厚生労働科学研究費補助金（化学物質リスク研究事業）分担研究報告書

# 胎児へのエピジェネティックな影響の解析
## （化学物質の胎盤機能と胎児発生におけるエピジェネティックな影響の解明）

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研究要旨
昨年度までの研究で、凍結保護剤や溶媒として、細胞への悪影響を意識せずに多用されているジメチルスルホキシド（DMSO）が、マウス初期胚のモデルである胚性幹細胞（ES 細胞）やその分化細胞に DNA メチル化プロファイルの変化を引き起こすことを明らかにしていた。この結果を受け、本年度は、細胞のエピジェネティック状態を変化させうる他の化学物質をスクリーニングする実験系を確立した。第１に、マウス Oct4 遺伝子の発現を GFP の蛍光によってモニターできるマウス ES 細胞株の樹立を行った。Oct4 遺伝子は ES 細胞の多分化能維持に必須であり、初期胚や ES 細胞を除くすべての体細胞でエピジェネティックに不活性化されている。この Oct4-GFP ES 細胞を様々な化学物質存在下で培養することにより、GFP 蛍光の有無を指標として、初期胚マスター遺伝子のエピジェネティックスを変化させうる化学物質をスクリーニングすることが可能である。第２に、DsRed 遺伝子を導入した組換え栄養膜幹細胞（DsRed-TS 細胞）の樹立にも成功した。DsRed-TS 細胞では、ゲノムに挿入された DsRed 遺伝子が不活性化されほとんど DsRed を発現していないが、ヒストン脱アセチル化酵素の阻害剤であるトリコスタチン A 存在下で培養すると、ほとんど全ての細胞が DsRed を発現するようになった。すなわち、DsRed-TS 細胞を用いることで、ヒストンアセチル化に影響を及ぼす化学物質のスクリーニングが可能である。以上の細胞系を確立するとともに、DNA メチル化プロファイルをハイスループットで解析する新たな手法も開発した。今後、これらの幹細胞・手法を用いることにより、化学物質が胎児体細胞、および、胎盤の細胞のゲノムに与えるエピジェネティックな影響を解析し、新たな毒性評価系の確立を目指す。

## A．研究目的

ヒト受精卵は 200 種もの異なる細胞へと分化する。この過程は「細胞は同じ DNA 塩基配列を持つが異なる性質を持つようになり、その性質は細胞分裂後も維持される」エピジェネティックなプロセスである。DNA メチル化は遺伝子サイレンシング・クロマチン構造の変換を伴う遺伝子発現の制御機構であるとともに、細胞分裂後も維持される、エピジェネティクスの分子機構である。各々の細胞はゲノム上の異なる座位がメチル化・脱メチル化され、細胞特異的な DNA メチル化プロファイルを持つようになる。環境中の化学物質が母体を通じて初期胚の DNA メチル化プロファイルに異常を生じさせ、個体発生にエピジェネティックな異常を生じさせている可能性がある場合、その異常は生後も引き継がれ、生涯を通じて遺伝子発現が異常になる可能性を秘めている。昨年度までの研究で、生殖医療の現場でもよく用いられる DMSO が、ES 細胞やその分化細胞に DNA メチル化プロファイルの変化を引き起こすことを明らかにした（Iwatani et al., 2006）。この結果は、これまで細胞への影響が未知であった物質、あるいは、形態や増殖能などの変化を起こさないことから無害だと思われていた化学物質の、細胞のエピジェネティック状態に対する影響を改めて見直す必要があることを示している。そこで本年は、種々の化学物質のエピジェネティック状態に対する影響を効率的に解析する実験系および手法の確立を目的とした。

## B．研究方法

### B-1　Oct4-GFP ES 細胞の樹立

マウス Oct4 遺伝子の上流約 18 kb の転写調節領域に緑色蛍光タンパク EGFP をコードする cDNA につないだトランスジーンと Neo 耐性遺伝子をもつコンストラクトを作製した。これを R1 ES 細胞に安定的に導入し、G418 によりこのコンストラクトの導入された ES クローンを樹立した。

### B-2　DsRed-TS 細胞の樹立

トリプシン処理により浮遊させた ICR 系統マウス胚由来 TS 細胞に、pDsRed-Express-N1 プラスミド DNA（Clontech）を Lipofectamine 2000（Invitrogen）を用いて導入した。導入プラスミド DNA がゲノムに組み込まれた安定形質転換株の選択には、G418 を用いた。得られた形質転換株 DsRed-TS に対するヒストン脱メチル化酵素阻害剤トリコスタチン A（TSA; Sigma-Aldrich）の効果は、200 nM TSA 存在下で 48 時間 DsRed-TS を培養後、蛍光顕微鏡下で確認した。

### B-3　倫理面への配慮

本研究に用いられた細胞は研究用マウス胚由来のものであり、倫理上の問題は全くない。

## C．結果

### C-1　Oct4-GFP ES 細胞の樹立

Neo 耐性をもつクローンのうち、ES 細胞で恒常的に EGFP を発現し、分化を誘導すると発現しな

くなるものを選択した結果、1クローンのOct4-GFP ES細胞の樹立に成功した。

C-2　DsRed-TS細胞の樹立

G418存在下で形成されたTS細胞コロニーを個別にピックアップし、G418を添加したまま継代維持した結果、安定的形質転換株（DsRed-TS）を1つ得ることができた。ゲノムPCRにより、導入したプラスミドが持つDs-Redタンパク質コード領域とその上流のCMVプロモーター配列もゲノムDNAに組み込まれていることが確認されたが、Ds-Redの発現は一様ではなく、Ds-Redの蛍光を有する細胞の割合は1％に満たなかった。TSA処理後、DsRed-TSにおけるDs-Redの発現は促進され、ほぼ全ての細胞がDsRedの蛍光を発していた。

C-3　新たなDNAメチル化プロファイル解析法の開発

細胞のDNAメチル化状態を解析する新たな手法として、Restriction tag-mediated amplification (REAM)法を開発した。REAM法ではメチル化感受性制限酵素で切断された末端を持つ断片が優先的に増幅される。増幅した断片を、12,800遺伝子の転写開始点から上流1 kbを高密度にタイリングしたDNAマイクロアレイとハイブリダイズさせ、これらの遺伝子のメチル化状況を測定することが可能となった。データ解析の結果、非常に再現性の高いデータが得られていることが確認出来た。また、Oct4、Stellaなどの上流から得られた結果は、すでに他の方法で明らかとなっているメチル化状況の結果や発現の状況に合うものであった。

D．考察

Oct4遺伝子は、発現のない分化誘導後のES細胞や体細胞において、上流転写調節領域のDNAメチル化によりその発現が抑制されている。本年度得られたOct4-GFP　ES細胞をある種の化合物で処理した際に、未分化条件下でGFPの蛍光を低下させるものがあれば、その化合物にはゲノムDNAのメチル化を誘導する作用があることが期待される。逆に、分化誘導後のOct4-GFP　ES細胞株を用いれば、ゲノムDNAの低メチル化を誘導する作用を持つ化合物をスクリーニングすることが可能になる。一方、DsRed-TSにおけるDs-Redの発現がTSAにより誘導されたことは、DsRed-TS細胞株においてはDs-Redの発現がヒストン脱アセチル化により抑制されていることを示唆する。従って、この細胞株を用いれば、ヒストンアセチル化に影響を及ぼす化合物のスクリーニングが可能となる。

これまで我々は、Restriction Landmark Genomic Scanning法（RLGS）によりメチル化感受性制限酵素であるNotIの認識部位のメチル化状態を解析してきた。CpGアイランドを持たない遺伝子であるOct4やCpGが極端に少ないIns2でもその発現はDNAのメチル化に依存することを明らかにしてきたが、このようなCpGが少ない領域はNotI認識配列が含まれることはまれであり、NotIを指標とした解析からは漏れてしまう。今回開発したREAM法を4塩基長の認識配列を持つメチル化感受性制限酵素を用いて行うことにより、DNAメチル化解析の解像度を大幅に向上し、従来の解析では対象となっていなかったゲノム領域のDNAメチル化も検出可能となった。

E．結論

Oct4-GFP　ES細胞、および、DsRed-TS細胞により、エピジェネティック制御の二つの主要な分子機構であるDNAメチル化とヒストンアセチル化に影響を与える化合物の効率的なスクリーニングが可能となった。さらに、高解像度なDNAメチル化解析法を確立することで、化合物の影響を、ゲノムワイドに詳細かつ迅速に解析することが可能となった。これらを利用することで、他研究グループが分析の対象としてきた化合物の、細胞への影響を検討することが今後の課題である

F．健康危険情報

該当なし

G．研究発表

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2. 学会発表

該当なし

H．知的財産権の出願・登録状況

該当なし

III. 研究成果の刊行に関する一覧表

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# Serum Levels of Volatile Organic Compounds in Patients with Sick Building Syndrome

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Volatile organic compounds (VOCs) pose possible health risks that could result from exposure to indoor airborne VOCs as suggested by the causal associations with symptoms of sick building syndrome (SBS) (Hodgson 2002). People can be exposed to VOCs through various products and processes, including building materials, paints, cleaning agents, pesticides, tobacco smoke, and personal care products in addition to the traditional sources of exposure such as occupation and ambient air pollution (Ashley et al. 1996). There is no universally accepted clinical definition of SBS and no adequate theory for its occurrence (Redlich et al. 1997). The characteristics of SBS are non-specific symptoms which include dryness and irritation of skin, eyes and air-ways and general symptoms. In Japan, SBS symptoms associated with indoor air VOCs in new or newly remodeled houses have been increasingly highlighted, and they are called 'sick house syndrome'.

Although past researchers have tried to understand the causes of SBS, they did not generally succeed in showing direct evidence that elucidates the relationship between SBS and VOCs (Hodgson 2002). For example, information concerning serum levels of VOCs in patients with SBS symptoms is not available because measuring sub ng levels of VOCs in serum with the exclusion of experimental contamination is very difficult. To address this issue, we examined (1) whether headspace gas chromatography/mass spectrometry (HS-GC/MS) was applicable to the measurement of serum VOC concentrations in SBS patients and volunteer controls, and (2) whether the elevation of serum VOC levels correlated with SBS symptoms.

## MATERIALS AND METHODS

For contamination control, blood-collecting equipment was treated as described below. Glass syringe barrels were sterilized in an autoclave and dried in a drying oven. Test tubes and screw caps were washed with detergent, water and methanol, and then heated at 180 °C for 5 hours. They were fitted with screw caps and then stored. A saturated saline solution was prepared by mixing sodium chloride and distilled water, which was subsequently subjected to aeration using helium gas and to degassing in an ultrasonic bath under reduced pressure. The prepared solution

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**Table 1.** Retention times, quantification and confirmation ions.

| Analyte | Retention time (min) | Quantification ion | Confirmation ion | Quantification ion (internal standard) |
|---|---|---|---|---|
| Benzene | 12.7 | 78 | 51 | 84 |
| Toluene | 15.3 | 91 | 92 | 98 |
| Ethylbenzene | 17.3 | 91 | 106 | 98 |
| m,p-Xylene | 17.4 | 91 | 106 | 98 |
| o-Xylene | 18.2 | 91 | 106 | 98 |
| Styrene | 18.3 | 104 | 78 | 112 |
| *p*-Dichlorobenzene | 21.2 | 111 | 146 | 115 |
| Naphthalene | 25.1 | 128 | 127 | 136 |

was immediately placed in headspace vials previously treated as for test tubes. Internal air space in the vial was replaced with helium gas. The headspace vials were sealed with Teflon-backed septa and crimp caps.

Fortified sample recovery was carried out using pig serum. Although human blood is easily available in hospitals, it is difficult to obtain it in quantity without contamination problems when baseline levels are measured. We therefore procured pig serum using the contamination control described above.

For the measurement of VOCs in serum, 1 mL of serum was diluted with 14 mL of saturated saline solution and analyzed by HS-GC/MS. VOCs were quantified using deuterated compounds as internal standards. Target VOCs (benzene, toluene, *o*-, *m*-, *p*-xylene, ethylbenzene, styrene, *p*-dichlorobenzene and naphthalene) were selected for analysis because they are frequently found in indoor air in Japan (Saijo et al. 2004). HS-GC/MS analysis was carried out under the following conditions:
Headspace sampler: Tekmar 7000 (Tekmar, USA), vial size: 22 mL, sample temperature: 60 °C, sample equilibrium time: 20 min, mixer: on (power 5, 3 min), sample loop size: 1 mL, sample loop temperature: 150 °C, transfer line temperature: 160 °C. GC/MS: AUTO MASS SYSTEM II (Jeol, Japan), Column: Vocol (60 m x 0.25 mm i.d., 0.1 μm film thickness, Supelco, USA), oven temperature: initial temperature 40 °C with 4 min hold, then 10 °C /min to 230 °C and post run at 230 °C for 5 min, ion source temperature: 210 °C, EI voltage: 70 eV, scan range: m/z 46-260. Table 1 shows the retention times and quantification masses used for each of the analytes and internal standards.

The study participants were 18 patients with SBS and 32 volunteer controls. Seven patients live in Sapporo, and 11 live in Aichi Prefecture, Japan. The patients were diagnosed according to the following criteria: 1) a typical setting for symptoms is a new or newly remodeled house; 2) symptoms generally improve when the patient is away from the house. The possible factors of SBS onset were moving into a new or remodeled house (56%, 10/18), use of chemicals such as insecticides, mothballs and bleach (33%, 6/18), exposure to organophosphorus pesticides (6%, 1/18) and unknown (6%, 1/18). The volunteer controls were recruited from the staff of Aichi Prefectural Institute of Public Health, Nagoya, Japan. All volunteer controls live in Aichi Prefecture, Japan. Five volunteer controls were excluded because they are smokers. Elevated serum VOC levels due to smoking have been reported

previously (Mannino et al. 1995), and smoking is the largest confounder in discerning the influence of other environmental exposure (Ashley et al. 1996). In fact, the detection rates and levels of toluene, styrene and benzene in the smokers' serum were higher than in nonsmoker volunteer controls (data not shown).

Of the patients, 89% (16/18) were women and 11% (2/18) were men. Previous studies have shown the same pattern (Stenberg et al. 1995). Among patients, 22% (4/18) were under 29 years of age, 44% (8/18) were 30-49 years of age, and 33% (6/18) were over 50 years of age. Representative symptoms were classified into 10 categories as follows: eye (irritation, dry eyes, eye congestion); nose (stuffy or runny nose); throat (sore throat, itchy throat); respiratory system (cough, shortness of breath); skin (itching and dry skin); general symptoms (fatigue, headache, dizziness); psychological symptoms (difficulty concentrating, insomnia, depression); musculoskeletal system (joint pain, numbness in the hands or feet); gastrointestinal system (nausea, stomachache, diarrhea); genitourinary system (increased urinary frequency, menstrual pain, menorrhagia). The prevalence of patients' symptoms was significantly greater than for controls as follows: (patients vs controls) eye, 67% vs 16%; nose, 83% 16%; throat, 56% vs 3%; respiratory system, 50% vs 3%; skin, 44% vs 9%; general symptoms, 72% vs 9%; psychological symptoms, 56% vs 3%; musculoskeletal system, 44% vs 0%; gastrointestinal system, 44% vs 0%; genitourinary system, 50% vs 0%. The data analyzed in this paper were collected in 2001 and 2002 in Japan. This study was conducted according to the Declaration of Helsinki and signed informed consent was obtained from all subjects.

For statistical evaluation of the prevalence of symptoms between patients and controls, the proportions of positive symptoms were compared using the Chi-square test or Fisher's exact test for the resulting 2x2 contingency table. To compare VOC concentrations between patients and controls, Mann-Whitney's U-test was used. Differences in the mean number of symptoms within the patient group and between the two groups dichotomized at the limit of quantification of serum VOCs were evaluated using Mann-Whitney's U-test. In all statistical analysis, a 5% level of significance was applied.

## RESULTS AND DISCUSSION

Reproducible calibration curves for all target VOCs were obtained with correlation coefficients greater than 0.998 (known concentration vs analyte/internal standard ratio) by HS-GC/MS analysis of standard VOCs. The method was sensitive with limits of detection between 0.1 and 0.5 ng in 1 mL of serum for all analytes. The method does not require complicated sample preparation procedures and takes only 1 hour to complete all procedures starting from sample dilution to quantification by HS-GC/MS analysis.

In order to examine the applicability of this method to real sample analysis, VOC recoveries were determined. The recoveries from blank pig serum spiked with target VOCs and their internal standards are summarized in Table 2. Spiked VOCs

**Table 2.** Recovery of VOCs from serum.[a]

| VOCs | Scan mode (with IS)[b] | | | SIM mode (with IS)[b] | | | Scan mode (No IS)[c] |
|---|---|---|---|---|---|---|---|
| | LOQ[d] (ng/mL) | Recovery (%) | CV[e] (%) | LOQ[d] (ng/mL) | Recovery (%) | CV[e] (%) | Recovery (%) |
| Benzene | 0.5 | 106 | 2.8 | 0.1 | 104 | 4.7 | 85.1 |
| Toluene | 0.5 | 103 | 1.4 | 0.1 | 101 | 6.0 | 65.6 |
| Ethylbenzene | 0.5 | 101 | 2.2 | 0.1 | 96.6 | 4.4 | 44.3 |
| *m,p*-Xylene | 0.5 | 97.3 | 1.7 | 0.1 | 100 | 4.5 | 42.1 |
| *o*-Xylene | 0.5 | 100 | 2.7 | 0.1 | 89.8 | 4.3 | 44.8 |
| Styrene | 0.5 | 101 | 1.9 | 0.1 | 105 | 4.9 | 48.6 |
| *p*-Dichloro-benzene | 2.0 | 98 | 6.4 | 0.5 | 114 | 6.3 | 35.2 |
| Naphthalene | 2.0 | 101 | 0.4 | 0.5 | 105 | 3.2 | 24.8 |

[a]A mixture of VOCs was added at 15 and 1.5 ng/mL for scan and SIM mode analysis, respectively.
[b]Results are the means of five replicate determinations. With IS; with internal standard correction
[c]Results were obtained from a single set of measurement. No IS: without internal standard correction
[d]LOQ, limit of quantification (S/N>5)
[e]CV, coefficients of variation

were satisfactorily recovered with the internal standard correction (scan mode; 97.3-106.3%, selected ion monitoring mode; 89.8-114%), whereas they were poorly recovered without the correction (24.8-85.1%). The coefficients of variation values were acceptable with the internal standard correction (scan mode; 0.4-6.4%, selected ion monitoring mode; 3.2-6.3%). The selected ion monitoring mode had greater recovery and coefficients of variation values than in scan mode, which might be derived from lower concentrations in the selected ion monitoring mode. These results indicate that internal standard correction is indispensable for the headspace analysis of VOCs in biological samples because headspace analysis tends to show less reproducibility than direct measurement procedures.

Measuring low levels of VOCs in human biological samples is very difficult because highly sophisticated techniques and contamination control are required (Ashley et al. 1996). VOCs are ubiquitous components in many consumer products and the laboratory environment. Thus, for contamination control, we carefully washed the blood-collecting equipment and removed VOCs from saturated saline solution as described in MATERIALS AND METHODS. These treatments reduced the VOC background to sub ng/mL level (data not shown).

Table 3 shows a summary of the serum VOC levels of 18 patients with SBS symptoms and 27 controls. Three of the most often detected VOCs among these 45 subjects were *p*-dichlorobenzene, toluene, and xylene which were found in 61%, 44%, and 44% of the patients, and 85%, 67%, and 15% of the controls, respectively.

**Table 3.** Serum VOC levels in patients (n=18) and controls (n=27).

| | No. of participants with indicated levels of VOCs (ng/mL) | | | | | No. of positives[b] | Mean conc. in positives (ng/mL) | Maximum conc. (ng/mL) |
|---|---|---|---|---|---|---|---|---|
| | <0.1 | 0.1-0.4 | 0.5-1.0 | >1.0 | | | | |
| Toluene | | | | | | | | |
| Patients | 10 | 6 | 2 | 0 | | 8 (44)[c] | 0.3 | 0.8 |
| Controls | 11 | 13 | 3 | 0 | | 18 (67) | 0.4 | 1.3 |
| Xylene[a] | | | | | | | | |
| Patients | 10 | 7 | 1 | 0 | | 8 (44) | 0.3 | 0.8 |
| Controls | 23 | 4 | 0 | 0 | | 4 (15) | 0.3 | 0.4 |
| Benzene | | | | | | | | |
| Patients | 16 | 1 | 1 | 0 | | 2 (11) | 0.4 | 0.7 |
| Controls | 26 | 1 | 0 | 0 | | 1 (4) | - | 0.1 |
| Ethylbenzene | | | | | | | | |
| Patients | 16 | 2 | 0 | 0 | | 2 (11) | 0.1 | 0.1 |
| Controls | 27 | 0 | 0 | 0 | | 0 (0) | - | - |
| Styrene | | | | | | | | |
| Patients | 17 | 1 | 0 | 0 | | 1 (6) | - | 0.1 |
| Controls | 25 | 2 | 0 | 0 | | 2 (7) | 0.2 | 0.2 |
| | No. of participants with indicated levels of VOCs (ng/mL) | | | | | No. of positives[b] | Mean conc. in positives (ng/mL) | Maximum conc. (ng/mL) |
| | <0.5 | 0.5-0.9 | 1.0-4.9 | 5.0-10 | >10 | | | |
| *p*-Dichlorobenzene | | | | | | | | |
| Patients | 7 | 4 | 5 | 0 | 2 | 11 (61) | 5.1 | 25.4 |
| Controls | 4 | 3 | 9 | 4 | 7 | 23 (85) | 16.8 | 171 |
| Naphthalene | | | | | | | | |
| Patients | 18 | 0 | 0 | 0 | | 0 (0) | - | - |
| Controls | 27 | 0 | 0 | 0 | | 0 (0) | - | - |

[a]Results are expressed as the sum of *o*-, *m*-, *p*-xylenes.

[b]*p*-Dichlorobenzene and naphthalene; >0.5 ng/mL, toluene, xylene, benzene,

[c]Number in parentheses indicate percentages. There is a significant difference in the ratio of positive samples of xylene ($P$ <0.05).

Among the most often detected VOCs, only xylene was significantly more prevalent in the patients. The positive rates of *p*-dichlorobenzene and toluene in the patients showed lower frequency than in the controls although there were no significant differences. The mean concentrations of *p*-dichlorobenzene, toluene and xylene in positive cases were 5.1, 0.3 and 0.3 ng/mL for the patients and 16.8, 0.4 and 0.3 ng/mL for the controls, respectively. The differences in the concentrations of *p*-dichlorobenzene and toluene were not statistically significant between the patients and controls. The low positive rates of other VOCs did not allow us to perform statistical analyses.

**Table 4.** Differences in the mean number of symptoms within the patient group and between the two groups dichotomized at the limit of quantification of serum VOCs.

| Compound | Mean No. of symptoms | | *p* Value |
|---|---|---|---|
| | Serum VOC positive | Serum VOC negative | |
| *p*-Dichlorobenzene | 5.5 | 7.0 | 0.3187 |
| Toluene | 7.5 | 5.0 | 0.0701 |
| Xylene | 6.0 | 6.2 | 0.8906 |

Table 4 shows a summary of the mean number of symptoms within the patient group. The differences in the number of symptoms are not statistically significant between the two groups dichotomized at the limit of quantification of serum VOCs.

These results indicate that it is difficult to determine the serum VOC levels responsible for inducing symptoms.

Several reports have suggested that SBS symptoms are related to VOCs in the indoor air environment (Norbäck et al. 2000; Kamijima et al. 2002; Saijo et al. 2004); however, there has been no report on the relationship between serum VOC levels and SBS symptoms to our certain knowledge. In this study we measured serum levels of VOCs in patients with SBS symptoms and volunteer controls. Measuring chemicals in blood is advantageous because we can calculate the body burden precisely. However, for nonoccupationally exposed people, it is generally difficult to clarify the relationship between adverse effects and VOC exposure or serum VOC levels because of the relatively low levels of VOCs found in serum (Ashley et al. 1994). The serum VOC levels of all subjects in this study were relatively low except for *p*-dichlorobenzene, which was consistent with previous reports (Ashley et al. 1996). The number of target VOCs was limited although there are more than one hundred VOCs in indoor air. The small subject population restricted our approach to include limited statistical analysis. Detailed information on the patients' history of exposure is lacking. Further investigation to overcome these problems is required.

In this study we intended to examine (1) whether HS-GC/MS was applicable to the measurement of serum VOC concentrations in SBS patients and volunteer controls, and (2) whether the elevation of serum VOC levels correlated with SBS symptoms. Despite the successful application of our HS-GC/MS method, we found no statistical differences in the concentrations of studied VOCs between the patients and controls. We also found no relationship between serum VOC levels and SBS symptoms in the patients studied. Therefore, we must consider that it is difficult to identify the responsible VOCs and their serum levels inducing SBS symptoms from the results of this study.

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# Two Sensitive Sick-building Syndrome Patients Possibly Responding to *p*-Dichlorobenzene and 2-Ethyl-1-Hexanol: Case Report

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**Sick-building syndrome (SBS) symptoms associated with indoor air volatile organic compounds (VOCs) in new or newly remodeled houses have been increasingly highlighted, and are known as "sick house syndrome" in Japan. In the course of the investigation of SBS patients, we found two sensitive patients who complained of severe symptoms and had elevated serum levels of *p*-dichlorobenzene and 2-ethyl-1-hexanol. One patient was a housewife, who complained of various symptoms such as headache, itching eyes, nasal irritation, and night sweats and had a high serum level of *p*-dichlorobenzene (25.4 ng/ml). She showed some improvement of symptoms in association with the gradual decrease in *p*-dichlorobenzene concentrations in both her bedroom and her serum. The other patient was a female professor who had experienced mainly respiratory symptoms, such as nonproductive cough, throat irritation, *etc.* when she entered her office, classrooms, and a faculty meeting room in a university building. Her serum 2-ethyl-1-hexanol concentration was 4.6 ng/ml, which was more than 7.7-fold higher than that in four other patients with other onsets. The elevation of her serum 2-ethyl-1-hexanol level was assumed to be due to daily exposure in the university building.**

**Key words**——sick building syndrome, *p*-dichlorobenzene, 2-ethyl-1-hexanol, volatile organic compounds, serum

## INTRODUCTION

Sick-building syndrome (SBS) has become a serious problem in indoor environments, in homes as well as in workplaces.[1] Volatile organic compounds (VOCs) pose possible health risks that could result from exposure to indoor airborne VOCs as suggested by the causal associations with symptoms of SBS.[2] There is no universally accepted clinical definition of SBS and no adequate theory for its occurrence.[1] The complaints are usually nonspecific, such as headache, nausea, irritated eyes, cough, dry and itchy skin, *etc.* For most people, the health problems disappear when leaving the building. In Japan, SBS symptoms associated with indoor air VOCs in new or newly remodeled houses have been increasingly highlighted, and are known as "sick house syndrome."[3] The Ministry of Health, Labour and Welfare of Japan provided guideline values (GLV) for indoor concentrations of 13 VOCs, such as formaldehyde, toluene, *p*-dichlorobenzene, *etc.*, and promulgated an advisable value of total VOCs.[4]

Although past research tried to understand the causes of SBS, it did not generally succeed in showing direct evidence that elucidates the relationship between SBS and VOCs.[2] Recently, Saijo *et al.* reported that indoor air concentrations of some VOCs were significantly related to the symptoms, and the sum of all VOCs was significantly related to throat and respiratory symptoms, although the concentrations of VOCs were relatively low.[5] However, to our knowledge, no study on the relationship between SBS and serum VOC concentrations has been conducted. To address this issue, we measured serum VOC concentrations in patients and volunteer controls; however, it was difficult to identify the responsible VOCs and their serum levels inducing the SBS symptoms because we did not find statis-

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tically significant differences in the concentrations of studied VOCs between the patients and controls and also found no relationship between serum VOC levels and SBS symptoms in the patients studied.[6] In the course of the investigation of SBS patients, we found two sensitive patients who complained of severe symptoms. In this study, we intended to examine the relationship between exposure to VOCs and SBS symptoms in the two patients by measuring serum and indoor air VOC concentrations.

## PATIENTS AND METHODS

**Patients** —— Patient A was a 44-year-old housewife from Sapporo, Japan. She complained of various symptoms such as headache, itching eyes, nasal irritation, night sweats, *etc.* Patient B was a 61-year-old female professor of Foreign Culture and Literature from Nagoya, Japan. She had been experiencing mainly respiratory symptoms, such as nonproductive cough, throat irritation, *etc.* when she entered her office, classrooms, and a faculty meeting room in a university building. 2-Ethyl-1-hexanol was suspected of being responsible for her respiratory symptoms because the indoor air concentrations of 2-ethyl-1-hexanol in the university building in summer were very high (max: approximately 1000 $\mu g/m^3$),[7] a level beyond the advisable value of total VOCs (400 $\mu g/m^3$).[4] Four other patients had other onsets such as moving into a new or newly remodeled house.

**Measurement of VOCs in Serum** —— For the measurement of VOCs in serum, 1 ml of serum was diluted with 14 ml of saturated saline solution and analyzed using headspace GC/MS.[6] VOCs were quantified using deuterated compounds as internal standards. Target VOCs were benzene, toluene, xylene, ethylbenzene, styrene, *p*-dichlorobenzene, and 2-ethyl-1-hexanol. HS-GC/MS analysis was carried out under the following conditions: headspace sampler, Tekmar 7000 (Tekmar, Mason, OH, U.S.A.); vial size, 22 ml; sample temperature, 60°C; sample equilibrium time, 20 min; mixer, on (power 5, 3 min); sample loop size, 1 ml; sample loop temperature, 150°C; and transfer line temperature, 160°C. GC/MS, Auto Mass System II (Jeol, Tokyo, Japan); column, Vocol (60 m × 0.25 mm *i.d.*, 0.1 μm film thickness, Supelco, Bellefonte, PA, U.S.A.); oven temperature, initial temperature 40°C with 4-min hold, then 10°C/min to 230°C and postrun at 230°C for 5 min; ion source temperature, 210°C; and electron ionization voltage, 70 eV.

**Measurement of VOCs in Indoor and Breathing-zone Air** —— For the measurement of VOCs in indoor and breathing-zone air, both active and passive sampling was used. Active sampling was carried out using personal pumps (PAS-500, Shibata Scientific Technology, Tokyo, Japan) attached to charcoal tubes (gas tube for organic solvents, Shibata Scientific Technology) with an air sampling rate of 100 ml/min. Passive sampling was carried out using a passive gas tube (Shibata Scientific Technology) or VOC-SD (Supelco, U.S.A.). Both active and passive sampling continued for 24 hr. The collected VOCs were extracted with carbon disulfide and analyzed using GC/MS, which was carried out under the following conditions: QP-5050A (Shimadzu, Kyoto, Japan); column, DB-1 (60 m × 0.25 mm *i.d.*, 1.0 μm film thickness, J&W Scientific, Santa Clara, CA, U.S.A.); oven temperature, hold at 40°C for 5 min, ramp at 10°C/min to 150°C, ramp at 20°C/min to 250°C, and hold at 250°C for 18 min; injector temperature, 250°C; and detector temperature, 280°C. Analysis was performed in a selected-ion monitoring mode.

**Ethics** —— This study was carried out as a part of the treatment of patients and was conducted according to the Declaration of Helsinki; signed informed consent was obtained from the patients.

## RESULTS AND DISCUSSION

### Case 1

The study design for patient A included a five-point series of measuring serum and/or indoor air VOCs and two types of countermeasures (Table 1). At time point 1, her serum level of *p*-dichlorobenzene (25.4 ng/ml) was high, but those of eight other VOCs (not detected 0.4 ng/ml) were low. We informed her of the results and conducted a follow-up investigation together with measures to reduce exposure to *p*-dichlorobenzene. At time point 2, the indoor air level of *p*-dichlorobenzene (0.35 ppm) was high, 8.8-fold higher than the GLV (0.04 ppm), while those of five other VOCs such as toluene and xylene were low (<0.001–0.004 ppm). As the first countermeasure, mothballs, which are *p*-dichlorobenzene products, were removed from drawers in her bedroom and ventilation of the living room and her bedroom was carried out as frequently

**Table 1.** Serum and Indoor Air Concentrations of *p*-Dichlorobenzene Sampled from Patient A

| Time point | Date | Concentration | |
|---|---|---|---|
| | | Serum (ng/ml) | Indoor air (ppm)[a] |
| 1 | January 2002 | 25.4 | —[b] |
| 2 | May 2002 | —[b] | 0.35 |
| 3 | September 2002 | 8.0 | 0.10 |
| 4 | December 2002 | 12.0 | 0.036 |
| 5 | June 2003 | 19.3 | 0.008 |

*a*) Samples were collected in the bedroom. *b*) Not measured.

as possible. At time point 3, the concentrations of *p*-dichlorobenzene in her serum and her bedroom decreased to 8.0 ng/ml and 0.10 ppm, levels 1/3.2 and 1/3.5 of those at time point 2, respectively. Some improvement in her symptoms was observed at time point 3. The concentration of *p*-dichlorobenzene in her bedroom still exceeded the GLV at that time.

As further countermeasures, clothes in drawers were aired together with facilitating the ventilation of the living room and her bedroom. At time point 4, the concentration of *p*-dichlorobenzene in her bedroom decreased to 0.036 ppm, a level 1/2.8 of that at time point 3, and less than the GLV, whereas the serum level was 12.0 ng/ml, 1.5-fold higher than at time point 3. Recovery continued at time point 4. At time point 5, she reported a relapse. The concentration of *p*-dichlorobenzene in her serum again increased to 19.3 ng/ml, although that in her bedroom decreased to 0.008 ppm. It became apparent that she had frequently visited a friend's house to babysit for 2 or 3 months before time point 5. When she entered the house, she always developed a headache due to the strong odor. The elevation of serum *p*-dichlorobenzene level was presumably caused by exposure at the friend's house. We advised her not to enter the house.

As *p*-dichlorobenzene is used widely in moth repellent, air fresheners, and deodorizers, elevated serum *p*-dichlorobenzene levels may be caused by the common use of these products. The excretion of *p*-dichlorobenzene may be slower than that of other VOCs, such as toluene, xylene, *etc.* At time point 4, the serum *p*-dichlorobenzene level was still high (12.0 ng/ml) although the level of *p*-dichlorobenzene in her bedroom was greatly decreased (from 0.35 to 0.036 ppm). One of the reasons for the elevation of her serum *p*-dichlorobenzene level is that she may be a poor metabolizer. Additionally, she was suspected of having multiple chemical sensitivity because her condition was unstable due to the occasional and accidental exposure to nonspecific odors. Multiple chemical sensitivity shows diverse symptoms triggered by extremely small quantities of variable chemicals in indoor air.

It has been reported that *p*-dichlorobenzene causes adverse effects among exposed populations, particularly in those with occupational exposure.[8] The Japan Society for Occupational Health recommends the Occupational Exposure Limits (OELs) as reference values for preventing adverse health effects on workers caused by occupational exposure to chemical substances, continuous or intermittent noise, *etc.*, in which the OEL of *p*-dichlorobenzene is 10 ppm.[9] On the other hand, nonoccupational GLV for an indoor concentration of *p*-dichlorobenzene in Japan is 0.04 ppm.[4] The relationship between SBS symptoms and *p*-dichlorobenzene exposure or serum level of *p*-dichlorobenzene has not been reported. The widespread exposure of populations to *p*-dichlorobenzene requires more detailed investigation.

### Case 2

Table 2 shows the summary results of serum VOC concentrations in patient B together with those of four other patients with other onsets such as moving into a new or newly remodeled house. The serum 2-ethyl-1-hexanol concentration of patient B was 4.6 ng/ml, the highest of the seven VOCs analyzed in this study, and was more than 7.7-fold higher than in the other four patients. Table 3 shows the summary results of sample analysis of patient B's breathing-zone air, and indoor air in an office and a seminar room mainly used by patient B together with her home, for seven of the 41 VOCs examined. 2-Ethyl-1-hexanol was detected in her breathing-zone air (18 $\mu g/m^3$), indoor air in her office (13 $\mu g/m^3$), and a seminar room (44 $\mu g/m^3$), while 2-ethyl-1-hexanol was not detected in indoor air in her home. These results suggest that her el-

**Table 2.** Serum VOC Concentrations of Patient B and Four Other Patients

| VOCs | Concentration (ng/ml) | | | | |
|---|---|---|---|---|---|
| | Patient B[a)] | Other patients | | | |
| | | 1 | 2 | 3 | 4 |
| 2-Ethyl-1-hexanol | 4.6 | 0.6 | <0.5 | 0.5 | 0.6 |
| *p*-Dichlorobenzene | 1.9 | <0.5 | <0.5 | 1.0 | <0.5 |
| Toluene | 0.4 | 0.2 | 0.5 | 0.1 | 0.1 |
| Benzene | 0.1 | <0.1 | <0.1 | <0.1 | <0.1 |
| Xylene | <0.1 | <0.1 | 0.2 | 0.1 | 0.1 |
| Ethylbenzene | <0.1 | <0.1 | 0.1 | <0.1 | 0.1 |
| Styrene | <0.1 | <0.1 | 0.1 | <0.1 | <0.1 |

*a*) Blood was drawn after the sampling of breathing-zone air and indoor air were finished.

**Table 3.** Selected VOC Concentrations of Patient B's Breathing-zone Air, and Indoor Air in an Office, a Seminar Room and Her Home

| VOCs | Concentration ($\mu g/m^3$) | | | |
|---|---|---|---|---|
| | Breathing-zone air | Indoor air | | |
| | | Office | Seminor room | Home (living) |
| 2-Ethyl-1-hexanol | 18 | 13 | 44 | <2.8 |
| *p*-Dichlorobenzene | 20 | <2.8 | 3.8 | 110 |
| Toluene | 54 | 49 | 49 | 23 |
| Benzene | 6.7 | 5.1 | 5.4 | 6.6 |
| Xylene | 10 | 9.9 | 9.8 | 18 |
| Ethylbenzene | 9.5 | 9.2 | 9.6 | 9.0 |
| Styrene | <2.8 | <2.8 | <2.8 | <2.8 |

evated serum 2-ethyl-1-hexanol level was due to daily exposure in the university building.

The increase in 2-ethyl-1-hexanol in indoor air is a sign of dampness-related alkaline degradation of di-(2-ethylhexyl) phthalate used in building material for glue or in carpets with a polyvinyl chloride backing.[10)] The presence of 2-ethyl-1-hexanol is recognized in European countries and the U.S.A. as an indoor air pollutant.[7)] A possible relationship between SBS symptoms and indoor air 2-ethyl-1-hexanol has been reported, although the maximum indoor air concentration of 2-ethyl-1-hexanol was relatively low (20–30 $\mu g/m^3$).[11,12)] Additionally, *p*-dichlorobenzene was detected both in patient B's serum (1.9 ng/ml) and indoor air in her home (110 $\mu g/m^3$) but not in indoor air in the university building. It is assumed that *p*-dichlorobenzene is not responsible for her symptoms because she does not usually experience from respiratory symptoms while at home.

Measuring chemicals in blood is advantageous because we can calculate the body burden precisely. Blood levels of VOCs are known to be good predictors of VOC exposure, even though metabolism and excretion decrease levels over time.[13)] It should also be noted that the half-life of VOCs in blood is generally short, indicating that the data reflect only recent exposure.[14)] We found no relationship between serum VOC levels and SBS symptoms in the patients studied in our previous report.[6)] In the present study, we found two sensitive patients who had elevated serum levels of *p*-dichlorobenzene and 2-ethyl-2-hexanol; however, it is not an indoor environmental evaluation as such. At present, there is no universally accepted clinical definition of SBS and no adequate theory for its occurrence, although there are several theories.[15)] It is likely that SBS is multifactorial in origin, related to various factors and exposures.[1)] Further investigations are needed to evaluate the relationship between serum VOC levels and SBS symptoms.

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報　文

# オンライン固相抽出-高速液体クロマトグラフィー/タンデム質量分析計を用いるヒト血しょう中有機フッ素系化合物の一斉分析法の開発

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# Development of Online Solid-Phase Extraction-HPLC/MS/MS Method for the Determination of Perfluorochemicals in Human Plasma

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A method for determining perfluorochemicals (PFCs) such as perfluorooctanesulfonic acid (PFOS), paerfluorooctane slufoneamide (PFOSA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA) and perfluorodecanoic acid (PFDA), in human plasma samples was developed by online solid-phase extraction-HPLC/MS/MS, only after deproteination with acetonitrile. The limits of detection of PFOS, PFOSA, PFOA, PFNA and PFDA in human plasma at a signal to noise (ratio of 3) were 0.08～0.14 ng/ml, and the limits of quantitation of PFOS, PFOSA, PFOA, PFNA and PFDA in human plasma were 0.50 ng/ml. The average recoveries of PFOS, PFOSA, PFOA, PFNA and PFDA ranged from 93.3 to 105% (RSD, 3.0～8.9%; $n = 6$). This method is more rapid and accurate, compared with the column-switching HPLC/MS method presented in previous reports[18)19)]. The developed method can be applied to the determination of PFOS, PFOSA, PFOA, PFNA and PFDA in human plasma samples for monitoring human exposure.

*Keywords*: perfluorooctanesulfonic acid; perfluorochemicals; MS/MS; human plasma; column-switching.

## 1　緒　　言

近年，新たな環境汚染物質として，パーフルオロオクタンスルホン酸（PFOS）を代表としたパーフルオロ化合物（PFCs）が注目されている．PFOSは，水にも油にも溶けやすい性質から界面活性剤として利用され，近年までに撥水剤，消火剤，潤滑油及び消泡剤等として用いられている．また，パーフルオロオクタン酸（PFOA）においては，テフロン加工製品にも応用されていることから，PFCsは，我々の生活環境中で広範囲に存在している．Fig. 1にこれらPFCsの構造を示す．直鎖状に並んだ炭素原子すべてにフッ素原子が結合しており，末端にスルホン酸基又はカルボン酸基を有する構造をしている．炭素原子とフッ素原子の結合は非常に強いため，PFOSは，極めて安定な化学物質であると考えられている．この安定性によりPFCsは，河川水，海洋性哺乳類，魚類及び鳥類等，生態系で分解することなく，長期にわたり残留することが報告されている[1)～5)]．また，毒性としては，実験動物に対する催奇形性，甲状腺ホルモンへの影響[6)7)]，ペルオキシソーム増殖作用[8)～10)]が報告されていることから次世代への影響や発がん作用，コレステロール代謝撹乱作用等が懸念されている．また，PFOAは実験動物において血しょうタンパク質と結

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$CF_3$-$(CF_2)_7$-$SO_3^-$
**PFOS**

$CF_3$-$(CF_2)_7$-COOH
**PFOA**

$CF_3$-$(CF_2)_7$-$SO_2NH_2$
**PFOSA**

$CF_3$-$(CF_2)_8$-COOH
**PFNA**

$CF_3$-$(CF_2)_5$-$CH_2CH_2SO_3^-$
**THPFOS(I.S.)**

$CF_3$-$(CF_2)_9$-COOH
**PFDA**

$CF_3$-$(CF_2)_6$-COOH
**PFHpA(I.S.)**

**Fig. 1** Structures of PFCs and internal standards
PFOS: perfluorooctanesulfonic acid; PFOSA: perfluorooctanesulfonamide; PFOA: perfluorooctanoic acid; PFNA: perfluorononanoic acid; PFDA: perfluorodecanoic acid; PFHpA: perfluoroheptanoic acid; THPOS: 1*H*,1*H*,2*H*,2*H*-perfluorooctaneslufonic acid

合して血液中に蓄積しているという報告がある[11)12)]．それ故PFCsによる生態系及びヒトへのリスク評価を行うためにサーベイランスが必要となっている．近年，国内においてPFCsは，環境モニタリングが実施されている[13)]が，ヒトへの暴露調査は，いまだほとんど行われていない．

現在，報告されている生体試料中PFCsの測定は，主に高速液体クロマトグラフィー/質量分析計（HPLC/MS）及び高速液体クロマトグラフィー/タンデム質量分析計（HPLC/MS/MS）が用いられている[14)~19)]．しかしヒトへの暴露実態を正確に把握するためには，より精度の高いHPLC/MS/MSを用いる方が望ましいとされている．また，試料前処理法には，液-液抽出法[5)14)15)]，固相抽出法[16)17)]，カラムスイッチング法[18)19)]が報告されている．液-液抽出法及び固相抽出法は，煩雑な操作を必要とし，多検体処理能に乏しい．また，先に報告したカラムスイッチング-HPLC/MS法[18)19)]では，簡便な操作ではあるが，分析時間が30分と長く，回収率及び分析精度がやや乏しい．そこで本研究では，簡便かつ多検体処理能を有する前処理法であるオンライン固相抽出法を採用し，高精度・高選択性な機能を有するHPLC/MS/MSを用いることにより，迅速かつ高感度・高精度な血しょう試料中PFCsの一斉分析法を開発した．

## 2 実　　験

### 2・1 試　薬

パーフルオロオクタンスルホン酸（PFOS，>98%），パーフルオロオクタン酸（PFOA，>90%），パーフルオロノナン酸（PFNA，>98%）はFulka製を用いた．パーフルオロオクタンスルホンアミド（PFOSA，97%）はABCR GmbH & Co.KG製，パーフルオロデカン酸（PFDA，97%）はLancaster製を用いた．内標準物質として用いたパーフルオロヘプタン酸（PFHpA，99%）はAldrich製，1*H*,1*H*,2*H*,2*H*-パーフルオロオクタンスルホン酸（THPFOS，>90%）はSynQuest製を用いた．精度管理用凍結乾燥プール血清コンセーラは，日水製薬製を用いた．超純水は日本ミリポア製Milli-Qの超純水装置で調製したものを用いた．アセトニトリル，メタノールは，和光純薬製HPLC用及び残留農薬試験用を使用した．ナイロンメンブランフィルター（0.2 μm，13 mm）は，日本ポール製を用いた．

### 2・2 標準溶液の調製

各標準品をアセトニトリルに溶解させ，1.0 mg/mlの溶液を調製し，0.50～100 ng/mlの範囲で標準溶液を水/アセトニトリル＝50/50（v/v）で適宜希釈して測定用試料を調製した．

### 2・3 装置及び分析条件

HPLC/MS/MSは，Waters製Quattro microシステムを用いた．インジェクションボリュームを50 μlとし，ガードカラムに関東化学製のMightysil RP-18 GPプレカラム（2.0 mm×5 mm，5 μm）を用い，分析カラムにGLサイエンス製Inertsil ODS-3（2.1 mm×50 mm，5 μm）を使用した．また，カラムオーブンは40℃に設定した．

オンライン固相抽出法の条件は，送液ポンプに島津製LC-10ADvp pumpを用い，固相カートリッジとしては，Waters製Oasis HLB extraction column（20 mm×2.1 mm，25 μm）を使用した．構築したオンライン固相抽出システムをFig. 2に，操作プログラムをTable 1に示す．オートサンプラーにより試料溶液を注入後，5分間50 mM酢酸・酢酸アンモニウム緩衝液（pH＝4.7）/メタノール（90/10，v/v）をPump 1より送液することで，固相抽出カートリッジ上で測定対象物質の濃縮とクリーンアップを行った．次に六方バルブを切り替え，Pump 2から1 mM酢酸アンモニウムを添加した水/アセトニトリル混液をバックフラッシュ法によりグラジエント溶出することで，測定対象物質を固相抽出カートリッジから溶出させ，分離部及び検出部に導入した．

MS/MSのイオン化法は，エレクトロスプレーイオン化法（ESI）のネガティブイオンモードを採用し，検量線及び実試料の測定は，Multipul Reaction Monitoring（MRM）モードで行った．MS/MS条件としては，デソルベーション温度及びソース温度をそれぞれ350℃，100℃とし，コーンガス流量及びデソルベーションガス流量を50 l/hr，

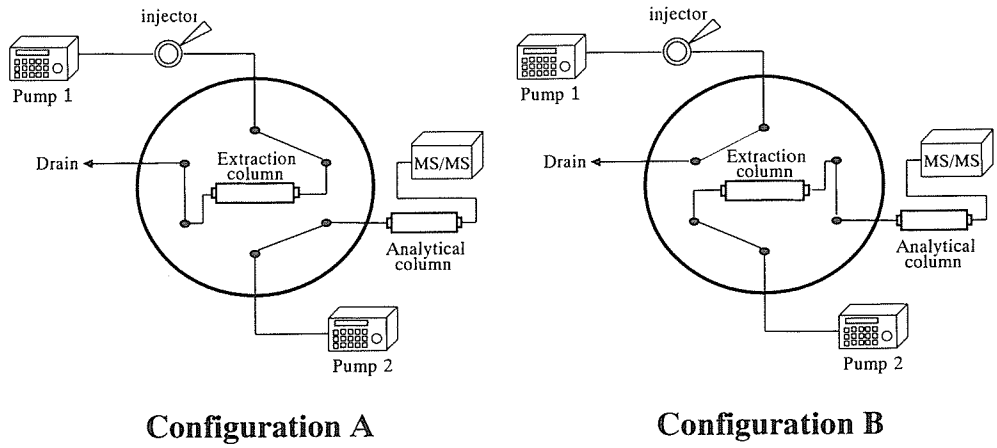


**Fig. 2**　Schematic diagram of the column-switching HPLC/MS/MS system
Configuration A: sample loading and washing; Configuration B: sample eluting

Table 1　Time program of column-switching HPLC/MS/MS coupled with on-line extraction condition

| Time/min | Column position [a] | Mobile phase | |
|---|---|---|---|
| | | Pump 1 [b] (A-B, v/v) | Pump 2 [c] (A-B-C, v/v) |
| 0.0 | Configuration A | 90 : 10 | 54 : 1 : 45 |
| 5.0 | Configuration B | 90 : 10 | 54 : 1 : 45 |
| 10.0 | Configuration A | | |
| 12.0 | | 90 : 10 | 14 : 1 : 85 |
| 14.9 | | 90 : 10 | 14 : 1 : 85 |
| 15.0 | | 90 : 10 | 54 : 1 : 45 |

a) Configuration A and B are shown in Fig. 2; b) Pump 1 solvent: (A) 50 mM Ammonium acetate buffer (pH-4.7), (B) Methanol; c) Pump 2 solvent: (A) Water, (B) 100 mM Ammonium acetate, (C) Acetnitrile

350 l/hr とした．また，キャピラリー電圧を－600 V に設定した．コーン電圧及びコリジョンエネルギーを PFOS: －60 V，65 eV，PFOSA: －45 V，35 eV，PFOA: －30 V，18 eV，PFNA: －30 V，20 eV，PFDA: －30 V，22 eV，PFHpA: －28 V，18 eV，THPFOS: －35 V，37 eV に設定した時，モニタリングイオンはそれぞれ，PFOS: $m/z$ 499 → 80，PFOSA: $m/z$ 498 → 78，PFOA: $m/z$ 369 → 169，PFNA: $m/z$ 419 → 169，PFDA: $m/z$ 469 → 169，PFHpA: $m/z$ 319 → 169，THPFOS: $m/z$ 427 → 81 となった．

測定対象物質の分離は，逆相分配モードの ODS (octadecyl silica) カラムを用い，1 mM 酢酸アンモニウムを添加した水/アセトニトリル (v/v) 混液を移動相として，流量 0.2 ml/min で送液し，測定時間 5～12 分にかけて，アセトニトリル含量を 45～85% にグラジエント溶出して行った．

### 2･4　ヒト血しょう試料

本研究遂行に当たり，血しょう試料の採取は提供対象者に対する人権擁護上の配慮，研究に対する利益・不利益等の説明を行い，インフォームドコンセントを得た．また，血しょう試料は，－80℃ で保存し，分析直前に室温で自然解凍した．ボランティアは，男女各 3 名ずつの健常人であった．

### 2･5　測定試料の調製法

0.1 ml のヒト血しょうに対して，内標準物質を含むアセトニトリル溶液を 0.2 ml 加えた．よくかくはん後，3000 rpm で 10 分間，遠心分離を行った．遠心分離後，上澄みをナイロンメンブランフィルター (0.2 μm) に通し，測定試料とした．

## 3　結果及び考察

### 3･1　オンライン固相抽出条件の検討

オンライン固相抽出法における溶離液の検討を行った．まず，水/メタノール (v/v) 混液について検討したところ，実試料と標準溶液の間において，保持時間にずれが生じた．この原因としては，固相抽出カートリッジ上での保持挙動が pH に依存するため，実試料中 PFCs の保持・溶出挙動が標準溶液と異なるためと考えられた．そこで，移動相に 50 mM 酢酸・酢酸アンモニウム緩衝液 (pH = 4.7)/メタノール (v/v) 混液を用いたところ，保持時間を一定に保つことが可能となった．また，Fig. 3 に 50 mM 酢酸・酢酸アンモニウム緩衝液 (pH = 4.7)/メタノール (v/v) の混合比率の検討を示す．メタノール含量が少ないほど，測定対象化合物のピーク面積は大きくなる傾向にあるが，メタノールを 10% 含む時点でほぼプラトーとなったので，最適混合比率を 50 mM 酢酸・酢酸アンモニウム

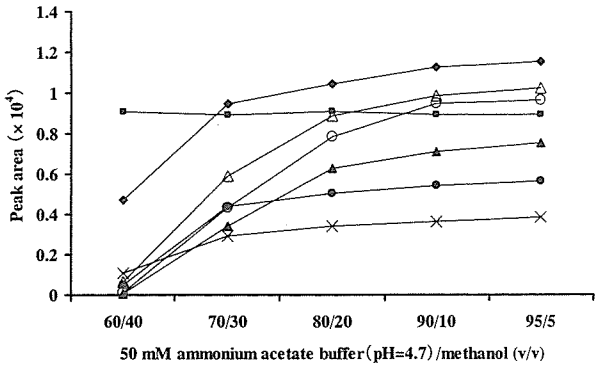


**Fig. 3** Effect of the mobile phase composition on the peak area of PFCs
◆: PFOS; ■: PFOSA; ▲: PFOA; ●: PFNA; ×: PFDA; ○: PFHpA; △: THPFOS

緩衝液（pH = 4.7）/メタノール = 90/10（v/v）とした．

### 3・2 MS/MS 測定条件の検討

PFCs5 種類の標準品を用いて，MS/MS のイオン化について検討した．測定対象物質のマススペクトルを Fig. 4 に示す．イオン化法に ESI 法を採用し，ネガティブイオンモードで測定したところ，PFOS においては $[M-K]^-$ イオンの $m/z$ 499，PFOSA，PFOA，PFNA，PFDA，PFHpA 及び THPFOS に関しては，$[M-H]^-$ イオンである $m/z$ 498，413，463，513，363，427 の分子量関連イオンピークがそれぞれ確認された．この結果より，PFOS，PFOSA 及び THPFOS に関しては，$m/z$ 499，498，427 をプレカーサーイオンとした．しかし，PFOA，PFNA，PFDA 及び PFHpA に関しては，$[M-H]^-$ イオンに比べ，$[M-COOH]^-$ イオンの強度が強かったため，プレカーサーイオンをそれぞれ，$[M-COOH]^-$ イオンである $m/z$ 369，419，468，319 に設定した．またプレカーサーイオンが開裂することで生じるプロダクトイオンは，それぞれ PFOS: $m/z$ 499 → 80，PFOSA: $m/z$ 498 → 78，PFOA: $m/z$ 369 → 169，PFNA: $m/z$ 419 → 169，PFDA: $m/z$ 469 → 169，PFHpA: $m/z$ 319 → 169，THPFOS: $m/z$ 427 → 81 とした．

設定したモニタリングイオンを用い，キャピラリー電圧のイオン強度に及ぼす影響について検討した．その結果を Fig. 5 に示す．キャピラリー電圧は −600 V の時，測定対象化合物すべてにおいて最大のピーク面積が得られた．また，一般的に MS や MS/MS を用いて測定をする際，揮発性の酸や塩基を少量加えることによりイオン化効率が上昇することが知られている．そこで今回，PFCs のイオン化効率を上昇させるため，酢酸アンモニウムの添加濃度を検討した（Fig. 6）．その結果，酢酸アンモニウムを 1 mM 添加した時に測定対象化合物のイオン強度が最大値を示した．

### 3・3 オンライン固相抽出-HPLC/MS/MS 測定条件の検討

得られた条件を用い，PFCs5 種類の標準品の測定を行った．Fig. 7（a）に示したクロマトグラムのようにすべての化合物を 15 分以内に良好に分離した．同様に血しょう試料に測定対象物質を 10 ng/ml となるように添加したクロマトグラムにおいても，他の夾雑物質の影響を受けることなく良好に相互分離することが可能であった｛Fig. 7（b）｝．また，血しょう試料における検出限界（$S/N = 3$）を求めたところ，それぞれ PFOS: 0.08 ng/ml，PFOSA: 0.11 ng/ml，PFOA: 0.11 ng/ml，PFNA: 0.10 ng/ml，PFDA: 0.14 ng/ml であった．定量限界は，すべての化合物を明瞭に測定することができる 0.50 ng/ml（$S/N > 10$）とした．

### 3・4 内標準物質の検討

近年，MS や MS/MS を用いた測定は，重水素置換体や $^{13}C$ 標識体等の安定同位体を用い，内標準法により測定が行われている．しかし，PFCs においては，重水素置換体や $^{13}C$ 標識体等の安定同位体の入手が困難なことから，内標準物質として PFOS 類似化合物が多く用いられている[15)17)]．そこで，今回は，従来多くの研究報告で用いられている PFHpA 及び THPFOS に着目した．標準溶液 0.1 ml に内標準物質含有アセトニトリル 0.2 ml を加え，よくかくはんした溶液を測定試料とし，検量線を作成したところ，0.50 ～ 100 ng/ml の範囲で良好な直線性（$r = 0.999$ 以上）を得ることができた．しかし，精度管理用プール血清コンセーラに測定対象物質が 10 ng/ml となるように添加して，回収率を求めた結果，PFHpA を内標準物質として用いた場合の回収率は，91.0% 以上と良好な結果を得ることができたが，THPFOS を用いた時，回収率 43.7 ～ 53.7% と良好な結果を得ることができなかった．THPFOS を用いた時に回収率が低減した原因としては，血清試料中の共存物質のマトリックス効果により，測定対象物質に比べ THPFOS のイオン強度が増加してしまったためと考えられる．これらの結果より，内標準物質として PFHpA を用いることにした．

### 3・5 添加回収試験

内標準物質に PFHpA を用い，ヒト血しょうにおける添加回収試験を行った．添加回収試験は，まず各測定対象化合物を異なる濃度レベルで添加した血しょう試料 0.1 ml に PFHpA 含有アセトニトリル 0.2 ml を加え，よくかくはんし，3000 rpm で 10 分間遠心分離を行った．次いで，上澄みをナイロンメンブランフィルター（0.2 μm）に通し，その沪液を測定して，回収率を算出した．その結果，平均回収率 93.3% 以上｛相対標準偏差（RSD）≦ 8.9%｝と良

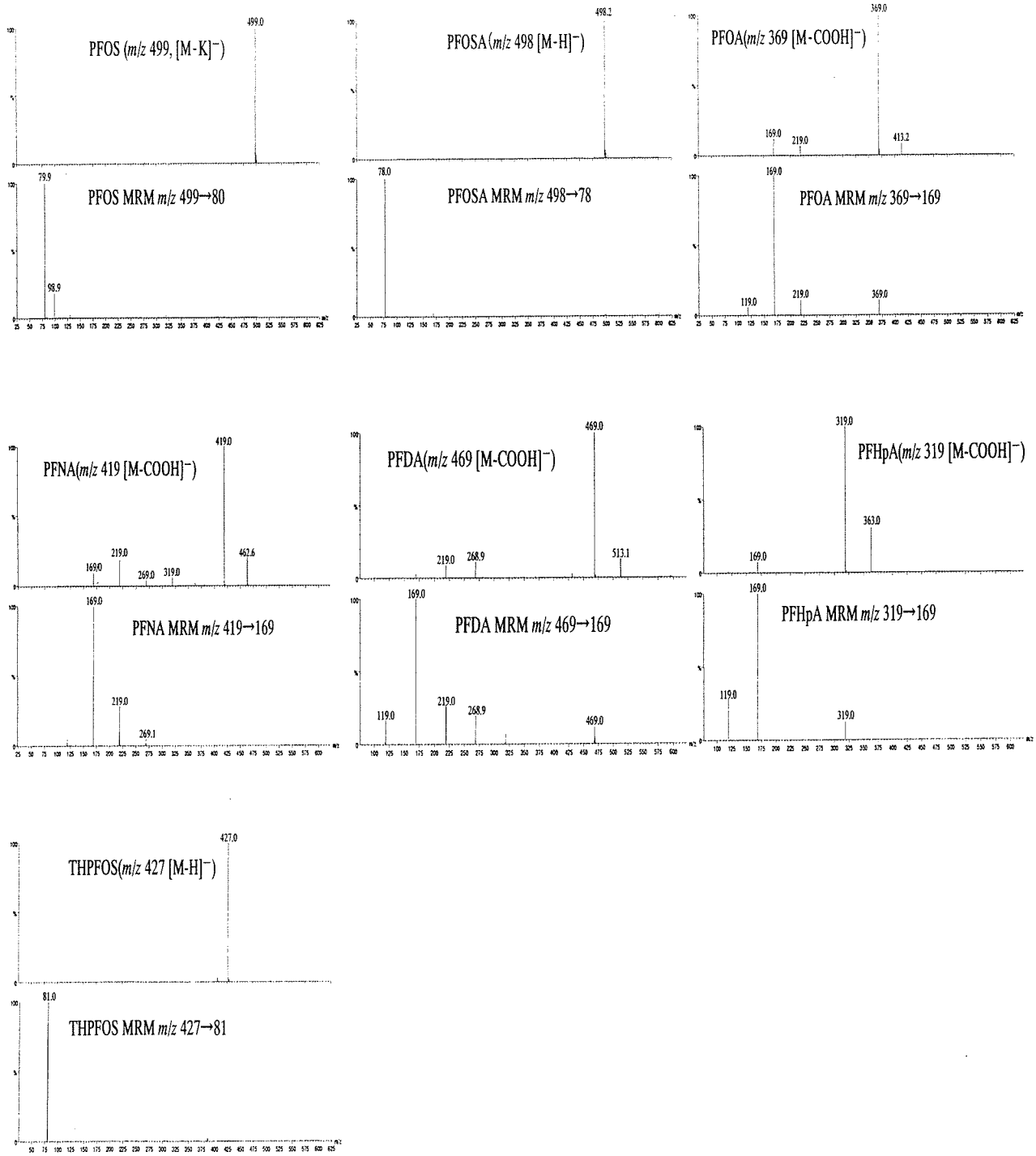


**Fig. 4**　Mass spectra of PFCs and internal standards
precursor ion spectrum: above; product ion spectrum: below

好な結果を得ることができた（Table 2）.

本法を健常人 6 人に適用し，血しょう中の PFCs を測定した（Table 3）. その結果，すべての検体から PFOS 及び PFOA を検出することができた. PFOS 濃度は 2.1～21.3 ng/ml の範囲で，PFOA 濃度は 0.7～4.6 ng/ml の範囲で存在した. また，PFNA に関しても 3 検体から 0.6～1.0 ng/ml の範囲で検出することができた. これらの濃度は，既に報告されているヒト血しょう中の PFCs の濃度[15]と比較し，ほぼ同レベルであったことから，本法は，ヒト血液試料中 PFCs の定量に応用可能であることが認められた.

## 4　結　　言

本分析法では，前処理法にオンライン固相抽出法を用いることで，除タンパクのみという簡便な操作で PFCs の測