Journal of Chromatography B 1104 (2019) 49–58

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/jchromb

Identification of volatile metabolites in human saliva from patients with oral squamous cell carcinoma via zeolite-based thin-film microextraction coupled with GC–MS

Hiroyo Shigeyama[a], Tao Wang[b], Masatoshi Ichinose[b], Toshihiro Ansai[a,**], Seung-Woo Lee[b,c,*]

[a] Division of Community Oral Health Development, Graduate School of Dentistry, Kyushu Dental University, 2-6-1 Manazuru, Kokurakita-ku, Kitakyushu 803–8580, Japan

[b] Graduate School of Environmental Engineering, The University of Kitakyushu, 1-1 Hibikino, Wakamatsu-ku, Kitakyushu 808–0135, Japan

[c] EnH Japan Co. Ltd., Kitakyushu Science and Research Park, 8-1 Hibikinokita, Wakamatsu-ku, Kitakyushu 808-0137, Japan

ARTICLE INFO

Keywords:
Volatile organic compounds
Biomarkers
Oral squamous cell carcinoma
Saliva
GC–MS
Thin-film microextraction
Zeolite

ABSTRACT

In recent years, volatile organic compounds (VOCs) discharged from the human body, of which some compounds exhibit strong correlations with pathological conditions, have attracted attention as a new means of disease diagnosis technology. The aim of this study was to establish the salivary metabolomic profiles of oral squamous cell carcinoma (OSCC) patients and healthy volunteers (control group) and to investigate VOCs as potential biomarkers in the diagnosis of oral cancer. We have demonstrated a method combining thin-film microextraction based on a ZSM-5/polydimethylsiloxane hybrid film coupled with gas chromatography–mass spectrometry and carried out a comparative analysis of salivary VOC profiles between OSCC patients and healthy controls. The results depicted that 42 and 73 VOCs were detected and identified in samples from the healthy control group (n = 50) and oral cancer group (n = 24), respectively. Among them, twenty-seven VOCs (ten were decreased, seven disappeared, and ten were newly produced in the oral cancer group) depict significant differences between both the sample groups, and they have relevance as candidate biomarkers for OSCC. Twelve salivary VOCs that were characteristic of oral cancer patients were finally extracted and used for pattern recognition analyses for oral cancer diagnosis. The proposed TFME approach for analyzing human saliva on the basis of a ZSM-5-loaded PDMS hybrid thin film has been performed for the very first time in the field of dentistry.

1. Introduction

Cancer has been the leading cause of death in Japan for > 30 years, although Japan has become the world's preeminent country for longevity as a result of progress in medical cures and public health. According to Vital Statistics of Japan published by the Ministry of Health, Labor, and Welfare, the annual number of deaths due to cancer has exceeded 370,000 and is increasing annually. Particularly, the numbers of both sufferers and deaths from oral cancer have increased in recent years; it has been reported that > 7600 people died from oral cancer in 2016 [1].

Oral cancer ranks sixth among cancers in worldwide occurrence, and 90% of all oral cancers are diagnosed as oral squamous cell carcinoma (OSCC). Additionally, it has been reported that there are nearly 300,000 new cases worldwide every year [2,3], of which 145,000 end in the death of the patient [4]. OSCC has a high mortality ratio in comparison with other carcinomas. OSCC has been known to develop from premalignant lesions and premalignant conditions of the oral mucosa, which are diseases that have a risk of malignant transformation. Although the oral cavity is easily accessible, OSCC is often asymptomatic in the early stages and its macroscopic findings are similar to those of other mucosal diseases or benign tumors [5]. Additionally, squamous cells travel through the lymphatic system and appear first in the nearby lymph nodes in the neck, and can also spread to the lungs and other parts of the body [6]. Furthermore, locoregional recurrence occurs at surgically treated sites, which is related to lower survival rates. Currently, the gold standard for the diagnosis of OSCC is biopsy followed by histopathological examination. The major drawback of this technique is delays in detection [7].

Biomarkers, which are measurable indicators of physiological and

* Correspondence to: S.-W. Lee, Graduate School of Environmental Engineering, The University of Kitakyushu, 1-1 Hibikino, Wakamatsu-ku, Kitakyushu 808–0135, Japan

** Correspondence to: T. Ansai, Graduate School of Dentistry, Kyushu Dental University, 2-6-1 Manazuru, Kokurakita-ku, Kitakyushu 803–8580, Japan.

E-mail addresses: [redacted]

https://doi.org/10.1016/j.jchromb.2018.11.002
Received 2 February 2018; Received in revised form 4 October 2018; Accepted 2 November 2018
Available online 06 November 2018
1570-0232/

pathological processes, are therefore useful in diagnosis and influence the prognosis of disease. A biomarker is defined as a characteristic that is an objectively measured and evaluated indicator of normal biological processes, pathogenic processes, or pharmacological responses to therapeutic intervention [8,9]. With recent advances in scientific technologies, biomarker research has benefited to the point where many metabolites are now recognized as an excellent diagnostic tools (for example, DNA, mRNA, proteins, and peptides) [10–12]. Additionally, volatile organic compounds (VOCs) are present in various excreted biological materials, and their analysis offers the possibility of screening for cancer [4,13–15].

VOCs can reflect metabolic changes in response to inflammation, necrosis, cancer, degeneration, or the alteration of microbiota or can be related to external factors such as environmental pollution, medication, and diet [16,17]. These metabolites are released into the bloodstream and reach the alveoli, salivary glands, renal tubules, and gut, where they are excreted via skin, exhalation, saliva, urine, and feces [18–22]. Recently, Haick et al. developed nanomaterial-based sensors and reported the possibility of detecting disease using VOCs [14,23,24]. Altomare et al. reported the possibility of screening for cancer by the analysis of VOCs in breath samples from colorectal cancer patients [25]. The origin of cancer-related VOC metabolites have also been investigated by several groups that attempted to detect the VOCs directly produced by the cancer cell lines in vitro, such as CALU-1 [26], NCI-H1666 [27], A549 [28], human bronchial epithelial cells (HBEpC) and human fibroblasts (hBF) [28], HepG2 [29], and HeLa [30]. All these results support the existence of small molecular cancer markers, and therefore the development of new materials and methods for reliable detection as well as studies in real patient groups for validation of biomarkers is strongly required.

On the other hand, despite the very attractive, non-invasive nature of the breath test, the methodology for breath sample collection is not homogenous through different research works [31], sometimes providing contradictory results from study to study for the same breath VOCs. This is because collection, handling, storage, concentration, and analysis of breath samples are technically challenging. An additional limitation is the use of only highly volatile organic compounds that can be excreted from the body via exhaled breath. A partial solution to these problems would be the use of more convenient sources of volatiles excreted from the body, for instance, urine (or saliva). Similarly to breath, urine (or saliva) includes both endo- and exogenous volatiles from sources such as diet and environmental exposure [32]. For instance, Hanai et al. [33] found that urine can be an alternative matrix for biomarker detection by comparing the VOC profile of A549 cell culture with that of urine from mice implanted with the same tumor morphology. Therefore, aqueous and fluidic biological samples such as urine and saliva can be important candidates to find semi-volatile or non-volatile molecular biomarkers under normal room conditions.

In this study, we focused on saliva as a representative biological sample. Saliva contains a wide spectrum of proteins, peptides, hormones, gingival exudates, and microbiota, etc., which can vary with high responsiveness and reflect bodily health [34]. VOCs are also transferred from blood to saliva, mainly via passive diffusion [22]. Furthermore, the collection of saliva provides a very easy, non-invasive, and cost-effective approach for the screening of large populations with less risk of infection than the collection of blood [6,7,35]. On the other hand, few reports on salivary VOCs have been published, and the use of salivary VOCs for disease diagnosis has made no progress. This is mainly because of the lack of highly sensitive systems for the detection of these analytes at concentrations that are 1000 times lower than those in blood [6], in combination with the lack of previous studies that separated highly pathogenicity oral bacterial flora and VOCs generated from inflamed tissues in the presence of > 300 kinds of oral bacteria.

This study demonstrates a novel thin-film microextraction (TFME) method based on a ZSM-5/polydimethylsiloxane (PDMS) hybrid film and carried out a comparative analysis of salivary VOC profiles between oral cancer patients and healthy controls. The identification of VOC profiles that have a high correlation with oral cancer is indispensable and will contribute to future work on the biochemical sources of VOCs and their metabolic pathways.

2. Experimental

2.1. Study subjects

This research was approved by the ethics committee of Kyushu Dental University, Kitakyushu, Japan (Approval Number 15-15). The study included the following two groups: patients with oral cancer and healthy volunteers (controls). Written informed consent was obtained from all subjects after a full explanation. Subjects were recruited from the Division of Oral and Maxillofacial Surgery of Kyushu Dental University Hospital, Kitakyushu, Japan. None of the oral cancer patients had received any prior to treatment such as chemotherapy, radiotherapy, surgery, or alternative remedies before sample collection. None of the controls had a history of malignancy, immunodeficiency, or underlying diseases. Histologically, all oral cancer patients were diagnosed as having OSCC.

2.2. Collection of saliva samples

The clinical characteristics of the subjects are summarized in Table 1. The subjects rinsed their mouths with water immediately prior to sample collection. On average, 2 mL of unstimulated whole saliva was collected in a 10 mL glass bottle over a period of 5–10 min. After collection, the samples were immediately stored at −80 °C. To confirm the reproducibility (continuity) and intraday fluctuations of analytes that were observed in the samples, we collected saliva several times from each subject. In the control group, saliva was collected continually for at least a period of 5 days between 7 am and 10 am while the subjects fasted. In the oral cancer patient group, saliva was collected for at least 1.5 h after meals for multiple (1–3) time periods up to surgery after hospitalization. For all subjects, smoking and the use of oral hygiene products such as toothpastes and mouthwashes were not permitted for at least 1 h prior to saliva collection.

2.3. Materials

ZSM-5 (SiO_2/Al_2O_3 molar ratio = 30, Lot 110421) was purchased from JGC Catalysts and Chemicals Ltd., Kawasaki, Japan. A PDMS

Table 1
Clinical characteristics of subjects.

Parameter	Classification	Healthy controls	Oral cancer patients
Number of subjects	–	8	12
Number of saliva samples	–	50	24
Age (mean ± SD)	Male	28.3 ± 10.3	64 ± 19
	Female	27	60 ± 16.8
Sex	Male	7 (87.5)	5 (41.7)
	Female	1 (12.5)	7 (58.3)
Smoking status	Yes	1 (12.5)	2 (16.7)
	Ex-smokers	0	1 (8.33)
	Never	7 (87.5)	9 (75.0)
Clinical stage	I	–	5 (41.7)
	II	–	6 (50.0)
	III	–	0
	IV	–	1 (8.33)
Histological type	Squamous cell carcinoma	–	12 (100)

SD: standard deviation.
The values in parentheses represent the percentage of each class of subjects for a given parameter.

solution kit (Sylgard 184 silicone elastomer kit) was purchased from Dow Corning Ltd., Tokyo, Japan. This kit includes a base solution A (Sylgard 184A) and a curing agent solution B (184B), which were mixed in a ratio of 10:1 to produce PDMS. Methanol was purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan. The following chemicals that were used as references compounds were purchased from: Wako Pure Chemical Industries (Osaka, Japan) for ethanol, undecane, and 1,3-butanediol; Tokyo Kasei (Tokyo, Japan) for 2-pentanone, butyrolactone, 3-heptanone, 1,2-pentanediol, and 1-hexadecanol; Nacalai Tesque (Tokyo, Japan) for benzyl alcohol and phenol; Kanto Chemical (Tokyo, Japan) for 1-octanol; Funakoshi (Tokyo, Japan) for hexadecanoic acid. Deionized pure water (18.3 MΩ cm) was obtained by reverse osmosis followed by ion exchange and filtration using a Direct-QTM purification system (EMD Millipore, Billerica, MA, USA).

2.4. Preparation of ZSM-5/PDMS hybrid films

A glass bottle (volume = ca. 50 mL) was used as the support substrate for the ZSM-5/PDMS hybrid film. PDMS was supplied as a two-part liquid component kit that comprised of a base and a curing agent to be mixed in a weight ratio of 10:1. Before the PDMS was solidified, it was mixed with ZSM-5 to give a content of 20 wt% ZSM-5 in a PDMS matrix [20], and 1.0 g of the mixture was placed in a glass bottle. The ZSM-5/PDMS hybrid film was left at 25 °C for 72 h and then heated at 100 °C for 1 h. Subsequently, the sampling bottles with a ZSM-5/PDMS film were thoroughly washed with methanol by shaking them for 3 days to remove the unreacted PDMS monomers.

2.5. Sample analysis

A 2 mL aliquot of saliva was taken from the collected samples and diluted with 3 mL deionized water. VOCs in saliva were extracted by shaking the sample for 3 h, and then the extraction bottle was gently rinsed with pure water and dried with nitrogen. Finally, the VOCs extracted onto the ZSM-5/PDMS film were condensed using 100 µL methanol, of which 1.0 µL methanol was subjected to GC–MS analysis using a JMS-Q1000GC (JEOL, Japan) GC–MS system (Fig. 1).

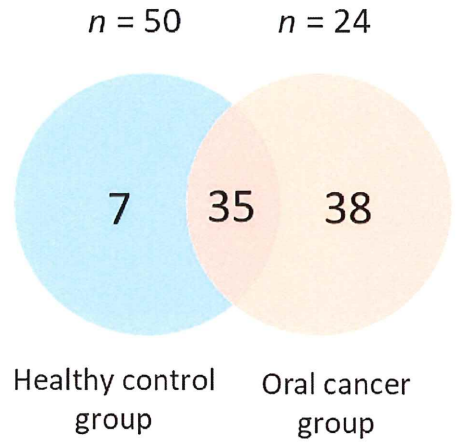


Fig. 2. Venn diagram depicting an overlap of VOCs between healthy controls and oral cancer patients. Thirty-five VOCs were common to both the groups.

2.6. GC–MS conditions

Chromatographic analysis with mass spectrometric detection was performed with a gas chromatography–mass spectrometry (GC–MS) system (JMS-Q1000GC, JEOL, Japan) consisting of an Agilent 7890A gas chromatograph coupled to a quadrupole mass spectrometer in electron ionization mode at 70 eV. The temperatures of the ion source and the GC interface were 200 °C and 230 °C, respectively. A DB-WAX capillary column (polyethylene glycol-based high-polarity stationary phase, 30 m length, 0.25 mm inner diameter, 0.5 µm film thickness, Agilent J&W, part number 19091J-413) was used for separation. Ultrahigh-purity helium gas (purity 99.999%) was flowed at a rate of 1.0 mL/min as a carrier gas. The GC injector temperature was kept at 230 °C. The oven temperature was programmed to be held at 40 °C for 3 min, increased at 10 °C/min to 230 °C, and held for 10 min. Retention time data were recorded from 5 min 20 s to 32 min. Data acquisition was performed in full scan mode with m/z = 25–310 and a scan time of 300 ms. Identification of the sample components was performed using

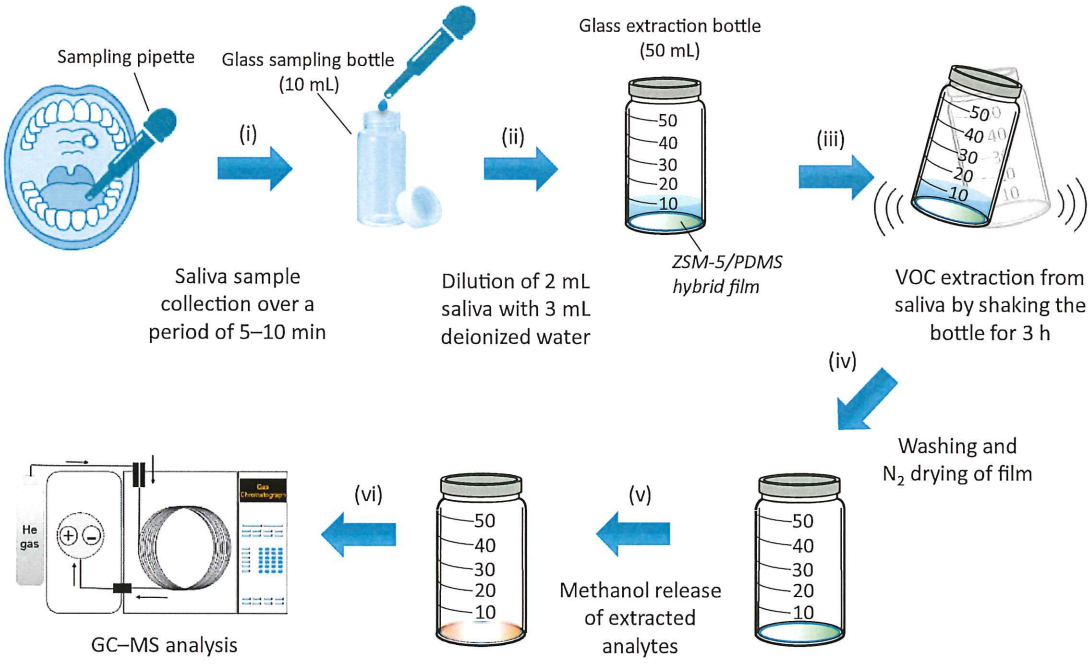


Fig. 1. Schematic of the GC–MS analysis of volatile metabolites via ZSM-5/PDMS hybrid thin-film extraction.

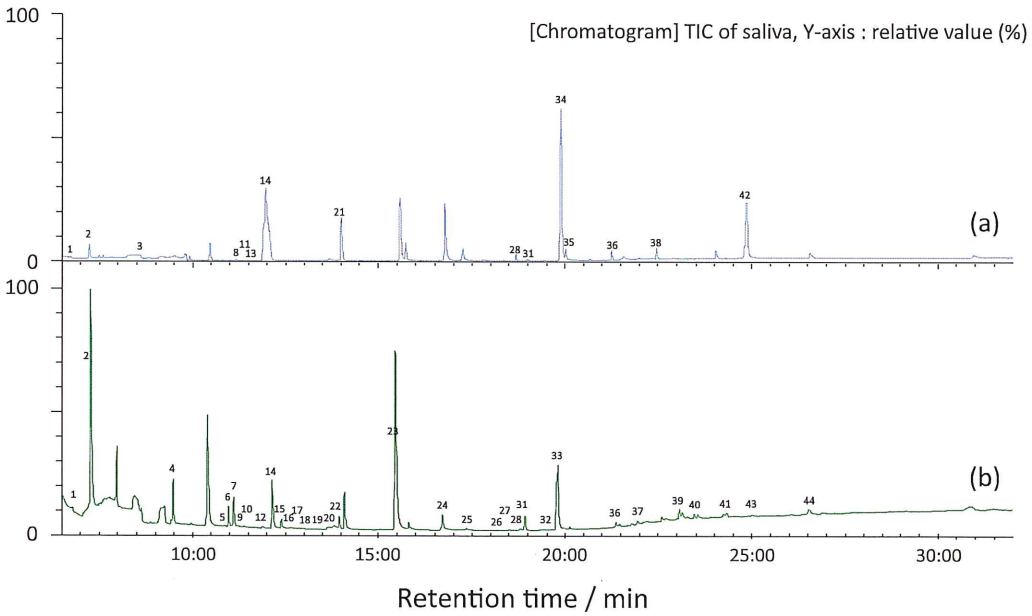


Fig. 3. Representative GC–MS TICs of salivary VOC profiles from two (a) healthy and (b) oral cancer patients. The analytical conditions are described in the text. The numbers indicate selected compounds as follows: (1) 2-pentanone; (2) 1-propanol; (3) 1-butanol; (4) 1-butanol, 3-methyl-; (5) thiocyanic acid; (6) octanal; (7) acetic acid; (8) 2-butanone, 3-hydroxy-; (9) cyclohexanone; (10) acetaldehyde, methoxy-; (11) 2-propanone, 1-hydroxy-; (12) propanoic acid, 2-hydroxy-; (13) propylene glycol; (14) 1-hexanol; (15) ethyl ether; (16) ethane, 1,2-diethoxy-; (17) 2-hexen-1-ol; (18) 1-undecane, 9-methyl-; (19) 2-decene, (*Z*)-8-methyl-; (20) 1-decene, 8-methyl-; (21) 1-octen-3-ol; (22) 1-hexanol, 2-ethyl-; (23) oxime, methoxy-phenyl-; (24) 1,3-butanediol; (25) 1,2-pentanediol; (26) propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester; (27) butanoic acid, butyl ester; (28) dimethyl sulfone; (29) 4-hexen-3-ol, 2-methyl-; (30) 2-furanmethanol, tetrahydro-; (31) phenylethyl alcohol; (32) 4-dodecanol; (33) 1-hexanol, 3-methyl-; (34) phenol; (35) phenol, 3-methyl-; (36) phenol, 4-methyl-; (37) 1-hexadecanol; (38) hexadecanoic acid; (39) 1-tetradecanol; (40) octadecanoic acid; (41) 9-octadecenoic acid, (*Z*)-; (42) indole; (43) 1H-indole, 3-methyl-; (44) *n*-pentadecanol.

Table 2
List of representative VOCs that were specifically newly produced (released) in samples from oral cancer patients.

No.	Compounds	Retention time, min	Selected ion, *m/z*	Classification	*FA* ($n = 24$)
1	3-Heptanone	8:47	57	Ketone	0.38
2	Acetaldehyde, methoxy-	11:16	45	Aldehyde	0.25
3	5-Hepten-2-one, 6-methyl-	11:35	43	Ketone	0.25
4	2-Butanol, 3-methyl-	14:59	45	Alcohol	0.33
5	Oxime, methoxy-phenyl-	16:31	77	Imine	0.29
6	1,3-Butanediol	16:48	43	Alcohol	0.79
7	1,2-Pentanediol	17:15	55	Alcohol	0.42
8	Butanoic acid, butyl ester	18:25	71	Ester	0.25
9	1-Hexadecanol	21:26	55	Alcohol	0.46
10	1-Tetradecanol	23:31	69	Alcohol	0.29

FA: frequency of appearance.

the National Institute of Standards and Technology (NIST) mass spectral library search software (JEOL version 1.5). For reliable VOC identification, compounds with structural similarity over 700 were chosen as candidate biomarkers.

2.7. Statistical analysis

Statistical significance was assessed with the use of non-parametric Mann-Whitney *U* test for each compound to compare samples from two different subject groups for independent observations. Data evaluation was carried out using Origin 9 software (Origin Lab Corporation, Northampton, MA, USA). In this study, p values < 0.05 were considered to be statistically significant. Principal component analysis (PCA), which potentially act as biomarkers for discriminating diseases, was conducted with a function implemented in the Origin 9 data analysis package and used to describe the total difference between two groups. GC peak areas under the curve in the selected ion chromatogram for individual analytes were used as PCA input variables. The statistical analysis was additionally conducted using a decision tree model based on supervised method, which was utilized to understand if the finally determined potential biomarkers can be used for describing the differences between both the heathy and cancerous sample groups. Decision tree was determined using the R software (R version 3. 4. 0, The R Foundation for Statistical Computing, Vienna, Austria).

3. Results and discussion

3.1. General features of VOCs excreted from saliva samples

To effectively detect the analytes that are present at trace levels, a preconcentration step is generally introduced during sample preparation while performing GC–MS-based analytical methods. Solid phase microextraction (SPME) is one of the most extensively used sample preparation techniques and it has achieved increasing success while being applied to various fields such as in environmental, food, and drug analyses [36,37]. Generally, the sensitivity of an SPME-based method is

Table 3
List of VOCs that displayed significant differences between samples from healthy controls and oral cancer patients.

No.	Compounds	Selected ion, *m/z*	Classification	*FA*		*p*-Value	↓ or ↑
				Healthy control (n = 50)	Oral cancer (n = 24)		
1	Ethanol	31	Alcohol	0.98	1.00	< 0.01	↓
2	2-Pentanone	43	Ketone	0.98	0.96	< 0.01	↓
3	1-Propanol	31	Alcohol	0.96	0.75	< 0.01	↓
4	Hexanal	56	Aldehyde	0.10	0.25	< 0.05	↓
5	3-Penten-2-one, 4-methyl-	83	Ketone	0.10	0.17	> 0.1	↑
6	Thiocyanic acid	73	Inorganic acid	0.37	0.13	< 0.05	↓
7	Cyclohexanone	55	Ketone	0.14	0.25	> 0.1	↑
8	2-Propanone, 1-hydroxy-	43	Ketone	0.82	0.75	< 0.01	↓
9	Propanoic acid, 2-hydroxy-	45	Acid	0.61	0.17	< 0.01	↓
10	Propylene glycol	45	Alcohol	0.61	0.21	< 0.05	↓
11	1-Hexanol	56	Alcohol	0.53	0.13	< 0.01	↓
12	Ethanedioic acid	59	Acid	0.39	0.17	< 0.05	↓
13	Acetic acid, hydroxy-	59	Acid	0.53	0.25	< 0.01	↓
14	1-Octen-3-ol	57	Alcohol	0.84	0.33	< 0.01	↓
15	Dimethyl sulfone	79	Organosulfur	0.49	0.08	< 0.05	↓
16	Phenylethyl alcohol	91	Alcohol	0.78	0.29	< 0.01	↓
17	Phenol	94	Phenol	1.00	0.92	< 0.01	↓
18	Phenol, 4-methyl-	107	Phenol	0.37	0.08	< 0.01	↓
19	2-Piperidinone	99	Amide	0.82	0.33	< 0.01	↓
20	Hexadecanoic acid	74	Acid	0.96	1.00	< 0.01	↓
21	Docosanoic acid	74	Acid	0.47	0.21	< 0.05	↓
22	Indole	117	Heterocyclic	0.78	0.29	< 0.01	↓

FA: frequency of appearance; ↓ = decreased and ↑ = increased.

observed to be proportional to the volume of the extraction phase. However, the diffusion of analytes in the coating material requires longer equilibration time. An optimal solution to improve sensitivity is to use a thin extraction phase that has a larger surface area to achieve a high ratio of the surface area to the volume. Recently, we have demonstrated the use of highly porous materials, such as zeolites that are a class of high-silica crystalline aluminosilicates with defined pore diameters that are observed to be smaller than 2 nm, along with porous polymer matrices. ZSM-5 is one of the most investigated zeolites during the past few decades because of its unique channel structure with pore opening sizes that are observed to be approximately similar in size to that of many industrially important organic molecules [38,39]. To the best of our knowledge, the proposed TFME approach for analyzing human saliva on the basis of ZSM-5-loaded PDMS hybrid thin films has been performed for the very first time in the field of dentistry.

Saliva samples were collected from eight healthy volunteers and 12 oral cancer patients to give a total of 50 and 24 samples, respectively (Table 1). Approximately 100 different VOCs were identified by the GC–MS analysis of saliva samples using a ZSM-5/PDMS hybrid film. Among them, totally 80 VOCs exhibited relatively good day reproducibility in the healthy and oral cancer patient groups (Table S1). It is clear that these analytes are originated from metabolic processes; namely, they are endogenous metabolites that were not influenced by daily activities or surrounding conditions. Based on the comparisons of both the sample groups that are depicted in Table S1, it is observed that 42 and 73 VOCs were detected for the healthy and oral cancer groups, respectively. An interesting observation is that a larger number of compounds could be detected from the group of patients. An overlap of 35 VOCs between the healthy and oral cancer groups can be depicted using a Venn diagram (Fig. 2). Consequently, we observed that 7 and 38 compounds were independently present in the healthy and oral cancer groups, respectively, which were further observed to not overlap between each other.

Fig. 3 compares typical GC–MS total ion chromatograms (TIC) obtained for two saliva samples from a healthy control and an oral cancer patient. Interestingly, many chromatographic peaks were found from oral cancer patients, and different salivary GC–MS profiles could be recognized for healthy controls and oral cancer patients. With a focus on the chemical classes of the detected VOCs, various chemical classes can be observed such as alcohols, ketones, hydrocarbons, aldehydes, organic acids, esters, phenol, etc. Alcohols can be absorbed rapidly from the gastrointestinal tract into the blood, and many of them are excreted in saliva [14]. Aldehydes are produced in the body as part of common physiological processes [14]. Ketones and organic acids are also affected by diet and metabolism. Heterocyclic compounds and phenols are among the substances that cause halitosis. Additionally, slight amounts of sulfur-containing compounds and nitrogen-containing compounds were detected.

3.2. Disease-specific VOCs detected from oral cancer saliva samples

Early detection of OSCC is of considerable importance in clinical practice because we observe that most of the oral cancer patients who are diagnosed during the advanced stage exhibit poor clinical results. This study investigates the volatile human salivary metabolome in patients undergoing OSCC, which may be an accurate and noninvasive tool to distinguish between healthy people and oral cancer patients. Transformation of normal cells into malignant cancer cells may be the primary cause for the metabolic changes that cause the production of new VOCs or that alter the concentration levels of normal metabolites. However, the metabolic origin and physiological function of most of the VOCs that were released from the human body have not yet been elucidated clearly. Generally, the aforementioned factors are derived using a variety of endogenous biochemical pathways and exogenous sources such as the surrounding environment, an unhealthy lifestyle, and oral bacterial species.

To verify the presence of disease-specific potential biomarkers among the 80 compounds that were identified from the groups of healthy control and oral cancer patients, a more detailed statistical analysis was additionally performed on two different data groups that can be represented as follows: (1) 38 VOCs that were detected from the oral cancer group and (2) 35 VOCs that overlapped between the healthy and oral cancer groups. Thus, the presence of both types of metabolites that were detected specifically in oral cancer patients and metabolites that were detected at significantly different levels in both the sample groups was suggested.

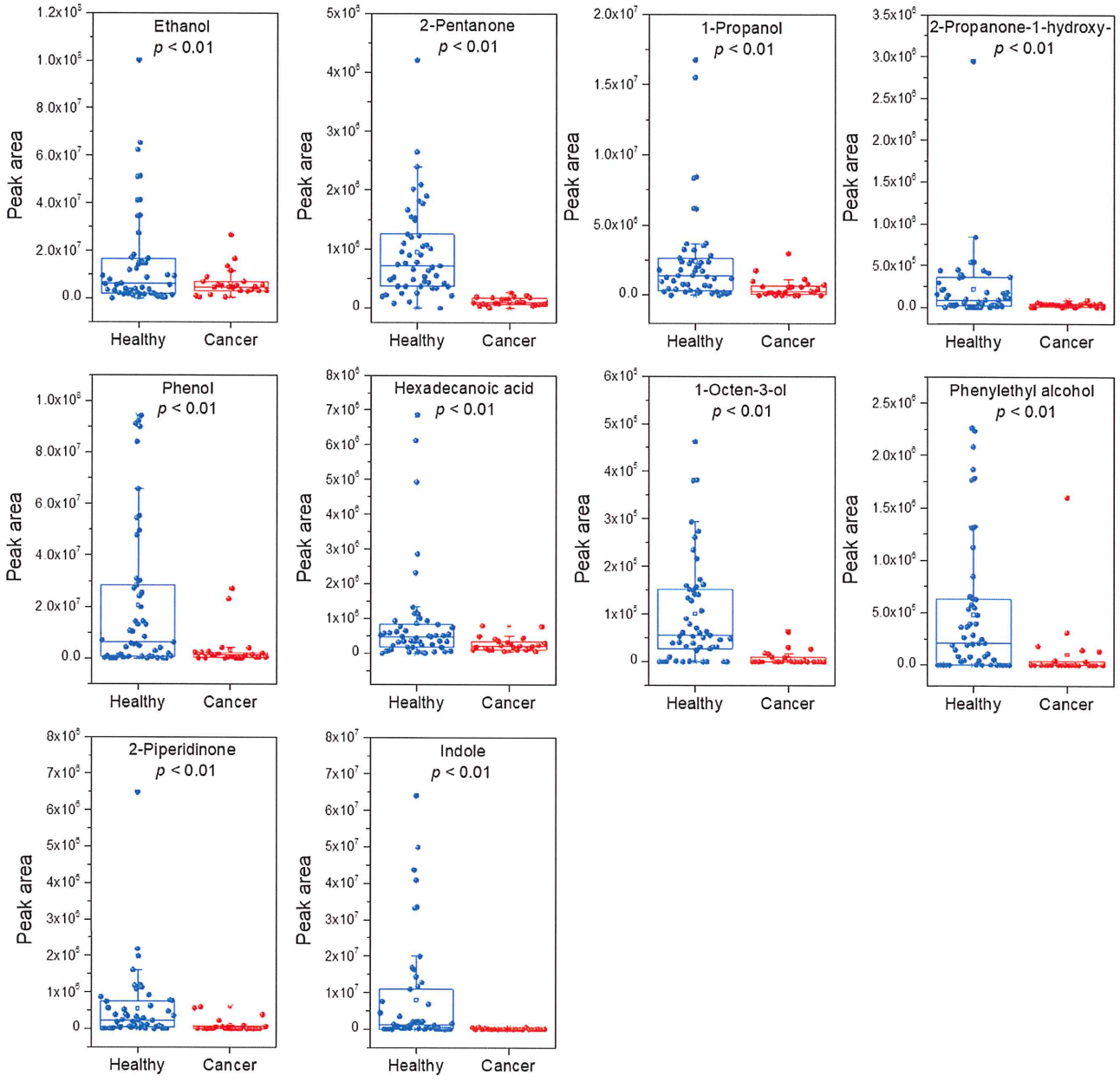


Fig. 4. Typical box whisker charts for comparison of peak areas for ten VOCs detected in saliva from healthy controls and oral cancer patients.

In this study, we used the term 'frequency of appearance (*FA*)' as a factor to represent both the *intra* and intersample *reproducibility* of each compound that was detected using samples that were obtained from cancer patients. The *FA* of each detected compound signifies the probability of its detection while performing each analysis, and it was estimated using the following Eq. (1):

$$FA = \frac{\text{Number of detections}}{\text{Total number of analyses}} \quad (1)$$

Among the 38 VOCs that were detected in the samples that were collected from cancer patients (Table S1), ten compounds were observed to exhibit high probability, and their *FA* values were observed to be > 0.25, which are depicted in Table 2. Aldehydes and ketones are generally produced in the body due to common physiological processes such as digestion and corporal odor emanation. They can be further formed by cell membrane lipid peroxidation. Particularly, recent studies have reported that aldehydes are potential cancer biomarkers of oxidative stress [4,40]. In some previous studies, 3-heptanone that is classified as ketone has been reported to be a potential biomarker for breast cancer, even though it was detected in urine [41]. In addition to 3-heptanone, the appearance of methoxyacetaldehyde and 6-methyl-5-hepten-2-one may be caused due to the chemical and nutritional variations that occur in OSCC or due to the occurrence of other oral diseases. Furthermore, it is possible that methoxy-phenyl-oxime can be a byproduct that is induced by aldehyde or ketone metabolites. Compounds such as 3-methyl-2-butanol, 1,3-butanediol, 1,2-pentanediol, 1-hexadecanol, and 1-tetradecanol are liable to be affected by confounding factors in the body such as age, gender, changes in water content, etc., because enzymes such as alcohol dehydrogenase and cytochrome P450, which are mainly active in the liver, are involved in the metabolism of alcohols [4,14]. It would be considered that biochemical cellular processes associated with tumor formation lead to an increase in the levels of these ten VOCs.

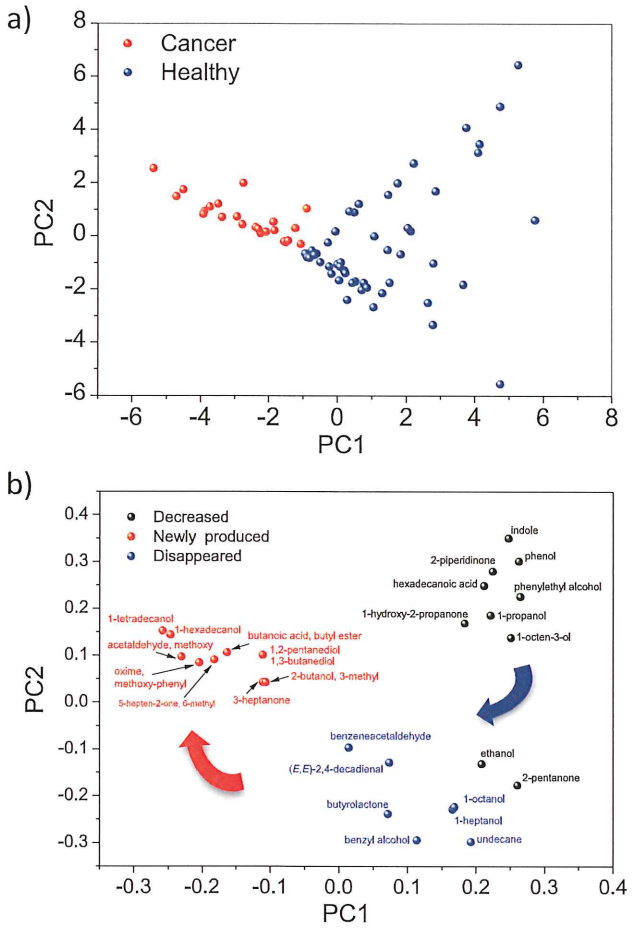


Fig. 5. (a) Reconstructed score plot obtained by PCA (PC1 vs. PC2) of a dataset consisting of GC peak areas for 27 VOCs obtained from healthy controls and oral cancer patients. (b) Loading chart indicating the contribution of variables in the total variability of the initial two principal components.

3.3. Common VOCs detected from healthy and oral cancer saliva samples

Biomarker molecules that can act as a screening tool for a particular disease are not necessarily restricted to the compounds that are observed to increase in cancer patients. Furthermore, it is also conceivable that certain metabolites may be present in decreased or increased amounts in cancer patients owing to tumor-specific metabolic changes. Among the 35 common VOCs that were observed (Fig. S1), 22 compounds displayed statistically significant differences in the distribution of chromatographic peak areas ($p < 0.05$ except for 4-methyl-3-penten-2-one and cyclohexanone) between both the groups, all of which exhibited a tendency to decrease in the group of oral cancer patients (Table 3). As can be observed from Fig. 4, we infer that six VOCs having *p*-values that were lower than 0.01 ($p < 0.01$), such as ethanol, 2-pentanone, 1-propanol, 1-hydroxy-2-propanone, phenol, and hexadecanoic acid, were observed to be highly reproducible ($FA > 0.70$) in both the groups. However, four VOCs, such as 1-octen-3-ol, phenylethyl alcohol, 2-piperidinone, and indole, were observed to exhibit relatively low *FA* values ($FA < 0.40$), despite their *p*-values lower than 0.01 ($p < 0.01$) (Fig. 4). We infer that this may be caused due to their reduced production that was observed in samples that were collected from oral cancer patients.

Generally, organic acids including hexadecanoic acid are considered to be main products that are produced by proteolysis inside the body. Phenol and 4-methyl-phenol are among the substances that cause halitosis, which are related to volatile sulfur-containing compounds such as hydrogen sulfide, methyl mercaptane, and dimethyl sulfide. Indole can be produced as a degradation product of the amino acid tryptophan by various kinds of anaerobic bacteria. Therefore, the aforementioned ten VOCs were additionally considered to be cancer biomarkers for the diagnosis of OSCC, although they depicted a reduced presence in the saliva samples that were collected from oral cancer patients.

Interestingly, we observed that only two compounds (4-methyl-3-penten-2-one and cyclohexanone) from among the 35 common VOCs illustrated an increased production in the oral cancer group, although they depicted relatively low statistical differences ($p > 0.10$); however, at the current stage it is difficult to explain the pathological relevance of these two compounds. Furthermore, another interesting feature was observed while using the seven VOCs (Table S1), such as undecane, 1-heptanol, 1-octanol, butyrolactone, benzeneacetaldehyde, (*E*,*E*)-2,4-decadienal, and benzyl alcohol, which was only observed in the healthy control group. In other words, this result would suggest that these seven VOCs either disappeared completely or were no longer metabolized in oral cancer patients.

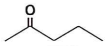
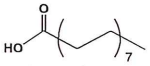
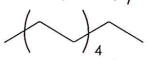
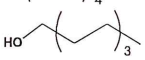
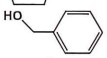
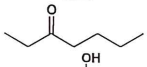
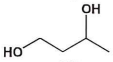
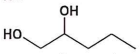
3.4. PCA analysis

PCA is a useful statistical approach to reduce the total variability that is present in a particular set of data to obtain more informative principal components and can be used for discrimination between the samples with and without cancer. Several parameters characterizing the saliva samples including age, sex, smoking status, clinical stage, and histological type (Table 1) were also available for PCA. Initially, multivariate analysis was performed on the detection peak areas for all the compounds that were detected in both healthy controls and oral cancer patients. However, there was no statistically significant correlation between the parameters that were considered to be variables (data not shown). Thus, the main comparison was carried out for the criteria cancer versus non-cancer without accounting for other sample parameters. The results of PCA are depicted in Fig. S2 for the initial two principal components of the variability of total VOCs, based on which the two sample groups (with and without cancer) are unambiguously separated, depicting negative and positive PC1 scores, respectively. This result is considered to be a critical evidence to verify that the biochemical changes in saliva composition may be caused due to the disease progression of the disease.

To further confirm the compounds that affect the distinction between these two groups, we investigated the precise contribution of independent variables in the total variability of the initial two principal components (PC1 and PC2). Similarly to the results that were observed in Fig. S2a, it was observed that all the 80 VOCs that were detected spread radially. Furthermore, these VOCs can be classified into two clusters on the PCA plot (Fig. S2b). This coincidence may be attributed to the fact that majority of the 42 metabolites that were detected in the healthy control group decreased (33 VOCs) or completely disappeared (seven VOCs) in the oral cancer group. Instead, it must be noted that 38 compounds were newly observed in the oral cancer group.

For a clearer distinction between the two groups, 27 compounds, which include ten compounds (exhibiting *FA* values > 0.25, Table 2) were newly produced by oral cancer, ten compounds (exhibiting *p* values smaller than 0.01, Table 3) were decreased, seven compounds (only observed in the healthy control group, Table S1) disappeared in the oral cancer group, were further extracted from the 80 VOCs and used to perform additional PCA. Fig. 5 depicts the reconstructed score plot and loading chart that were obtained using the PCA of variability of 27 metabolites. Both the sample groups (with and without cancer) can be clearly separated using PC1 scores that depict positive values for most of the samples collected from the healthy control group, whereas all the samples that were collected from the oral cancer group depicted negative PC1 values (Fig. 5a). Therefore, we can conclude that these 27 VOCs mainly contribute to the distinction between the healthy and oral cancer groups.

Table 4
List of twelve potential biomarkers that were extracted from ten-decreased, seven-disappeared, and ten-newly produced VOCs in the oral cancer group.

Tentative compound name		Chemical structure	Probable metabolic origin (CAS number)
Decreased	Ethanol		Related to enzymes such as alcohol dehydrogenase (ADH) activity and cytochrome *p*450 (64-17-5) [4,14]
	2-Pentanone		Lung cancer (107-87-9) [42]
	Phenol		Produced by aerobic intestinal micro flora acting on tyrosine metabolism (108-95-2) [43,44]
	Hexadecanoic acid		Fatty acid biosynthesis (57-10-3) [45]
Disappeared	Undecane		Oxidative stress (1120-21-4) [14,46]
	1-Octanol		Leukemia, colorectal, and lymphoma caner (111-87-5) [47]
	Butyrolactone		Endogenous compound made from gamma-aminobutyrate (96-48-0) [48]
	Benzyl alcohol		Toluene metabolism (100-51-6) [44]
Newly produced	3-Heptanone		Mitochondrial-oxidation (106-35-4) [49]
	1,3-Butanediol		Dietary energy in liver cytoplasm (107-88-0) [50]
	1,2-Pentanediol		Unknown (5343-92-0)
	1-Hexadecanol		Colorectal cancer (36653-82-4) [51]

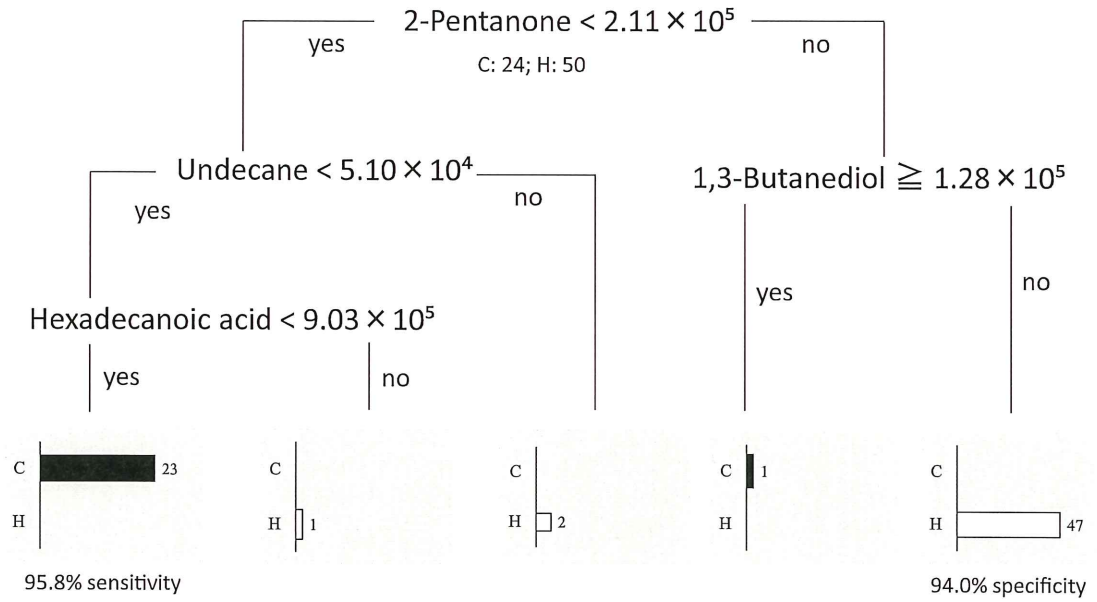


Fig. 6. Decision tree for prediction of promising oral cancer biomarkers, which is based upon GC peak areas for twelve potential biomarkers listed in Table 4. Numbers 24 and 50 indicate a total of samples that were collected from healthy volunteers (H) and oral cancer patients (C), respectively. Predicted category "yes/no" for oral cancer diagnosis is based on classification matrices, that is, 2-pentanone over 2.11×10^5 is less probable to be associated with oral cancer.

Interestingly, the 27 VOCs that were used to perform PCA provide useful information on the basis of their correlation with metastasis to cancer. As illustrated in Fig. 5b, the ten-decreased and seven-disappeared VOCs are located at the positive PC1 area of the PCA plot and are grouped, although ethanol and 2-pentanone are observed to be quite far from the remaining compounds in the decreased VOC group. Probably, this fact that these two compounds deviated from their own group and approached the different VOC group that includes disappeared VOCs may be explained by the following reasons: 1) they decrease more prominently compared to the other compounds that exhibited a tendency to decrease in the oral cancer group and 2) they depict a strong correlation with the disappeared compounds. Furthermore, the newly generated ten VOCs are observed to gather densely in the opposite negative area. Obviously, this result suggests that the aforementioned 27 compounds are strongly involved in cancer progression and that they can be used for early detection or prediction of cancer. With the growth of cancer, metabolites vary specifically, which is observed on the PCA plot, moving in a clockwise direction from the upper right to the lower right and further to the left.

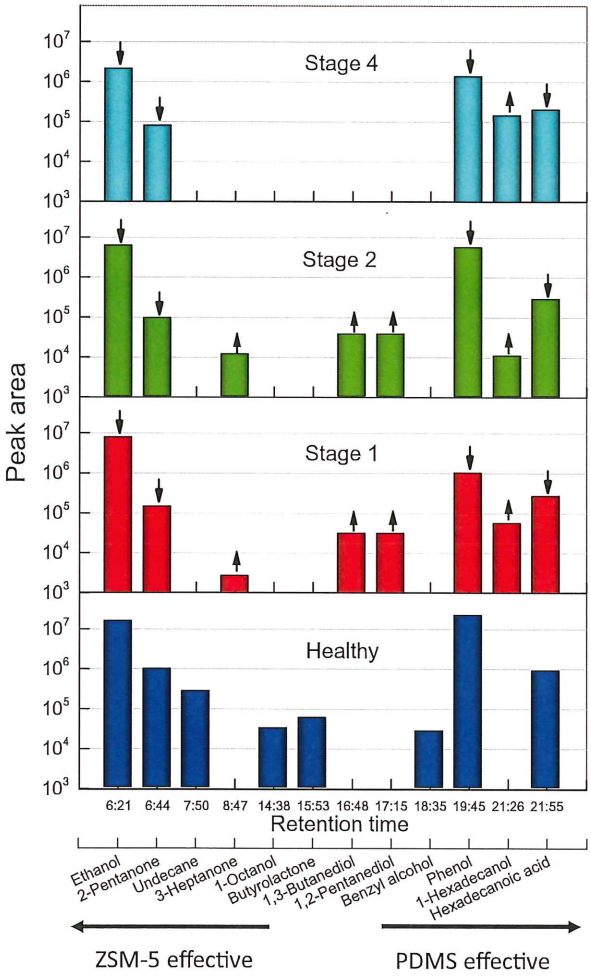


Fig. 7. Comparison of VOC profiles of twelve potential biomarkers for the distinction between healthy controls and oral cancer patients in different stages.

3.5. Selection and profiling of potential biomarkers

The aforementioned 27 VOCs were finally concentrated to twelve compounds that can be potential OSCC biomarkers, which are summarized in Table 4, depicting the most remarkable variations that are observed among the 27 VOCs. This selection methodology of VOCs confirms the presence of general molecular cancer markers that are observed in literature, i.e., there is a strong evidence of oral cancer-related VOCs that are present in the samples that were collected from oral cancer patients. Retention times and MS profiles of these final candidate biomarkers were well consistent with those that were obtained using the reference compounds, as compared in Fig. S3.

Generally, a decision tree as a predictive model is a useful tool for mapping observations on items to conclusions on their target values [52]. To select the most relevant features for this classification model, we utilized a pruning algorithm to screen the presence of oral cancer from salivary samples. As depicted in Fig. 6, 2-pentanone, undecane, 1,3-butanediol, and hexadecanoic acid were determined as the main variables that distinguish the two sample groups with and without oral cancer. As can be seen from Fig. 5b, 2-pentanone depicts a more prominent decrease compared to the other compounds that exhibited a tendency to decrease in the oral cancer group, which is rather close to the group of the disappeared compounds on the PCA plot. In addition, it is clear that undecane (completely disappeared in the oral cancer group) and 1,3-butanediol (newly generated in the oral cancer group) can be considered as promising biomarkers. Through this decision tree classification, we could identify a classifier that has mathematical capacity to diagnose oral cancer with 95.8% sensitivity and 94.0% specificity.

Finally, we compared the average values of GC peak areas for the twelve VOCs that were detected in the saliva samples that were collected from both the sample groups to clarify the fact that they can be considered to be tumor-specific candidate biomarkers that serve as screening tools to detect oral cancer (Fig. 7). Furthermore, the healthy control group is compared with the oral cancer group at different stages (stages I, II, and IV) that depict different VOC profiles as compared to that of the healthy control group. Interestingly, the average GC peak areas for the twelve metabolites that were detected in the healthy control group were observed to be obviously different compared to those that were detected in the samples at stage I ($n = 10$) and stage II ($n = 12$). Unfortunately, a very small set of samples ($n = 2$) at stage IV could be used for this study. For more detailed data analysis, a sufficient number of saliva samples from patients with oral cancer at advanced stages (stage III and stage IV) are required.

Fig. 7 depicts additional information on affinity (selectivity) of the ZSM-5/PDMS hybrid film to the metabolites that were identified in this study. Among the twelve VOCs, compounds detected in the range of longer retention time after approximately 18 min, such as benzyl alcohol, phenol, 1-hexadecanol, and hexadecanoic acid, are relatively hydrophobic in the form of aromatic or long-carbon-chain aliphatic molecules. As illustrated in our previous study [30], the PDMS film may be considered to be effective for concentrating these hydrophobic compounds owing to their hydrophobic interactions with the PDMS polymer. However, smaller hydrophobic compounds, such as ethanol, 2-pentanone, and 3-heptanone, are observed in the opposite range of shorter retention time before approximately 10 min. These compounds exhibiting smaller sizes than the pores of ZSM-5 can be selectively adsorbed using the zeolite that depicts equilibrated retention. The remaining compounds can be effectively detected in the ZSM-5/PDMS hybrid film, since they have adequate affinities that are induced by both ZSM-5 and PDMS components.

4. Conclusions

This study depicted that a technique for the analysis of VOCs that were extracted using a ZSM-5/PDMS hybrid film was useful for the establishment of metabolomic patterns of salivary VOCs that were characteristic of oral cancer patients and healthy volunteers. Eighty kinds of volatile metabolites were detected and identified between the samples from the oral cancer group ($n = 24$) and the control group ($n = 50$), which were classed as alcohols, ketones, hydrocarbons, aldehydes, organic acids, esters, phenols, etc. Among them, twenty-seven metabolites (ten were decreased, seven disappeared, and ten were newly produced in the oral cancer group) were considered to be tumor-specific candidate biomarkers that serve as screening tools for oral cancer. Furthermore, the results of multivariate analysis suggested that these VOCs can be considered to contribute to the distinction between both the sample groups. Eventually, twelve metabolites were selected as potential oral cancer biomarkers and their VOC profiles revealed the possibility of being used for distinction between different stages of oral cancer. For future clinical practice, the actual population of OSCC patients must be extended. In the light of our present results, more detailed correlations between VOC levels and tumor growth or proliferation at different stages should be also investigated in future.

Acknowledgments

This work was supported by the grant-in-aid for scientific research (B) (25293429) from the Japan Society for the Promotion of Science. S. W. Lee also acknowledges the grant-in-aid for scientific research (A) (15H01713) from the Japan Society for the Promotion of Science and

the financial support from Shinkou Seiki Co. Ltd. as a special research project. Finally, authors would like to thank all the participants who provided salivary samples for this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2018.11.002.

References

[1] Ministry of Health, Labor and Welfare, Summary report of vital statistics of Japan, Available at: Mhlw.go.jp, http://www.mhlw.go.jp/toukei/saikin/hw/jinkou/geppo/nengai16/index.html, (2018) , Accessed date: 3 January 2018.

[2] M. Nagata, H. Kurita, K. Uematsu, S. Ogawa, K. Takahashi, H. Hoshina, R. Takagi, Diagnostic value of cyclin-dependent kinase/cyclin-dependent kinase inhibitor expression ratios as biomarkers of locoregional and hematogenous dissemination risks in oral squamous cell carcinoma, Mol. Clin. Oncol. 3 (2015) 1007–1013.

[3] S. Blatt, M. Krüger, T. Ziebart, K. Sagheb, E. Schiegnitz, E. Goetze, B. Al-Nawas, A.M. Pabst, Biomarkers in diagnosis and therapy of oral squamous cell carcinoma: a review of the literature, J. Craniomaxillofac. Surg. 45 (2017) 722–730.

[4] M. Bouza, J. Gonzalez-Soto, R. Pereiro, J.C. De Vicente, A. Sanz-Medel, Exhaled breath and oral cavity VOCs as potential biomarkers in oral cancer patients, J. Breath Res. 11 (2017) 016015.

[5] A. Jensen, P.S. Nolet, M.A. Diwan, Oral squamous cell carcinoma: an atypical presentation mimicking temporomandibular joint disorder, J. Can. Chiropr. Assoc. 48 (2004) 266–272.

[6] T. Pfaffe, J. Cooper-White, P. Beyerlein, K. Kostner, C. Punyadeera, Diagnostic potential of saliva: current state and future applications, Clin. Chem. 57 (2011) 675–687.

[7] T. Radhika, N. Jeddy, S. Nithya, R.M. Muthumeenakshi, Salivary biomarkers in oral squamous cell carcinoma – an insight, J. Oral Biol. Craniofacial Res. 6 (2016) S51–S54.

[8] N. Spielmann, D. Wong, Saliva: diagnostics and therapeutic perspectives, Oral Dis. 17 (2011) 345–354.

[9] M. Mal, Noninvasive metabolic profiling for painless diagnosis of human diseases and disorders, Future Sci. OA 2 (2016) FSO106.

[10] M. Sugimoto, D.T. Wong, A. Hirayama, T. Soga, M. Tomita, Capillary electrophoresis mass spectrometry-based saliva metabolomics identified oral, breast and pancreatic cancer-specific profiles, Metabolomics 6 (2010) 78–95.

[11] E.C. Porto-Mascarenhas, D.X. Assad, H. Chardin, D. Gozal, G. De Luca Canto, A.C. Acevedo, E.N.S. Guerra, Salivary biomarkers in the diagnosis of breast cancer: a review, Crit. Rev. Oncol. Hematol. 110 (2017) 62–73.

[12] S. Saxena, B. Sankhla, K.S. Sundaragiri, A. Bhargava, A review of salivary biomarker: a tool for early oral cancer diagnosis, Adv. Biomed. Res. 6 (2017) 90.

[13] R. Gasparri, M. Santonico, C. Valentini, G. Sedda, A. Borri, F. Petrella, P. Maisonneuve, G. Pennazza, A. D'Amico, C. Di Natale, R. Paolesse, L. Spaggiari, Volatile signature for the early diagnosis of lung cancer, J. Breath Res. 10 (2016) 016007.

[14] H. Haick, Y.Y. Broza, P. Mochalski, V. Ruzsanyi, A. Amann, Assessment, origin, and implementation of breath volatile cancer markers, Chem. Soc. Rev. 43 (2014) 1423–1449.

[15] J. Rudnicka, M. Walczak, T. Kowalkowski, T. Jezierski, B. Buszewski, Determination of volatile organic compounds as potential markers of lung cancer by gas chromatography-mass spectrometry versus trained dogs, Sensors Actuators B Chem. 202 (2014) 615–621.

[16] M. Di Lena, F. Porcelli, D.F. Altomare, Volatile organic compounds as new biomarkers for colorectal cancer: a review, Color. Dis. 18 (2016) 654–663.

[17] P. Mochalski, K. Unterkofler, G. Teschl, A. Amann, Potential of volatile organic compounds as markers of entrapped humans for use in urban search-and-rescue operations, TrAC Trends Anal. Chem. 68 (2015) 88–106.

[18] R. Jiang, E. Cudjoe, B. Bojko, T. Abaffy, J. Pawliszyn, A non-invasive method for in vivo skin volatile compounds sampling, Anal. Chim. Acta 804 (2013) 111–119.

[19] V. Ruzsanyi, P. Mochalski, A. Schmid, H. Wiesenhofer, M. Klieber, H. Hinterhuber, A. Amann, Ion mobility spectrometry for detection of skin volatiles, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 911 (2012) 84–92.

[20] M.J. Traynor, S.C. Wilkinson, F.M. Williams, Metabolism of butoxyethanol in excised human skin in vitro, Toxicol. Lett. 177 (2008) 151–155.

[21] Q. Liang, H. Liu, L.X. Xie, X. Li, A.H. Zhang, High-throughput metabolomics enables biomarker discovery in prostate cancer, RSC Adv. 7 (2017) 2587–2593.

[22] A. Amann, B.D.L. Costello, W. Miekisch, J. Schubert, B. Buszewski, J. Pleil, N. Ratcliffe, T. Risby, The human volatilome: volatile organic compounds (VOCs) in exhaled breath, skin emanations, urine, feces and saliva, J. Breath Res. 8 (2014) 034001.

[23] Y.Y. Broza, H. Haick, Nanomaterial-based sensors for detection of disease by volatile organic compounds, Nanomedicine 8 (2013) 785–806.

[24] M.K. Nakhleh, H. Amal, R. Jeries, Y.Y. Broza, M. Aboud, et al., Diagnosis and classification of 17 diseases from 1404 subjects via pattern analysis of exhaled molecules, ACS Nano 11 (2017) 112–125.

[25] D.F. Altomare, M. Di Lena, F. Porcelli, L. Trizio, E. Travaglio, M. Tutino, S. Dragonieri, V. Memeo, G. De Gennaro, Exhaled volatile organic compounds identify patients with colorectal cancer, Br. J. Surg. 100 (2013) 144–150.

[26] W. Filipiak, A. Sponring, T. Mikoviny, C. Ager, J. Schubert, W. Miekisch, A. Amann, J. Troppmair, Release of volatile organic compounds (VOCs) from the lung cancer cell line CALU-1 *in vitro*, Cancer Cell Int. 8 (2008) 17.

[27] A. Sponring, W. Filipiak, C. Ager, J. Schubert, W. Miekisch, A. Amann, J. Troppmair, Analysis of volatile organic compounds (VOCs) in the headspace of NCI-H1666 lung cancer cells, Cancer Biomark. 7 (2010) 153–161.

[28] W. Filipiak, A. Sponring, A. Filipiak, C. Ager, J. Schubert, W. Miekisch, A. Amann, J. Troppmair, TD-GC-MS analysis of volatile metabolites of human lung cancer and normal cells *in vitro*, Cancer Epidemiol. Biomark. Prev. 19 (2010) 182–195.

[29] P. Mochalski, A. Sponring, J. King, K. Unterkofler, J. Troppmair, A. Amann, Release and uptake of volatile organic compounds by human hepatocellular carcinoma cells (HepG2) *in vitro*, Cancer Cell Int. 13 (2013) 72.

[30] T. Nozoe, S. Goda, R. Selyanchyn, T. Wang, K. Nakazawa, T. Hirano, H. Matsui, S.-W. Lee, In vitro detection of small molecule metabolites excreted from cancer cells using a Tenax TA thin-film microextraction device, J. Chromatogr. B 991 (2015) 99–107.

[31] A. Amann, P. Spanel, D. Smith, Breath analysis: the approach towards clinical applications, Mini-Rev. Med. Chem. 7 (2007) 115–129.

[32] K. Matsumura, M. Opiekun, H. Oka, A. Vachani, S.M. Albelda, K. Yamazaki, G.K. Beauchamp, Urinary volatile compounds as biomarkers for lung cancer: a proof of principle study using odor signatures in mouse models of lung cancer, PLoS ONE 5 (2010) e8819.

[33] Y. Hanai, K. Shimono, H. Oka, Y. Baba, K. Yamazaki, G.K. Beauchamp, Analysis of volatile organic compounds released from human lung cancer cells and from the urine of tumor-bearing mice, Cancer Cell Int. 12 (2012) 7.

[34] X. Wang, K.E. Kaczor-Urbanowicz, D.T.W. Wong, Salivary biomarkers in cancer detection, Med. Oncol. 34 (2017) 7.

[35] S. Ishikawa, M. Sugimoto, K. Kitabatake, M. Tu, A. Sugano, I. Yamamori, A. Iba, K. Yusa, M. Kaneko, S. Ota, K. Hiwatari, A. Enomoto, T. Masaru, M. Iino, Effect of timing of collection of salivary metabolomic biomarkers on oral cancer detection, Amino Acids 49 (2017) 761–770.

[36] W. Wardencki, M. Michulec, J. Curyło, A review of theoretical and practical aspects of solid-phase microextraction in food analysis, Int. J. Food Sci. Technol. 39 (2004) 703–717.

[37] J. Lee, H.K. Lee, K.E. Rasmussen, S. Pedersen-Bjergaard, Environmental and bioanalytical applications of hollow fiber membrane liquid-phase microextraction: a review, Anal. Chim. Acta 624 (2008) 253–268.

[38] S. Goda, R. Selyanchyn, T. Nozoe, H. Matsui, S.W. Lee, Development of a thin-film microextraction device based on ZSM-5/Tenax TA for VOC detection in liquid samples, J. Anal. Bioanal. Tech. S12 (2014) 004.

[39] T. Wang, T. Ansai, S.W. Lee, Zeolite-loaded poly(dimethylsiloxane) hybrid films for highly efficient thin-film microextraction of organic volatiles in water, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 1041-1042 (2017) 133–140.

[40] K. Schallschmidt, R. Becker, C. Jung, W. Bremser, T. Walles, J. Neudecker, G. Leschber, S. Frese, I. Nehls, Comparison of volatile organic compounds from lung cancer patients and healthy controls – challenges and limitations of an observational study, J. Breath Res. 10 (2016) 046007.

[41] C.L. Silva, M. Passos, J.S. Câmara, Solid phase microextraction, mass spectrometry and metabolomic approaches for detection of potential urinary cancer biomarkers – a powerful strategy for breast cancer diagnosis, Talanta 89 (2012) 360–368.

[42] M. Li, D. Yang, G. Brock, R.J. Knipp, M. Bousamra, M.H. Nantz, X.A. Fu, Breath carbonyl compounds as biomarkers of lung cancer, Lung Cancer 90 (2015) 92–97.

[43] T.A. McDonald, N.T. Holland, C. Skibola, P. Duramad, M.T. Smith, Hypothesis: phenol and hydroquinone derived mainly from diet and gastrointestinal flora activity are causal factors in leukemia, Leukemia 15 (2001) 10–20.

[44] B. De Lacy Costello, A. Amann, H. Al-Kateb, C. Flynn, W. Filipiak, T. Khalid, D. Osborne, N.M. Ratcliffe, A review of the volatiles from the healthy human body, J. Breath Res. 8 (2014) 014001.

[45] K. Magnuson, S. Jackowski, C.O. Rock, J.E. Cronan, Regulation of fatty acid biosynthesis in *Escherichia coli*, Microbiol. Rev. 57 (1993) 522–542.

[46] M. Phillips, R.N. Cataneo, J. Greenberg, R. Gunawardena, A. Naidu, F. Rahbari-Oskoui, Effect of age on the breath methylated alkane contour, a display of apparent new markers of oxidative stress, J. Lab. Clin. Med. 136 (2000) 243–249.

[47] C.L. Silva, M. Passos, J.S. Cmara, Investigation of urinary volatile organic metabolites as potential cancer biomarkers by solid-phase microextraction in combination with gas chromatography-mass spectrometry, Br. J. Cancer 105 (2011) 1894–1904.

[48] F. Vaiano, G. Serpelloni, S. Furlanetto, D. Palumbo, F. Mari, A. Fioravanti, E. Bertol, Determination of endogenous concentration of γ-hydroxybutyric acid (GHB) in hair through an ad hoc GC-MS analysis: a study on a wide population and influence of gender and age, J. Pharm. Biomed. Anal. 118 (2016) 161–166.

[49] G. Gamez, L. Zhu, A. Disko, H. Chen, V. Azov, K. Chingin, G. Kramer, R. Zenobi, Real-time, in vivo monitoring and pharmacokinetics of valproic acid via a novel biomarker in exhaled breath, Chem. Commun. 47 (2011) 4884–4886.

[50] D.R. Romsos, C. Sasse, G.A. Leveille, Effect of dietary 1,3-butanediol on in vitro fatty acid synthesis and malic enzyme activity in rat liver and adipose tissue, J. Nutr. 104 (1974) 202–209.

[51] E.C.Y. Chan, P.K. Koh, M. Mal, P.Y. Cheah, K.W. Eu, A. Backshall, R. Cavill, J.K. Nicholson, H.C. Keun, Metabolic profiling of human colorectal cancer using high-resolution magic angle spinning nuclear magnetic resonance (HR-MAS NMR) spectroscopy and gas chromatography mass spectrometry (GC/MS), J. Proteome Res. 8 (2009) 352–361.

[52] I.M. Modlin, B.I. Gustafsson, I. Drozdov, B. Nadler, R. Pfragner, M. Kidd, Principal component analysis, hierarchical clustering, and decision tree assessment of plasma mRNA and hormone levels as an early detection strategy for small intestinal neuroendocrine (carcinoid) tumors, Ann. Surg. Oncol. 16 (2009) 487–498.