C146-E463

SHIMADZU
Excellence in Science

Technical Report

Profiling Volatile Compounds from Culture Supernatants of Periodontal Bacteria Using "MonoTrap" and GC/MS/O

Moyu Taniguchi[1], Asuka Mori[2], Masae Kubota[3], Atsuo Amano[3], Eiichiro Fukusaki[1, 2, 4]

Abstract:

Volatile compounds produced by periodontal bacteria are thought to be the main cause of halitosis. This study comprehensively analyzed the volatile compounds produced by the periodontal bacteria *Fusobacterium nucleatum* and *Porphyromonas gingivalis* using MonoTrap silica monolithic adsorbent and a gas chromatograph/mass spectrometer/olfactometer (GC/MS/O).

Keywords: halitosis, periodontal bacteria, volatile compounds, MonoTrap, GC/MS/O

1. Introduction

It is generally understood that halitosis is caused by volatile compounds produced by microbes within the oral cavity. In particular, volatile sulfur compounds (VSCs) such as hydrogen sulfide, methanethiol, and dimethyl sulfide are the main substances responsible for halitosis. These VSCs are produced by the gram-negative anaerobic periodontal bacteria *Fusobacterium nucleatum* and *Porphyromonas gingivalis*. Diagnosis of halitosis frequently relies on an analysis of these sulfur compounds. However, in existing study reports, there has yet to be an example of a comprehensive analysis of the volatile compounds produced by periodontal bacteria. New findings related to the volatile components produced by periodontal bacteria may suggest approaches to the noninvasive analysis of the bacterial flora in the oral cavities of patients with periodontal disease and for the search for new biomarker compounds for the measurement of halitosis. The aim of this study was the comprehensive analysis of the volatile components produced by *F. nucleatum* and *P. gingivalis*.

GC systems are suitable for the analysis of volatile compounds. Volatile compounds, which have low olfaction thresholds, are a cause of halitosis even in trace quantities. Accordingly, a concentration treatment prior to GC analysis is required for a high-sensitivity analysis of substances responsible for halitosis. MonoTrap silica monolithic adsorbent is an adsorbent with a monolithic structure. It enables large sample loading and efficient compound collection. Additionally, with the GC/MS/O analysis method, the mass spectrum of the compounds acquired by MS is used in combination with evaluations of odors by human olfaction. Using this method makes it possible to identify which compounds are responsible for which odors.

This article describes the results of a comprehensive analysis using GC/MS/O of the volatile compounds in the headspace of a culture solution of *F. nucleatum* and *P. gingivalis*, collected using MonoTrap.

1 Osaka University Shimadzu Omics Innovation Research Laboratories
2 Graduate School of Engineering, Osaka University
3 Graduate School of Dentistry, Osaka University
4 Institute for Open and Transdisciplinary Research Initiatives, Osaka University

2. Procedure

2-1. Cultivation Method

The gram-negative anaerobic bacteria *F. nucleatum ATCC 25586* and *P. gingivalis ATCC 33277* were cultivated at 37 °C under anaerobic conditions (80 % N_2, 10 % H_2, 10 % CO_2). They were first cultivated for 3 days in a sheep blood agar medium for CDC anaerobes (Nippon Becton Dickinson Company, Ltd.), after which the colony was collected and transferred to a 10 mL liquid culture medium. The structure of the liquid culture medium for *F. nucleatum* consisted of yeast extract (BD Biosciences Advanced Bioprocessing) at 10 g/L, trypticase peptone at 10 g/L, biosate peptone at 10 g/L, brain heart infusion broth at 19.2 g/L, hemin (Sigma-Aldrich) at 5 mg/L, menadione (Sigma-Aldrich, Missouri, USA) at 1 mg/L, $CaCl_2$ at 0.008 g/L, $MgSO_4$ at 0.008 g/L, K_2HPO_4 at 0.04 g/L, KH_2PO_4 at 0.04 g/L, $NaHCO_3$ at 0.4 g/L, and NaCl at 0.08 g/L. The structure of the liquid culture medium for *P. gingivalis* consisted of trypticase soy broth (Becton, Dickinson and Company) at 30 g/L, yeast extract at 1 g/L, hemin at 5 mg/L, and menadione at 1 mg/L.

After cultivation for 12 hours, each was centrifuged (4 °C, 7670 × g, 7 minutes) and transferred to new culture media after removing the supernatant, ensuring that the absorbance ($OD_{600\ nm}$) was 0.1, and the amount of culture solution was 15 mL. After cultivation for 24 hours, the $OD_{600\ nm}$ was measured, and after centrifugation, it was filtered, and the supernatant was transferred to a new tube. (Fig. 1)

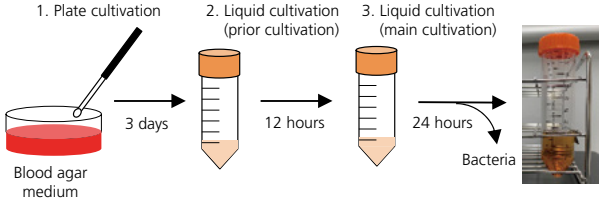


Fig. 1 Cultivation Sequence for Periodontal Bacteria

2-2. Preparation of Measurement Samples

15 mL of the culture supernatant obtained with the cultivation method in 2-1 was transferred to a 40 mL glass vial. MonoTrap RGPS TD (GL Sciences) was positioned in the headspace, and the vial was sealed. After warming at 40 °C for 2 hours, the volatile compounds were collected, and then MonoTrap was placed into a special glass tube for thermal desorption instruments (Fig. 2).

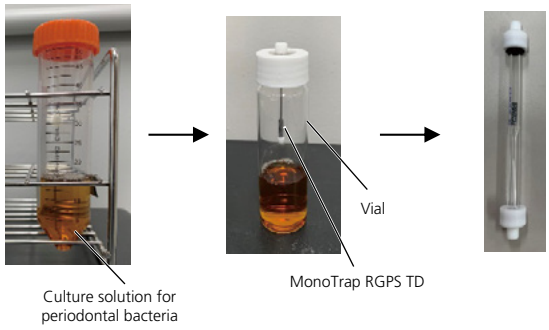


Fig. 2 Collecting Volatile Compounds Using MonoTrap

2-3. Analytical Conditions

The GCMS-TQ8050 NX gas chromatograph mass spectrometer, AOC-6000 Plus multifunctional autosampler, OPTIC-4 multimode inlet, and OP275 Pro II sniffing port (GL Sciences) were used for the GC/MS/O analysis (Fig. 3). The analytical conditions are listed in detail in Table 1.

The GCMSsolution Ver. 4.52, MS-DIAL Ver. 4.70, and GC-Analyzer (MsMetrix) were used for the analysis of the data obtained with the MS analysis. The file format was converted with GCMSsolution, and baseline correction, peak detection, and alignment were performed using MS-DIAL. After this, compound annotation was performed using GC-Analyzer. Compound annotation was automatically performed for any items with a similarity of 80 % or higher using the NIST20 MS spectral library. The minimum peak height value for automatic detection was set to 1,000. However, even if their peak intensity was smaller than this, compounds that had an odor in the sniffing analysis were annotated manually by performing a similarity search with GCMSsolution.

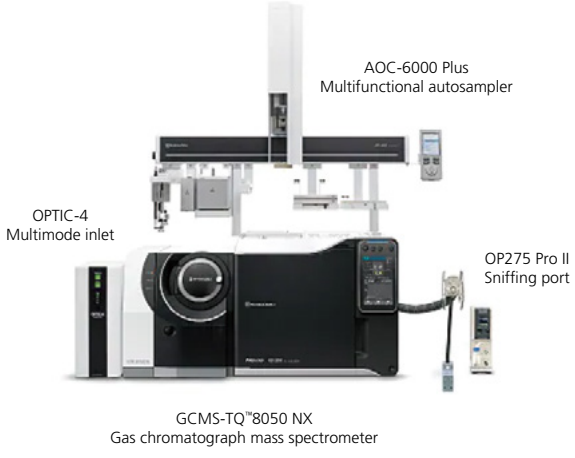


Fig. 3 Configuration of the Analysis System

Table 1 GC/MS/O Analytical Conditions

System Configuration	
Multimode inlet	: OPTIC-4 (with Cryotrap)
Autoinjector	: AOC-6000 Plus
	LINEX + CDC Station
GC-MS	: GCMS-TQ™8050 NX
Sniffing port	: OP275 Pro II
Column	: InertCap FFAP
	(60 m × 0.32 mm i.d., df = 0.50 µm)
Software	: GCMSsolution™ Ver. 4.52
	: Evolution Workstation Ver.4.6.3
	: Olfactory Voicegram V2.3.0
OPTIC-4	
Vent time	: 0 sec
Equilibration time	: 10 sec
End time	: 98.2 min
Carrier gas	: He
Injection port temp.	: 40 °C → 5 °C/sec → 250 °C (8 min)
Injection mode	: Split
Column flowrate	: 4.6 mL/min
Septum purge flowrate	: 5.0 mL/min
Split flowrate	: 4.6 mL/min
Cryofocus temp.	: -110 °C
GC	
Column oven temp.	: 40 °C (3 min) → 3 °C/min → 250 °C (17 min)
MS	
Ion source temp.	: 250 °C
Interface temp.	: 250 °C
Ionization method	: EI
Ionization voltage	: 70 V
Measurement mode	: Scan
Event time	: 0.2 sec
Scan mass range	: *m/z* 24 - 350
Olfactometer	
Sniffing port temp.	: 100 °C (23 min) → 3 °C/min → 240 °C (21 min)

3. Analysis Results and Discussion

Volatile compounds were collected with MonoTrap from the headspace of the supernatant of a culture solution in which the periodontal bacteria *F. nucleatum* and *P. gingivalis* were cultivated and then analyzed using GC/MS/O. The total ion current chromatograms (TICC) obtained are shown in Figs. 4 and 5. The peaks detected with the TICC after cultivation are possibly due to impurities in MonoTrap or the culture solution. In order to analyze only the volatile compounds produced by the periodontal bacteria, a differential analysis was performed (data not shown) with blank measurements using the culture solution. As a result, 110 compounds likely produced by *F. nucleatum* and 100 compounds likely produced by *P. gingivalis* were annotated, including VSCs, ketones, alcohols, and aromatic compounds. Those that were also detectable with a sniffing analysis and their odors are shown in Tables 2 and 3. 17 compounds with an unpleasant odor, including 9 VSCs, were detected from *F. nucleatum*, and 20 compounds with an unpleasant odor, including 6 VSCs, were detected from *P. gingivalis*.

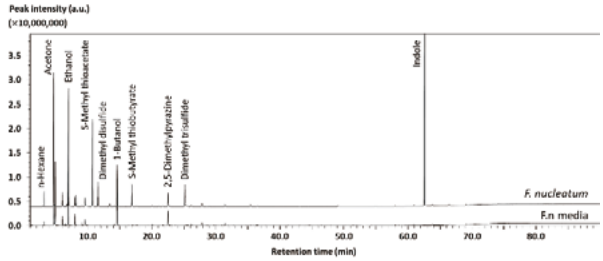


Fig. 4 TICCs of *F. nucleatum* before Cultivation (F.n media) and after Cultivation (*F. nucleatum*)

Table 2 Volatile Compounds (n = 4) Detected from the Culture Solution in which *F. nucleatum* was Cultivated

Compound	RT (min)	Quant MS	Area	Odor
Hydrogen sulfide *	3.0	-	< 1000	Weak
Methanethiol	3.5	47	100833	Medium
Valeraldehyde	6.5	44	194075	Medium
S-Methyl thioacetate	10.7	43	34383063	Strong
Dimethyl disulfide	11.6	94	7002566	Weak
1-Butanol	14.6	56	8260392	Weak
4-Methyl-2-pentanol	15.4	45	26919	Medium
S-Methyl thiobutyrate	16.9	43	6452957	Strong
2,5-Dimethylpyrazine	22.5	108	4261051	Medium
2-Ethylpyrazine	23.0	107	46941	Weak
Dimethyl trisulfide	25.1	126	5526234	Medium
S-Methyl thiohexanoate	26.0	43	37535	Weak
2-Ethyl-3,6-dimethylpyrazine	28.5	135	36962	Weak
Butyric acid	35.4	60	593285	Strong
2-Acetylthiazole	36.3	43	65136	Weak
2,3,5-Trithiahexane	36.8	61	47626	Medium
Indole	62.6	117	61341848	Strong

The data indicates the average values.
*: Lower than minimum peak height

Indole increased significantly during cultivation with both *F. nucleatum* and *P. gingivalis*. Indole has a low unpleasant odor threshold of 0.0003 ppm and is known to be a cause of halitosis[1]. The results of this study suggest that *F. nucleatum* and *P. gingivalis* contributed to the production of indole. In addition, butyric acid increased significantly with *F. nucleatum* and *P. gingivalis*. Butyric acid, valeric acid, propionic acid, and other short-chain fatty acids are known to cause halitosis[2], and the results of this study suggest that *F. nucleatum* and *P. gingivalis* contributed to the production of butyric acid. S-methyl thioacetate, which increased during the cultivation of *F. nucleatum*, and dimethyl disulfide, which increased during the cultivation of *F. nucleatum* and *P. gingivalis*, are VSCs, and have also been detected from saliva incubated from patients with halitosis[3]. S-methyl thiobutyrate, which increased during the cultivation of *F. nucleatum* and *P. gingivalis*, has not been reported to date as a halitosis causing substance. However, this study suggests that it is produced by periodontal bacteria and is a cause of halitosis.

In addition, this study was implemented with a repeat count of n = 4 (biological replicates), and the relative standard deviations RSD (%) of the areas of the peaks detected were evaluated. The RSD for methanethiol, which is a halitosis marker and an important halitosis causing substance, was 70 % or higher for *F. nucleatum* and *P. gingivalis*. The amount of methanethiol produced by periodontal bacteria possibly shows significant biological variability. These results should be considered when using methanethiol as a biomarker with quantitative objectives.

4. Summary

Compounds with a low unpleasant odor threshold could contribute to halitosis even in trace quantities, so it is important to detect trace volatile compounds in expiration analysis.

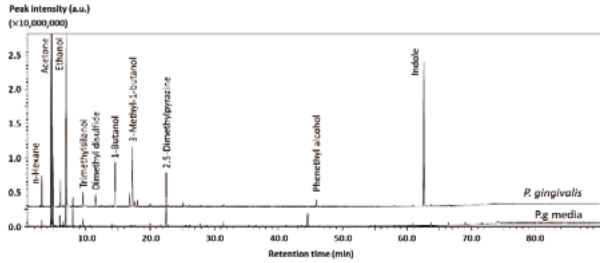


Fig. 5 TICCs of *P. gingivalis* before Cultivation (P.g media) and after Cultivation (*P. gingivalis*)

Table 3 Volatile Compounds (n = 4) Detected from the Culture Solution in which *P. gingivalis* was Cultivated

Compound	RT (min)	Quant MS	Area	Odor
Methanethiol	3.5	47	103446	Weak
Valeraldehyde	6.5	44	117050	Weak
1-Butanol	14.6	56	5864899	Medium
4-Methyl-2-pentanol	15.4	45	24523	Medium
S-Methyl thiobutyrate	16.9	43	2521647	Medium
3-Methyl-1-butanol	17.3	55	7611208	Strong
Pyrazine	17.6	80	526746	Weak
S-Methyl isovalerate	18.1	57	978477	Weak
2-Methylpyrazine	20.0	94	836691	Weak
2,5-Dimethylpyrazine	22.5	108	7852577	Weak
Dimethyl trisulfide	25.1	126	954592	Medium
Trimethylpyrazine	26.1	122	323248	Weak
2-Ethyl-3,6-dimethylpyrazine	28.5	135	11947	Weak
Butyric acid	35.4	60	214348	Strong
2-Acetylthiazole	36.3	43	28204	Weak
2,3,5-Trithiahexane	36.8	61	33159	Medium
Isovaleric acid	37.0	60	157841	Medium
Geraniol	43.5	69	6712	Weak
Phenethyl alcohol	45.9	91	1917824	Weak
Indole	62.6	117	37592405	Strong

The data indicates the average values.

In this study, a comprehensive analysis of volatile compounds produced by periodontal bacteria was implemented using MonoTrap, which easily enriches and collects compounds, and GC/MS/O. The volatile compounds detected in this study might prove to be measurement targets for diagnosing halitosis.

For details on this application, refer to the following article.
Mori, A., Taniguchi, M., Kuboniwa, M., Amano, A., Fukusaki, E.: Profiling volatile compounds from culture supernatants of periodontal bacteria using gas chromatography/mass spectrometry/olfactometry analysis with a monolithic silica gel adsorption device, *J. Biosci. Bioeng.,* (2022)(e-pub)

References

[1] Hampelska, K., Jaworska, M. M., Babalska, Z. Ł., and Karpiński, T. M.: The role of oral microbiota in intra-oral halitosis, pp. 1-17, in, Journal of Clinical Medicine, vol. 9 (2020).
[2] Porter, S. R., and Scully, C.: Oral malodour (halitosis), BMJ, 333, 632-635 (2006).
[3] Monedeiro, F., Milanowski, M., Ratiu, I. A., Zmysłowski, H., Ligor, T., and Buszewski, B.: VOC profiles of saliva in assessment of halitosis and submandibular abscesses using HS-SPME-GC/MS technique, Molecules, 24, 2977 (2019).

Triple Quadrupole Gas Chromatograph Mass Spectrometer

GCMS-TQ8050 NX

Superior Performance

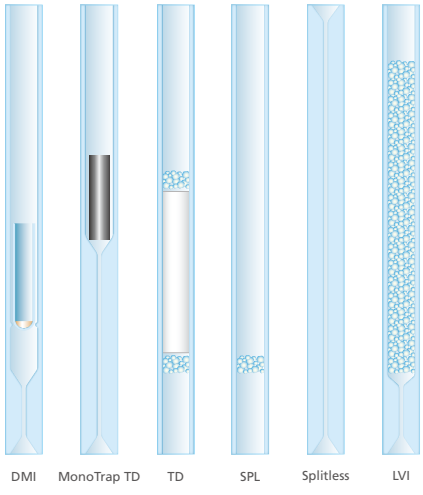
High Efficiency for a Variety of High-Sensitivity Analysis

A new turbomolecular pump with higher evacuation performance achieves a superior vacuum state in the MS unit, which also provides high sensitivity in the single GC-MS mode. With the high-efficiency collision cell (UFsweeper™) installed as before, high-sensitivity detection is possible even when mass resolution is increased.
The new Active Time Management™ function achieves further operating efficiencies by appropriately managing the wait times that occur during maintenance, while switching systems, or when the system is used by multiple users, to extend the operating time.

The OPTIC-4 multimode inlet

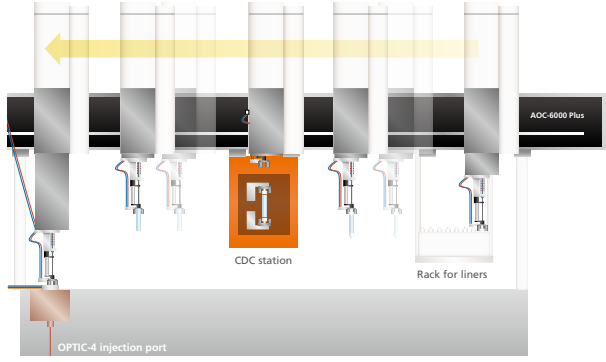
Accommodates a Wide Range of Sample Forms

The wide range of injection modes offered by the OPTIC-4 multimode inlet makes it possible to accommodate many different sample forms. So, in addition to split/splitless injections, many other analyses, such as the pyrolysis analysis of solid samples and the thermal desorption analysis of odor components, can be performed.



Various liners

The liner placed in the rack, after removing its caps at the CDC station, is installed in the OPTIC-4 injection port.



GCMS-TQ, GCMSsolution, UFsweeper and Active Time Management are trademarks of Shimadzu Corporation or its affiliated companies in Japan and/or other countries.

SHIMADZU

Shimadzu Corporation

www.shimadzu.com/an/

For Research Use Only. Not for use in diagnostic procedures.
This publication may contain references to products that are not available in your country. Please contact us to check the availability of these products in your country.

The content of this publication shall not be reproduced, altered or sold for any commercial purpose without the written approval of Shimadzu.
Company names, products/service names and logos used in this publication are trademarks and trade names of Shimadzu Corporation, its subsidiaries or its affiliates, whether or not they are used with trademark symbol "TM" or "®".
Third-party trademarks and trade names may be used in this publication to refer to either the entities or their products/services, whether or not they are used with trademark symbol "TM" or "®".
Shimadzu disclaims any proprietary interest in trademarks and trade names other than its own.

The information contained herein is provided to you "as is" without warranty of any kind including without limitation warranties as to its accuracy or completeness. Shimadzu does not assume any responsibility or liability for any damage, whether direct or indirect, relating to the use of this publication. This publication is based upon the information available to Shimadzu on or before the date of publication, and subject to change without notice.

© Shimadzu Corporation, 2022 / First Edition: July, 2022, 3655-06214-PDFIT, C146-E463