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技術報告
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GC-MSを用いた大気中多環芳香族炭化水素酸化物の分析法開発に関する基礎検討

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Determination of Oxygenated Polycyclic Aromatic Hydrocarbons in the Atmosphere using Gas Chromatograph-Mass Spectrometer

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Summary

A determination method of atmospheric oxygenated polycyclic aromatic hydrocarbons (Oxy-PAHs) in particulate matter and the gaseous phase using gas chromatograph (GC)-mass spectrometer (MS) was studied in the present study. First, we obtained fundamental data of GC-MS analyses (mass spectra and calibration curves) of twelve Oxy-PAHs as follows: acenaphthenequinone (AceQ), 1,4-naphtoquinone (1,4-NQ), 9-fluorenone (9-F-one), 1,4-phenanthrenequinone (1,4-PQ), 1,4-, 9,10-anthaquinone (1,4-, 9,10-AQ), 1-hydroxyanthrquinone (1-OHAQ), 1,2-benzanthraquinone (1,2-BAQ), benz[*c*]phenanthrene[1,4]quinine (BcP-1,4Q), 3,4-dihydrobenzo[*a*]anthracene-1(2*H*)-one (BaA-one), 1,4-chrysenequinone (1,4-CQ) and 9,10-dihydrobenzo[*a*]pyrene-7(8*H*)-one (BaP-one). Subsequently, to evaluate collection efficiency of Oxy-PAHs, air was passed through a quartz-fiber filter (QFF) spiked with authentic Oxy-PAHs and then through cleaned polyurethane form (PUF) plugs using a mini-pump or a low-volume air sampler for 24 h in the dark at a constant room temperature (20 or 35 ℃). Oxy-PAHs retained on the QFF and those trapped within PUF plugs were simultaneously Soxhlet extracted, purified with silica by gel column chromatography and determined using GC-MS.

The calibration curves for the determination of the twelve Oxy-PAHs were proportional in the concentration range between 0.02 and 1.0 μg/mL with r^2 values of 0.960-0.999. Among the twelve Oxy-PAH compounds examined, recoveries of six compounds (1,4-NQ, 9-F-one, 9,10-AQ, 1,2-BAQ, BaA-one and BaP-one) during sampling, Soxhlet-extraction and clean-up procedures sufficiently yielded 79-127% at a room temperature of 20 ℃ and 57-127% at 35 ℃. The method detection limits (MDLs) of the six compounds ranged between 0.61-1.07 ng/m^3.

The proposed method was applied to the determination of the six Oxy-PAH compounds in the atmosphere. Air sample was collected at an urban location of Osaka, Japan for 12-13 February 2007. 1,4-NQ, 9-F-one and 9-F-one were detected in the sample at concentration level of approximately 0.7-3 ng/m^3.

Key words: GC-MS, Oxygenated polycyclic aromatic hydrocarbons, Particulate matter, Gaseous phase, Osaka

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1. はじめに

トラックなどの大型車に搭載されているディーゼルエンジンは，ガソリンエンジンと比較して，窒素酸化物や粒子状物質（Particulate matter［PM］）を多量に排出する[1]。PMについては，がん，呼吸器あるいは心臓血管系疾患への寄与が指摘されている[1]が，中でも粒径2.5 μm以下の微小粒子（PM2.5）は，その粒径の小ささから肺の深部に進入しやすく，人体への影響が危惧されている。日本においては，2009年9月，環境省がPM2.5の環境基準について告示を行う[2]など，国および地方自治体は，現在，その規制と対策に取り組んでいるところである。しかしながら，PMの表面上あるいはその内部に存在する化学物質の生体への影響についての情報は比較的少ないと思われる。

粒子状化学物質の代表的なものとして，多環芳香族炭化水素類（Polycyclic Aromatic Hydrocarbons［PAHs］）が挙げられる。これらは，発がん性あるいは変異原性など人体に悪影響を及ぼす[3]ことが知られており，世界中で調査研究が実施されている[4-18]。この他，ニトロ化PAHs[19-22]やPAH酸化物（Oxygenated PAHs［Oxy-PAHs］）[23-29]についても，PAHsと同様，人体への健康影響などが報告されている。中でも，Oxy-PAHsは，生体内においてレドックスサイクリングを介して活性酸素種を生成し，DNA損傷などの酸化的ダメージを与えたりすることが明らかになっている[1, 25]。このことから，大気中Oxy-PAHsの濃度レベルやその動態を把握することは非常に重要である。

粒子状Oxy-PAHsの分析には，一般的に高速液体クロマトグラフ（High-performance liquid chromatograph［HPLC］）が使用されており，検出器として蛍光検出器[23-25]，化学発光検出器[26]あるいはタンデム質量分析計（Mass spectrometer［MS］）[27]などが用いられている。一方，ガスクロマトグラフ（Gas chromatograph［GC］）-MSを用いたOxy-PAHsの分析については，これらの化合物は蒸気圧が比較的低く，極性が比較的強いため，感度が不十分である[23]と考えられており，使用頻度はHPLCと比較して非常に低い[28, 29]。しかし，GC-MSは多成分一斉分析に適していることから，基礎的な検討が必要であると思われる。

粒子状Oxy-PAHsのサンプリングについては，ハイボリューム（High volume［HV］）エアーサンプラーを用いて石英繊維ろ紙（Quartz-fiber filter［QFF］）又はガラス繊維ろ紙上に捕集するのが一般的である。しかし，Oxy-PAHsのうち比較的分子量の低い9,10-anthraquinone等については，蒸気圧が比較的高い[30]ため，粒子のみならず気相にも存在している可能性がある。しかし，Oxy-PAHsのPM-気相間分配に関する情報はほとんどない。また，HVエアーサンプラーを用いた試料採取時には，ろ紙を通過する多量のオキシダントによるOxy-PAHsの分解やBlow-off等のアーティファクトが懸念される[18, 31]。

本研究では，粒子状および気体状Oxy-PAHsのGC-MSを用いた分析手法の開発を目的として，12化合物を対象に，QFFとポリウレタンフォーム（Polyurethane form［PUF］）を用いた捕集効率，クリーンアップ及びGC-MS測定について基礎的な検討を行った。捕集効率の検討については，20℃あるいは35℃に設定した恒温室内にて遮光された状態で実施した。さらに，試料採取時におけるアーティファクトを最小限に抑えるため，ミニポンプ又はローボリューム（Low volume［LV］）エアーサンプラーを用いて低流速（5 L/min）で大気を吸引した。各実験手法については化学物質分析法開発マニュアル[32]を参考とした。

2. 実験方法

2. 1 Oxy-PAHs標準試薬

本研究で検討したOxy-PAHsは，acenaphthenequinone（AceQ），1,4-naphtoquinone（1,4-NQ），9-fluorenone（9-F-one），9,10-phenanthrenequinone（9,10-PQ），1,4-，9,10-anthaquinone（1,4-，9,10-AQ），1-hydroxyanthrquinone（1-OHAQ），1,2-benzanthraquinone（1,2-BAQ），benz[*c*]phenanthrene[1,4]quinine（BcP-1,4Q），3,4-dihydrobenzo[*a*]anthracene-1（2*H*）-one（BaA-one），1,4-chrysenequinone（1,4-CQ），9,10-dihydrobenzo[*a*]pyrene-7(8*H*)-one（BaP-one）の12化合物で，東京化成製，Aldrich製，Avocado Research Chemicals製，ICN Biomedicals製およびFluka BioChemika製を使用した。

2. 2 大気中Oxy-PAH化合物の捕集

2. 2. 1 捕集の概要

前段にQFF（直径47 mm，東京ダイレック），後段にPUF（直径50 mm，高さ50 mm，GLサイエンス）2個を小型サンプラー（特注品，GLサイエンス）に直列に装着し，QFFで大気中PMを，PUFで気体状物質を捕集した。本研究では，ミニポンプ（MP-Σ500，柴田科学）又はLVエアーサンプラー（SP208 LV-30 L，GLサイエンス）を用いて流速5.0 L/minで24時間，大気を通気した。QFFは600℃で4時間加熱したものを，PUFはアセトンで8時間，続いてジクロロメタンで16時間ソックスレー抽出にて洗浄し，真空デシケーターで24時間以上乾燥させたものを使用した。

2. 2. 2 測定方法の検出下限値（Method detection limit [MDL]）算出用及び大気調査用試料の捕集

測定方法の検出下限値（Method detection limit［MDL］）算出用として，洗浄したQFFとPUFを装着したサンプラーを8組用意し，2.2.1に従い，同時に大気を吸引した。大気試料は大阪市東成区に位置する大阪府環境農林水産総合研究所（以下，研究所と記す）で採取した。この地点は，近くに交通量の多い阪神高速道路や主要幹線道路があり，種々の大気中化学物質の調査が実施されている[8,9,33-35]。捕集後，8組のうち7組のQFFに各Oxy-PAH化合物を30 ng添加した。添加方法は，1 ng/μLのOxy-PAHs混合標準溶液（溶媒：ヘキサン）をマイクロシリンジで30 μL分取し，QFF上に円を描くように滴下した。ヘキサンは室温にてすぐに蒸発した。添加後，QFFはPUFと併せて前処理に供した（2.3参照）。無添加の1組についてはブランク試験用とした。

大気調査用の試料は，2007年2月12－13日の24時間，研究所にて2.2.1に従い採取した。採取後，QFFとPUFはそのまま前処理に供した（2.3参照）

2. 2. 3 添加回収実験

洗浄したQFFに各Oxy-PAH化合物を300 ng添加した。添加方法は，10 ng/μLのOxy-PAHs標準ヘキサン溶液をマイクロシリンジで30 μL分取し，QFF上に滴下した。そのQFFと洗浄済みのPUFをサンプラーに装着し，研究所内の恒温室にて，一定温度の下，消灯した状態で，2.2.1に従い大気を吸引した。この試験については，20℃および35℃で実施し，添加回収実験用として4-5組，ブランク試験用として1組を実験毎に用意した。その後，QFFおよびPUFは前処理に供した（2.3参照）。

2. 3 前処理及びGC/MS測定

2.2の各実験において用意したQFFおよびPUFを併せてソックスレー抽出管に挿入し，ジクロロメタンを用いて24時間抽出を行った。粗抽出液は，ヘキサンへ転溶後，5 gの5％含水シリカゲ

ルでクリーンアップを行い，内標準物質（fluoranthene-d_{12} および perylene-d_{12}，和光純薬製）をそれぞれ 25 ng ずつ添加後，窒素吹き付けにより 1 mL に濃縮した。本実験で使用した 5 %含水シリカゲルは，シリカゲル（C-200，和光純薬製）47.5 g と蒸留水 2.5 mL を三角フラスコに入れ，約 10 分間激しく振とうして調製し，3 時間程度デシケーター内で放置したものを使用した。有機溶媒は和光純薬製の農薬分析用を使用した。

調製した濃縮液 1 mL のうち 1 μL を GC-MS（HP6890 A; Agilent, 5973 N Mass Selective Detector; Agilent）に注入し，Oxy-PAH 化合物を定量した。測定条件は Table 1 に示す。

3. 結果と考察

3. 1 Oxy-PAH 化合物のマススペクトル

Oxy-PAH 化合物および内標準物質のトータルイオンクロマトグラム（Total ion chromatogram［TIC］）を Fig. 1 に示す。検討したすべての化合物のピークが検出された。

各化合物のマススペクトルを Fig. 2 に示す。AceQ 以外の 11 化合物については，定量用イオンとして分子イオンを示した。Oxy-PAH 化合物の定量用および確認用イオン（定量用イオンは下線）は，1,4-NQ（m/z 158, 102），9-F-one（m/z 180, 152），AceQ（m/z 126, 182），1,4-PQ（m/z 208, 152），9,10-AQ（m/z 208, 180），1,4-AQ（m/z 208, 152），1-OHAQ（m/z 224, 139），1,2-BAQ（m/z 258, 202），BaA-one（m/z 270, 212），1,4-CQ（m/z 258, 202）および BaP-one（m/z 270, 214）となった。

3. 2 検量線及び装置の検出下限値（Instrumental detection limit [IDL]）

各 Oxy-PAHs の標準品 10 mg を 10 mL のヘキサンに溶解し，1.0 mg/mL としたものを標準原液とし，ヘキサンで適宜希釈して，標準溶液が 0.02-1.0 μg/mL，内標準物質が 0.5 μg/mL となるように標準溶液を調製した。この標準溶液から得られた各 Oxy-PAHs の

Table 1 Analytical conditions of GC-MS for the determination of Oxy-PAHs

GC	
Column	SLB-5ms (Supelco, PA, USA)
	30 m × 0.25 mm I.D., 0.25 μm f.t.
Column temp.	70 °C (1 min. hold), 30 °C/min. to 130 °C, 5 °C/min. to 310 °C (6 min. hold).
Carrier gas	He (constant flow: 1.2 mL/min)
Injection temp.	300 °C
Injection mode	splitless
MS	
Ion source	EI positive
Ion source temp.	300 °C
Interface temp.	200 °C
Ionization voltage	70 eV

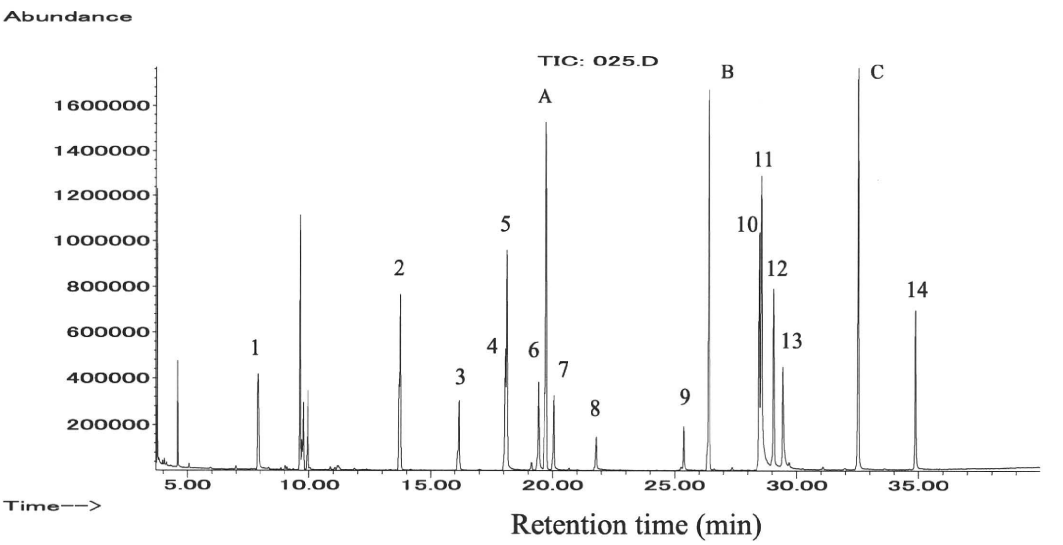

Fig. 1 Total ion chromatogram of authentic Oxy-PAH compounds and deuterated PAHs

Concentration of each Oxy-PAH and deuterated PAH were 1.0 and 0.5 ng/mL, respectively. 1: 1,4-NQ, 2: 9-F-one, 3: AceQ, 4: 1,4-PQ, 5: 9,10-AQ, 6: 1,4-AQ, 7: 1-OHAQ, 8: 9,10-PQ, 9: 2-OHAQ, 10: 1,2-BAQ, 11: BcP-1,4-one, 12: BaA-one, 13: 1,4 -CQ, 14: BaP-one, A: fluoranthene-d_{12}, B: chrysene-d_{12}, C: perylenre-d_{12}. Peak nos. 8 and 9 were not examined, and C was not used in the current study

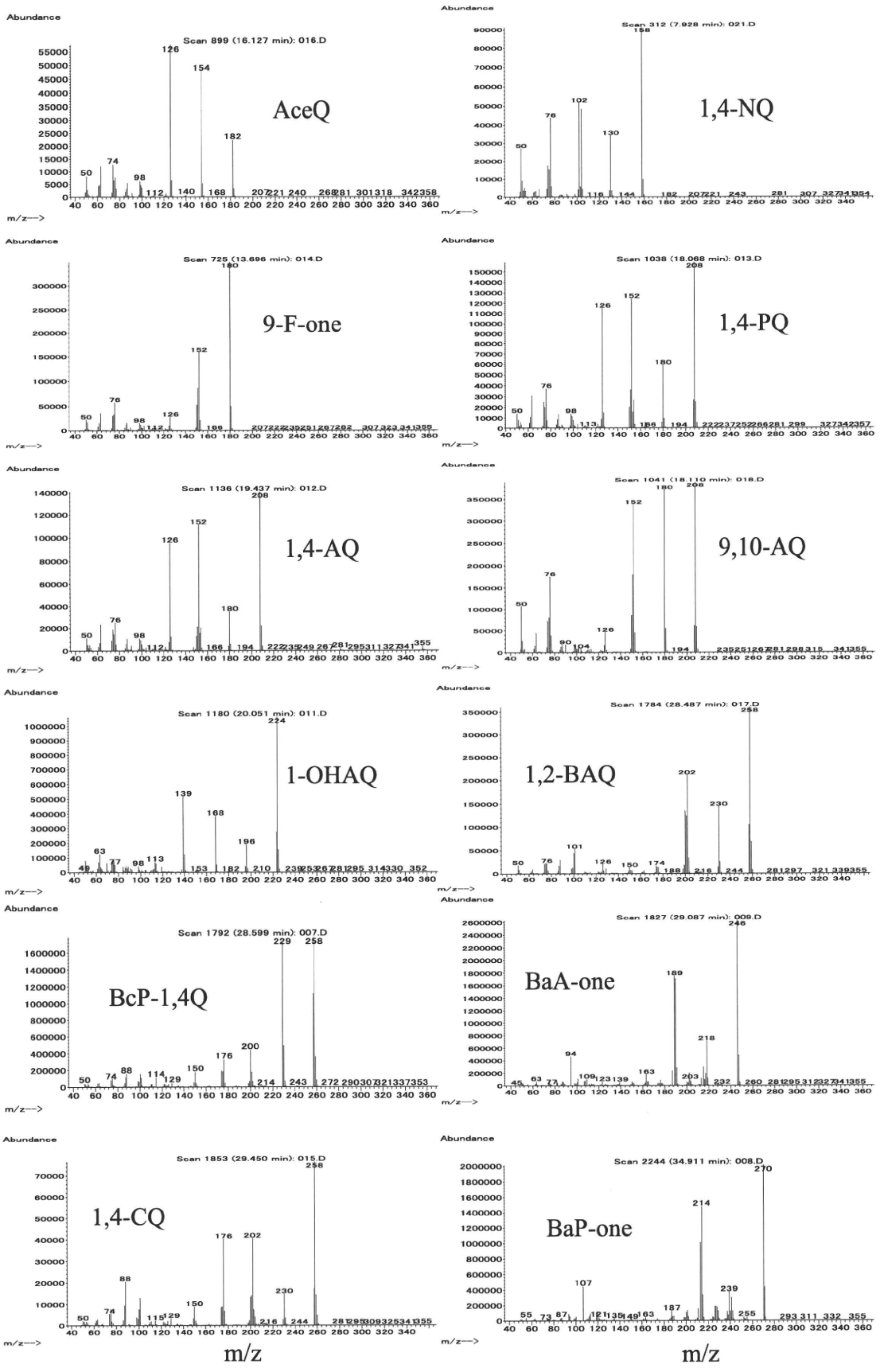

Fig. 2 Mass spectra of twelve authentic Oxy-PAH compounds

ピーク面積と内標準物質 (fluoranthene-d_{10} 又は perylene-d_{12}) のピーク面積比と濃度比から検量線を作成した (Fig. 3 および Table 2)。検討したすべての Oxy-PAH 化合物について, 0.02-1.0 μg/mL の間で直線関係が認められた (r^2 : 0.9604-0.9997)。

続いて, 検量線の最低濃度である 0.02 μg/mL 標準溶液を 7 回繰り返し測定することにより, 装置の検出下限値 (Instrumental detection limit [IDL]) を求めた (Table 2)。算出方法は次式[32] に従った。

$$IDL = t(n\text{-}1,\ 0.01) \times Sc \quad (1)$$

ここで, Sc は n 回繰り返し分析での標準偏差, t (n-1, 0.01) は危険率 1 %, 自由度 n-1 の t 値 (片側) を指す。検討した Oxy-PAH 化合物の IDL 値は 2.5-8.9 pg/μL(相対標準偏差:3.4-1.4 %)であった。これらの値は, LC-化学発光検出器による 1,2-NQ, 1,4-NQ, 9,10-AQ および 9,10-PQ の検出下限値 (4.1-9.5 pg/μL : S/N = 3 より算出)[26] や LC-蛍光検出器による 1,2-BAQ の検出下限値 (1.3 pg/μL : S/N = 3 より算出)[23] と比較して同程度あるいは若干上回っている程度であったが, LC-タンデム MS による benzo[*a*] pyrene-dione 等の定量下限値 (0.03-1.7 pg/μL : S/N = 10 より算出)[27] を大きく上回っていた。引用したこれらの文献とは調査対象物質が必ずしも一致しておらず, ここでは詳細は解らないが, 1,4-NQ, 9,10-AQ および 1,2-BAQ の感度については, GC-MS と各種 HPLC 法との間に顕著な差はないと推察される。

3. 3 シリカゲルカラムクロマトグラフィーの分画試験

各 Oxy-PAH 化合物 100 ng をカラムクロマト管 (内径 1.5 cm) に充填した 5 g の 5 %含水シリカゲルの上端に添加し, 第一画分としてヘキサンを 25 mL ずつ計 100 mL 滴下し, 続いて第二画分として 5 %アセトン／ヘキサンを 20 mL ずつ計 100 mL 滴下した。その後, 2.5 に従い各分取液に内標準物質を添加後, 窒素吹き付けにより 1 mL に定容し, GC-MS にて各分取液中 Oxy-PAHs を定量した。Fig. 4 のとおり, 第一画分には Oxy-PAH 化合物はほとんど溶出せず, 大半が第二画分の 20-80 mL に溶出した。これらの結果から, ヘキサンを 100 mL 溶出させた後, 5 %アセトン含有ヘキサンを 80 mL 溶出させることとした。しかし, AceQ と BcP-1,4 Q についてはほとんど回収されなかった。

3. 4 添加回収試験 (Oxy-PAHs の捕集効率)

本研究ではサロゲート化合物を使用していないため, 2.2.2 および 2.3 の操作で得られた回収率は, サンプリング操作に加えて, 抽出・前処理操作を含めた値となる。ここで, Oxy-PAH 化合物の捕集効率を把握するため, 初めに AceQ と BcP-1,4 Q を除いた 10 化

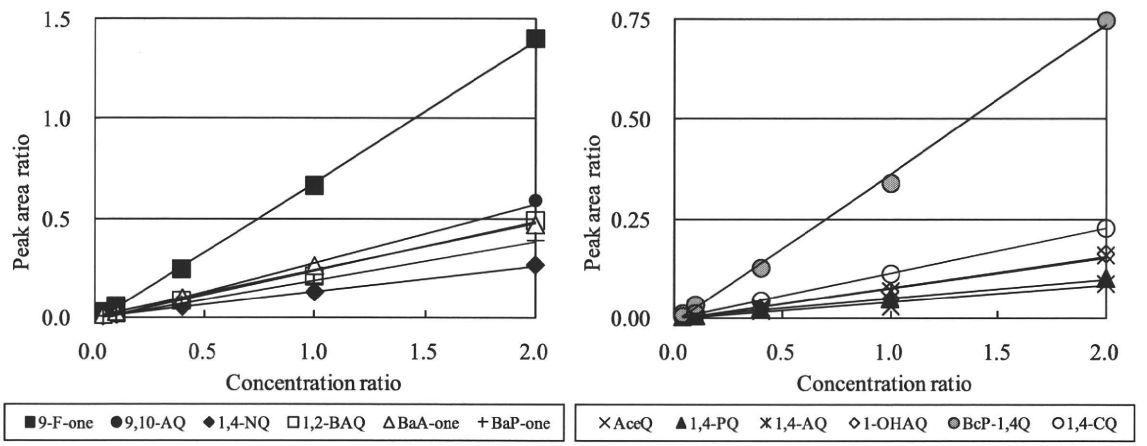

Fig. 3 Calibration curves for the determination of twelve Oxy-PAH compounds

Table 2 Calibration curves for the determination of twelve Oxy-PAH compounds and their instrumental detection limits (IDLs)

Oxy-PAHs	Calibration curves			IDL (pg/μL)	RSD (%)
	Slope	Intersept	R^2		
1,4-NQ	0.1309	−0.0005	0.9994	3.7	4.6
9-F-one	0.7036	−0.0214	0.9992	2.5	3.5
AceQ	0.0411	−0.0011	0.9604	6.4	7.9
1,4-PQ	0.0488	0.0007	0.9970	7.4	9.9
9,10-AQ	0.2984	−0.0235	0.9899	3.0	3.7
1,4-AQ	0.0782	−0.0034	0.9937	6.9	9.0
1-OHAQ	0.0808	−0.0059	0.9904	8.9	11.4
1,2-BAQ	0.2455	−0.0094	0.9942	4.2	5.6
BcP-1,4Q	0.3759	−0.0141	0.9979	6.8	8.3
BaA-one	0.239	0.0029	0.9968	3.3	4.1
1,4-CQ	0.1148	−0.0021	0.9997	5.8	7.3
BaP-one	0.1943	−0.0067	0.9973	2.5	3.4

合物の抽出・前処理操作における回収率を確認した（Table 3）。実験方法は，Oxy-PAH 化合物を添加した QFF と洗浄済み PUF を 2. 3 に従って抽出・前処理し，GC-MS にて定量した。ここで，回収率は添加した Oxy-PAHs の量に対する GC-MS により定量された量の割合と定義する。10 化合物のうち 1,4 -PQ と 1,4-CQ の平均回収率はそれぞれ 12 %と 29 %であり，抽出・前処理過程における損失が特に顕著であったが，他の 8 化合物については 57 %（1,4 -AQ）-108 %（9-F-one）の範囲内であった。

続いて，1,4-PQ と 1,4-CQ を除く 8 化合物のサンプリング操作から前処理操作における回収率を Table 3 に示す。サンプリング操作は室温を 20 ℃又は 35 ℃に維持して実施したが，9-F-one および 9,10-AQ の回収率については両温度において顕著な差は認められず，回収率は 117 -127 %であった。これらの値は，抽出・前処理操作における回収率と比較して若干増加しているが，これらの化合物が強い極性を有するため，サンプリング時に捕集した共存物質（マトリックス）の影響を受けて定量値が過大となったと思われる[36, 37]。1,4-NQ および 1,4-AQ については，20 ℃での回収率は抽出・前処理操作の値とほぼ同程度であったが，35 ℃における回収率は 20 ℃の値を下回っていた。これは，分子量の低い 1,4-NQ と 1,4-AQ の蒸気圧は比較的高く，そのため，高温時には QFF 表面から揮散しやすくなること等が原因であると考えられる。中でも 1,4-AQ の 35 ℃における回収率は 24 %であり，捕集時における損失は大きいと思われる。一方，1-OHAQ，1,2-BAQ，BaA-one および BaP-one については，20 ℃における回収率は抽出・前処理操作における値とほぼ同程度であったが，35 ℃における回収率は 20 ℃の値を上回った。これについてもマトリックスの影響[36, 37] と推察される

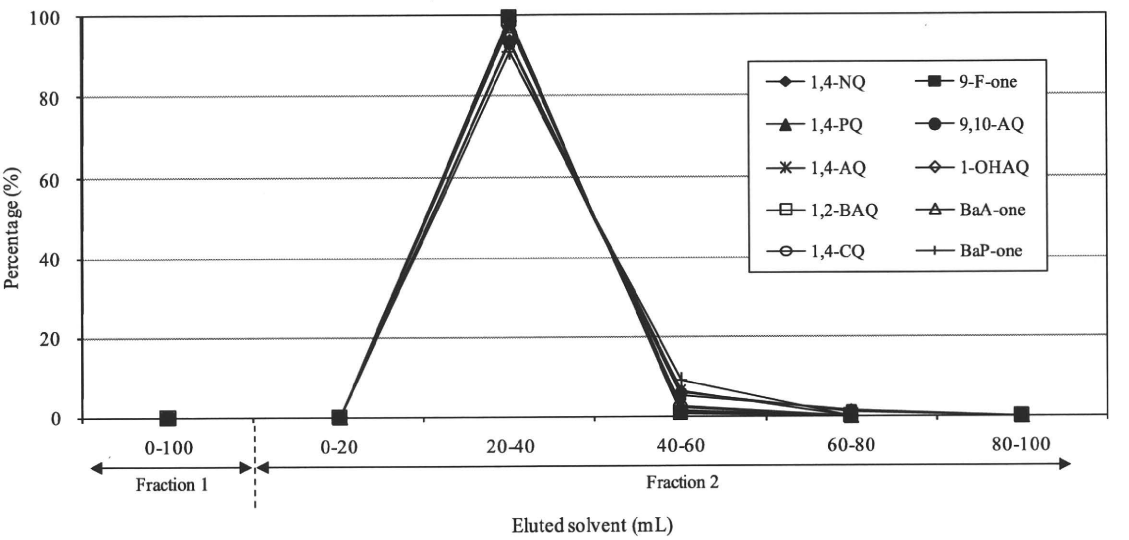

Fig. 4 Elution patterns of Oxy-PAH compounds through 5 g of silica (5 % deactivated by distilled water) by gel chromatography
Fraction 1 : hexane,
Fraction 2 : 5 % acetone/hexane

Table 3 Average recoveries[a] of ten Oxy-PAH compounds during extraction and clean-up procedures and those during sampling, extraction and clean-up procedures

Oxy-PAHs	During extraction and clean-up[b]	During sampling, extraction and clean-up	
		20 °C[c]	35 °C[d]
1,4-NQ	74 ± 2	79 ± 3	57 ± 2
9-F-one	108 ± 3	117 ± 4	121 ± 4
1,4-PQ	12 ± 2	-	-
9,10-AQ	102 ± 5	127 ± 3	127 ± 5
1,4-AQ	57 ± 1	50 ± 2	24 ± 4
1-OHAQ	101 ± 2	125 ± 5	200 ± 1
1,2-BAQ	93 ± 3	98 ± 5	122 ± 3
BaA-one	98 ± 1	84 ± 5	100 ± 7
1,4-CQ	29 ± 3	-	-
BaP-one	95 ± 2	82 ± 3	92 ± 4

[a]Average amount of each Oxy-PAH compound determined by GC-MS relative to the amound added on the QFF.
[b]Mean±SD (n=3).
[c]Mean±SD (n=5).
[d]Mean±SD (n=4).

が，Oxy-PAHsについて捕集時の気温とマトリックス効果の関係を研究した事例はほとんどなく，今後，詳細な検討が必要であろう。これらの4化合物のうち1-OHAQについては，35℃での回収率は20℃の場合と比較して75％も上昇しており，マトリックス効果が他の物質と比較して際立っていた。以上より，1,4-NQ，9-F-one，9,10-AQ，1-OHAQ，1,2-BAQ，BaA-oneおよびBaP-oneの6化合物については，捕集時の気温に関わらず，検討した分析手法にて十分に回収できるものと判断した。

3. 5 測定方法の検出下限（MDL）及び大気中Oxy-PAH化合物の測定

1,4-NQ，9-F-one，9,10-AQ，1-OHAQ，1,2-BAQ，BaA-oneおよびBaP-oneの6化合物について，検討した測定方法（ソックスレー抽出，シリカゲルによるクリーンアップおよびGC-MSによる分析）における検出下限値を算出した（Table 4）。算出方法はIDLと同様，(1)式に従った。検討した6化合物のMDL値は0.61-1.07 ng/m^3（相対標準偏差：2.3-8.9％）であった。

検討した分析手法を用いて，大阪市内の都心部における大気中1,4-NQ，9-F-one，9,10-AQ，1-OHAQ，1,2-BAQ，BaA-oneおよびBaP-oneの測定を実施した（Table 4）。検出されたOxy-PAH化合物は，1,4-NQ，9-F-oneおよび9,10-AQの3化合物であり，他は検出下限値未満であった。以上の結果から，検討した分析手法により1.0 ng/m^{3}程度の大気中Oxy-PAHsを測定することが可能であることがわかった。

最後に，大阪市内の測定結果を文献値（Table4）と比較すると，大阪市内における1,4-NQおよび9-F-oneの濃度はサンティアゴ（チリ）[29]やミュンヘン（ドイツ）[39]の値を上回っていたが，9,10-AQについてはサンティアゴが最も高濃度であり，大阪市内の値はミュンヘンと同程度であった。一方，1,2-BAQについては、サンティアゴとミュンヘンで検出されたが，大阪市内では検出されなかった。本研究では，アーティファクト低減のため低流速で大気試料を捕集したことから，本法のMDL値はHVエアーサンプラー・GC-MS法[29]の値を上回っており，このことが1,2-BAQ等が検出されなかったことの一因であると考えられる。大気中Oxy-PAHsの挙動を詳細に把握するためには，より高感度な機器を使用する等，さらに感度を上げる必要があると思われる。

近年，Kishidaらは，主にダイオキシン類分析用[34, 39-41]として使用されている高分解能MSを用いて，ミニポンプで捕集したカトマンズ[4]，ハノイ[6, 7]および大阪府域[42, 43]における大気中PAHsを定量し，各地域における汚染状況を調査した。また，Nakaoら[44]はニトロ化PAHsを誘導体化後，高分解能MSで定量する方法を開発した。このように，高分解能MSの使用が感度向上の最善の方策と考えられる。現在，研究所では高分解能MSを用いた大気中Oxy-PAHsの詳細調査が実施されているところである[45]。

謝　辞

本研究の一部は，化学物質分析法開発調査として，環境省の委託を受けて実施したものである。

要　約

本研究では，GC-MSを用いた大気中に存在する粒子状および気体状Oxy-PAHsの分析手法の検討を行った。大気中Oxy-PAHsは，ミニポンプ又はLVエアーサンプラーを用いて流速5.0 L/minで24時間，大気をQFFとPUFに通気して捕集した。その後，QFFおよびPUFは併せてジクロロメタンでソックスレー抽出を行い，シリカゲルによるクリーンアップの後，GC-MSにて定量した。

検討したすべてのOxy-PAH化合物について，0.02-1.0 μg/mLの間で直線関係が認められた（r^2：0.960-0.999）。しかし，12化合物のうちサンプリング操作・ソックスレー抽出・前処理の全工程を通じて十分に回収されたのは6化合物（1,4-NQ，9-F-one，9,10-AQ，1-OHAQ，1,2-BAQ，BaA-one及びBaP-one）であった。この6化合物について，MLD値を算出し，大阪市内において環境大気の調査を実施した結果，本法によりng/m^{3}程度のOxy-PAHsの測定が可能であることがわかった。

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Table 4 Method detection limits (MDLs) of Oxy-PAHs and their atmospheric concentrations at an urban location of Osaka, Japan

Oxy-PAHs	MDL (ng/m^3)	RSD (%)	Atmospheric concentration (ng/m^3) [a]		
			This study [b]	Santiago [c]	Munich [d]
1,4-NQ	0.76	5.0	1.6	0.27	N.M.
9-F-one	0.69	2.3	3.2	0.62	0.35
9,10-AQ	0.61	3.2	0.72	1.58	0.96
1,2-BAQ	0.86	6.0	N.D.	1.37	0.41
BaA-one	1.07	8.0	N.D.	N.M.	N.M.
BaP-one	1.05	8.9	N.D.	N.M.	N.M.

[a] N.D.: Not detected. N.M.: Not measured.
[b] Sampled on 12–13 February, 2007.
[c] See a reference no.29.
[d] See a reference no.37.

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Cigarette smoke condensate extracts augment collagen-induced arthritis in mice

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ABSTRACT

Although cigarette smoking is a solid environmental risk factor for rheumatoid arthritis (RA) as revealed by epidemiological studies, the scientific basis has not been provided. Proinflammatory cytokines produced by synoviocytes are implicated in the pathogenesis of RA. As cigarette smoke condensate (CSC) is able to up-regulate the production of proinflammatory cytokines from human fibroblast-like synoviocytes, we studied the effect of CSC on induction of arthritis in the mouse model of collagen type II-induced arthritis (CIA). When mainstream CSC or sidestream CSC was administered into DBA/1J mice at the time of immunization with collagen and complete Freund adjuvant, CSC dose-dependently augmented the induction and clinical development of arthritis at both young and older mice. Peritoneal injected mainstream CSC one day before immunization also exhibited the augmenting effect, suggesting the systemic effect of CSC. These results support the etiological role of cigarette smoking in RA.

1. Introduction

Rheumatoid arthritis (RA) is a systemic disease associated with a chronic inflammatory condition in multiple joints. The disease is characterized by proliferation of synoviocytes in inflamed synovia, formation of pannus and production of proinflammatory cytokines and chemokines by synoviocytes [1]. These cytokines contribute to the disease by production of proteases and reactive oxygen intermediates, induction of proliferation of synovial fibroblasts, cartilage degradation, infiltration of inflammatory cells and angiogenesis [2,3]. Fibroblast-like synoviocytes as well as synovial tissue-infiltrating macrophages are major cells producing the proinflammatory cytokines. Fibroblast-like synoviocytes or transformed cell clones derived from RA patients secrete, constitutively or in response to interleukin-1 (IL-1) or tumor necrosis factor α (TNFα), proinflammatory cytokines, including IL-1α, IL-1β, IL-6 and IL-8 [4,5]. The critical role of these proinflammatory cytokines in RA has been verified in both RA patients and animal models of arthritis. An improvement of synovial inflammation and decreased joint destruction in RA patients have been reported following treatment with neutralizing anti-TNFα antibody [6], soluble TNF receptor [7], IL-1 receptor antagonist (IL-1ra) [8] or neutralizing anti-IL-6 antibody [9]. However, the etiology and the mechanisms responsible for the cytokine induction and subsequent development of arthritis remain unknown.

Epidemiological studies indicate an association of cigarette smoking with disease outcome in patients with early inflammatory polyarthritis [10], the increase of rheumatoid factor and nodule formation in patients with RA [11], and a strong association between heavy cigarette smoking and RA, particularly in patients without a family history of RA [12]. Especially the risk of smoking for the disease is quite high in individuals, either men or women, with shared epitope (SE) in HLA-DRB1 [13]. In addition, maternal smoking in pregnancy is a determinable factor of infant rheumatoid arthritis and other inflammatory polyarthritis [14]. However, the scientific basis supporting the epidemiological studies has not been provided.

Polycyclic aromatic hydrocarbons (PAHs) such as 3-methylcholanthrene (3-MC), benzo[*a*]pyrene (B[*a*]P) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) are much contained in tobacco smoke. We have previously reported that 3-MC, B[*a*]P and TCDD up-regulated IL-1β mRNA in RA patient-derived SV40 T antigen-transformed human fibroblast-like synoviocyte line MH7A [15], which has similar characteristics as parental synoviocytes [16,17]. We also reported that cigarette smoke condensate (CSC), either mainstream or sidestream, also induced IL-1α, IL-1β, IL-6 and IL-8 at both mRNA and protein levels in the cells [18]. As proinflammatory cytokines produced by synovial cells are critical for RA, in this study we determined the effects of CSC on induction and clinical development of arthritis in the mouse model of collagen type II-induced arthritis (CIA), an experimental model of human chronic rheumatoid arthritis.

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2. Materials and methods

2.1. Reagents

Polymyxin B was purchased from SIGMA-ALDRICH Co. (St. Louis, MO, USA). Bovine type II collagen was from KOKEN Co. Ltd. (Tokyo, Japan). Incomplete Freund adjuvant (ICFA) and *Mycobacterium butyricum* were from BD (Tokyo, Japan). Endotoxin test kit, Endospecy ES-24S Kit was from SEIKAGAKU BIOBUSINESS CORPORATION (Tokyo, Japan). Detection limit was 0.001 EU/ml, where one EU indicates 0.1 ng/ml of *Escherichia coli* 055:B5-derived endotoxin.

2.2. Preparation of cigarette smoke condensate (CSC)

CSC was prepared as described previously [19]. A common American brand of cigarette was used in this study. Each cigarette was 84 mm long, 25 mm in circumference, and had a charcoal filter that adsorbs normally 9 mg of tar, and 0.8 mg of nicotine. Both particulate matters from mainstream and sidestream smoke were collected using a cigarette smoke collection apparatus as described previously with several modifications [20]. Briefly, cigarette smoking was performed in a glass chamber (40 cm high × 25 cm i.d.). Cigarettes were smoked at a condition of 90 ml of puff volume per 5 s, once every 15 s. The mainstream smoke was collected on a glass fiber (Shibata, Tokyo, Japan, T60A20 55 mm). This filter system is effective for the collection of only the particulate matter. On the other hand, the sidestream smoke was collected at about 170 ml/s. Smoke from 100 cigarettes was collected. After the cigarettes were consumed, the weights of the filters with trapped particulate matter were determined, and the particulate matter was extracted by sonication with benzene/ethanol (1/3, v/v) four times for 15 min. The extract was filtered and evaporated to dryness under reduced pressure, and then the residue was redissolved in ethanol (cigarette smoke condensate: CSC). The yield of mainstream CSC and sidestream CSC were 9.25 mg and 18.0 mg per cigarette, respectively. One mg of CSC, either mainstream or sidestream, did not contain endotoxin as determined by Endotoxin test.

2.3. Animals

Specific pathogen-free DBA1/J male mice were purchased from Charles River, Kanagawa, Japan, and the mice were kept in a specific pathogen-free condition. Standard laboratory food and water were available to the mice ad libitum. This study was approved by the animal ethics committee of Nagoya City University.

2.4. Collagen -induced arthritis

Bovine type II collagen (CII) (3 mg/ml) solution in 0.01 M acetic acid containing vehicles (ethanol) and polymyxinB (0.4 mg/ml) with or without CSC were emulsified with an equal volume of complete Freund's adjuvant (CFA), which consists of incomplete Freund's adjuvant (ICFA) supplemented with *M. butyricum* (8 mg/ml). In some experiments, the antigen was emulsified with ICFA without *M. butyricum*. Mice were subcutaneously injected with the antigen emulsion (100 μl) at several sites into the base of the tail. After 3 weeks, the mice were intraperitoneally injected with 100 μl CII in 0.01 M acetic acid at the concentration of 0.75 mg/ml.

2.5. Assessment of clinical disease activity

The severity of clinical disease activity in the mice was determined by examining each of the four paws and scoring on a scale of 0-4, as follows: 0 = normal joint, 1 = erythema and swelling in one finger, 2 = erythema and swelling in more than 2 fingers or one big joint, 3 = swelling with below 4 mm thickness and erythema in one entire paw, 4 = swelling with over 4 mm thickness and erythema in one entire pow and joint rigidity. The total score for clinical disease activity was based on all 4 paws and was a maximum of 16 for each mouse.

2.6. Histology

Mice were sacrificed on day 40 and hindpaws were removed, skinned, fixed in 4% formalin, decalcified, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

2.7. Statistic analysis

Differences between group means were assessed by Mann–Whitney test. *P* values less than 0.05 were considered significant.

3. Results

3.1. Mainstream CSC augments induction and clinical development of arthritis in older mouse

In order to examine the effect of CSC on collagen-induced arthritis, mice were immunized with antigen emulsified with ICFA or CFA with or without mainstream CSC (200 μg per mouse) on day 0, boosted with the antigen alone on day 21, and then the development of arthritis was measured. Male mice aged 27 weeks were used for the experiment because male and older mice are prone to develop arthritis as compared to female and young mice. As shown in Fig. 1A, none of the mice immunized with antigen in ICFA developed arthritis. However, the mice immunized with antigen in CFA (positive control) developed arthritis with the duration of time. Mainstream CSC augmented the incidence of arthritis. Especially on day 28 only one mouse in a group immunized with antigen in CFA developed arthritis, but CSC treatment induced arthritis in all the mice. CSC also significantly augmented the clinical development of arthritis (Fig. 1B). This augmentation remained up to 42 days.

3.2. Mainstream CSC augments induction and clinical development of arthritis in young mouse

Next young male mice aged 6 weeks were used. Dose-dependency of mainstream CSC was also examined. As compared to older mice incidence of arthritis was low in mice immunized with antigen in CFA (positive control) (Fig. 2A). In contrast, treatment with 100 μg CSC per mouse markedly augmented the induction of arthritis, and the treatment with 200 μg CSC per mouse further augmented the induction. CSC also dose-dependently ameliorated the arthritis (Fig. 2B). Although the difference of scores between positive control and CSC-treated groups was not statistically significant, this was due to the very severe arthritis in only one mouse in the positive control group. The mean body weight of CSC treated mice was low as compared to control mice, although the difference was not statistically significant (Fig. 2C).

3.3. Histologic evaluation

Histologic evaluation of joints of mice was performed on day 40 at the same experimental condition with Fig. 2. The finger joints of the mice immunized with CII emulsified with ICFA exhibited no destruction, and were the same as non-immunized mice (Fig. 3A). In contrast, those of mice, exhibiting mild arthritis, immunized with CII in CFA had moderately destroyed cartilage and subchondrial bone accompanied by infiltration of inflammatory cells (Fig. 3B). More severe destruction of the joints accompanied by a large number of inflammatory cells was observed in mice immunized with CII in the presence of CSC (Fig. 3C).

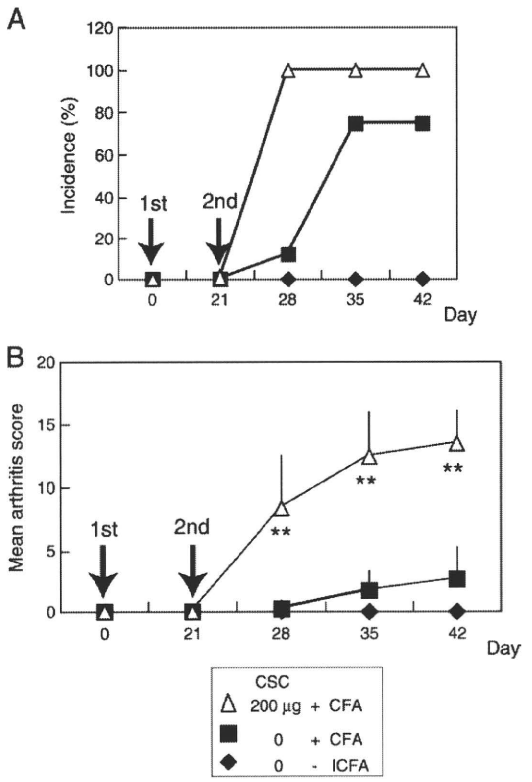

Fig. 1. Mainstream CSC augments clinical development of collagen induced arthritis (CIA) in mice (27w). DBA/1J mice (27w, male, $n=8$) were subcutaneously immunized with bovine type II collagen (CII) emulsified with CFA or ICFA with or without mainstream CSC (200 μg per mouse). After 3 weeks, all the mice were boosted by an intraperitoneal injection with CII, and then clinical development of arthritis was determined. (A) Arthritis incidence. (B) Arthritis score. Each point and vertical bar represents mean ± S.D. of 8 mice per group. $^{**}P<0.01$: significantly different from the positive control value.

3.4. Sidestream CSC augments induction and clinical development of arthritis in young mouse

The effect of sidestream CSC was examined by using 6 weeks aged male mice. As shown in Fig. 4, sidestream CSC at either 100 μg or 200 μg per mouse dose-dependently augmented the induction of arthritis and ameliorated the arthritis. However, sidestream CSC at 50 μg per mouse could not exhibit the augmenting effect.

3.5. Intraperitoneal administration of mainstream CSC augments induction and clinical development of arthritis in young mouse

In order to exclude the possibility of direct effect of CSC on antigen, mainstream CSC (100 μg per mouse) was intraperitoneally injected into 6 weeks aged male mice on day −1, immunized with antigen and CFA on day 0, boosted with the antigen alone on day 21, and then the development of arthritis was measured. In this experiment CSC also augmented the induction and development of arthritis (Fig. 5).

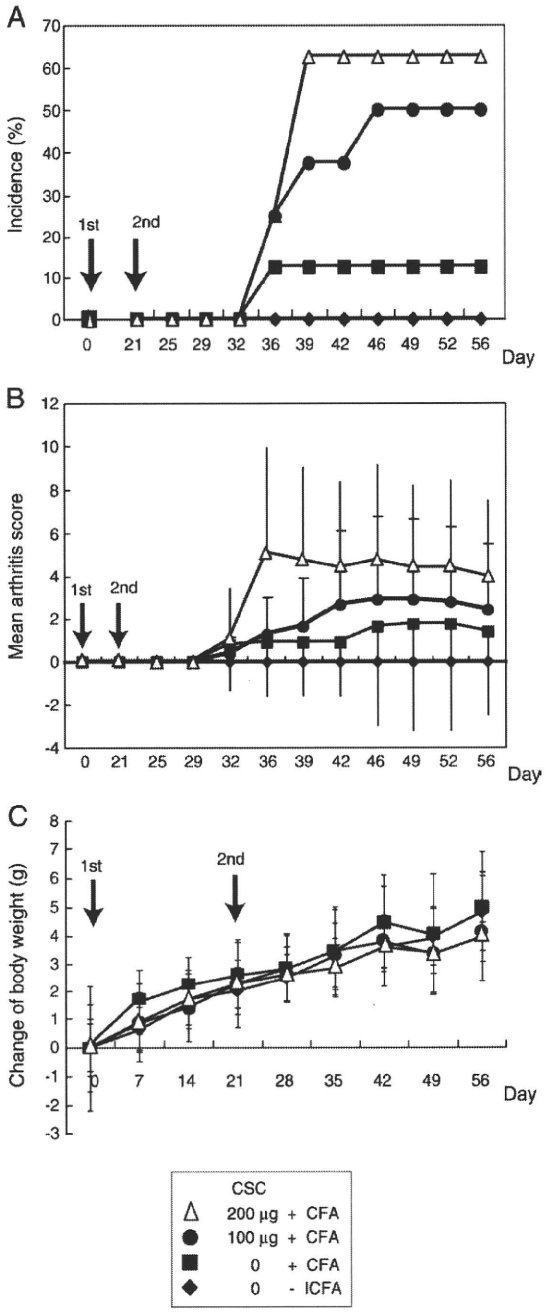

Fig. 2. Mainstream CSC augments clinical development of CIA in mice (6w). DBA/1J mice (6w, male, $n=8$) were subcutaneously immunized with CII emulsified with CFA with or without mainstream CSC (200 μg or 100 μg per mouse). After 3 weeks, all the mice were boosted by an intraperitoneal injection with CII, and then clinical development of arthritis was determined. (A) Arthritis incidence. (B) Arthritis score. Each point and vertical bar represents mean ± S.D. of 8 mice per group. (C) Body weight change. Each point and vertical bar represents mean ± S.D. of 8 mice per group.

4. Discussion

In this study we showed for the first time that mainstream CSC or sidestream CSC augmented the induction and clinical development of arthritis in collagen-induced arthritis. In this study we used DBA1J male mice for the experiment because male and older mice are prone to develop arthritis as compared to female and young mice. First, mainstream CSC appeared to augment the induction and severity of arthritis in older mice. In human onset of RA is most frequent in 40 to 50 years. However, younger people aged 10–20 years are also affected. Therefore, we also examined the effect of CSC in young mice. As expected the incidence of

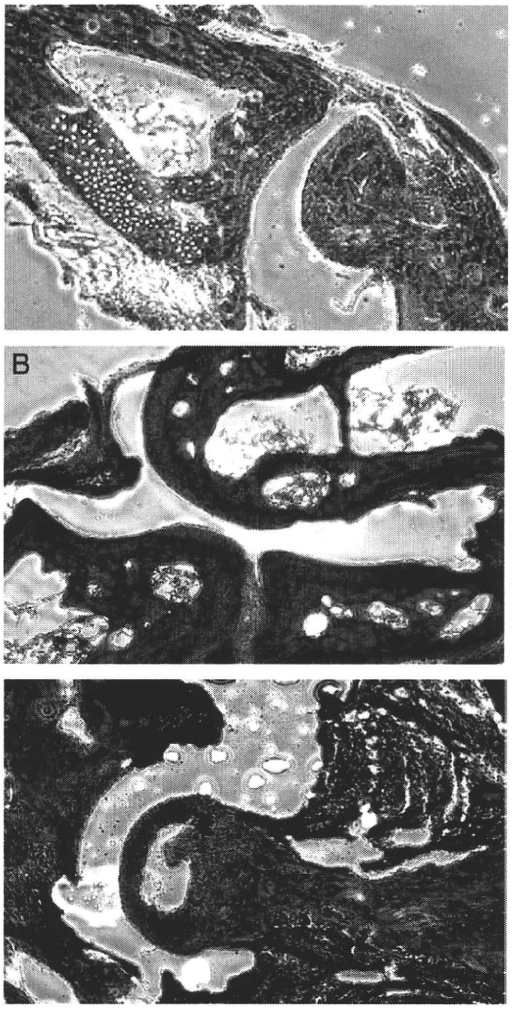

Fig. 3. Histologic changes of tarsal joints. All sections stained with H&E. A to C are sections of finger joints of representative mice from the experiment described in Fig. 2. (A) Synovitis of non-immunized mice. (B) Synovitis of mice immunized with CII emulsified with CFA. (C) Synovitis of mice immunized with CII emulsified with CFA in the presence of mainstream CSC (200 μg per mouse). Original magnification, ×40.

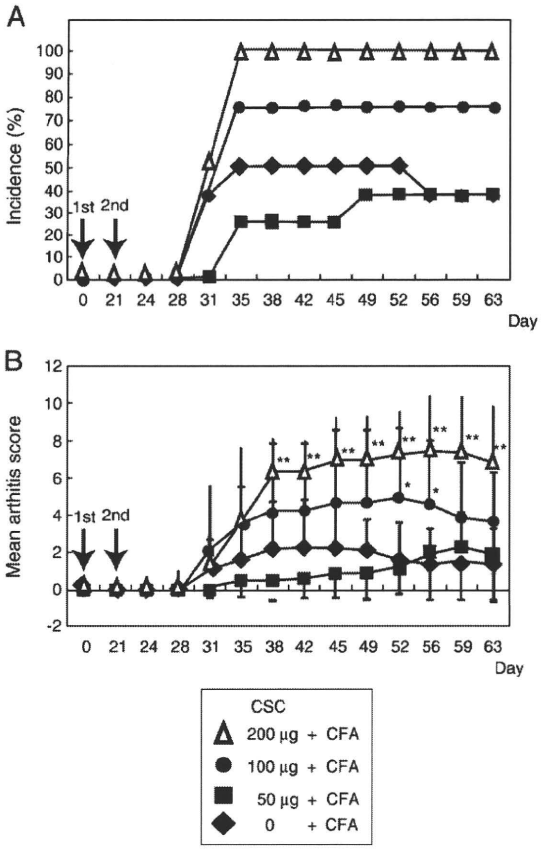

Fig. 4. Sidestream CSC augments clinical development of CIA in mice. DBA/1J mice (6w, male, $n=8$) were subcutaneously immunized with CII emulsified with CFA with or without sidestream CSC (200 μg,100 μg or 50 μg per mouse). After 3 weeks, all the mice were boosted by an intraperitoneal injection with CII, and then clinical development of arthritis was determined. (A) Arthritis incidence. (B) Arthritis score. Each point and vertical bar represents mean ± S.D. of 8 mice per group. $^{*}P<0.05$, $^{**}P<0.01$: significantly different from the positive control value.

arthritis in the positive control of young mice was low as compared to older mice. However, mainstream CSC augmented the induction and clinical severity of arthritis in the young mice as well. Sidestream CSC also exhibited the same augmenting effect as mainstream. Although the data were not shown, we could not observe the augmenting effect of mainstream CSC at 50 μg per mouse. Therefore, there were no differences between mainstream CSC and sidestream CSC. Sidestream CSC contains more carcinogenic compounds than mainstream CSC. Probably chemicals other than carcinogenic compounds in CSC are responsible for the augmenting effect. We added CSC into the emulsion of antigen and CFA in order to chronically expose the mice to CSC, which is thought to mimic the daily intake of cigarette. However, CSC might have directly modified the antigen or has adjuvant activity. In addition, smokers are exposed to cigarette smoke at the remote site from synovium. Therefore, we administered intraperitoneally CSC 1 day before immunization, and found that CSC also exhibited the augmenting effect, excluding the direct effect of CSC on antigen. Rather our findings suggest the systemic effect of CSC on mice.

Our study is in contrast to the recent report showing that cigarette smoke and nicotine exposure delayed development of collagen-induced arthritis in mice [21]. Although the reason is not clear, the discrepancy may be due to the experimental conditions. We collected the particulate matter in the smoke from cigarette equipped with a charcoal filter and injected CSC with antigen while Lindblad exposed mice with smokes from unfiltered cigarette. Therefore, CSC we used may contain less amount of nicotine than unfiltered smoke, and the balance between the active molecule and nicotine may be important for augmenting the induction of RA. If so, the cigarette containing less amount of nicotine will be more hazardous in induction of RA. The active molecular entity in CSC responsible for the augmenting effect is not known. CSC may contain endotoxin (LPS), which is able to induce proinflammatory cytokines from macrophages, subsequently it may contribute to the augmenting effect. However, one mg of CSC, either mainstream or sidestream, did not contain LPS (detection limit 0.001 EU). In addition, we avoided the effect of undetectable level of LPS by adding polymyxin B into the antigen mixture. We could not find the augmenting effect in the extract from filters alone (data not shown). We have previously reported that PAHs such as 3-MC, B[*a*]P and TCDD up-regulated IL-1β mRNA in RA patient-derived human fibroblast-like synoviocyte line MH7A [15]. We also reported that CSC induced IL-1α,

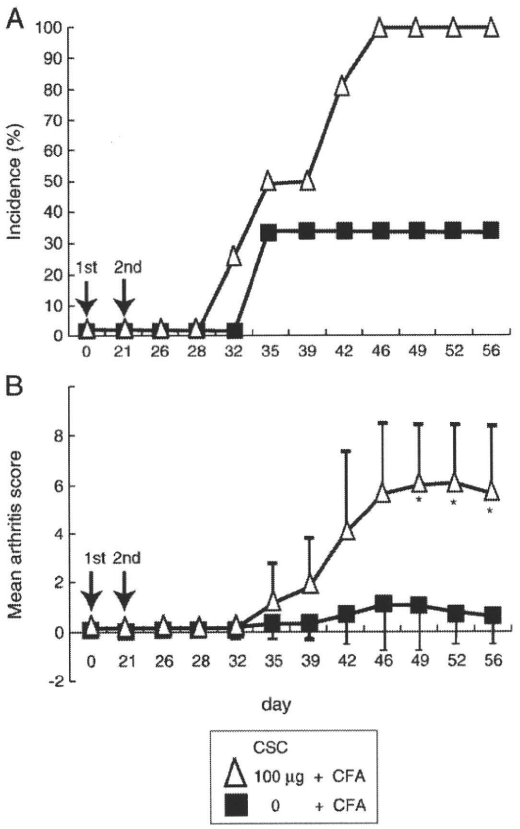

Fig. 5. Peritoneal injection of mainstream CSC augments clinical development of CIA in mice. DBA/1J mice (6w, male, positive control $n=6$, CSC $n=8$) were subcutaneously immunized with CII emulsified with CFA. One day before administration of the antigen mainstream CSC was intraperitoneally injected into mice (100 μg per mouse). After 3 weeks, all the mice were boosted by an intraperitoneal injection of CII, and then clinical development of arthritis was determined. (A) Arthritis incidence. (B) Arthritis score. Each point and vertical bar represents mean ± S.D. of 6 or 8 mice per group. $^{**}P<0.01$: significantly different from the positive control value.

IL-1β, IL-6 and IL-8 at both mRNA and protein levels in the cells [17]. As the effect of CSC *in vitro* was partially inhibited by an antagonist for the aryl hydrocarbon receptor (AhR), PAHs as well as other compounds are thought to contribute to the augmenting effect. This may also be true for the effects of CSC *in vivo*. In human higher AhR mRNA and protein levels were expressed in RA synovial tissue than *osteoarthritis* (OA) tissue, and AhR expression was up-regulated by TNFα [22]. In conjunction with our earlier studies, these findings suggest that an exposure to AhR ligands in cigarette smoke may exacerbates RA. Indeed, cigarette smoke exposure is able to induce AhR activation *in vivo* in AhR-dependent reporter gene transgenic mice [23].

Currently about 1% of the world's population is affected by the disease. Quite interestingly, however, studies on document, excavation, examination of skeletons and paintings suggest that RA has not been found until 17 century in old world (Europe) [24–28]. Guillaume Baillou (1558–1616) and Thomas Syndenham (1624–1689) first identified RA and distinguished it from the related disease, such as gout and rheumatic fever. However, the reason why RA has not been found in the old world until the 17th century is a big mystery[29], and RA is thought to be imported from the new world (America), where RA was present from 3000 to 5000 years ago [24]. Tobacco is a plant native in North and South America and imported from the new world to the old world [30]. Rothschild, Turner and DeLuca [24] included tobacco among variables that could be responsible for the appearance of RA in Europe.

These historical contexts support the epidemiological studies that tobacco smoking, especially heavy smoking, is a solid environmental risk factor for RA. In our study the yields of mainstream CSC and sidestream CSC were 9.25 mg and 18.0 mg per cigarette, respectively. Most importantly, the dose of mainstream CSC (100 μg per mouse), which is able to augment induction of arthritis in mice, is reachable if individual with 60 kg body weight takes only 32 cigarettes (manisteam) or exposed to 17 cigarettes (sidestream) in assuming that all the smokes were adsorbed. Actually heavy smokers daily intake much more cigarettes and the number reaches up to uncountable level for a long period of time. Therefore, significant amount of CSC, especially because PAHs and other hydrophobic chemicals are readily absorbed, can be accumulated over a long period of time.

Although further studies are needed to clarify the mechanism of augmenting effect of CSC on induction and development of arthritis, our findings support the etiological role of cigarette smoking in RA.

Acknowledgments

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Determination of particle-associated hydroxynitropyrenes with correction for chemical degradation on a quartz fibre filter during high volume air sampling

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A correction method for the determination of atmospheric monohydroxylated derivatives of 1-nitropyrene (hydroxy-1-nitropyrenes, OHNPs) based on their degradation rates during high volume air sampling was established. OHNPs adsorbed directly on a quartz fibre filter (QFF) or on airborne particles collected on a QFF were exposed to ambient air passively or actively in a high volume air sampling system. The influence of ozone flux and exposure time on the degree of degradation of OHNPs was investigated. Up to 50% of OHNPs degraded over 1 h of exposure to ambient air containing ~60 ppbv of ozone in the active system. The degradation rate constants of OHNPs were found to correlate with the number of ozone molecules passing through the QFF in a unit time (N_{O3}) during high volume air sampling. The chemical loss of OHNPs under high volume air sampling conditions was successfully evaluated by the exposure time and the pseudo-first-order rate constant for OHNP degradation estimated from the correlation with N_{O3}. Concentrations of 3-, 6-, and 8-hydroxy-1-nitropyrenes in airborne particles collected in Osaka, Japan were determined using the established correction method.

Keywords: polycyclic aromatic hydrocarbons; nitropyrene; airborne particles; sampling artifact; ozone; oxidation

1. Introduction

Polycyclic aromatic compounds (PACs), including polycyclic aromatic hydrocarbons (PAHs) and nitrated polycyclic aromatic hydrocarbons (NPAHs), are a class of atmospheric mutagens/carcinogens. In recent years, several kinds of PAHs and their derivatives have also been found to act as endocrine disruptors that may cause dysfunction of human and wildlife endocrine systems, abnormalities associated with developing reproductive systems and deficiencies in immune systems. 1-Nitropyrene (1-NP) is a representative NPAH formed through combustion processes of fossil fuel, such as diesel fuel combustion, and one of the most abundant NPAHs in the atmosphere [1,2]. We recently found that the hydroxylated derivatives of 1-NP (3-, 6-, and 8-hydroxy-1-nitropyrenes; 3-, 6-, and 8-OHNPs) show estrogenic, antiestrogenic and antiandrogenic activities in yeast two-hybrid assay systems [3]. 8-OHNP in particular exhibits strong

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antiestrogenic and antiandrogenic activities, e.g. 1.0×10^{-6} M of 8-OHNP inhibited 32 and 90% of β-galactosidase activity induced by 1.0×10^{-9} M of 17β-estradiol and 1.0×10^{-8} M of 5α-dihydrotestosterone in the assay systems, respectively. Gibson *et al.* [4] previously reported that OHNPs were observed in ambient airborne particles. However, the details of their sources or sinks in the atmosphere are still uncertain. In order to clarify the health impacts of OHNPs on humans, their monitoring in the atmosphere is urgently required.

Most of the OHNPs in the atmosphere are expected to be distributed in the particle phase, because the vapour pressure of OHNPs should be lower than that of the parent 1-NP due to hydrogen bonds derived from hydroxyl groups in their structures. Therefore, it is necessary to collect airborne particles for determination of atmospheric OHNPs. Many reports indicated that particle-associated PACs may degrade on glass- or quartz fibre filters (GFF and QFF) during the collection of airborne particles due to oxidation reactions with oxidants such as O_3, OH radical, NO_3 radical, etc. [5–8]. Since OHNPs have reductive phenolic hydroxyl groups in their structures, they are expected to decompose more easily than the parent PACs during high volume air sampling. The heterogeneous chemical reaction of PACs with O_3 is an especially important decomposition process of particle-associated PACs in the atmosphere [9–11]. In fact, O_3 can be regarded as a tracer for atmospheric oxidising power that drives the chemical degradation of PACs during air sampling [8]. It is commonly accepted that the substrate material on which PACs are deposited also affects the degradation of PACs by the reaction with O_3. For example, the decomposition of PACs on GFF and QFF occurs more easily than on Teflon filters [9]. On the other hand, PACs adsorbed onto airborne particles, especially soot-rich particles, are protected from chemical transformations [12,13].

In this study, we investigated the effect of O_3 flux, i.e. the number of O_3 molecules passing through QFF in a unit time (N_{O3}/molecules min^{-1}), on the loss of OHNPs under high volume air sampling. We also established a correction method for the determination of atmospheric particle-associated OHNPs. This was accomplished by the calculation of the decomposed fraction of OHNPs on airborne particles during high volume air sampling based on the degradation rate and exposure time.

2. Experimental

2.1 *Reagents and chemicals*

3-, 6- and 8-OHNPs and deuterated 3-OHNP (3-OHNP-d_8) were synthesised according to the previously reported procedure [14]. Briefly, acetoxypyrene, which was prepared from pyrene by treatment with lead tetraacetate in benzene/acetic acid (9/1, v/v), was nitrated using concentrated HNO_3 in acetic acid. The obtained mixture of three isomers of acetoxynitropyrenes was treated with CH_3ONa in methanol/THF (1/1, v/v) to obtain a mixture of OHNPs. Each OHNP isomer was purified by preparative normal phase HPLC (SUPELCO, Supelcosil PLC-SI, 21.2 mm ID $\times$ 250 mm, eluted with CH_2Cl_2 containing 0.5 mM CH_3COOH at 10 mL/min). To identify the synthetic compounds, their GC-MS and proton NMR spectra were compared with literature data [14,15]. 1-NP and deuterated 1-NP (1-NP-d_9) were obtained from Sigma-Aldrich Co. and C/D/N Isotopes, respectively. All solvents and other chemicals used were HPLC or analytical grades from Wako Pure Chemical Ind.

2.2 *Chemical analysis of OHNPs by HPLC*

The filter samples were cut into fine pieces before extraction. The soluble organic fractions (SOF) from the filter samples were extracted twice with 100 mL of ethanol under sonication for 20 min. The extract solution was filtered with a cellulose acetate filter to remove solid residue, followed by adding 100 μL of dimethyl sulphoxide (DMSO) into the filtrate to avoid complete dryness of the solvent during the concentration steps. After concentration using a rotary evaporator to ca. 5 mL and filtration with a 0.45 or 0.22 μm membrane filter, the samples were concentrated to 100 μL under a nitrogen stream to leave only DMSO, and then 400 μL of methanol was added. An aliquot of each of the sample solutions was subjected to HPLC analysis. An HPLC system with column-switching and chemiluminescence detection [16–18] was employed for OHNPs and 1-NP analysis, with several modifications to the column type and size in the previously reported system [19]. Briefly, the system consists of four HPLC pumps, a 6-port switching valve, a clean up column (GL Sciences, Inertsil ODS-P, 3.0 mm ID × 250 mm), separation columns (GL Sciences, Inertsil ODS-EP, 3.0 mm ID × 250 mm or Inertsil ODS-3, 3.0 mm ID × 250 mm × 2), a reducer column (Jasco, NPpak-RS, 4.6 mm ID × 10 mm), a trapping column (GL Sciences, Inertsil ODS-3, 4.0 mm ID × 30 mm), and a chemiluminescence detector (Soma Optics, S-3400). The chemiluminescence reagent solution was an acetonitrile solution containing 0.03 mmol L^{-1} bis(2,4,6-trichlorophenyl)oxalate and 15 mmol L^{-1} H_2O_2. Mobile phases were methanol/water (3 : 1, v/v) for the clean up and reduction of OHNPs and/or 1-NP, and acetonitrile/imidazole-perchloric acid buffer (45 : 55, v/v) for the separation. The reduction of OHNPs and 1-NP into the corresponding amino compounds, which are strongly fluorescent, was performed at 373 K in the reducer column. In order to exclude interfering compounds, specific fractions for the analytes eluted from the clean up column were introduced into the separation column: two different injections were necessary to determine all the OHNP isomers for a sample. The injection volume was 20 μL. To clarify the origin of the peaks observed in the HPLC chromatograms, the SOF sample washed with 5% NaOH/water was also analysed by the HPLC system. For the calibration curves of the standard OHNPs, the chemiluminescence intensities were proportional to the concentrations of the three compounds in the range from 10 to 2000 fmol per injection, and the calibration curves showed good linearity ($r^2 > 0.999$). Quantification limit of the HPLC system employed for each OHNP was 2 fmol (S/N = 10).

2.3 *Airborne particle collection for the exposure experiment of OHNPs*

Prior to the evaluation of the degradation of OHNPs on airborne particles during high volume air sampling, ambient particles were collected on the QFF every 3 hours at the rooftop level of a three-story building approximately 10 m above ground level at Osaka Prefecture University, Sakai, Osaka, Japan (34°55′N, 135°51′E). This sampling site is located in a polluted residential area. Traffic on moderately busy roads Route 310 and Hanwa-Highway is the only substantial source of air pollutants throughout the year and no large potential stationary source of airborne particles is located near the site. Sampling was conducted using a high volume air sampler (Kimoto Electric, Model 120) having no cut-off stage with the QFF (Advantec MFS, QR100), i.e. total suspended particulate matters (TSP) were collected, at a flow rate of 1500 L min^{-1} during 12–16 May 2003. The mass of ambient particles was determined by measuring the weight of the QFF, before and

after sample collection, after equilibrium weight was attained for each filter stored in desiccators at constant relative humidity of ca. 40% under 295 ± 3 K, resulting in 14 ± 6 mg (mean $\pm$ S.D., $n = 36$). All the QFF samples were stored at 253 K until subjected to the exposure experiments or analysis.

2.4 *Passive exposure of OHNPs to indoor air on QFF and on airborne particles*

Ten pmol of 3-, 6- and 8-OHNPs dissolved in methanol were uniformly deposited on a QFF directly by the following procedure: 2 mL of the stock solution (5 nM) was evenly dribbled onto the QFF with a microsyringe, and then the solvent was evaporated at room temperature in the dark. The air-dried QFF was passively exposed to indoor air containing trace levels of O_3 (less than 1 ppbv) for 1 and 18 hours in the dark at room temperature. In order to avoid interference of OHNPs originally contained in the airborne particulate samples with the HPLC analysis, 10 pmol of 3-OHNP-d_8 dissolved in methanol was deposited onto airborne particles collected on a QFF as described above. 3-OHNP-d_8 adsorbed on airborne particles was also exposed to indoor air for 24 hours according to the same procedure. The remaining OHNPs and 3-OHNP-d_8 were analysed by HPLC after extraction from the QFF or airborne particle samples as described above.

2.5 *Active exposure of 3-OHNP-d_8 to ambient air on airborne particles under the high volume air sampling condition*

Decomposition of 3-OHNP-d_8 on airborne particles during high volume air sampling was evaluated according to the following procedure. Ten pmol of 3-OHNP-d_8 and 1-NP-d_9, which was added in order to determine the recovery during the sample pretreatment, dissolved in methanol were uniformly deposited on airborne particles that were collected on a QFF as described above. After being installed in a high volume air sampler, the QFF was exposed to ambient air containing up to 60 ppbv of O_3 at a flow rate of 1500 L min^{-1} for 1–9 hours. The remaining 3-OHNP-d_8 and 1-NP-d_9 were analysed by HPLC after extraction from the airborne particles as mentioned above. To clarify the unexpected formation of 3-OHNP-d_8 by oxidation of 1-NP-d_9 during the high volume air sampling, the active exposure of 1-NP-d_9 in the flow system was also performed independently.

2.6 *Airborne particle collection for the determination of the atmospheric particle-associated OHNPs with the correction method*

TSP were collected every 3 hours at the same site, as is described in Section 2.3. Sampling was conducted using a high-volume air sampler with a QFF at a flow rate of 1500 L min^{-1} during 26–27 November 2001 for 24 hours. A total of 8 samples were prepared. The filter samples onto which 0.5 mL of 10 nM 3-OHNP-d_8 solution was added as an internal standard were subjected to the extraction process.

2.7 *Measurement of gases*

The concentration of O_3 in the air was monitored using a UV spectrophotometric O_3 analyser (Dylec, Model 1150) or obtained by public environment monitoring stations in Sakai, Osaka, Japan.