majority of research to date has been divided between: i) analytical assessment of the e-liquids, ii) analytical assessment of the vapour phase, iii) toxicity of the e-liquids and/or vapour in animal models and/or animal cells, iv) toxicity of the e-liquids and/or vapour in human cells (primary and immortalised both cancer and non-cancerous, 2-dimension and 3-dimension) and v) clinical studies on cigarette (traditional and/or e-cigarettes) smokers. Though the analytical assessment seems to be more reproducible due to standardised methods used in the chromatographic method, eluents, and detection, more variability appears in the biological work. This might be related to the dosing, concentration of ingredients, sample variation from same or different manufacturers, flavourings, cells and even the media.

**Composition**

Tobacco smoke comprises many classes of chemicals including polycyclic aromatic hydrocarbons, benzo(a)pyrenes, and tobacco specific nitrosamines such as 4-

(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and N'-nitrosonornicotine (NNN)[3]. E-cigarettes do not have a source of combustion, and this is a reason why the health risks of vaping are assumed to be less harmful compared with traditional smoking. Therefore manufacturers have shown a growing interest to produce e-cigarettes, for indoor use, whereas the traditional cigarettes have been banned[4,5]. Nevertheless, the components in the e-cigarette aerosol and e-liquid refills contain the carbonyls formaldehyde (up to 9.0 µg/g of e-liq), acetaldehyde (up to 10.2 µg/g of e-liq), acrolein (up to 5.5 µg/puff), propionaldehyde (up to 1500 ng/puff), as well as the Volatile Organic Compounds (VOCs) toluene (up to 6.3 µg/150 puff), N-nitrosonornicotine (NNN) (up to 16.7ng/mL e-liq), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (up tp 10.8ng/mL e-liq), glycols such as propyelene glycol and glycerine (variation), nicotine (depending on the manufacturer's label), traces of polycyclic aromatic hydrocarbons and the metals Ni (up to 0.29 µg/150 puff), Cd (up to 0.22 µg/150 puff) and Pb (up to 0.57 µg/150 puff) with traces of Ag, Al, Zn and Cr (Kaiser, PisingersNumbers for reference?).

*Nicotine.* From vapours containing tobacco, tobacco specific nitrosamines (TSNAs) including N'-nitrosonornicotine (NNN) and 4-(methylonitrosoamino)-1-(3-pirydyl)-l-butanone (NNK) can be formed in the combustion process in traditional cigarettes (Callahan) and are considered to be highly toxic[17,18]. There is evidence that these toxic carbonyl compounds have been found in the vapour of e-cigarettes (Besaratinia Number?). Some studies have demonstrated that impurities and nicotine degradation products such as nicotine-cis-N-oxide, nicotine-trans-N-oxide, myosmine, anabasine, and anatabine, which are very carcinogenic, can be found in e-cigarettes refill liquids[20]. The molecules can lead to mutations in genes such as Ras (vital function in signal transduction of cell proliferation), p53 and Retinoblastoma (with roles as tumour suppressors) as these molecules can form adducts with cellular DNA[21-24]. Nicotine can

be absorbed through different routes such as inhalation, ingestion, skin, and mucous membranes. Therefore it is feasible that the vapour from e-cigarettes users could cause secondary exposure of nicotine and other toxins to the individuals in the surrounded area[3]. Nicotine is a stimulant and side effects can include death.

A danger of e-cigarette refills are for those bottles containing fruity or sweet flavours and aromas which children can mistake for fruit juices. A fatality has been reported of a 2 year old child after drinking an unknown amount of e-cigarette refill (Breland). Concentrations of nicotine in the air have been studied for conventional and e-cigarettes. It has been reported that e-cigarettes with a refill liquid of nicotine concentration of 24 mg/ml emitted nicotine concentrations between 0.82 μg/$m^3$ to 6.23 μg/$m^3$, with the mean concentration of nicotine from regular cigarettes ten times higher (31.60 ± 6.91 μg/$m^3$)[25]. A threshold limit of nicotine exposure in the work place is published by The American Conference of Governmental Industrial Hygienists and is established for an eight-hour time-weighted average (TWA), as 500 μg/$m^3$ [26]. Manufacturers normally indicate the expected level of nicotine in the e-cigarette refills. Though they are often very close to the label value, some samples seem to not reflect the label value (Callahan). We observed in our studies that the origin of nicotine is also an important factor. Nicotine can be used in the chemical industry, and this grade of nicotine is not as pure as pharmaceutical nicotine which should be employed in the tobacco industry.

Alkaloids are present in plants and one of the most notorious examples is nicotine. Other alkaloids from tobacco can include cotinine, myosmine and anabasine, with most of them being present in e-cigarette refills. A comprehensive study on the alteration of gene expression on CCL-185 (human lung carcinoma cell line) upon exposure to these four alkaloids (Marlowe) showed an increase of CEACAM6 (an adhesion molecule

involved in carcinogenesis and metastasis) when the cells were treated with nicotine and myosmine, and a decrease when exposed to anabasine and cotinine. In the case of ALDH3A1 (an enzyme involved in the detoxification of reactive aldehydes), the treatment with myosmine showed an increase, while for PIR (transcription regulator for apoptosis and oxidative stress) a decrease was observed in the cases of nicotine, anabasine and cotinine. Mysomine had little effect on PIR. Only nicotine showed an increase of TLR4 (a ligand involved in the immune response) while the other compounds showed a decrease thereof (Marlowe).

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*Various metals* such as nickel, cadmium, lead and silica particles can be present in the aerosols produced from e-cigarettes. They could arise from the wick and heating coil constituents. These metals are considered to be carcinogenic, nephrotoxic, neurotoxic, and hemotoxic[19].

*Glycerine (glycerol) and propylene glycol.* Glycerine, also called glycerol, is an intermediate in carbohydrate and lipid metabolism. It is used as a solvent, emollient, pharmaceutical, and sweetening agent in food industry[27]. Both glycol and glycerine are used in manufacturing industries, as well as aviation and are well knowns respiratory irritants (Callahan Lyon). Glycerine and propylene glycol are chemical compounds both used in normal and e-cigarette liquids to control the moisture content[28]. However, they may be pyrolysed (burned) to acrolein and formaldehyde at higher temperatures[14]. Acrolein and formaldehyde have been found in e-cigarette vapour even though the levels detected were 15 times smaller than in conventional cigarettes. This is due to the fact that the evaporation temperature of e-liquids at 60$^{o}$C-120$^{o}$C is lower than that of the combustion temperature of up to 650$^{o}$C in regular tobacco cigarettes[29].

*Flavourings and their toxicities.* The sensation of flavours is determined by chemical substances that can interact with the senses of taste and smell[30]. There are over 2500 flavouring substances being employed in the food industry. Safety procedures have been introduced to control their use (Munro), though they are directed to consumption of food rather than e-cigarettes, where the uptake is different. In general, oral consumption, and the quantities in the food need to be considered. These chemicals/flavouring substances (aldehydes, esters, acids) tend to be metabolised very rapidly through active enzymes in the liver and intestine (Phase I and Phase II enzymes, including the CYP450 family and glutathione transferase) (Munro). A decision tree is followed based on the chemical structure and is based on data from human and animal studies. The Flavour and Extract Manufacturers Association (FEMA) assess the safety of chemical compounds used as flavouring ingredients, but cannot regulate the use the flavour ingredients in e-cigarettes, because the use of the flavourings in e-cigarettes has not been approved[31]. Adding flavours to traditional cigarettes has been practised in some countries. Some anti-tobacco groups claimed this could attract new smokers. Different flavours can be added from oils to natural extracts, with the majority of them in the fruity range, such as mint and menthol. In the case of traditional cigarettes, the combustion temperature could produce pyrolysis or oxidation of these compounds and convert them into toxic carbonyls (Baker). There is often no more information given about the composition or source of such additives, other than that these flavours are “natural“[4]. As the most widely available sources of flavourings are for food products, we could expect some of the e-cigarette manufacturers could be using food flavouring products. For example, diacetyl (butanedione or butane-2,3-dione) is a by-product of the transformation of glucose to ethanol by yeast during the beer fermentation process and is extensively used in the food industry to flavour dairy products (batarfi). It is safe as food flavouring in popcorn,

but when inhaled it has been shown to produce “popcorn lung syndrome” or *bronchiolitis obiliterans*[32]. Animal studies of diacetyl exposure has shown morphological changes in the liver (Batarfi). Studies of cells exposed to butterscotch flavoured e-cigarettes have also shown toxicity[33].

Menthol is one of the most widely used flavours in both e-cigarettes and traditional cigarettes. These mentholated (e)-cigarettes seem to mask some early signs of respiratory diseases as menthol has antitussive properties. Nevertheless, this seems to be more a hypothesis than being backed by real data (Heck) and very limited information of toxicological data is available even on traditional cigarettes (Wang). Menthol is a volatile compound and it could be ready vapourised rather than suffer pyrolysis. As a food additive, menthol has been subjected to many toxicological research, but little has been done on the respiratory tract with very little findings besides irritation *in viv. N*evertheless, menthol is present in many products directed to treat respiratory problems such as the case of Vicks Vaporub.

An interesting study (MRVA) on an adenocarcinomic human alveolar basal epithelial cell (A549) showed that exposure to vapours of several flavouring agents, e.g cinnamladehyde, benzaldehyde, diacetyl, 2,3-pentadione, vanillin, acetoin and triacetin, for 24 hours, proved to be very toxic to the cells. This was especially the case for cinnamaldehyde and benzaldehyde. This is in accordance with other studies in which cinnamon flavour in e-liquids has shown high cytotoxicity levels in other cells[34]. Interestingly, vanillin, acetoin and triacetin proved to be the less toxic. Another study employing the same cell line, but different varieties of e-liquids, found no toxicity though there was an increased level on the release of IL-8 (Misra) at a very high dose.

A549 is a cancer cell line, generally used for anticancer drug screening and safety profiling of new drugs. Immortalisation of human cells using telomerase or SV40 virus are good options for cytotoxic studies. In the case of bronchial cells, some examples

are Beas-2B and 16-HBE14o. A study testing individual flavours for chocolate (2,5-dimethypyrazine), vanillin, apple/citrus (damascenone), floral (linalool), raspberry (α-ionone), caramel (ethyl maltol) and strawberry furaneol challenged the cells for 24 hours. At the concentrations tested findings shown vanillin and furaneol were relatively non-toxic (in agreement with other studies of vanillin). The rest of the flavours showed activity on the cells, with the chocolate flavour showing a reduction of the capability of producing/communicating signalling molecules (Sherwood).

As the e-cigarette industry is growing, more needs to be done to assess the quality of the ingredients as well as the biological effects thereof. Some groups compared both the analytical composition and the toxicological effect of the e-liquid and the vapours associated with it (Costigan, Kosmider). The different temperatures that can be achieved in the vapourisation chamber (up to 350°C), can modify the functionality of the chemical ingredients and transform them into dangerous carbonyls such as formaldehyde and acetaldehyde. The concentration in the vapour seems to be dependent on the voltage and temperature (Kosmider). The amount of nicotine found in the aerosol has been found to be 85% lower than traditional cigarettes (Tayyarah), which implies that the smoker and passive smokers of e-cigarettes are exposed to less damage.

A very interesting decision tree for carcinogenicity, mutagenicity and teratogenicity for flavourings proposed by Costigan (Costigan) highlights the need to compare both what the seller informs the buyer about the ingredients and their quantities with the results from the e-liquid and breakdown products employing GC-MS. From here the ingredients can be compared to existing data bases for biological information. If more and/or new ingredients are found, then they will need to be assessed.

**Analytical method of assessment**

Gas-chromatography coupled to mass spectrometry, GC-MS, is the most popular method for analytical detection in majority of the articles published. Other variants include gas chromatography coupled to thermal energy analysis (GC-TEA) which is very sensitive to nitrosamines (Tuyyarah) and liquid chromatography with tandem mass spectrometry (LC-MSMS). Each technique has benefits and disadvantages. The ingredients in the e-liquids are volatile compounds, and some e-liquids have a simple formulation (single flavouring agents, propylene glycol, nicotine), while others have complex mixtures (natural extracts for flavour, sweeteners, tobacco and more)ref. GC relies on the volatility of each chemical and when this is not possible a process of derivatisation can be used. LC on the other hand, does not require the compounds to be volatile but to dissolve in the elution system, generally using acidified (0.1% formic acid) water/methanol or water/acetonitrile. Columns used in both methods are generally based on carbon 18. In the case of LC, polar columns such as HILIC have been very useful. We have found that some compounds, such as menthol, are not detected very well in LC-MS though it is easily observed when using GCMS. The tandem quadruple MSMS or time of flight ToF allows for quantification if a patron was used for compound monitoring (optimisation technique that works as a fingerprint). Limits of detection and sensitivity apply to all techniques as well as accuracy (Tayyarah). Some groups have used both GC-MS and LC-MS but for different purposes (Kavvalakis). For example, GC-MS were used for the analysis of solvents/humectants (propylene glycol and glycerine) and polycyclic aromatic hydrocarbons, while LC-MS were employed for the quantification of nicotine, nitrosamines and flavours.

From the review data, there is no “one fits all” analysis technique, but more modern equipment seem to perform better than some of the older techniques. Infra-red (IR) technology can detect the functional groups in small molecules, and can differentiate

if there is a carbonyl in a sample, a hydroxyl or a nitrile group. New and more sensitive equipment using IR is emerging and allowing analysis of materials used in the cosmetic, food, and forensic industries (Deconinck ALL). Techniques like Attenuated Total Reflectance – Fourier Transform – IR (ATR-FT-IR) and near IR (NIR) alongside modelling methods like K-nearest neighbours (k-NN), partial least squares-discriminant analysis (PLS-DA), software independent modelling by class analogy (SIMCA), classification and regression tress (CART) and randon forests with Matlab as data processing software have been widely used to determine if an e-liquid has nicotine or not (Deconinck).

Heating propylene glycol can produced the toxic carbonyls formaldehyde (600°C), acetaldehyde (600°C), and acrolein (traces at 350°C) (Uchiyama). A free-radical dehydration of glycerol yields 3-hydroxyl-1-propen-1-ol and through tautomerisation 3-hydroxylpropionaldehyde can be obtained. The latter one can lose one water molecule through free-radical formation to give rise to acrolein (Gillman). If the temperature is >400°C, 3-hydroxylpropionaldehyde can be converted to formaldehyde and acetaldehyde by a retro-Aldol reaction (Gillman). An interesting study (Gillman) trapped aerosols at different vapour conditions and monitored the formation of aldehyde by means of trapping with 2,4-dinitrophenylhydrazine and assessing it by HPLC-UV. The coil in the electronic compartment will heat the e-liquid when power is applied, and this is measure in watts. It is noticeable that different electronic designs produce a different output. While some designs produce a steady increase in the three aldehydes when more power (producing more temperature) is applied, in some other cases the amount remains at low levels and with no increase.

Metal content is a known issue in traditional cigarettes and traces of metals have been found in e-cigarettes, ICP-MS methods have also been used to assess the heavy metal content in e-liquids (Beauval).

**Health**

E-cigarettes are getting more widely used due to promotion by manufacturers as a healthier alternative to conventional smoking. Amongst the complaints e-cigarette users describe, mouth and throat irritations have a high incidence. This could be due to carbonyls formed during vaping[15]. It is important to notice that burns are also important consequences from the electronic devices due to faulty or fake batteries and/or mechanisms (monks).

Other volatile organic compounds (VOCs) such as toluene and m,p-xylene which can be produced in the process are considered to be carcinogenic, hemotoxic, neurotoxic and irritants[16]. More harmful side effects are continuously being found[35-40] through *in vitro* and animal models. The vapour heating process can produce carbonyls, though not in as high concentrations as traditional smoking. There is biological evidence that aldehydes are toxic to mammalian cells by acting as mutagens, producing DNA single-strand breaks and chromosomal aberrations (Golzer). Toxicity comes in different shades, and in the case of human and animal subjects, toxicological studies imply the assessment of biomarkers such as pro-inflammatory cytokines, development of cancer, teratogenicity, plasma nicotine concentration and effect on metabolism (Golli). For animal models a lethal dose can be easily assessed. In the case of cell culture, toxicity initially appears as cell viability, followed by cell health, metabolic pathways, mutagenicity, release of cytokines and signalling (golli). Studies in mice, which are indicators of acute exposure due to high concentrations and short, but persistent contact with the vapour, have shown an increase in inflammation markers such as IL-6, IL-1α and IL-13, especially in the lung area (Lerner) and reduction in immune defence towards bacterial and viral infections as the phagocytosis by alveolar macrophages was compromised upon challenge with e-cigarette smoke (Sussan). In the case of rats (Golli), e-cigarettes with nicotine affected the body weight and energy

intake, and alteration in the lipid profiling (though some effect was observed when nicotine was not present). Nevertheless, with or without nicotine, the e-cigarettes depleted the hepatic glycogen producing hyperglycemia and affected the kidneys by altering the anti-oxidant response in both cases. This implyies that the rest of the ingredients have toxicological effects on renal ducts (Golli, Golli).

Research on e-cigarette toxicity is not very extensive as the market is relatively new. Nevertheless, the area is not free of controversy. Several studies have been carried out on human and animal cells, animal models, stem cells and there were also some short clinical trials, using vapours and smoke extract obtained from e-cigarette devices as well as the e-liquid refills (Lerner). There is an increasing amount of research dedicated to the toxicity of the contents of the e-cigarette refills, looking at the biological activity of nicotine, the vehicle and flavours. (Bahl, Farsalinos 2013, Lerner). Studies on human bronchial airway epithelial cells and human foetal lung fibroblast showed that exposure to different flavours of e-liquids (lerner) exhibited high levels of stress in the form of reactive oxidative species (ROS). Furthermore, the cell morphology changed to enlarged cells, cell viability decreased and inflammatory markers were raised and responses occurred in neutrophils (lerner, lerner 2016, highman).

Published research is trying to shed light on the hot topic of “are they toxic” and more and more studies are focusing on comparing e-cigarettes to tobacco cigarettes. In a study comparing both types of smokes on HaCat (non-cancerous human keratynocytes) and A549 found that pro-inflammatory cytokines and chemokines (PDGf-BB, basic FGF, IL-8, IL-12, IL-17, GM-CSF, IP-10, MCP-1, MIP-1β in both cells and IL-1rα, IL-10, G-CSF, IFN-γ, RANTES, TNF-α and VEGF in HaCat) were released upon exposure to e-liquids, with cell death more preponderant in the traditional tobacco smoke (cervellati).

A great majority of the biological studies focus on the lung and cardiovascular functions, with the result that the morphology of the nasal epithelia is being overlooked. A study was conducted on this topic by collecting biopsies and fluids from the nasal passages from non-smokers, as well as cigarette and e-cigarette smokers (Martin Not in ref list?). The changes in the expression of mRNA of key genes were used to monitor the health of the cells and the metabolic pathways. The findings include a decrease in the expression of immune related genes for electronic and traditional cigarette smokers, and in some cases the response was stronger in e-cigarettes. This indicates that this type of smoking changes the immune composition at the nasal mucosa. A review by Biyani focused on the area of otorhinolaryngology (Biyani) and looked at the implications of e-cigarettes in a paediatric clinic where 80% of adult smokers started smoking before age 18. Though they did not present any clinical trials to determine the effect of passive e-cigarette smoking, they described the problem of liquid poisoning. As young adults, they commence to smoke, believing e-cigarettes to be non-toxic.

A decrease in cardiovascular function has been linked to the use of traditional cigarettes, with the main side effect being inflammation, thrombosis and oxidation of low-density lipoprotein that can affect the myocardial activity (Grana, Jasper, Molina). A clinical study sponsored by the Lorillard Tobacco Company, used human subjects to compare limited exposure to e-cigarettes to traditional cigarettes (Yan) (for standardisation, 1 refill of 16mg/mL providing 50 puffs vs 1 Marlboro® Gold King size with both yielding around 0.8mg, though in real subjects this might vary). Unlimited exposure found the nicotine plasma level to be increased (with the traditional cigarette having higher concentrations and acting faster after 5 minutes). In addition, the combination of propylene glycol with glycerine in the e-liquid helps to deliver more nicotine than propylene glycol alone. The mechanism for heart rate increase due to nicotine has been elucidated and ascribed to the activation of the sympathetic nervous

system with release of norepinephrine and epinephrine upon exposure to nicotine (Cryer, Benowitz). As the traditional cigarette increased nicotine in plasma more and faster than the e-cigarette, the heart rate increased in correlation to the amount of nicotine in plasma (Yan). Though the e-cigarettes increased the nicotine content in plasma which affected the systolic and diastolic blood pressure and increase the heart rate, it did so much less than traditional cigarettes. Other studies seem to validate the notion that switching from traditional cigarettes to e-cigarettes (and hopefully then quitting completely) will assist to lower the systolic blood pressure (Farsalinos 2016, emergency paper, Burbank). However some of them found that the nicotine plasma level were equal in both e-cigarettes and traditional cigarettes (St Helen).

More recently studies are using different systems to assess toxicity, such as a *C. elegans* model (panitz), in which refill components (nicotine, propylene glycol, flavourings) were tested. Oxidative stress, growth and brood size were affected in the same way when tests were conducted with liquid and vapours.

It is important to note that many of the studies arrive to the same conclusion regarding the biological activity, as well as the analytical composition. Parallels are difficult to draw amongst the many different studies as the concentration of the dosing sample varies, as well the test as conditions (such as feeding media, time and type of exposure). An excellent review published in ATLAS (Manupello) comments on the majority of the *in vitro* methods used (2D, 3D) and different types of assays to study toxicology, risk assessment, cell transformation and cell health assays and genomic analysis of tobacco products. This could be extremely important when planning biological research.

**Marketing and metrics**

With a world-wide market reaching over £35 billion by 2025 (Hartung long) not much emphasis can be found on the marketing that e-cigarettes receive, but a presentation

by Monks and Crawford (Texas Tech University Health Sciences Center, El Paso) obtained on the USA Environmental Protection Agency website (Monks), provides some interesting numbers. In the UK figures obtained from Action on Smoking and Health (ASH) showed that e-cigarette user numbers rose from 700,000 in 2012 to 2.1 million by 2014 (Hartung). For 2014, in the USA, around 13.4% (2 million) teens smoke them. This highlights the growing tendency of this habit. On the other hand, this teen population fell from 15.8% of tobacco smokers to 9.2% by the same year (Hartung long). As this overall population is under 18, and banning laws apply, more disguises (tic-tac boxes, juice bottles) are found for the e-cigarettes to be smoked. Calls related to e-cigarette poisoning in the state of Texas showed that 57% was related to children younger than 5 years old. These were unintentional, with 96% of cases occurring in their houses. Of these, 85% was from ingestion and 11% dermal. This is an important aspect as the marketing directed to adults is also affecting small children. E-cigarette marketing has been very aggressive, with many adverts containing a strong sexual content, or trying to relate to foods/diets or traditional cigarettes, as well as using celebrities. For this reason the advertising expenses have increased from $6.4 million in 2011 to a staggering $112.9 million in 2014 (Monks).

In this paper we screened the web for information on the composition and toxicity of e-cigarettes, with an emphasis on the flavour activities and health profiles. We decided to compare some of the published results with our own studies. We purchased around 18 samples of e-liquids and exposed the human bronchial cell line Beas2B to different concentrations of the e-liquids. We also analysed the content of these samples using LC-MSMS. We found our data to be in agreement with other published material.

**Materials and methods**

*Materials.* The following materials and chemicals were obtained from Thermo Fisher Scientific, Altrincham, Greater Manchester, UK: 0.25% Trypsin-EDTA (GIBCO), sterile phosphate buffered saline, acetonitrile optimal LCMS grade (ACN), ammonium acetate analytical grade, formic acid optimal LCMS grade. Lonza, Slough, Berkshire, UK: BEGM Single-Quot kit. Sigma-Aldrich, Dorset UK: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), sterile phosphate saline buffer (sPBS), nicotine, propylene glycol, dimethyl sulfoxide (DMSO), chlorpromazine. Other supplies include ECACC, Porton Down, Salisbury, UK: Beas2B cell line (immortalised cells obtained from autopsy of normal human bronchial epithelia from non-cancerous patients) VWR West Sussex, UK: plastic ware. Anachem, Luton, UK: pipettes and pipette tips. Amazon UK: e-cigarette refills. Superdrug, Manchester, UK: Nicolite refills. Hichrom, Reading, Berkshire, UK: 0.2µm polypropylene syringe filters.

*Cell maintenance*. Cells were grown as adherent monolayer culture in 75cm$^3$ flasks in Bronchial Epithelial Growth Medium (BEGM) using Lonza's BEGM SingleQuot kit, at 37°C, and under a humidified atmosphere containing 5% $CO_2$ and 95% air. Cells were changed twice a week after reaching 70% confluence.

*MTT assay*. Two types of e-liquids are available on the market. These are synthetic ones, containing artificial flavours and natural ones, containing extracts of tobacco leaves and natural flavours extracted from plants. Pre-packed cartridges can have varying nicotine concentrations (ranging between 0-18 mg/ml nicotine/cartridge) with diverse flavourings, for example tobacco, menthol, mint, chocolate, apple, cherry, caramel and many more (12,13). We used different suppliers that were commercially available over the counter and through the internet. We tested a variety of flavours and nicotine content, as well as synthetic nicotine and propylene glycol which is used generally as carriers for the production of the vapours. All e-cigarette refills, nicotine and propylene glycol were tested at a range of concentrations (0, 4, 10, 20, 40, 80,

120, 160 and 200% puff, with each puff being 5 µl (100%) for e-cigarettes. Dilutions were made up in sterile water from 0-1.64mg/ml for the nicotine stock solution, 0-0.1g/ml for propylene glycol stock solution, and 0-100 µM for chlorpromazine stock solution (this is the positive control in all assays). Cell death percentage was determined by the colorimetric MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] micro-culture assay. Cells were detached from the 75cm$^3$ flasks (at a confluence of 70%) by trypsinisation, seeded in 100µl aliquots into 96-well clear micro-culture plates. Cell densities of 40,000; 30,000 and 20,000 cells/ml for 24, 48 and 72 hours of incubation were used respectively. This method was chosen in order to ensure exponential growth of untreated controls throughout the experiment. Cells were allowed to grow in the 96-well micro-culture plate for 24 hours prior to dosing. Stock solutions of the test compounds in water were appropriately diluted in complete culture media to make up the required concentrations, and then added in 10µL aliquots into the 96-well micro-culture plate. Cells were exposed to the test compounds for 72 hours. Plates were maintained at 37°C in a humidified atmosphere containing 95% air and 5% $CO_2$. At the end of the incubation period, 30µl/well MTT solution in sPBS (3 mg/ml) were added, then incubated for a further 3 hours. After the end of the incubation, the supernatants containing medium and MTT were removed and the formazan crystals formed by the viable cells were dissolved in 100µl of DMSO per well. Optical densities at λ = 540 nm were measured with LUMIistar Omega multi-mode plate reader (Edinburgh, UK). The colorimetric MTT assay was used to determine the cell death percentage at a serial diluted concentration of the tested compounds and the concentration at which 50% of cell growth was inhibited ($IC_{50}$). In comparison to the control wells which did not contain any test component, was determined from a dose–response curve using OriginPro 9.1 (Northampton, MA, USA) data analysis and graphing software. Chlorpromazine was used as positive control in the MTT assay.

Data were collected as duplicates and statistical analysis calculated as standard deviation (SD) using Excel Microsoft (Reading, Berkshire, UK). Pictures were taken with a Axio Vert.A1, PE-300 microscope from Zeiss, Cambridge, Cambridgeshire, UK. *Mass spectrometry.* The analysis was performed on an Agilent 6540 LC-MSMS Q-ToF Jet Stream ESI (Greater Manchester, UK). Measurement conditions were: +2500V, CE (collision energy) 80eV, Sheaf gas 350$^{o}$C at 10l/min, drying gas at 325$^{o}$C at 10L/min. Nebuliser gas pressure was at 18psi. The chromatography was performed on an Agilent 1260 series (Greater Manchester, UK) with auto sampler and thermal controlled column chamber. The separation was done on a Thermo Scientific Accucore HILIC 50x2.1mm particle size 2.6µm (Thermo Fisher Scientific, Altrincham, Greater Manchester, UK) column kept at a stable 20$^{o}$C. The flow rate was set at 0.4ml/min using a gradient profile of ACN (acetonitrile) 95%:$H_2O$ 5% (0.1% formic acid / 5mM ammonium acetate) 0min: 100%. 20min: 60%. 25min 100% end 30min, with the remainder being $H_2O$ (0.1% formic acid / 5mM ammonium acetate). The column was prepared and stabilised for 6hours before running using ACN 95%:$H_2O$ 5% (0.1% formic acid / 5mM ammonium acetate) with a flow rate of 1.0 ml/min. The samples were prepared by diluting the e-cigarette fluid of 10µl with 990µl of ACN and filtering it through 0.2µm polypropylene syringe filters (this is known as “dilute and shot methodology”).

**Results and Discussions**

We tested 18 different e-cigarette refill flavours for their toxicity on human derived bronchial cells (Beas2B). In our studies we exposed the cells at different concentrations and times to the cells. The definition of “puff”, its volume and quantity seems to be different in various publications, as made with reference to the total reservoir and expressing it into nicotine content[1,41-43]. The puff is also dependent on the user, with some puffing a larger volume than others. Based on literature evidence

of amount of nicotine used[41-43] and the given value per cartridge of nicotine at an average of 18mg/ml, we calculated that 1 inhalation might be equal to 5µl, and this "puff" would contain around 90µg of nicotine.

**Biological data**

In this work we will equate 1 puff to 5µl of the e-liquid refill. The Beas2B were exposed to 0, 4, 10, 20, 40, 80, 120, 160 and 200% puffs. This in effect means 0, 0.2, 0.5, 1, 2, 4, 6, 8, 10 µl of refill respectively (dilutions were made with distilled sterile water). Exposure periods were for 24, 48 and 72 hours. The bronchus conducts the air into the lungs where the surface area[44] can varied in the range of 40-80$m^2$. Although 1 puff (5µL) appears to be a large volume, smokers rarely would have only 1 puff. Instead there would be a continuous flow. Therefore our aim is to study how it can affect the viability of the bronchial cells.

In Table I, we show the $IC_{50}$ values obtained from duplicate results. At 24 hours, the $IC_{50}$ values ranged from 1.12 to 70%, making some e-cigarettes based on menthol, tobacco and butterscotch flavours the most toxic ones (Figure 1). The same pattern seems to be repeating itself at 48 and 72 hours with ranges between 6.3-40% and 1-92% respectively. Propylene glycol seems to show more toxicity when the cells were exposed at longer times (48 and 72 hours), while nicotine was quite consistent through the total study. Flavours like grape, blueberry, cherry and some menthol blends produced the lowest toxicity.

To have a clearer understanding of the results, we plotted the $IC_{50}$ values (Figure 1). It is interesting to note that at 24 hours the majority of the samples tested are very toxic with the $IC_{50}$ values in the lower 1/3 band on the y axis. At 48 hours this tendency changes to be more in the middle band and at 72 hours there is a clear tendency for higher toxicity (lower band in the y axis). It is interesting to note the e-cigarettes

flavoured with vanilla and grape are the less toxic samples. Icemint can be a complex mixture and this is observed in the increasing toxicity it showed on the cells.

Pictures of each sample at dosages of 200, 120, 10, 4 and 0% puffs (a summary in Figure 2 and the remaining pictures and $IC_{50}$ curves in the Appendix) were taken. In Figure 2, the pictures show the cells exposed to a low puff concentration (10% of puff) for the longest period of time (72 hours).

In our set of 18 e-cigarette refill samples, we studied different fruit flavours as well as candy flavours such as butterscotch and bubblegum. The butterscotch flavour when inhaled has been found to be responsible for a particular lung condition in employees working in popcorn factories, called popcorn lung[44]. Exposure to diacetyl in the butterscotch flavour in the working environment affects the middle and lower airways producing cough, dyspnea, and bronchiolitis obliterans (Maier). This flavour has been discontinued in the market of electronic cigarettes. However, reports (Hartun short) express concerns as this is found in around 75% of all e-liquids samples. Alternatives have been proposed (such as 2,3-pentadione, 2,3-hexanedione, 3,4-hexanedione and 2,3-heptanedione) and studied on murine models. Results indicate that they might not be completely safe (Anderson). The sample we obtained were shown to be very toxic, with $IC_{50}$ values for 1, 2 and 3 days of exposure around the value of 10% of a puff (0.5µL). The pictures clearly show how cell numbers are low and the cells are very elongated when compared to cells exposed to the media only. The other candy flavour, bubblegum, though toxic, had $IC_{50}$ values in the range of 20 to 30% of a puff, with cell numbers higher and displaying a slightly rounder shape.

A popular flavour, vanilla, seems to be gaining territory in the market. In our testing of a sample of vanilla refill, we found the $IC_{50}$ values to be moderately toxic at 24 and 48 hours of exposure (~20%) and much less toxic at 72 hours (80%). This implies that the cells can recover with time if not exposed continuously. The cell numbers in the

photographs (appendix) not only showed higher survival rates, but the cells were forming islands which is characteristic of lung type cells. Menthol and mint are very popular flavours, so we tested one sample of mint (icemint) and three samples of menthol. We found that the mint flavour was low in toxicity after 1 day of exposure ($IC_{50}$ = 70%), but became more toxic the longer the cells were exposed ($IC_{50}$ in the range of 30-20%). Furthermore, the cells also looked very unhealthy after the initial times of exposure, but showed remarkable recovery towards the 72 hour period of incubation. It is possible that the cells managed to metabolise the toxic contents to less damaging agents. The menthol samples were in general very toxic with $IC_{50}$ values for all incubation times lower than 20%. The cells appeared elongated and in the majority of cases quite isolated. It is interesting to notice that samples from different suppliers have different toxicity, giving rise to questions as to what the ingredients are or at least the percentages in those refills.

Electronic cigarettes, as a relatively healthier option, have much less ingredients than a tobacco based cigarette. Nevertheless, because the tobacco flavoured e-cigarette is popular amongst consumers, we tested 4 samples of tobacco based e-cigarettes with different concentrations of nicotine. We found them all to be quite toxic. For example, the classic tobacco flavour has $IC_{50}$ values around 10%, and for tobacco with nicotine it was around 10-30% in a sample of virgin tobacco. It also became very toxic the longer the cells were exposed to the liquid. The cells looked much damaged at high concentrations and short exposure times, showing a very flat and elongated appearance towards the end of the experiment.

Fruit based flavours are very popular with younger generations. Suppliers might use natural or synthetic flavours to produce the desired flavour. From all the samples tested, except Dekang Cherry Blossom, they did not have nicotine in the ingredients list. This could explain why in general these samples were less toxic. We tested refill

flavours of banana, blueberry, grape, apple, strawberry and cherry. We found them to be moderately toxic ($IC_{50}$ values in the vicinity of 30%), with the grape flavour being the least toxic one. Nevertheless, at high concentrations of refill liquid and short exposure times the cells look disperse, elongated and damaged. Towards the end of the trial, the cells looked healthier and formed some islands, thus showing better recovery.

We tested stock solutions of nicotine and the propylene glycol carrier control. We found nicotine to be moderately toxic in the range of what it would be expected to appear in puffs ($IC_{50}$ values between 15-30%). We also found that propylene glycol became increasingly toxic the higher the volumes of the puff and the longer the exposure times were ($IC_{50}$ values between 5-15%). In both cases cells looked unhealthy with a tendency to recover.

Interestingly, in a study performed on HaCat (normal human immortal keratinocytes), HN30 (human neck squamous cell carcinoma from a primary laryngeal tumour) and UMSCC10B (human neck squamous cell carcinoma from a metastatic lymph node) for which vapour of e-cigarettes with and without nicotine were tested, found a ~1.5 fold for samples without nicotine and up to 3 folds for samples with nicotine increased cell death when the DNA strand breaks were tested (Yun). Extrapolating the results from *in vitro* to *in vivo* does not seem to be an easy subject, as there are many variabilities in the e-cigarette delivery due to different electronic devices. Some research points in the direction of the nicotyrine hypotheses (Abramovitz). This chemical, a product of the oxidation of nicotine, seems to accumulate in e-liquids with time when it is exposed to air. It is a reversible inhibitor for CYP2A13 in the nasal and respiratory epithelia, and irreversible inhibitor of CYP2A6 in the liver. The hypothesis postulates that nicotine is delivered more effectively if nicotyrine is present because it facilitates the absorption in the airway epithelia (by inhibiting CYP2A13) and inhibites nicotine's metabolism in

the liver (by inhibiting CYP2A6). It therefore raises the nicotine's plasma concentration and hence relieve the nicotine craving (Abramovitz). Though data seems to support it, more evidence needs to be acquired.

**Analytical data**

We studied the 18 samples for their composition as well as nicotine content. Using state-of-the-art mass spectrometry equipment, we developed new liquid-chromatography methodologies to test the ingredients and analyse the content of the main toxicant. An Accucore HILIC column was employed, and in it, nicotine showed a retention time of 7.55min. The sample was measured using MS/MS fragmentation 163.1230m/z → 131.0650 m/z with CE of 30eV. A dilution curve with a highest amount of 25 mg/ml was prepared to quantify the areas related to the nicotine content. The results (Figure 3) showed that the samples which according to the manufacturer's labels should be nicotine free, had quantifiable levels of nicotine within them. The majority of them had extremely low amounts in the low ppm level (part per million), though some such as butterscotch had 0.015% and juicy apple 0.03%. On the other hand, the levels of nicotine in samples which have stated nicotine contents (according to the manufacturer's labels), vary depending upon the producer. The Nicolite brand, showed a large variation in the analysed to stated amounts, e.g the 11mg/ml sample showed values close to 9mg/ml. The 16 mg/ml samples that had different flavours such as tobacco and menthol, ranged from approximately 6mg/ml to almost 12 mg/ml. The other manufacturers, Dekang and Vapouriz, for which the labels described a content of 18 mg/ml, varied in the range of 16 mg/ml to 18 mg/ml. The levels of nicotine are likely indicators that GMP (good manufacturing practices) is not being followed by some manufactures of the e-cigarette fluids, and may run afoul of the current manufacturing guide lines set by the European Union[45]. It is interesting to notice that

nicotine based e-cigarette refills showed the highest toxicity with $IC_{50}$ values ranging from 3 to 25 % puff for the 72 hours period of incubation on the Beas2B cells.

One of the most concerning flavour ingredient in e-fluids is the butterscotch flavouring, i.e diacetyl flavouring (butane-2,3-dione, also a diketone), (1.2min retention time)) which is known to produce lung disease when inhaled[44,46]. We analysed the AV Butterscotch flavour e-cigarette refills, and we found the content of diacetyl (presented at a retention time of 1.2min) was 10625 molecular count which in real terms means traces. This comes as good news for e-cigarette smokers, as other flavourings can be used to mimic the butterscotch flavour or aroma. Nevertheless in this particular e-cigarette refill sample, the biological data showed high toxicity in the biological assessment, implying that the flavouring agent, maybe another member of the diketones family, is also toxic[47].

Tobacco flavours are extremely popular as it might give the e-cigarette smoker the sensation of a real cigarette, but without the toxins. We investigated four samples of refills containing tobacco flavour and we found (Figure 5, the results are presented as molecular counts and they are actually traces in the low ppm of flavours only) they contain traces of several other chemicals, including flavouring agents such as vanillin, ethyl butyrate (tropical flavour), ethyl vanillin, ethyl-methyl-maleimide (tobacco), β-damascone (fruit), butanedione (butter) and benzyl alcohol (fruit). Investigation of the flavour profile of the tobacco flavoured e-fluids showed that it is possible to “fingerprint” the different manufacturer batches. While the number investigated was small it does open up the possibility of a data base for forensic analysis of e-cigarette analysis. Overall we found the e-cigarette refills contain around 99% of the carrier, with this being generally propylene glycol, up to 0.8% of nicotine (near 0% in the free nicotine refills), 0.018% sweetener (in the form of maltol / ethyl maltol and other sweetening flavours) and 0.002% of flavouring agents, including unknowns. It is this 0.002% that would help

to fingerprint a sample. Our data is in good agreement with other studies (Tayyarah) that have reported e-cigarette liquid to contain glycerol or propylene glycol ≥75%, water ≤18%, nicotine ~2% and flavours ~10%. All the tobacco samples proved to be highly toxic with the Vapouriz ones presenting the highest cell death rate. Incidentally, they have less ethyl-methyl-maleimide though they have higher levels of vanillin based flavour. The small difference in the tobacco samples for the $IC_{50}$ could be due to unknown ingredients in the refill, as many times manufacturers use natural or complex extras.

**Conclusion**

Research by Action on Smoking and Health (ASH) showed e-cigarettes users has rapidly increased[48] with the teenage group increasing 800% (Kaisar). E-cigarettes are considered as one of the options helping people to quit smoking. However, the safety and reliability of e-cigarettes have to be reviewed extensively. Many of the literature reports reviewed, indicate that e-cigarettes are not free of emissions[13,14,16], as they release an aerosol containing acetaldehyde, formaldehyde, nicotine, propylene glycol, glycerol and flavourings. Users and those who are exposed to second-hand inhalation can be affected[16,36]. Our work supports the opinion that e-cigarettes and especially the ingredients of the e-liquid, which can change in structure after the process of heating, have not been thoroughly characterised or evaluated for safety[37]. The evaluation of the results of this investigation supports our hypothesis that certain flavours of e-liquids, like menthol, tobacco and coffee are more toxic than others such as banana or apple, which show less toxicity on Beas2B cells by direct liquid exposure.

In a previous study the cytotoxicity of e-cigarette refill samples using human embryonic and adult cells, showed that majority of samples were moderately to highly toxic to the embryonic cells, but less toxic on the adult cells. Also, the cytotoxicity was correlated to the other components of the fluids rather than the presence of nicotine[37]. In another

study, the cytotoxicity of liquid (smoke) flavourings was assessed and compared with that of cigarette smoke condensate. It was found that the cigarette smoke condensates were generally less toxic than liquid smoke flavourings on Chinese Hamster Ovary cells (CHO)[38]. Published results have shown in *in vitro* studies that human bronchial cells exposed to different e-cigarette vapours had mutations in the gene patterns, similar to exposure to tobacco smoke[49].

The existing research does not indicate that e-cigarettes are completely safe, even though the delivery of nicotine without the toxins found in tobacco cigarettes makes them a safer option. E-cigarette vaping is less toxic than smoking normal cigarettes, and this group of users benefit from this new technology. Nevertheless, e-cigarettes contain toxicants, including nicotine, flavourings and volatile compounds, and their thermal degradation products.

We have clearly shown that flavours such as menthol, tobacco, and butterscotch can be considered toxic. However, the assumption that e-liquids with nicotine, especially with higher concentrations of 16 mg/ml plus, could be more toxic than the one without nicotine, could not be proven. Nevertheless, e-liquids such as blueberry and tobacco are more toxic with a lower $IC_{50}$-value then e-liquids with nicotine.

Public Health England (PHE) has endorsed the use of e-cigarettes to help smokers to quit the habit (McNeil). Evidence seems to indicate that smoking electronic cigarettes is healthier than traditional tobacco cigarettes, so for the traditional cigarette smoker this is a good option, especially if it allows overall quitting. However, concerns have been raised for the passive smoker and the younger generations who find smoking e-cigarettes an exciting new habit (Monks, McKee). Politics, policies and funding seem to play an important role in the evaluation of the safety of e-cigarettes. Therefore, more independent, long-term research needs to be conducted to determine how safe e-cigarettes really are (McKee).

The work reported in this paper further contributes useful and new information to debate on the safety of e-cigarettes and the different flavouring liquids consumed by users in the devices, and clearly indicate some areas of concern which warrant closer attention in future. This in agreement with a recent clinical trial in where several toxicants biomarkers (nicotine and metabolites) from both traditional and e-cigarettes were monitored and shown the exposure was reduced upon switching.Reference?

Acknowledgements

This research was funded by the University of Salford and Manchester Metropolitan University through their Bidding Research Support. We would like to express our gratitude to: Dr Nanda Puspita, Ms Basma Al-Sudani and Ms Nasrin Ahmed for their help with tissue culture at the University of Salford, Professor Marija Krstic-Demonacos for their comments and the Erasmus Exchange Programme for part-funding the stipendiary of Ms Jasjot Singh and Ms Emilie Luquet.

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Table I: $IC_{50}$ values of different e-cigarette refills tested on Beas2B at 24, 48 and 72 hours. (Results for duplicate determinations). *: these samples do not contain nicotine. [1]: ($IC_{50}$ in µM, ± SD)

| **$IC_{50}$ (% of puffs, ± SD)** | **24 hours** | **48 hours** | **72 hours** |
|---|---|---|---|
| Aulola Butterscotch* | 7.4 ± 5.2 | 10.1 ± 3.6 | 9.2 ± 1.7 |
| Vapouriz Bubblegum* | 28.3 ± 0.6 | 26.3 ± 10.5 | 17.1 ± 2.1 |
| Vapouriz Vanilla Velvet* | 25.5 ± 1.6 | 19.3 ± 0.9 | 79.0 ± 0.3 |
| Vapouriz Banana* | 12.5 ± 1.7 | 39.8 ± 0.4 | 26.0 ± 0.9 |
| Vapouriz Grape* | 32.7 ± 5.8 | 29.7 ± 9.7 | 91.6 ± 0.2 |
| Dekang CherryBlossom18mg/ml | 20.9 ± 4.0 | 22.8 ± 1.8 | 24.7 ± 0.8 |
| Vapouriz Blueberry* | 28.5 ± 1.8 | 12.3 ± 3.8 | 30.1 ± 8.8 |
| Dekang Blueberry Mist* | 37.6 ± 0.1 | 24.5 ± 0.1 | 21.0 ± 1.1 |
| Vapouriz Strawberry Bliss* | 20.8 ± 0.3 | 17.3 ± 0.1 | 18.2 ± 0.6 |
| Vapouriz Juicy Apple* | 29.8 ± 6.3 | 21.5 ± 3.5 | 28.7 ± 0.6 |
| Nicolite Menthol 16mg/ml | 8.7 ± 0.9 | 6.3 ± 2.6 | 2.8 ± 14.4 |
| Dekang Menthol 18mg/ml | 21.7 ± 0.6 | 10.8 ± 2.4 | 12.3 ± 1.2 |
| Vapouriz Menthol Special blend | 1.1 ± 1.6 | 18.4 ± 1.7 | 22.2 ± 4.1 |

| | | | |
|---|---|---|---|
| Vapouriz Icemint | 68.9 ± 0.1 | 32.9 ± 0.9 | 19.3 ± 0.8 |
| Vapouriz Classic Tobacco | 4.3 ± 7.3 | 9.5 ± 3.9 | 11.9 ± 6.5 |
| Vapouriz Virgin Tobacco | 30.9 ± 5.8 | 15.2 ± 0.8 | <1 ± 2.19 |
| Nicolite Tobacco 11mg/ml | 21.9 ± 1.2 | 36.9 ± 0.3 | 11.5 ± 2.5 |
| Nicolite Tobacco 16mg/ml | 31.0 ± 3.2 | 11.9 ± 14.7 | 24.7 ± 7.6 |
| Nicotine stock 18mg/ml | 32.3 ± 1.5 | 27.2 ± 1.8 | 13.9 ± 2.9 |
| Propylene glycol stock 1g/ml | 14.7 ± 5.4 | 9.5 ± 19.2 | 5.7 ± 2.2 |
| Chlorpromazine [1] | 3.1 ± 9.0 | 1.5 ± 4. 7 | 1.0 ± 0.2 |

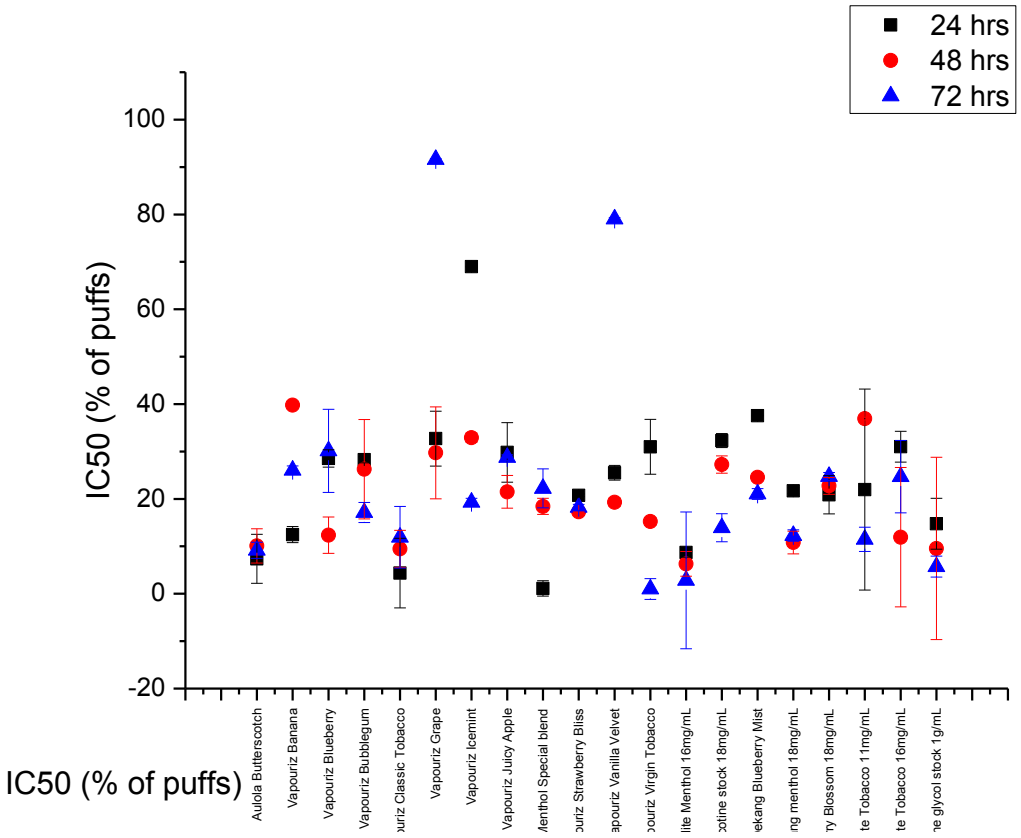


Figure 1: $IC_{50}$ values at 24, 48 and 72 hours.

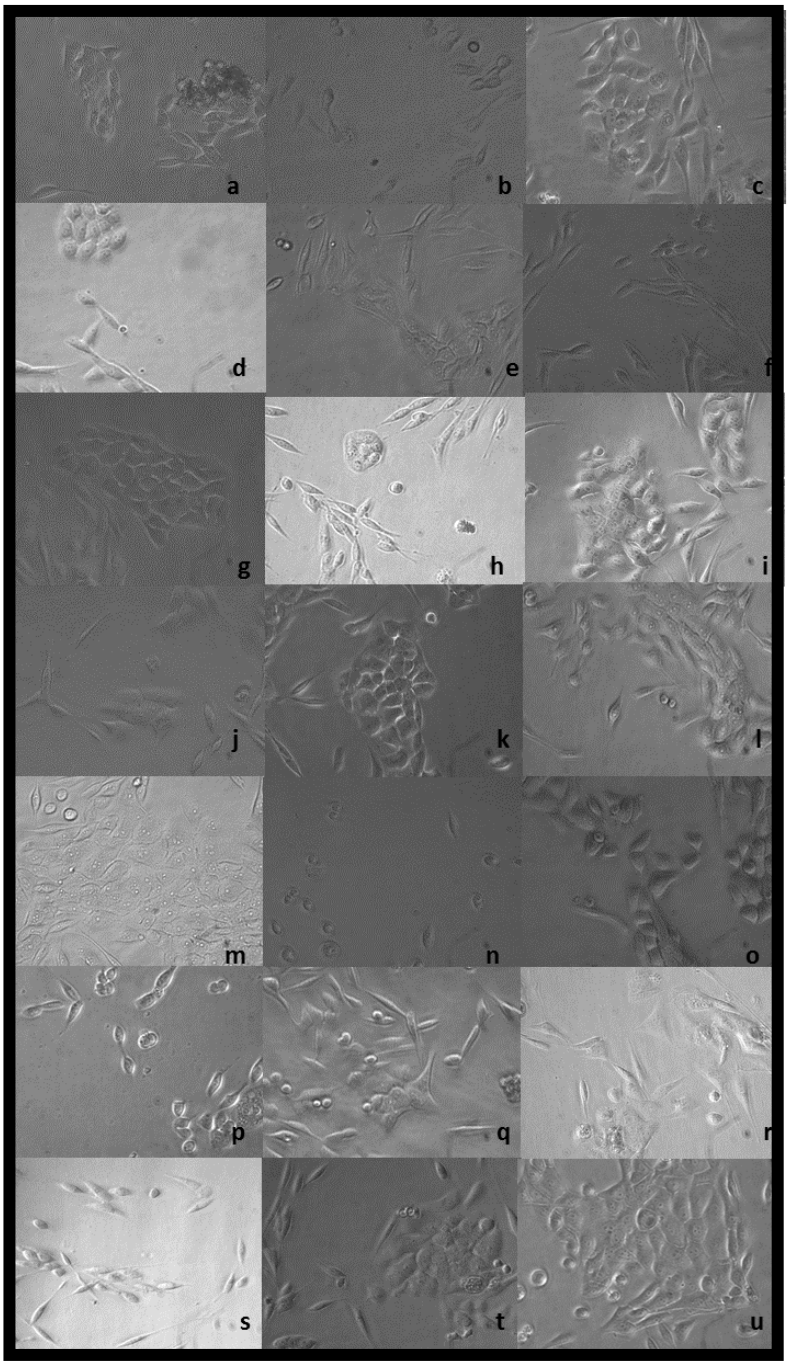


Figure 2: Cells exposed to 10% (0.1) puff for 72 hours: a: Aureola Butterscotch, b: Vapouriz Banana, c: Dekang Blueberry Mist, d: Vapouriz Juicy Apple, e: Vapouriz Bubblegum, f: Vapouriz Grape, g: Vapouriz Strawberry Bliss, h: Nicolite Menthol 16mg/ml, i: Vapouriz Vanilla Velvet, j: Dekang Cherry Blossom, k: Vapouriz Blueberry, l: Dekang Menthol, m: Nicolite Tobacco 16 mg/ml, n: Vapouriz Virgin Tobacco, o: Nicolite Tobacco 11mg/ml, p: Vapouriz Classic Tobacco, q: Vapouriz Icemint, r: Vapouriz Menthol Special Blend, s: Nicotine stock (pharmaceutical grade) (0.08mg/ml), t: Propylene Glycol stock (0.005g/ml), u: Media

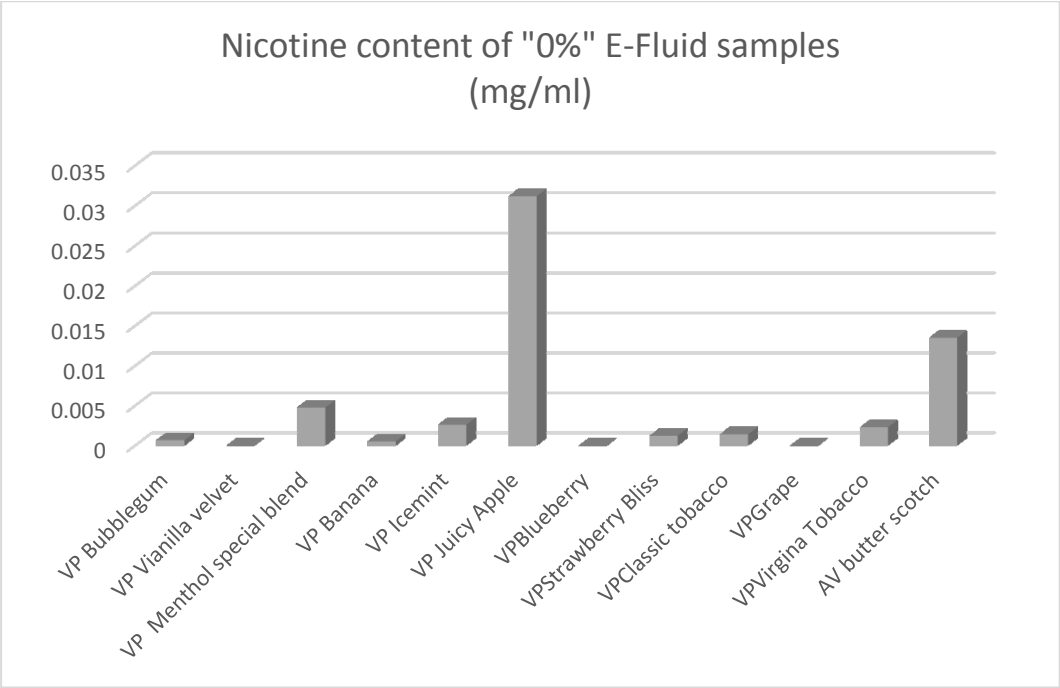


Figure 3: Analytical determination of nicotine in the e-cigarette refills for which the nicotine content is 0 (zero) according to the package information.

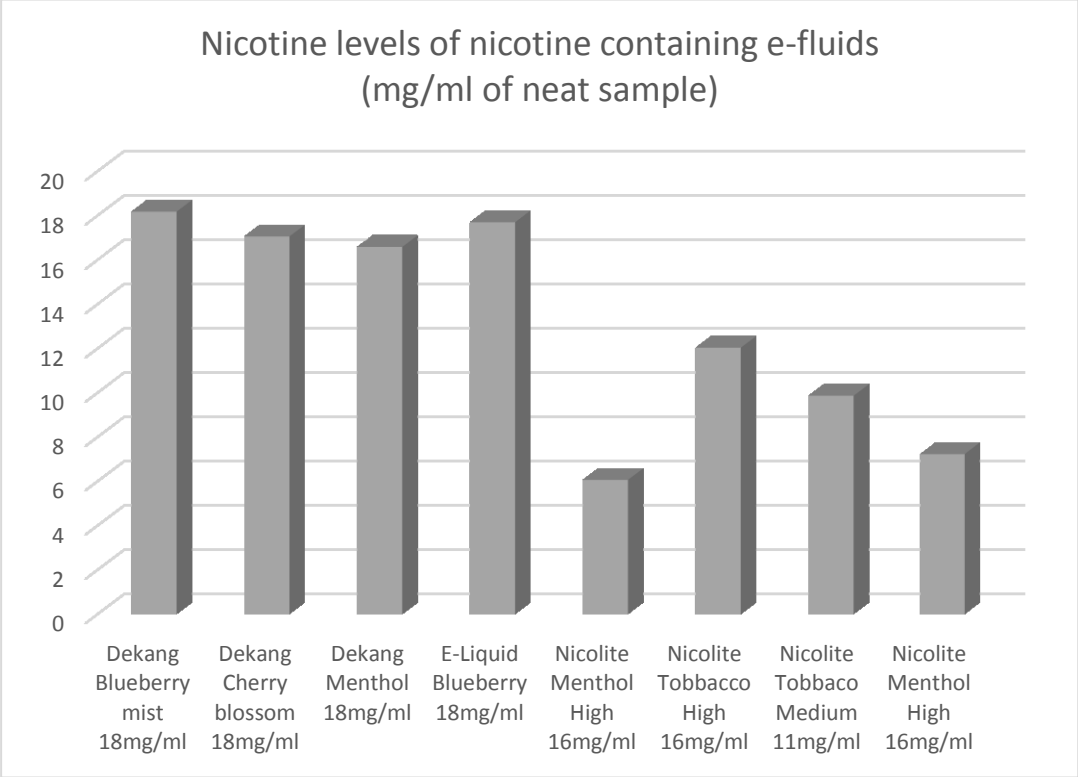


Figure 4: Analytical determination of nicotine in the e-cigarette refills for which the nicotine content varies from 11 mg/ml to 18 mg/ml according to the package information.

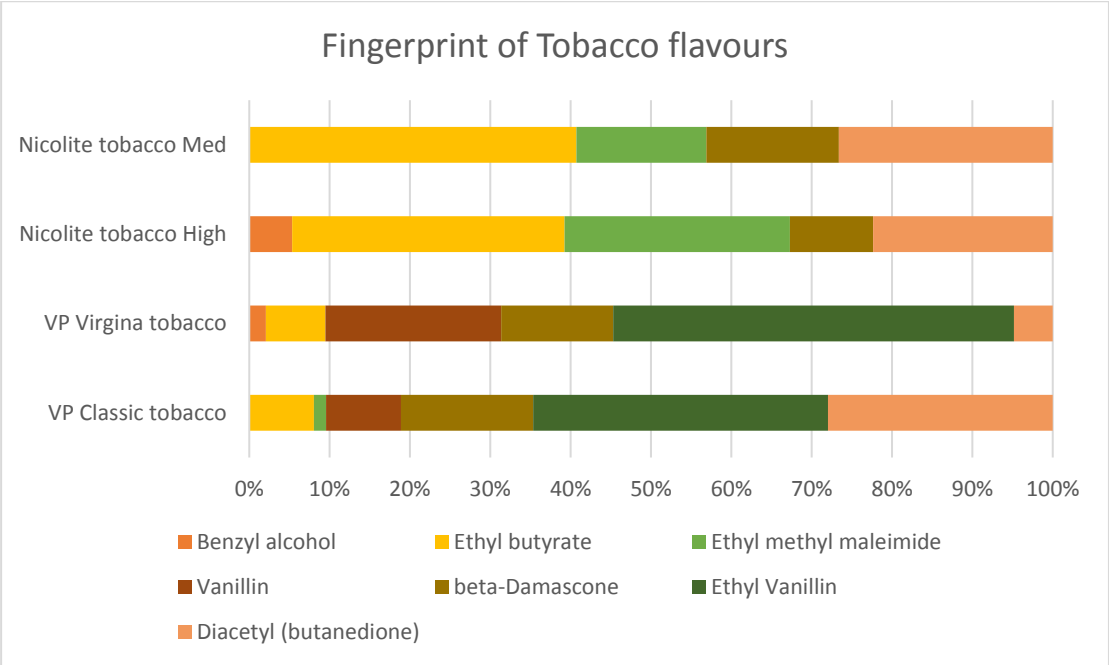


Figure 5: Analytical determination and fingerprint analysis of tobacco flavour in the e-cigarette refills.