Characterisation of the volatile sensometabolome in human breath of cigarette smokers, electronic cigarette users and non-smokers by Aroma Extract Dilution Analysis

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Abstract

Smokers are known to have a characteristic smelling and long-lasting breath after the consumption of a cigarette. However, the responsible compounds for this malodour have not been fully explored yet. Therefore, the aim of the present study was to characterise key aroma compounds in the breath of cigarette smokers and compare the resulting aroma profile to electronic cigarette (EC) users'- and non-smokers' (NS) breath before and after product consumption by application of aroma extract dilution analysis (AEDA) in combination with gas chromatography olfactometry (GC-O).

Interestingly, the breath of cigarette smokers revealed a significantly higher intensity in the overall aroma, resulting in higher flavour dilution factors, in comparison to the breath of non-smokers or electronic cigarette users. This was predominantly caused by a high number of aroma-active pyrazines. Exhibiting an earthy, musty smell, these combustion products can still be found in breath one hour after smoking and are hypothesised to be responsible for the characteristic 'ashtray' smell. These findings align well with results of a study on cigar smokers' breath by Bazemore et al. who suggest that due to their structure, pyrazines are trapped in both mucosa and saliva leading to a long-lasting 'smokers-breath. In comparison, the breath of EC users revealed similar aroma profiles to the ones of NS even immediately after consumption. In doing so, these data suggest that EC use may have personal and social consideration benefits.

Keywords: Aroma, GC-O, Breath, Vaping, Smoking

Introduction

Tobacco is a stimulant which was known to the inhabitants of Mesoamerica long before it was first described in literature over 400 years ago [1]. The reason for the enduring popularity of the tobacco plant (*Nicotiana Tabacum*) is its high content of nicotine, a highly addictive drug exhibiting positive psychological effects [2]. Today, the most common form of tobacco consumption is as cigarette, with more than one billion cigarette smokers worldwide [3].

Cigarette combustion is a complex process, which leads to a vast spectrum of chemical compounds in the aerosol of smoked tobacco [4]. In fact, more than 5000 substances have been identified in cigarette smoke [5]. It is common knowledge that cigarette aerosol contains a high number of harmful substances, however, pyrolysis of tobacco also leads to large variety of aroma active compounds that can be found in cigarette smoke.

Some of these substances cause an undesired malodour in the breath of smokers after the consumption of a cigarette which is long-lasting and often described as 'ashtray-like' or 'smokers' breath'. Bazemore et al. identified several pyridines and pyrazines in the saliva of cigar smokers, which may contribute to the characteristic breath odour [6].

In recent years, a new technology for nicotine consumption by inhalation was developed [7]. Electronic cigarettes (or e-cigarettes) are electronic nicotine delivery systems (ENDS) that do not contain tobacco, nor undergo combustion [8, 9]. E-cigarettes have become increasingly popular over the past decade as alternative to conventional tobacco smoking [9, 10]. Instead of pyrolysing natural material, these battery-operated devices heat up a cartridge which typically but not exclusively contains nicotine, humectants and a flavour mixture to create an aerosol that is chemically very different to tobacco smoke [9, 10]. Even though e-cigarette users do not seem to exhibit a characteristic aroma profile in their breath such as smokers, no scientific data have been reported to date to verify this hypothesis.

Therefore, the aim of the present study was to identify differences in the aroma profiles of breath in e-cigarette users (EC) and cigarette smokers (CS) by gas-chromatography-olfactometry (GC-O) and compare them to non-smokers (NS) who do not use either of both products.

When it comes to the sampling of volatiles in human breath, several methods have been deployed. Breath volatile organic compounds (VOC) are commonly present in very low levels, and thus, efficient sampling and concentration is crucial [11]. A frequently reported method to collect and concentrate breath VOCs describes exhalation into polymer bags with consequent trapping of volatiles onto thermal desorption (TD) tubes [11]. This approach, however, is not suitable for most GC-O instruments, which are not commonly set up with a thermal desorption unit. A different methodology that has been described to identify aroma active compounds in human

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breath by GC-O used nylon mesh coated swabs on the subject's tongue surface, which was consequently concentrated via solid phase micro extraction (SPME) [6]. While this approach is simple and fast, substances that are not trapped in the saliva or the mucosa of the tongue may be neglected.

Thus, a new method involving the trapping of breath VOCs on SPE cartridges with consequent elution, concentration and GC-O analysis has been developed for the aim of the present study.

Experimental

Panellist Recruitment

A group of nine healthy individuals, both male and female between 26 – 52 years old, three of each group: non-smokers (NS), electronic cigarette users (EC) and cigarette smokers (CS) were recruited to participate in the study. Panellists were asked to use their own preferred product for the study. Furthermore, they were asked not to use their product, eat or drink anything apart from water within 60 min prior to breath sampling.

Prior to the study, participants gave their informed consent for participation.

Sample Preparation

Breath samples were taken at time points T_0 (prior to consumption), T_1 (5 minutes post consumption) and T_2 (60 minutes post consumption), with NS following the same sampling pattern without consuming a product. After the first sampling step, participants were asked to consume their product in an allocated testing room for 10 minutes.

In order to trap the volatile fraction in breath samples volunteers were asked to breathe normally for 10 minutes and exhale during this period into a Teflon mouthpiece that was connected to an S15 sorbent cartridge (FlavoLogic, Germany). The cartridge was connected via tubing to a vacuum pump. A constant flow rate of 1.6 ml/min was maintained and controlled with a flowmeter that was connected between pump and cartridge.

Following volatile trapping, the compounds were eluted off the sorbent cartridge using 10 ml methylene chloride (p.A., Merck, Germany) and dried over sodium sulphate (anhydrous, Sigma Aldrich, Germany). Samples were transferred into a pointed flask, and individual samples per consumer group and time point were pooled to eliminate inter-subject variability. Finally, the sample extracts were concentrated to 250 µl via gentle distillation at 50 °C. Consequently, the concentrate was diluted in 1:1 steps with methylene chloride. Breath extracts were stored at -16 °C and analysed within 48h of sampling.

Each dilution was assessed at the olfactory detection port (ODP) of the gas chromatograph by two trained assessors in altering order until no smell could be perceived anymore.

Gas Chromatography-Olfactometry/Mass spectrometry

Chromatography was performed using an Agilent 7890 Gas chromatograph fitted with a low thermal mass (LTM Series II) column module coupled to an Agilent 5977 A mass spectrometer (MSD) and a Gerstel olfactory detection port (ODP 3). Liquid samples volumes of 1µl were injected in splitless mode and the inlet was held at 250 °C. Helium was used as carrier gas with a constant flow rate of 1.5 ml/min. Separation of compounds was achieved using a DB-FFAP fused silica capillary column with the following dimensions: 30 m x 0.25 mm i.d. x 0.25 µm film thickness. The column module was programmed to run the following temperature gradient: 40 °C (1 min), with 6 °C/min to 200 °C (0.1 min), then with 10 °C/min to 250 °C (6.5 min). MSD source was held at 230 °C, ODP and GC oven holding uncoated fused silica capillaries were held at 250 °C and the split ratio between the two detectors was 1:1. The MSD was operated in electron impact ionizing mode with an ionizing energy of 70 eV.

Identification of compounds was achieved by matching odour quality and retention index with an internal standard database, at a minimum. Additionally, all aroma active compounds were checked for their mass spectrum and optionally identified using MS NIST library. Compounds that only matched odour quality and a hit within the NIST library were marked as 'tentatively identified'.

Results and discussion

The developed method for breath sample collection in combination with GC-O was efficient for the purpose of the present study. The breath odour of three consumer groups at three different time steps has been assessed.

In total, 113 compounds have been identified as aroma active across samples. A range of compounds have been detected with similar flavour dilution (FD) factors in all samples, such as 3-Ethylphenol, 3-Phenylpropionic acid as well as a range of 'earthy' smelling pyrazines, thus suggesting that these compounds are present in human breath naturally.

Interestingly, all three groups also exhibited differences in aroma profiles in their unstimulated breath, most of which can be explained as individual biological variability in combination with a low number of participants. Though, it is remarkable that CS exhibited a differentiated control breath compared to EC and NS, showing a higher variety in substances with 'earthy' odour characteristics.

Figure 1 shows, however, that key driver for differentiation between samples is stimulation, with the largest shift within CS at T_1, immediately after product consumption. Interestingly, this shift is predominantly caused by high FD factors as well as higher numbers of 'earthy' and 'roasted' smelling compounds, thus exhibiting the same odour impression as the previously mentioned unique compounds of CS T_0. Additionally, some 'creamy' and 'savoury' smelling substances were perceived at the ODP that could not be detected pre cigarette consumption, nor in any other consumer group.

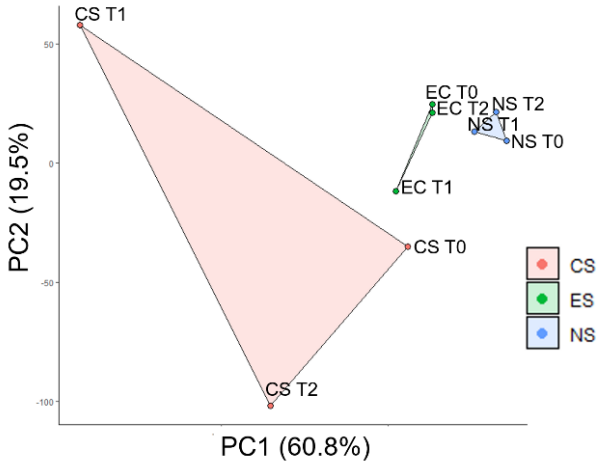

Figure 1*: PCA Score Plot of all samples based on compounds flavour dilution factors.*

The shift observed in EC at T_1 was less prominent. Apart from a 'fruity' smelling compound with an FD factor of 32 that was not found in any other samples, the aroma profile showed only minor changes compared to T_0, which can be explained by the biological variability of assessing sniffers.

The compounds with the highest impact towards the aroma profiles of breath samples in T_1 across groups are listed in Table 1.

Table 1: Key compounds (FD >16) in T_1 across groups.

Compound	RI (FFAP)	Perceived Odour quality	FD in T_1 samples		
			CS	EC	NS
2-Ethyl-3-methylpyrazine	1424	Roasted, Earthy	128	n.d.	n.d.
3-Ethylphenol	2204	Phenolic	64	64	32
Unknown	1118	Earthy	64	n.d.	n.d.
Ethyl Lactate[1]	1237	Creamy, Fruity	64	1	n.d.
4-Methylphenol	2092	Faecal, Horse	n.d.	n.d.	64
2-Isobutyl-3-Methoxypyrazine	1536	Green bell pepper, Earthy	32	32	4
Unknown	2773	Earthy	32	n.d.	n.d.
Unknown	2044	Sweet, Fruity	n.d.	32	n.d.
2-Ethyl-3,5/6-dimethylpyrazine	1442	Earthy	8	32	2

[1] *Tentatively identified*

While in EC no major difference could be detected at T_2 anymore, CS still show a differentiation to T_0, as seen in Figure 1. The reason for this is again persisting 'earthy' smelling compounds, many of which have been identified as various pyrazines. Compounds of this chemical class have previously shown potential for repartition in the mucosa of the upper airways [12] and they may also be retained by lipocalins in saliva, proteins which have been proven to bind small hydrophobic molecules [13].

Their physiochemical properties, as well as the fact that pyrazines are vastly found in cigarette smoke, are in accordance with the increased detection as well as the elongated duration in CS breath. Additionally, these findings align well with the initially mentioned study, which also suggested pyrazines to have a major impact on the aroma of cigar smokers' breath [6].

Some pyrazines exhibit an extremely low odour perception threshold, such as the in CS identified 2-Isobutyl-3-methoxypyrazine with a threshold of 0.002 ppb in water [14], which may ultimately lead to olfactory detection in the exhaled breath of smokers over time, even at low retention and release levels in the oral cavity.

Finally, it is worth noting that none of the in literature described as ‘bad breath’ causing sulphur-containing volatiles could be detected. These compounds have extremely low boiling points and therefore, the described method may not be suitable for their detection.

Conclusion

The aroma profile in cigarette smokers’ breath showed alterations within at least the first hour of smoking. Most of these changes resulted in an increase of ‘earthy’ smelling compounds which may lead to the distinctive ‘smokers’ breath’. Neither electronic cigarette users, nor non-smokers exhibited those long-lasting changes. However, the panel of participants in the study was rather small and changes in breath aroma could be detected even in non-consumers. Furthermore, some unique compounds were seen in each group, which leads to the conclusion that the biological inter-subject variability is large.

In addition, GC-O methods are strongly dependent on the individual perception and performance of sniffers, which leads to large error margins. Though, GC-O is crucial for the identification of aroma active compounds and the present study led to the identification of chemical classes that play a role in the breath aroma of the three consumer groups, which delivers the basis to further targeted investigations with a larger cohort of participants as well as a robust quantitation method that includes volatile sulphur compounds to provide detailed aroma profiles across consumers.

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