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Original Article

Metabolic Profiling of Oxidized Lipid-Derived Volatiles in Blood by Gas Chromatography/ Mass Spectrometry with In-Tube Extraction

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Once lipids are oxidized, various volatiles are produced by cleavage of the fatty acid side chain. Considering the variety of lipids present in the body, a large number of possible volatiles might originate from oxidized lipids. However, only specific volatiles such as aldehydes are exclusively examined in current studies, and there is no reported method for the exhaustive analysis of all volatiles. We developed a sensitive analytical method for the detection of all possible volatiles for multimarker profiling, applying a new extraction method called in-tube extraction. Oxidized phosphatidyl choline standards were prepared *in vitro* and analyzed in order to determine the potential variety of volatiles. Over 40 compounds, including alcohols, ketones, and furanones, were identified in addition to the aldehydes reported previously. Based on this result, we applied our analytical method to mouse plasma and identified 12 volatiles, including 1-octen-3-ol, which is correlated to disease states. To determine the volatile profile after oxidation, we oxidized plasma *in vitro* under various conditions and identified 27 volatiles, including 1-octen-3-ol and benzaldehyde. The generation capacity of each volatile was different. This method allows sensitive and exhaustive analysis of various volatiles in addition to aldehydes.

Keywords: in tube extraction, volatile, lipids, oxidation, metabolic profiling

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INTRODUCTION

Lipids are major components of biological membranes and play important roles in signaling as biologically active agents. This has motivated the development of lipidomic analyses focusing on phospholipids.[1)] Lipids are also known to be correlated with the development of some diseases such as arterial sclerosis and diabetes. In response, oxidized lipid analyses have received significant attention in recent years.[2)] Oxidized lipids are produced through oxidation at the carbon double bond, including branched-chain fatty acids. During this process, short-chain oxidized lipid compounds are also produced by cleavage of adjacent carbon double bonds. Some compounds, including aldehydes and carbonic acids, are also produced as volatile short-chain oxidized lipids.[3)]

Various volatiles from peroxidized lipids have been studied. For example, volatiles produced from autoxidized lipids were analyzed by gas chromatography, revealing the production of esters and alcohols as well as aldehydes.[4)] In addition, the volatile profile was different depending on the nature of the lipids initially present, including the degree of unsaturation and length of the carbon chain. Moreover, it has been reported that the released volatiles react with amino acids and nitrogen-containing compounds to produce new cyclic compounds.[5,6)] Accordingly, various volatile compounds are expected to be produced by the oxidation of lipids or their reaction with other metabolites.

Recently, volatile compounds in biological samples have generated a great deal of interest. For example, it has been reported that volatile compounds such as malondialdehyde (MDA) and formaldehyde (FA) are produced during up-regulation of oxidation in the body.[7)] These compounds have been recognized as markers of oxidative stress. Some researchers have attempted to distinguish or predict diseases by analyzing unique volatile compounds released in the blood. The determination of volatile profiles (that is, determination of the difference in the concentration of the volatiles) has been applied for the identification of cancer in patients.[8,9)] 4-Hydroxy-2-nonenal (4-HNE) and 1-octen-3-ol, which are correlated with diseases, have been reported

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as volatile biomarkers.[8,10] In addition, volatiles can react with DNA and proteins to form adduct compounds, which have also been analyzed.[11,12] Recently, aldehyde-modified phosphatidylethanolamine (al-PEs) was reported as a bioactive compound,[13] motivating studies focused on volatiles such as aldehydes in the body. However, it is thought that various volatiles are released and that many classes of compounds in addition to aldehydes exist in the body. Simultaneous analysis of volatiles would enable the search for new biomarker candidates. Moreover, it is possible to perform multicomponent analysis by expanding the types of subjects analyzed. Multimarker profiling is expected to improve diagnostic accuracy. In the past, such analysis has been used to characterize disease states that are notoriously difficult to characterize. For example, some cancers such as colorectal and lung cancer with increasing prevalence have no established biomarkers for the early phases. Adapting multimarker profile techniques that exhaustively profile metabolites correlated with these diseases may help in distinguishing or predicting diseases in earlier phases.[14,15]

For the analysis of the volatile components, headspace-solid phase microextraction (HS-SPME) has been performed, resulting in the extraction of the volatiles onto SPME fibers. However, different fibers were required for each target compound, making exhaustive analysis of the volatiles difficult. In addition, the SPME-coated fibers were fragile, had a very limited volume,[16,17] and generated a background peak. In contrast, in-tube extraction (ITEX) accommodated a much larger volume than SPME because the sorbent was contained in a syringe needle. The background peak intensity was lower than that of SPME because the desorption process was carried out by flash heating of the needle. We found that volatile compounds could be exhaustively extracted and identified using the ITEX method, allowing us to perform multimarker volatile analysis in a method similar to the current profiling analysis.

If we can analyze volatiles in the body, we can perform multimarker volatile analysis, similar to the current profiling analysis. Volatile multimarker profiling is expected to improve the accuracy of diagnosis and enable the discovery of new volatile biomarkers. However, current volatile analyses focus on only specific compounds. Thus, the development of a method for the exhaustive analysis of volatiles is needed for multimarker profiling.

In this study, we developed such a method for the exhaustive analysis of volatiles for multimarker profiling. We prepared some oxidized lipid samples by adding an oxidant to phosphatidylcholine, which has various polyunsaturated fatty acids in its side chain, and analyzed the resulting volatiles *via* gas chromatography/mass spectrometry (GC/MS).

EXPERIMENTAL

Materials

Methanol (HPLC grade) was purchased from Kishida Chemical Co., Ltd. (Osaka, Japan). Sodium chloride (pesticide residue-PCB analysis grade), distilled water (HPLC grade), and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Sodium phosphate as well as monobasic and disodium hydrogen phosphate 12-hydrate were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). 1,2-Diacyl-*sn*-glycero-3-phosphocholine (PC) standards, including 1-palmitoyl-2-stearonyl-*sn*-glycero-3-phosphocholine (16:0/18:0 PC, PSPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (16:0/18:1 PC, POPC), 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (16:0/18:2 PC, PLPC), 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (16:0/20:4 PC, PAPC), and 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine (16:0/22:6 PC, PDPC), were purchased from Avanti Polar Lipids Inc. (Alabaster, Al, USA). Mouse plasma was purchased from Kohjin Bio Co., Ltd. (Saitama, Japan). The pooled plasma originated from a mix of male and female mice that were 8–12 weeks old and sexually mature.

Preparation of oxidized lipid samples

PC standard solutions, including PSPC, POPC, PLPC, PAPC, and PDPC, were dissolved in methanol to a concentration of 20 mmol/L. Oxidation treatment was conducted based on the method described by Takebayashi *et al.*[18] Phosphate buffered saline (PBS) was prepared and used to dissolve AAPH to a final concentration of 0.5 mol/L. This solution was prepared just before use.

PC standards were oxidized using AAPH as a radical initiator. A hundred microlitters of PCs were dried under a nitrogen stream in a 2-mL microcentrifuge tube. Five-hundred microlitters of AAPH was added, and the mixture was incubated at 1400 rpm for 4 h at 37°C under aerated conditions. This mixture was used as the oxidized lipid sample. In mouse plasma sample, 500 μL sample was oxidized in same method without drying under a nitrogen stream. This mixture was used as the oxidized mouse plasma sample.

Analytical methods

GC/MS was performed with a TRACE DSQ system coupled with a TRACE GC 2000 system (Thermo Finnigan, USA). The GC apparatus was equipped with a CTC Analytics CombiPal autosampler with ITEX (Zwingen, Switzerland). The GC/MS parameters were as follows: an Omegawax 250 fused silica capillary column (30 m×0.25 mm I.D.×0.25 μm, SUPELCO, USA) connected to a CP-Sil 8 CB low-bleed column (0.75 m×0.25 mm I.D.×0.25 μm, Varian Inc., Palo Alto, CA, USA) as a transfer line. The carrier gas was helium with a flow rate of 1 mL/min, and the injector unit and transfer line temperatures were set to 250°C. The oven temperature was kept at 35°C for 2 min and then increased by 4°C/min to 280°C and maintained at this temperature for 15 min. The mass detector was operated in electron ionization and full scan modes over the m/z range 35–300. The GC/MS system was operated with Xcalibur software (v. 1.3, Thermo Finnigan) with integrated CTC control software, Cycle composer (v. 1.5.4, CTC Analytics).

The in-tube extraction method was carried out using a Tenax TA. One-hundred microlitters of the oxidized sample or normal mouse plasma was transferred to the vial and combined with 40 mg of sodium chloride that had been baked at 200°C for 48 h to reduce possible residuals. All ITEX experiments were performed in 10-mL vials with screw caps and silicone blue transparent/PTFE white septa.

The samples were stored in a sample tray. After the sample was transferred to the agitator, the first extraction step was performed by stirring the sample at 500 rpm for 30 s and heating to the extraction temperature of 50°C. Ten extrac-

tion strokes with an aspiration and dispensing volume of 1 mL were performed, at an extraction speed of 200 μL/s. The trap temperature was 35°C, and the syringe temperature was 45°C. After this extraction, the sample was heated and stirred again. This extraction step was performed by stirring the sample at 500 rpm for 5 min and heating to the same temperature. Ten extraction strokes were performed. This secondary extraction process was repeated five times. Next, 500 μL of the gas phase was aspirated as desorption gas from the headspace of vial. Desorption was performed in the injector after the ITEX trap was heated to 230°C at a desorption speed of 20 μL/s. The autosampler returned to the initial position for trap cleaning. To avoid carryover, the trap was flushed for 10 min at a temperature of 250°C under flowing helium gas after the plunger of the syringe was moved above the side port. Afterward, the plunger was moved down and the trap temperature was set to 35°C to prepare the trap for the next sample.

Data analysis was also performed using Xcalibur software.

Identification of volatile compounds

The compounds detected in the oxidized phosphatidylcholine were checked using Xcalibur's Qual Browser. The peaks detected in the total ion current chromatograms were compared with control, oxidized PSPC, and oxidized lipid standards containing different unsaturated fatty acid side chains. We confirmed these peaks manually in order to identify the compounds derived from the oxidized lipids. A compound search was performed by comparing the

Table 1. Volatile compound candidates from oxidized phosphatidylcholine standards.

Class	RT [min]	Compound	Phosphatidylcholine			
Alkanals	3.7	Pentanal		LA	AA	
	6.14	Hexanal		LA	AA	
	9.03	Heptanal	O		AA	
	12.38	Octanal	O			
	15.79	Nonanal	O		AA	
Alkenals	5.3	2-Butenal				DHA
	6.87	2-Pentenal				DHA
	7.65					
	10.34	2-Hexenal			AA	DHA
	13.72	2-Heptenal		LA	AA	
	16.26	2-Octenal		LA	AA	
	17.11					
	20.42	2-Nonenal		LA		
	26.85	2-Undecenal		LA		
Alkane dienals	18.51	2,4-Heptadienal				DHA
	22.38	2,4-Octadienal		LA		
	25.5	2,4-Nonadienal		LA	AA	DHA
	28.55	2,4-Decadienal			AA	
Ketones	5.74	2,3-Pentanedione				DHA
	12.33	2-Octanone			AA	
	12.9	1-Octen-3-one		LA	AA	
	16.46	3-Octen-2-one		LA	AA	
	21.65	3,5-Octadien-2-one				DHA
	29.06	5-Decanone			AA	
Alcohols	3.02	Isopropyl alcohol	O	LA	AA	DHA
	7	2-Methyl-1-propanol	O	LA	AA	DHA
	8.9	1-Butanol			AA	
	9.21	1-Penten-3-ol				DHA
	12.07	1-Pentanol		LA	AA	
	14.21	2-Penten-1-ol				DHA
	15.14	1-Hexanol			AA	
	18.04	1-Octen-3-ol		LA		
	18.24	Heptanol	O		AA	
	19.29	2-Ethyl-1-hexanol	O			
	21.32	1-Octanol	O			
	23.02	2-Octen-1-ol			AA	
	23.84	1-Nonen-4-ol			AA	
Furans	7.34	2-*n*-Butyl furan		LA	AA	
	10.04	2-Pentyl furan		LA	AA	
	12.63	*trans*-2-(2-Pentenyl)furan				DHA
Furanone	17.43	5-Methyl-2(3*H*)-furanone				DHA
	20.72	5-Methyl-2(5*H*)-furanone				DHA
Others	16.56	5-Etylcyclopent-1-enecarboxaldehyde			AA	
	20.18	Benzaldehyde	O	LA	AA	DHA
	30.86	2-Cyclopentylethanol			AA	

O: oleic acid side chain, LA: linoleic acid side chain, AA: arachidonic acid side chain, DHA: docosahexaenoic acid side chain.

experimental mass spectra with those in the NIST library (NIST/EPA/NIH Mass Spectral Library Version 2.0) using NIST MS Search 2.0. The compounds identified through this search are listed in Table 1. We defined the highest mass peak as the calculated mass and calculated each peak area from the mass chromatogram. However, some peaks did not generate a hit from the library search even though they were the only peaks detected in their respective oxidized lipid samples; these peaks were defined as unknowns. Table 1 lists the results of the oxidized lipid-derived volatiles analysis and we applied these data, including the retention time and mass spectral pattern information, to biological sample analysis.

RESULTS AND DISCUSSION

Analysis of volatiles derived from oxidized phospholipid standard *in vitro*

Various lipids having different carbon numbers and degrees of unsaturation are found in the body, and hence, many kinds of volatiles are expected to be released upon the oxidation of these lipids. During oxidation, volatiles are produced by radical reactions, making it difficult to predict the profiles of each lipid. It was necessary to check the entire volatile profile of the standard, but it was difficult to prepare all the possible volatiles from the oxidized lipids.

Hence, we prepared volatile oxidized lipid compounds by oxidizing phospholipid standard samples *in vitro* in the course of developing our analytical method, anticipating the formation of many kinds of volatiles in addition to aldehydes. In this study, we applied ITEX, a solventless, headspace extraction method in which a syringe attached to a needle filled with adsorbent material is used.[19,20] A heater surrounding the syringe needle was used, and the needle was flash-heated into the GC injector to desorb the volatiles in the sorbent. The lipid sample was phosphatidylcholine, which had palmitoyl acid and unsaturated fatty acids with different carbon numbers and degrees of unsaturation, including oleic, linoleic, and arachidonic acid, or docosahexaenoic acid, as the side chain. These standards were oxidized, and the resulting volatiles were analyzed *via* ITEX. By searching the NIST database, over 40 volatile oxidized lipid compounds were identified (Table 1).

Twelve peaks were detected in oxidized POPC, which has an oleic acid side chain. Nine compounds were annotated and three compounds were unknown. Cyclic volatiles such as benzaldehyde and hydroxy compounds such as heptanol were identified, as well as aldehydes. Among these, nonanal was strongly detected from oxidized POPC. Oleic acid is a ω-9 fatty acid and a monounsaturated fat, suggesting that nonanal was strongly detected because of cleavage at the adjacent carbon double bond in oleic acid during the oxidation reaction. In previous studies, it was reported that nonanal was one of the main volatile compounds produced from heating triolein,[21] and it was thought that octanal and nonanal were significantly produced from oxidized oleic acid as a side chain in phosphatidylcholine.

Twenty-two peaks were detected in oxidized PLPC. Five compounds were unknowns and 17 were annotated, including alkenal compounds such as 2-heptenal and 2-octenal, suggesting that linoleic acid was cleaved not at the ω-6 but at the ω-9 carbon double-bond position. Once linoleic acid-rich oil was oxidized, some aldehydes such as pentanal, hexanal, and 2-octenal were detected,[22] and the volatile profile was almost identical to those obtained in previous studies. It was reported that 2,4-nonadienal and 2,4-decadienal, which were also detected from oxidized PLPC, were detected from heated trilinoleic oil.[23] In addition, 2-heptenal was not produced by lipids with oleic acid but was significantly produced from lipids with linoleic acid[23]; similar results were obtained with phosphatidylcholine standards.

Moreover, phosphatidylcholine with the polyunsaturated fatty acids (PUFAs) arachidonic and docosahexaenoic acids generated 34 and 24 peaks, respectively, including 8 and 9 unknown compounds, respectively. Alcohol compounds such as 1-octen-3-ol and 2-octen-1-ol as well as aldehydes and alkanals were detected, except from phosphatidylcholine with oleic acid or linoleic acid. We expected that alcohol would be produced,[24] but cyclic compounds such as 2-butylfuran and 5-methyl-2(5*H*)-furanone have not been detected before. Arachidonic and docosahexaenoic acids possess numerous double bonds in their carbon chain, all of which are cleavable, raising the expectation that some volatiles with multiple unsaturated bonds would be released. 2,4-Decadienal, which was released by lipid oxidation and reacted with other metabolites and cyclic compounds such as 2-pentylfuran, was produced,[5] suggesting that cyclic compounds were produced by these reactions in phosphatidylcholine with PUFAs. In the volatiles produced, we identified ketones, cyclic volatiles such as furanone, and aldehydes with plural unsaturated bonds, as well as aldehydes and alcohols from oxidized lipid standards.

In this study, over 40 compounds were detected from the oxidized lipid standard samples with ITEX-GC/MS. In previous studies, HS-SPME has been applied, a method that suffers from disadvantages such as the fragility microfibers during thermal desorption and background peaks derived from the fibers.[16,17] In contrast, ITEX is more robust and offers longer extraction-phase lifetimes as well as a method with lower detection limit compared to SPME.[19] These advantages led us to use ITEX for volatile extraction, thereby enabling to identify furans as well as aldehydes. In addition, the SPME-coated fiber was fragile with a very small volume,[16,17] and it generated a background peak. In contrast, ITEX used a much larger volume than SPME because the sorbent was contained in a syringe needle. The background peak intensity was lower than that of SPME because the desorption process was carried out by flash heating of the needle. Therefore, volatile compounds could be exhaustively extracted and identified by applying the ITEX method.

Analysis of volatiles in normal mouse plasma

Various volatile compounds as well as aldehydes were released from oxidized phosphatidylcholine samples, and by applying the ITEX-GC/MS method, we were able to identify these volatiles. There are fatty acids with various carbon numbers and degrees of unsaturation in the body, implying that oxidation results in the formation of various volatiles from lipids. We exhaustively analyzed the volatiles from biological samples by applying this method. The sample used was normal commercial mouse plasma. Compound identification was carried out by comparison of the retention times and mass spectra to those obtained by previous oxidized lipid analyses. A database search *via* the NIST library was

Table 2. Analysis of mouse plasma by ITEX-GC/MS.

	RT [min]	Compound	calc. Mass.	RSD [%] (n=3)	
				RT [min]	Area
1	3.08	Isopropyl alcohol	45	1.29	6.60
2	6.18	Hexanal	44	0.58	4.22
3	7.08	2-Methyl-1-propanol	43	0.24	4.07
4	8.91	1-Butanol	56	0.41	13.40
5	9.21	1-Penten-3-ol	57	0.27	11.16
6	11.90	1-Pentanol	42	0.77	2.70
7	15.06	1-Hexanol	56	0.29	3.58
8	18.05	1-Octen-3-ol	57	0.08	4.47
9	18.23	Heptanol	70	0.09	8.29
10	19.26	2-Ethyl-1-hexanol*	57	0.03	6.33
11	20.27	Benzaldehyde	77	0.08	5.96
12	24.06	Acetophenone*	105	0.10	7.38

*Identified *via* NIST library.

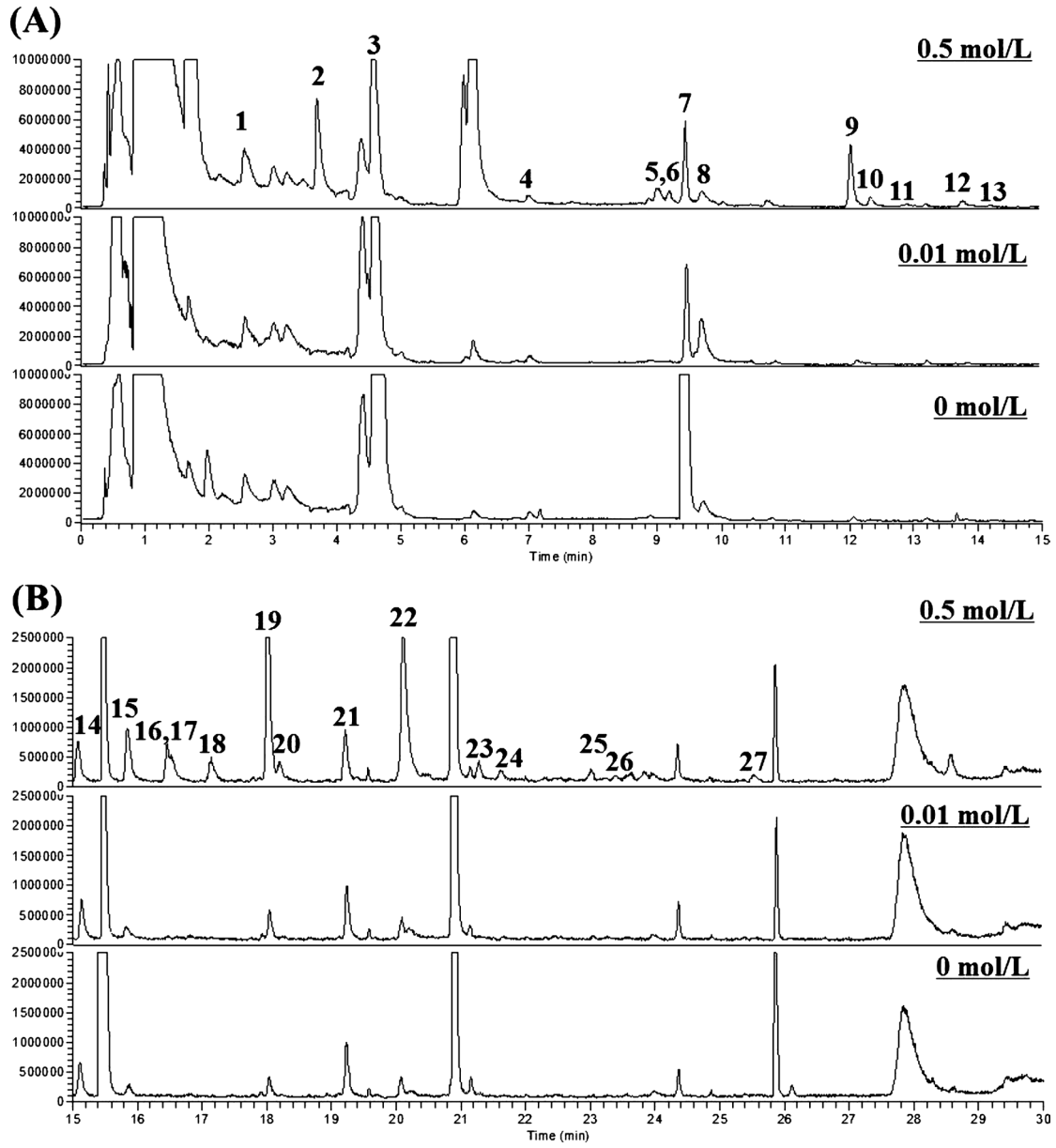

Fig. 1. Total ion current chromatogram of oxidized mouse-plasma under different oxidant concentrations. The peak labels correspond to those in Table 3. (A) 0–15 min, (B) 15–30 min.

also performed. Thus, 12 volatile compounds were identified from normal mouse plasma (Table 2).

Table 2 lists the low-molecular-weight compounds such as isopropyl alcohol and hexanal detected and identified from mouse plasma. 1-Octen-3-ol, a compound that has been reported to be significantly elevated in the blood of liver cancer patients, was also identified by this method.[8] In addition, benzaldehyde was identified from normal mouse plasma. This compound has been reported to be produced from oxidized lipids and is a possible marker of lung cancer.[25] The results of previous studies suggest that this compound is produced from the metabolism of 2,4-decadienal or phenylethyl alanine.[26,27] Acetophenone has not been reported in either biological samples or oxidized lipids. The structure of this compound is similar to that of benzaldehyde, suggesting that benzaldehyde reacted *in vivo* to produce acetophenone. In the compounds identified from this sample, isopropyl alcohol, 2-methyl-1-propanol, heptanol, 2-ethyl-1-hexanol, and benzaldehyde were produced from phosphatidylcholine with oleic acid, as revealed in a previous study. The others were produced from lipids with highly saturated fatty acids such as linoleic or arachidonic acid. Thus, some volatiles derived from highly saturated fatty acids would exist under normal conditions.

We also calculated the relative standard deviation of the retention times and peak areas for each compound to determine the reproducibility of ITEX-GC/MS. We defined the highest mass peak as the calculated mass and calculated the peak area in the mass chromatogram. The RSD of the retention time was approximately 1%. The RSD values of the peak areas of 1-butanol and 1-pentanol were 13% and 11%, respectively, and those of the other compounds were lower than 10%, even for trace volatiles.

Volatile analysis of oxidized mouse plasma

Various volatiles were detected and identified by applying this method to a biological sample. Some compounds that have not been reported were detected, suggesting that they may be notable compounds in volatile multimarker profiling.

We oxidized normal mouse plasma *in vitro* and determined the volatile profiles after enhanced oxidative reaction. We prepared 0.001, 0.01, 0.1, and 0.5 mol/L solutions of AAPH as an oxidant. We also prepared PBS as a control sample, defining this sample as 0 mol/L.

The total ion current chromatogram was shown in Fig. 1. Twenty-seven compounds in total were identified from oxidized mouse plasma (Table 3). Some compounds were also identified from the control sample, even though the analysis conditions were different from those used for the normal mouse plasma. There were no differences between the oxidized mouse plasma with 0 mol/L oxidant and that containing 0.001 mol/L oxidant. However, 3,5-octadien-2-one (RT=21.61 min) was identified in the sample containing over 0.01 mol/L oxidant. This compound was produced from phosphatidylcholine with docosahexaenoic acid as a side chain, suggesting that volatile compounds were released in biological samples only when using 0.01 mol/L oxidant. Oxidant concentrations greater than 0.1 mol/L produced greater numbers of identifiable volatiles, including alcohols and furans, as well as aldehydes. The ratio of isopropyl alcohol, 2-methyl-1-propanol, 1-hexanol, and 2-ethyl-1-hexanol

Table 3. Variation of volatile compounds in each oxidant concentration.

	RT [min]	Compound	Oxidant concentration [mol/L]				
			0	0.001	0.01	0.1	0.5
1	3.02	Isopropyl alcohol	0.90	0.74	0.86	1.08	1
2	3.7	Pentanal	—	—	—	0.05	1
3	6.14	Hexanal	0.01	0.01	0.02	0.57	1
4	7.01	2-Methyl-1-propanol	0.90	0.84	0.86	0.95	1
5	8.9	1-Butanol	0.58	0.53	0.55	0.98	1
6	9.03	Heptanal	—	—	—	0.55	1
7	9.21	1-Penten-3-ol	—	—	—	0.91	1
8	10.03	2-Pentylfuran	—	—	—	0.42	1
9	12.12	1-Pentanol	—	—	—	0.30	1
10	12.32	Octanal	—	—	—	—	1
11	12.87	1-Octen-3-one	—	—	—	0.80	1
12	13.72	2-Heptenal	—	—	—	0.97	1
13	14.28	2-Penten-1-ol	—	—	—	0.83	1
14	15.12	1-Hexanol	0.82	0.78	0.85	0.78	1
15	15.87	Nonanal	0.16	0.17	0.17	0.69	1
16	16.46	3-Octen-2-one	—	—	—	0.62	1
17	16.55	5-Ethylcyclopent-1-enecarboxaldehyde	—	—	—	—	1
18	17.11	2-Octenal	—	—	—	0.53	1
19	18.04	1-Octen-3-ol	0.08	0.07	0.11	0.75	1
20	18.24	Heptanol	—	—	—	0.23	1
21	19.29	2-Ethyl-1-hexanol	1.00	0.85	1.01	0.98	1
22	20.22	Benzaldehyde	0.04	0.04	0.06	0.53	1
23	21.31	1-Octanol	—	—	—	0.31	1
24	21.61	3,5-Octadien-2-one	—	—	0.20	1.54	1
25	22.99	2-Octen-1-ol	—	—	—	0.59	1
26	23.78	1-Nonen-4-ol	—	—	—	0.81	1
27	25.5	2,4-Nonadienal	0.90	0.74	0.86	1.08	1

The variation was calculated from each peak area divided by the corresponding area detected in 0.5 mol/L AAPH. The unit is "/peak area in 0.5 mol/L AAPH." Some volatiles not detected are marked as "—."

in each oxidant sample did not change. However, the ratio of the other 22 compounds increased according to oxidant concentration (Table 3). Lipid autoxidation increased with increasing concentrations of radical initiators such as water-soluble AAPH resulting in the release of various short-chain volatiles. In addition, benzaldehyde and 2-pentylfuran, compounds known to be disease biomarkers, were identified.[25)]

2-Pentylfuran and 3,5-octadien-2-one were produced from oxidized linoleic acid and Strecker degradation of 2,4-decadienal,[5,24)] respectively; however, there are no reports of these compounds in biological samples. In addition, the volatile oxidized lipid (*E*)-4,5-epoxy-(*E*)-2-heptanal was shown to react with nitrogen-containing compounds to produce propanal and alkyl pyrrol compounds. These volatiles then formed furan compounds.[6)] Through these kinds of reactions, various volatile compounds were produced in addition to aldehydes.

Volatile profiles were expected to depend on oxidant concentration, that is to say, the degree of oxidation enhancement in the body. Previous studies required the utilization of pretreatment steps such as extraction or derivatization in volatile analysis. A serum-metabolomics-based diagnostic method for colorectal cancer was developed by exhaustively analyzing derivatized metabolites,[14)] in addition to a method that relies on manual identification of compounds related to disease states, by analyzing derivatized volatiles in samples collected from lung cancer patients.[28)] Both of these methods require pretreatment steps such as purification or derivatization. In contrast, the analytical method presented here is designed to exhaustively detect and identify volatiles in biological samples, as do current metabolomics profiling approaches without the requirement of any pretreatment steps.

CONCLUSION

In this study, we developed an analytical method for the exhaustive analysis of oxidized lipid-derived volatiles using ITEX-GC/MS. By oxidizing the lipids, various volatile compounds as well as aldehydes were produced through the cleavage of the carbon double bonds in the fatty acid side chains. Because previous studies focused heavily on only a small number of specific volatiles, we desired a way to exhaustively analyze volatiles using ITEX-GC/MS. Using our method, we simultaneously identified alcohols, ketones, and cyclic compounds as well as aldehydes from oxidized lipids. We also applied the method to a biological sample, in which we identified various volatiles; the resulting RSD values for retention time and peak areas were very low. Moreover, some of the compounds we detected are considered to be disease biomarkers, such as benzaldehyde,[25)] demonstrating that our method enables volatile screening of biological samples. This method is expected to be suitable for multimarker profiling based on exhaustive volatile analysis and current metabolomics approaches. Using multimarker profiling, disease states could be predicted or distinguished through the relative variation of various volatiles.

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