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Volatile composition of the morning breath

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Abstract

We have measured the composition of volatile organic compounds (VOCs) in the morning breath of 30 healthy individuals before and after tooth brushing. The concentrations of VOCs in the breath samples were measured with proton-transfer-reaction time-of-flight mass spectrometry (MS) and further identification was performed with a combination of solid phase microextraction and offline gas chromatography–MS. We hypothesize that compounds, whose concentrations significantly decreased in the breath after tooth brushing are largely of microbial origin. In this study, we found 35 such VOCs. Out of these, 33 have been previously connected to different oral niches, such as salivary and subgingival bacteria. We also compared the concentrations of the 35 VOCs found in increased amounts in the morning breath to their respective odor thresholds to evaluate their ability to cause odor. Compounds that could contribute to the breath odor include many volatile sulfur compounds, such as methanethiol, hydrogen sulfide, dimethyl sulfide, and 2-methyl-1-propanethiol, but also other VOCs, such as acetic acid, butyric acid, valeric acid, acetaldehyde, octanal, phenol, indole, ammonia, isoprene, and methyl methacrylate.

1. Introduction

The origin of volatile organic compounds (VOCs) in the human exhaled breath is complex. Exogenous sources, such as smoking, or the intake of food can affect breath concentrations of many compounds. Similarly, many regular metabolic processes in the human body produce volatile compounds, which can enter exhaled breath. In addition, impaired function of organs can change the breath volatile composition as well [1, 2]. Another important source of breath VOCs is the oral cavity, where bacteria metabolize sugars and amino acids to, among other things, volatile sulfur compounds (VSCs), volatile nitrogen compounds and volatile fatty acids [3–8]. Majority of these compounds are thought to be malodorous and to cause halitosis, known as 'bad breath' [3, 9–11]. In most early reports, VSCs are recognized as the main contributors of halitosis, especially two small, distinctively odorous compounds hydrogen sulfide (H_2S) and methanethiol (CH_3S) [12–15]. In addition, some other sulfur compounds, such as dimethyl sulfide (DMS), have been suggested as minor contributors to intra- and extra-oral halitosis [15, 16]. Malodorous breath in the morning, better known as 'morning breath', has been connected to the excess amount of these sulfur compounds accumulated in the oral cavity [17, 18]. Only a few early reports discuss the role of other VOCs, sulfur or non-sulfur, in halitosis and even fewer demonstrate it [19, 20]. Consequently, most of the later research on halitosis has targeted only these few VSCs, instead of the complete volatile composition of bad breath. As a result, reports suggesting the importance of non-sulfur compounds in halitosis are few and not confirmed *in vivo*. Motivated by this, we wanted to investigate the composition of morning breath in more detail and to evaluate the potential role of different VOCs in oral malodor, including but not limited to VSCs.

Aside from examining oral malodor, VOCs emanating from the oral cavity could also be used to assess its health. Many of the bacterial species connected to such oral diseases as periodontitis and dental abscesses can produce significant amounts of VOCs *in vitro* [3–5]. Salivary and cariogenic bacteria, as well as the tongue microbiome, are also known to produce a variety of VOCs [5–8]. Compounds such as butyric and propionic acid have been found in increased amounts from the gingival crevicular fluid and plaque of patients with chronic periodontitis, with the concentrations correlating with the severity of periodontal disease [21, 22]. Higher levels of methanethiol and hydrogen sulfide in breath can indicate poor periodontal health [15], and these compounds have also been connected to abscess formation [23]. Bacterial volatile compounds have potential as biomarkers for oral infections, especially because they can be measured non-invasively from the exhaled breath or the salivary headspace. However, we must first confirm the bacterial origin of the potential biomarkers and connect them to correct microbiomes both *in vitro* and *in vivo*. Consequently, we aimed to investigate the possible connection between the VOCs of morning breath and oral bacterial activity.

In this study, we connected the results of our previous *in vitro* VOC measurements from oral bacteria to *in vivo* measurements of human exhaled breath. We examined the changes in breath volatiles of 30 healthy individuals in the morning, before and after tooth brushing, to identify which compounds were significantly affected by oral cleaning. Proton-transfer-reaction time-of-flight mass spectrometry (PTR-ToF-MS) was used for measuring the concentrations of VOCs in the samples, and solid phase microextraction (SPME) combined with gas chromatography (GC)–MS was used for further identification of the compounds. We concentrated specifically on compounds, whose concentrations decreased significantly after tooth brushing as these were most likely of oral bacterial origin. We also examined whether any of these compounds have been previously connected to oral microbiomes *in vitro* or *in vivo*. In addition, we evaluated the contribution of these compounds to oral malodor.

2. Material and methods

2.1. Participants

A total of 30 participants (15 men, 15 women) took part in this study. All participants were generally healthy, non-smoking individuals between the ages of 21 and 63 (age mean 35 years). The participants' oral health was not assessed prior to enrolment by a dentist, however, people with diagnosed prior or existing periodontitis were excluded. Other exclusion criteria included any general health issues, such as diabetes, kidney disease, or heart disease, as well as smoking. In addition, people over 65 years old were excluded from the study, due to putatively increasing oral health problems with age.

All participants gave their written informed consent to participate in the study. The research was approved by the coordinating ethics committee of internal medicine in hospital district of Helsinki and Uusimaa (reference numbers 491/E5/2006 and 238/13/03/00/15).

2.2. Breath sample collection

One sample of the morning breath and one sample after brushing the teeth were collected from each participant. Participants did not eat or drink before the sample collection. The first breath sample was taken straight after waking in the morning and the second sample after brushing the teeth the same morning. Participants were instructed to brush their teeth as they normally would without any restrictions or additional instructions. The breath samples were collected by the participant themselves at home. Afterwards, the participant delivered the samples to the laboratory for analysis. Samples were stored in room temperature.

Breath samples were collected by having participants exhale into commercial aluminum-coated sampling bag of 1.3 l volume (Wagner Analysen Technik, WT 8004). Bags were cleaned with pure air prior to sample collection. The concentrations in the cleaned bags were checked after each cleaning. Participants were asked to hold their breath for 5 s and then to exhale into the collection bag through a disposable one-way mouthpiece until the bag was full. The mixture of breath gas from the oral cavity, trachea, lower airways, and alveoli was thus collected. All breath samples were analyzed within 8 h from the collection.

2.3. Breath sample analysis

2.3.1. PTR-ToF-MS for measurement of VOC concentrations

Breath samples were analyzed with a commercial PTR-ToF-MS instrument (PTR-TOF 1000, Ionicon). PTR-ToF-MS is an online MS technique, which uses chemical ionization to enable the measurements of individual VOCs down to parts per trillion (ppt, 10^{-12}) levels. The PTR-ToF-MS analysis methodology and operation conditions have been previously reported in detail [3, 4]. Field density ratio (E/N) of 116 Td (corresponding to drift tube voltage of 500 V) was used in this study.

Sample bags were connected to the PTR-ToF-MS instrument via polytetrafluoroethylene (PTFE) tubing. A flow of 96 ml min^{-1} was used to transfer the breath sample from the bag to the instrument. Each bag was measured until almost empty, which took roughly 6 min. Concentrations were averaged over this 6 min period. Mass scans were performed from the mass-to-charge-number ratio (m/z) 17–239 u, with a sampling frequency of one spectrum per second.

The spectral data from each breath sample were used to calculate the mean concentration of different molecular species in the sample, as well as to obtain the measured accurate masses of these species. The accurate masses were compared to their calculated exact masses to aid in the assignment of spectral peaks to correct molecular species. This method was used to distinguish compounds with the same nominal mass (isobaric compounds) but could not be used for compounds with the same exact mass (structural isomers). Information from complementary GC–MS measurements, discussed in the next section, was used for further identification.

To obtain accurate concentrations, the PTR-ToF-MS instrument should be calibrated separately for each individual compound, which in the case of breath samples are numerous. The PTR-ToF-MS instrument in this study was not calibrated specifically for each analyzed compound, and therefore, the concentrations presented in this work should be considered estimates.

2.3.2. SPME and GC–MS for identification of VOCs

The method used has been previously reported in detail [3]. A gas chromatographic instrument (Agilent 6890A) combined to a quadrupole mass spectrometer (Agilent 5973N MSD) using electron ionization was used for all breath samples collected before and after tooth brushing. The specifications of the GC-column used are as follows: the length of 30 m, the inner diameter of 0.25 mm, and the film thickness of 0.15 μm (DB-1701, J&W Scientific). For separation, initial temperature of 40 °C with a 2 min hold, was ramped up to 250 °C with 5 min hold, at 10°C min^{-1} rate. Splitless injection was used, with splitless time of 1.0–3.0 min from injection. The injection port temperature was 240 °C. Helium (99.996% from Linde Gas, Espoo, Finland) was used as a carrier gas at a flow rate of 1.0 ml min^{-1}. The MS operation parameters were as follows: the mass scan range of 20–300 u, ion source temperature of 230 °C, quadrupole temperature of 150 °C, ionization energy of 70 eV, and GC–MS transfer line temperature of 250 °C.

Pre-concentration of volatiles from the breath samples was performed using a polydimethylsiloxane/divinylbenzene SPME Arrow (1.1 mm outer diameter, 120 μm phase thickness, CTC Analytics AG, Zwingen, Switzerland). The SPME Arrow was directed into the sampling bag through the rubber tubing in the bag. The SPME Arrow was revealed for 30 min, during which the compounds in the headspace were concentrated on the sorbent. After this, the SPME Arrow was concealed, removed from the bag, and immediately introduced to the GC-instrument injection port for 2 min for desorption. The GC–MS measurement protocol was simultaneously initiated. At least two empty runs with the SPME Arrow were done in between every individual measurement to avoid contamination from the previous measurement. National Institute of Standards and Technology NIST14 Mass Spectral Library and Analysis Tools were used for the identification of compounds from the GC–MS data.

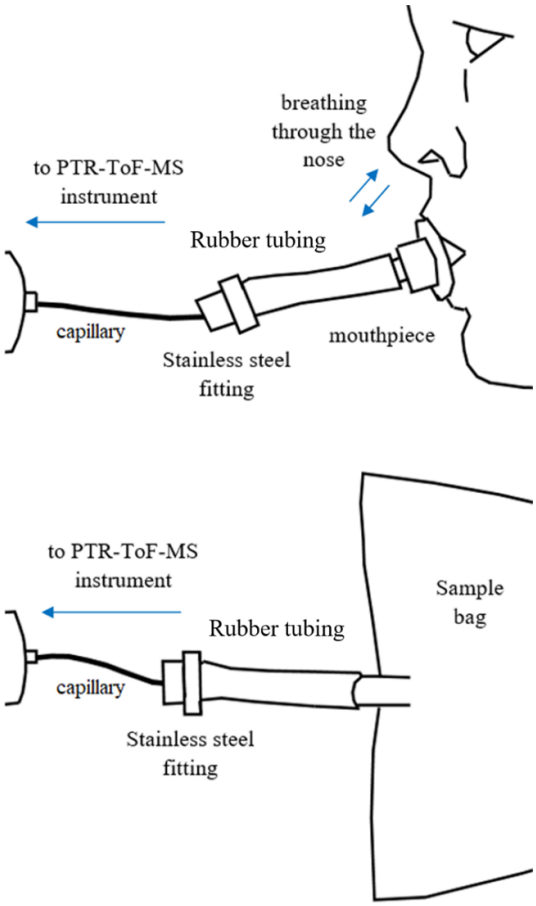

Figure 1. Possible losses of oral VOCs in the sampling bags due to dilution, absorption, or dissolution were tested by comparing two different sampling methods, (a) oral headspace and (b) breath sample bags. Otherwise, the two measurements setups were identical. The individual giving the oral headspace sample kept the mouthpiece in their mouth and breathed through their nose, so that mostly the oral headspace was sampled.

2.3.3. Sampling method validation

The possible losses of breath VOCs in the sampling bag due to adsorption and dissolution, as well as the dilution effect of collecting the mixed breath, were tested by comparing the concentrations measured from sampling bags to those measured straight from the headspace of the oral cavity. Figure 1 describes the two sampling methods. The breath samples were collected as described above, containing mixed air from oral cavity, trachea, lower airways, and alveoli. The mouth headspace samples were collected by connecting a mouthpiece to the measurement instrument with rubber tubing. The mouthpiece was then used as with the bag samples, but instead of blowing into it, the participant held the mouthpiece in their mouth and breathed normally through their nose. The oral

cavity headspace was then measured for 2 min. Concentrations were averaged over this 2 min period. A total of 20 samples for each sampling method were analyzed for two different individuals.

We also evaluated the diurnal variation of VOCs in the breath of four individuals. Bag samples were collected hourly throughout the day, with a total of nine samples per person. The first sample was the morning breath, followed by tooth brushing. The second sample was taken immediately after this. Participants ate breakfast between the second and third samples, and lunch between the sixth and seventh samples. Eating and drinking was not regulated.

2.4. Statistical methods

The paired *t*-test was used to determine, which compounds differed significantly (at confidence level of 99% or 95%) between the breath samples taken before and after tooth brushing.

3. Results

The complete data of all compounds significantly affected by tooth brushing, as well as their respective mean concentrations and standard deviations before and after tooth brushing, p-values, and accurate masses, can be found from the supplementary information (table S1). An example of the PTR-MS measurement from before and after samples and the diurnal variation of breath VOCs of one individual are provided in the supplementary information (figures S2 and S3).

Out of the total 136 compounds identified with GC–MS from the morning breath of 30 generally healthy individuals, 35 were found to significantly decrease in concentration after tooth brushing according to the *t*-test statistics and measured with PTR-MS. Table 1 lists these 35 compounds, and presents their mean percentual decrease after tooth brushing. Table 1 also presents the minimum and maximum concentration of these 35 compounds found among all participants, as well as their respective odor thresholds collected from the literature.

According to the sampling method validation, compounds significantly underestimated in the sampling bags are methanethiol (28% of the concentration in mouth headspace), indole (35%), phenol (46%) hydrogen sulfide (50%), acetic acid (53%), hydrogen cyanide (56%), ethanol (74%) and 2-methyl-1-propanethiol (81%). Compounds significantly overestimated in the sampling bags were isoprene (310%), 1-tetradecene (220%) and 3-methylbutanal (175%). Compounds, whose concentrations were overestimated in the bags are most likely released largely from the lungs, and therefore, are not as abundant in the mouth headspace.

4. Discussion

4.1. Oral bacterial VOCs

One of the main objectives in this study was to search for oral bacterial VOCs (bVOCs) from the breath samples to establish their background levels for healthy individuals and to confirm their microbial origin. According to our hypothesis, the morning breath contains the highest amounts of bVOCs in a healthy subject's breath, because of their accumulation and the undisturbed bacterial activity during the night's sleep. Thus, exhaled breath or the oral cavity headspace in the morning can be used to sample bVOCs *in vivo*. It should, of course be noted that the oral cavity is not static throughout the night and aspects such as salivary flow, swallowing, opening of the mouth etc can affect the concentrations and variety of bVOCs in the breath. Also, the oral cavity cannot be controlled in the same way as the *in vitro* bacterial cultures, where the environment and nutrients can be strictly regulated. Therefore, connecting the *in vivo* situation to the earlier findings from the *in vitro* bVOC measurements [3, 4] can be challenging. However, we believe that examining the composition of the morning breath provides a convenient way to evaluate the bVOCs generated in the oral cavity.

We hypothesize that those compounds whose amount significantly decreases in the breath after tooth brushing are of oral origin. To further examine this, we compare the compounds found in this study in increased amounts in the morning breath to those reportedly produced by oral bacteria *in vitro*. By combining the *in vivo* results from the current study to the *in vitro* results from earlier research, we aim to show which compounds could be of oral bacterial origin. We concentrate here on the bVOCs produced by cariogenic and salivary bacteria, as well as those connected to the tongue microbiome and the periodontal anaerobes. However, it should be noted that studies investigating the VOC production of oral bacteria, pathogenic or otherwise, are still lacking. Therefore, some of the compounds not discussed here could still be from a bacterial origin, but their production has not yet been confirmed *in vitro* or *in vivo*. It should also be noted that tooth brushing affects the VOC profile of the oral cavity via multiple ways. Mechanical cleaning and increased fluid flow are most likely the largest factors, however, aspects such the oral pH and temperature can also play a significant role. For example, changes in the oral pH can affect the solubility of certain VOCs. The protonation of compounds with a higher pKa is increased when the oral pH decreases, which leads to increased solubility, and therefore, the concentrations of these compounds may decrease in the breath and oral headspace. Ammonia is an example of such a compound [31].

Table 1. Compounds found from the morning breath in significantly increased amounts. Estimated maximum and minimum concentrations found amongst all participants, the known odor thresholds, and the mean percentual decrease after brushing are presented. For compounds significantly underestimated/overestimated in the sampling bags, the corrections are given in parenthesis. The concentrations of bolded compounds exceed their respective odor thresholds for some individuals. Overlapping signals in PTR-ToF-MS are shown in parenthesis next to compound name.

Compound	Mean conc. decrease (%) after tooth brushing	Max. conc. (ppb) in morning breath	Min. conc. (ppb) in morning breath	Odor threshold (ppb) in air	Ref. for odor threshold
Sulphur compounds					
Hydrogen sulfide	23	**4.3 (8.6)**	**0.6 (1.2)**	**0.4**	[24]
Methanethiol	62	**38 (136)**	**1.0 (3.6)**	**0.07**	[24]
Dimethyl sulfide	13	**69**	**11**	**3.0**	[24]
Dimethyl disulfide	20	2.1	0.7	2.2	[24]
Dimethyl trisulfide	3.2	1.0	0.3	1.0	[25]
2-methyl-1-propanethiol	4.3	**13 (16)**	**1.7 (2.1)**	**0.0068**	[24]
S-Methyl pentanethioate	4.2	0.54	0.088	1.0–3.0[b]	[26]
Carboxylic acids					
Acetic acid	12	**90 (170)**	**4.6 (8.7)**	**6.0**	[24]
Butanoic acid	6.2	**18**	**1.5**	**0.19**	[24]
Pentanoic acid	4.5	**10**	**0.33**	**0.037**	[24]
Octanoic acid (3-methylbutyl propanoate)	2.8	0.58	0.094	0.86	[27]
Aldehydes					
Acetaldehyde	8.2	**140**	**42**	**50**	[28]
Pentanal; 3-methylbutanal (2-pentanone)	16	15 (8.6)	1.2 (0.69)	28; 49[a]	[28, 29]
Heptanal (2-heptanone, 5-methyl-2-hexanone)	6.0	6.6	0.38	7.0[a]	[29]
Octanal	3.6	**3.6**	**0.37**	**0.17**	[30]
Ketones					
Acetone	4.7	3000	240	42 000	[24, 28]
2-pentanone (pentanal, 3-methylbutanal)	16	15	1.2	28	[24]
3-penten-2-one	22	28	0.86	n/a	
Cyclohexanone	4.3	4.6	0.4	880	[28]
2-heptanone, 5-methyl-2-hexanone (heptanal)	6.0	6.6	0.38	6.8	[24]
6-methyl-5-hepten-2-one	2.1	2.1	0.33	83[a]	[29]
Alcohols					
Methanol	8.0	3600	130	33 000	[24]
Phenol	8.7	**3.6 (7.8)**	**0.81 (1.8)**	**5.6**	[24]
Esters					
3-methylbutyl propanoate (octanoic acid)	2.8	0.58	0.094	35[a]	[26]
Methyl methacrylate	5.6	**17**	**1.3**	**3.5**	[24]
Vinyl methacrylate	3.9	3.0	0.25	n/a	
Nitrogen compounds					
Hydrogen cyanide	31	12 (21)	1.7 (3.0)	580	[29]
Ammonia	15	**8 000**	**70**	**1500**	[24]
Indole	29	**3.9 (11)**	**0.1 (0.29)**	**0.3**	[24]

(Continued.)

Table 1. (Continued.)

Compound	Mean conc. decrease (%) after tooth brushing	Max. conc. (ppb) in morning breath	Min. conc. (ppb) in morning breath	Odor threshold (ppb) in air	Ref. for odor threshold
Hydrocarbons					
Isoprene (1,3-pentadiene)	29	**760 (245)**	**110 (35)**	**48**	[24]
Styrene	3.5	1.2	0.19	35	[24]
Naphthalene	4.5	3.9	0.35	84	[29]

[a] Calculated from the odor threshold in water using Henry's law.

[b] Typical for S-thioesters, n/a information not available.

Out of the 35 compounds that significantly decreased in the breath samples after tooth brushing, 33 have been connected in earlier reports to the oral bacteria [3–8, 32–34]. The two exceptions are isoprene and vinyl methacrylate. Figure 2 describes the distribution of these compounds between the different oral niches. The relationship between the bVOCs emanating from the different niches of the oral cavity can be complex. Many of the VSCs, including methanethiol, hydrogen sulfide, dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS), are produced by multiple niches in the oral cavity, which makes it challenging to use them as markers for specific pathogens. Same applies to several other compounds, such as indole, ammonia, phenol, 3-methylbutanal, acetaldehyde, 2-heptanone and acetone. Especially the tongue and subgingival microbiomes seem to have many VOCs in common, similarly to their shared core bacterial species [35, 36].

There are, however, some compounds that could be connected specifically to the subgingival anaerobes from the *Treponema, Porphyromonas, Tannerella,* and *Prevotella* genera, which increase in periodontitis compared to a healthy mouth [35]. These include methanol, pentanal, 3-penten-2-one, 1,3-pentadiene, methyl methacrylate, 3-methylbutyl propanoate, and 2-methyl-1-propanethiol.

Supragingival bacteria with cariogenic properties from *Streptococcus, Lactobacillus and Propionibacterium* genera could be connected to cyclohexanone, 2-pentanone, octanal, and DMS. Salivary bacteria seem to produce especially short- to medium-chain fatty acids, as well as some ketones. S-methyl pentanethioate is the only VSCs connected *in vitro* to salivary bacteria. As mentioned, compounds connected to multiple niches, such as indole and many of the VSCs, might not be ideal markers for specific pathogens, however, they could be used as markers for the bacterial load and activity level of the oral cavity in general. This information could prove helpful, for example, when accessing the effectiveness of antimicrobial treatment, such as antibiotics.

Even though halitosis is a separate condition from such oral infectious diseases as periodontitis, there is a significant correlation between malodor and the increased number of periodontal pathogens on the tongue surface, increased number and depth of periodontal pockets, and increased oral bone loss and bleeding [37–39]. Clearly, oral diseases increase the production of odorous bVOCs in breath and these bVOCs are important in relation to halitosis and vice versa. Consequently, many of the compounds connected here to different oral bacteria are also markers for halitosis.

4.2. Oral malodor

In this section we discuss the odor potential of the 35 compounds, whose concentrations were found to significantly decrease after tooth brushing. We concentrate especially on those compounds found in the breath samples in concentrations surpassing their respective odor thresholds. These include several sulfur compounds, fatty acids, and nitrogen compounds, and some aldehydes, hydrocarbons, esters, and alcohols. Ketones are a major constituent of human breath, but their concentrations mainly did not reach odorous values. Compounds, whose concentrations increased significantly after tooth brushing include different terpene derivatives from toothpastes and mouthwashes and are not discussed further.

4.2.1. Sulfur compounds

Compounds mostly discussed in earlier works concerning halitosis are methanethiol, DMS and hydrogen sulfide [12–16]. In fact, most of the research done on halitosis is limited to these compounds. In this study, methanethiol and DMS were present in the morning breath in large amounts, clearly observable to the human sense of smell. Hydrogen sulfide was found in smaller concentrations compared to methanethiol and DMS, but still well over its odor threshold. These VSCs also remained in the breath in concentrations above their respective odor thresholds after tooth brushing. It should be noted that hydrogen sulfide has a proton affinity only slightly larger than that of water, which affects the PTR-ToF-MS ionization process and can cause underestimation of the hydrogen sulfide concentration. Our results corroborate the earlier consensus that methanethiol is

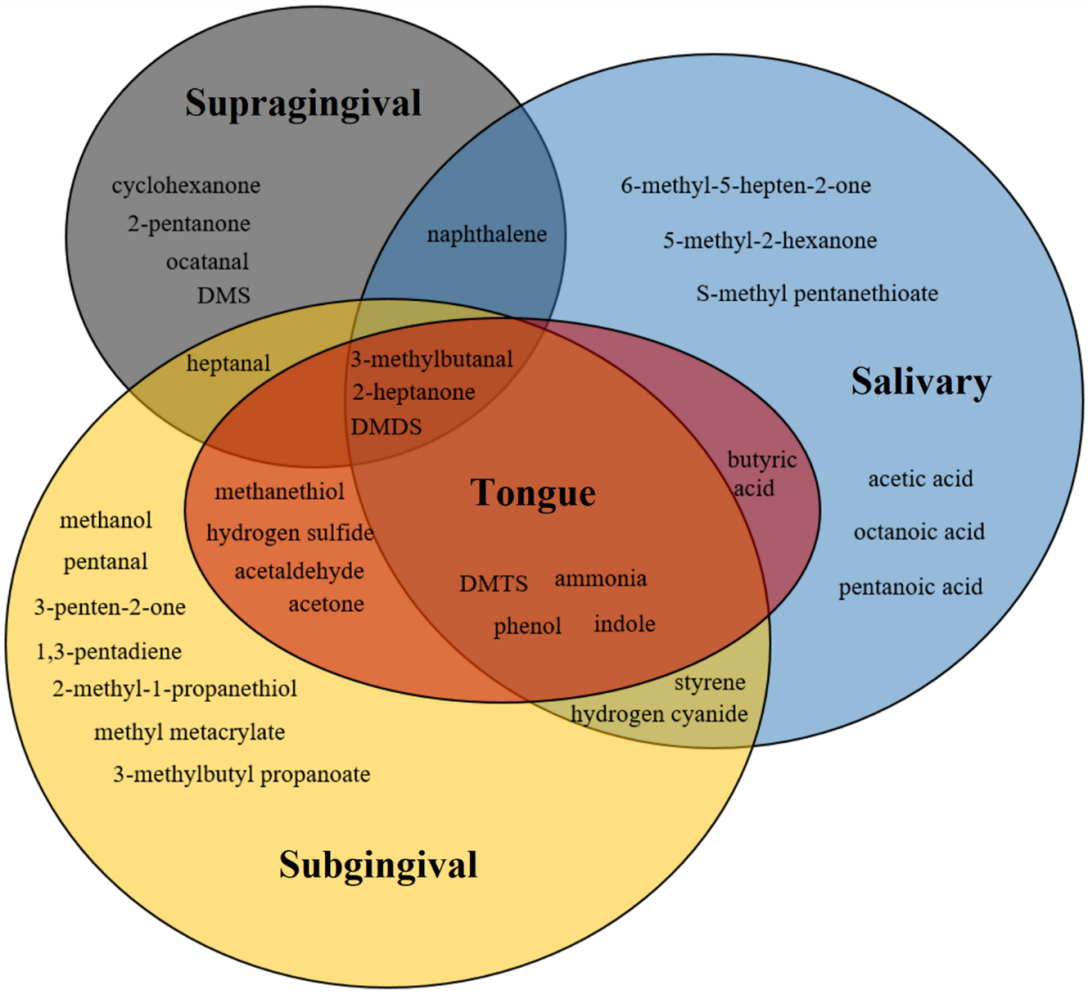

Figure 2. Summary of the compounds that significantly decreased in the breath of healthy individuals after tooth brushing and that have also been connected to different oral niches *in vitro*. In total, 33 compounds were found for cariogenic bacteria (*S. mutans, Lactobacillus salivarius, Propionibacterium acidifaciens*) [6], salivary bacteria (*Klebsiella oxytoca, Staphylococcus saccharolyticus*) [7, 32] and saliva headspace [8, 33], tongue scrape [5, 34], and subgingival anaerobes (*P. gingivalis, Treponema denticola, P. intermedia, P. nigrescens, T. forsythia*) [3, 5].

one of the main culprits of oral malodor. In fact, methanethiol is the compound most significantly affected by tooth brushing and among the few compounds that decreased after tooth brushing in every participant. Hydrogen sulfide seems to have smaller contribution to oral malodor, which is also in agreement with earlier reports. However, according to our results, DMS seems also to be an important component in oral malodor, contrarily to some earlier reports. In earlier works, DMS has been suggested as one of the main components of extra-oral halitosis [16], but our results suggest at least a partial intra-oral origin.

We also identified several other VSCs that can contribute to oral malodor. Isobutanethiol (2-methyl-1-propanethiol) has the lowest odor threshold of all the discussed compounds, but it has not been previously connected to halitosis. In this study, isobutanethiol was found from the morning breath of every participant in concentrations well above the threshold. It is not as significantly affected by tooth brushing as some of the other sulfur compounds, and thus, could contribute to the development of persistent halitosis. It also remains in the breath in levels above the odor threshold even after tooth brushing. DMDS and DMTS were also found from the morning breath, but not in levels above their respective odor thresholds. S-methyl pentanethioate has been identified recently as a potential biomarker for halitosis as it was found from the salivary samples of subjects with halitosis [11]. In our study it was also found in small amounts from the breath of all individuals, however, at levels not exceeding the odor threshold.

4.2.2. Carboxylic acids

Acetic, butyric and valeric acids were found from the morning breath of healthy individuals in levels above their respective odor thresholds. In fact, they were found from the breath of most participants in levels above the threshold even after tooth brushing. The possible role of fatty acids in halitosis has been mentioned before [9, 11, 37], but no reports have demonstrated it *in vivo*. Our results suggest that short- and

medium-chain fatty acids can be important contributors to oral odor and halitosis, alongside with sulfur compounds. As mentioned earlier, fatty acids have been connected to salivary bacteria *in vitro*, whereas VSCs have been linked to the subgingival and tongue microbiomes. This may suggest a different mechanism for the development of halitosis in individuals suffering mostly from the effects of VSCs compared to those suffering from the effects of fatty acids.

4.2.3. Nitrogen compounds

The concentrations of most odorous nitrogen containing compounds were under their respective odor thresholds, except for ammonia and indole. Ammonia is present in the morning breath of healthy individuals in large concentrations, which has also been recorded before [33]. It is not as significantly decreased after tooth brushing as, for example, some of the VSCs, and could be a component of persistent halitosis. Indole is also found from the morning breath in levels high enough to cause odor. As mentioned earlier, indole has been identified as one of the volatile metabolites of certain oral bacteria, and therefore, it is reasonable to assume that people with poor oral hygiene or oral infection could have even higher amounts of indole in their breath. As such, indole could have an important role in halitosis. We did not find other malodorous nitrogen compounds, such as cadaverine or putrescine [12], from the morning breath of healthy individuals.

It should be noted that the PTR-MS signal for protonated ammonia (NH_4^+) can be affected by a contribution from the ion source. However, during measurements and data analysis we confirmed that the background signal was always smaller than the sample signal. In addition, the contribution from the background has been subtracted from the reported ammonia values in the breath samples.

4.2.4. Other compounds

Phenol is the only alcohol, and methyl methacrylate the only ester, significantly decreased by tooth brushing and found from the breath of healthy individuals in levels surpassing their respective odor thresholds. Aldehydes present in the morning breath in levels above their respective odor thresholds were acetaldehyde and octanal. In fact, octanal is one of the compounds remaining in the breath of healthy individuals in levels above its odor threshold even after tooth brushing. Acetaldehyde, on the other hand, is an abundant component of the breath of healthy individuals in general [2] and is likely important for the overall breath odor. The only hydrocarbon found in the breath of healthy individuals in levels over its odor threshold is isoprene. Isoprene is one of the most abundant compounds in the human exhaled breath [2], and in this study, it was found from the morning breath in levels greatly exceeding the odor threshold. A structural isomer of isoprene, 1,3-pentadiene, overlaps with the isoprene signal in the PTR-ToF-MS measurements. However, as isoprene is abundant in exhaled breath, the contribution of 1,3-pentadiene is difficult to discern.

4.3. Limitations of the study

Here we discuss some of the limitations of this study, mostly concerning the sample collection, standardization, and participants' oral health status.

In order to make the sample collection as convenient as possible for the participants, the breath samples were collected by the participant themselves at home into breath bags provided to them. Consequently, the samples collected contained mixed breath from the mouth, the airways, and the lungs, instead of simply the mouth headspace, which would have been ideal for the analysis of oral VOCs. However, collecting only the mouth headspace at home would have required more complicated sampling procedures, and therefore, mixed breath was chosen as a compromise. The sampling method validation measurements proved that the concentrations of VOCs between mixed breath and mouth headspace samples differed only in respect to a few compounds, and the correction factors were considered when analyzing the results. Furthermore, the results obtained from the mixed breath measurements correlate well with previous findings. For example, several sulfur compounds previously proven to cause morning breath and halitosis [9–18], were found in increased amounts from our mixed breath samples before tooth brushing. Also, 33 of the 35 compounds that significantly decreased in our study after tooth brushing have been connected to oral bacteria in literature [3–8, 32–34]. In our opinion, these findings prove that the mixed breath samples used in this study represent the morning breath sufficiently well.

Another limitation of the study is that no rigorous restrictions were imposed on the participants regarding oral cleaning, or the food eaten the previous day from sample collection. Participants were instructed to collect the first breath sample straight after waking, to brush their teeth the way they were used to, and to collect the second breath sample straight after that. The paired *t*-test was used to compare each individual's breath before tooth brushing to the same individual's breath after tooth brushing, after which the compounds most significantly affected between all participants were chosen for further examination. As the aim of this study was simply to observe which VOCs exist in the morning breath of generally healthy individuals and what happens to those VOCs after tooth brushing, the lack of restrictions could even be beneficial. We observed consistent changes in breath VOCs between different individuals regardless of the oral cleaning method or food consumed, which suggests that these changes are true in general, not just in a highly standardized situation. Consequently, the

protocol used in this study was suitable for our purposes, while a more standardized approach should be chosen, for example, when assessing the effectiveness of oral cleaning or its clinical relevance regarding malodor.

The oral health status of the participants was not assessed during this study, which is possibly the largest limitation. Clinical oral examination or sampling requiring microbiological work, such as collection of salivary samples, were not accessible for this work. We chose participants, who did not report a history of halitosis or periodontitis, who did not smoke, and who did not have any general health issue, such as diabetes. All the participants were also under 65 years old. While combining clinical information about the participants' oral conditions and the oral microbiota with the VOC data would be valuable, the present study provides vital information on the methodological details and requirements for more detailed studies warranted in the future.

5. Conclusions

We have measured the VOC profiles of the morning breath of healthy subjects before and after tooth brushing. From these profiles, we identified compounds whose concentrations significantly decreased by the oral cleaning. These compounds were assumed to be linked to oral bacterial activity and possibly oral malodor. We found 35 such compounds. Out of these, 33 have been earlier connected *in vitro* to either supragingival bacteria with cariogenic properties, salivary bacteria, saliva headspace, tongue scrapes, or subgingival bacteria. Some of the compounds are connected to overall bacterial activity of the oral cavity, such as indole and several sulfur compounds, while others could be specific to an oral niche or a single pathogen. Studies reporting on the effects of oral cleaning on breath VOCs in general are few [40]. To our knowledge, our study is the first to investigate the VOC composition of the morning breath in specific, as well as the changes in VOCs of the morning breath after oral cleaning. We found this type of methodology a simple and convenient way to examine the connection between the breath volatiles and bacterial activity.

Several of the 35 compounds found to significantly decrease after tooth brushing were present in the morning breath of healthy individuals in levels surpassing their respective odor thresholds. Many of them are also malodorous. These compounds include many sulfur compounds, such as methanethiol, hydrogen sulfide, DMS, and 2-methyl-1-propanethiol. However, we also found several other compounds that likely contribute to the odor profile of exhaled breath. They include acetic, butyric and valeric acid, acetaldehyde, octanal, phenol, methyl methacrylate, indole, ammonia, and isoprene. We conclude that many of these compounds can affect the oral odor of generally healthy people and can most likely be found in increased amounts from the breath of people suffering from halitosis or oral infections. We also found that several odorous compounds remain in the breath in levels above their respective odor thresholds even after tooth brushing, including most of the identified sulfur compounds, butyric and valeric acid, isoprene and octanal. These compounds could cause persistent halitosis.

Our results shed light to the complex nature of oral odor beyond VSCs and define some threshold values for VOCs in the morning breath of healthy subjects. Most of the odorous VOCs found from the morning breath are likely of bacterial origin. Further work is needed to investigate the levels of these compounds in the breath of people suffering from disease, such as oral infections or halitosis, as several of the compounds discussed in this study are potential breath biomarkers for these conditions.

Data availability statement

The data generated and/or analysed during the current study are not publicly available for legal/ethical reasons but are available from the corresponding author on reasonable request.

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Author contribution

K R and M M conceived the study idea and hypothesis; K R, M M, P P and M L designed the experiments; K R performed the breath measurements; K R and M M analyzed the V O C data; K R wrote the manuscript; all authors reviewed the manuscript.

Conflict of interest

Authors declare no conflict of interest.

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