報　文
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定量分析値の信頼性確保のためのqNMRを用いた市販試薬の純度決定

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Determination of the Purities of Commercial Reagent Products using qNMR for the Ensuring the Reliability of Quantitative Analysis

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Summary

In environmental analysis, the commercial reagent and reference material products of analyte compounds are indispensable for chromatography such as GC/MS and LC/MS. However, most of their purities are not certificated traceability to the International System of Units (SI). Hence the possibility that their obscure purities greatly ruin the reliability of the quantitative value is incontrovertible. In this study, the purities of forty one commercial pesticide reagent products (new or old) were determined by a quantitative analytical method which is traceable to SI using nuclear magnetic resonance (qNMR). qNMR is a rapid and simple quantitative analysis method and no reference compound of analyte is needed. The purities of ten commercial reagent products among our measured forty one products are different more than 5% to their labeled purities by the manufacturers, and the values were found in 47.9-94.8%. Therefore it consequently seems that the differences between SI traceable purities and labeled purities cause the error of 5.1-50.8% to the quantitative values of analytes. This result represents that qNMR analysis has potential to work as a bridge of SI traceability and the quality control of reagent product using qNMR is greatly important to secure the accuracy of analytical data.

Key words: quantitative NMR, standard, purity, reliability

1. はじめに

環境分析における水や大気等の時間軸上で流動的な試料は，同一の試料を再度採取することができないため，定量分析値の信頼性確保が特に重要である。現在，環境中の農薬等の有機物質の定量分析法には，分解能や微量分析能に優れた液体クロマトグラフィーやガスクロマトグラフィー等のクロマトグラフィーが広く採用されている。クロマトグラフィーは標準被検物質を用いて相対的に定量するため，測定対象化合物の標準物質が不可欠である[1,2]。一方，物質量の絶対値は普遍的な国際計量標準である国際単位系(SI)にトレーサブルな測定によって得られると定義されているが[3,4]，有機化合物については，この定義に従い，計量計測トレーサビリティの確保された純度が保証された標準物質として市販されているものは少ない。そのため，市販試薬が定量用標準物質の代用品として使用される。しかし，市販試薬の多くは，試薬メーカー独自の方法で調製および管理を行っており，これらの製品のラベルや成績書には，品質保証として，JIS規格に準拠したクロマトグラム上に観察される主成分のピーク面積百分率，あるいは吸光光度法による比吸光度等よ

り算出したものを「純度%」と示している場合が多い。ピーク面積百分率は，クロマトグラム上に観察されるすべての成分のピーク面積の総和に対する測定対象化合物のピーク面積を比率として表したものであり[1,2]，元素組成比の異なる不純物を含む混合物の純度は原理的に精確な測定が困難である。また，吸光光度法では，化合物の吸光係数が正確であることを大前提としているが，純度100 %の上位標準となる化合物が存在しなければ，精確な純度値を求めることが不可能と思われる。したがって，SIへの計量計測トレーサビリティが確保された標準物質が定量分析に用いられていない以上，得られた定量分析値は厳格なトレーサビリティの連鎖から外れ，試薬の純度値の誤差が定量分析値の信頼性を大きく損なっている可能性を否定できない。

実際に，我々は，純度が計量計測トレーサビリティの確保されていない市販試薬を定量分析に用いた場合，得られた定量分析値に非常に大きなばらつきがあり，結果として，メーカー間にある純度の差が定量分析値の不確かさを大きくし，室間精度に影響を与えていることを報告している[3]。また近年，各国の標準物質の同等性，化学計測の国際的整合性が議論されるようになり，得られた定量分析値について，計量計測トレーサビリティの欠如が問題視されるようになってきた。国際標準化機構(ISO)発行のISOガイドにおいても，測定値および分析結果の精確さの問題についての勧告があり，分析手順や校正手順，適切な認証標準物質の選択，トレーサビリティの確保等，トレーサビリティを実現し，精確な特性値を得ることを含んだ標準物質に関する指針が示されている[6-11]。このような背景から，環境分析の分野における分析精度の更なる向上のために，有機化合物の純度を計量学的に精確に測定する方法が早急に必要とされている。

我々は，これまでに定量分析値の高精度化を目指し，SIに基づく計量計測トレーサビリティが確保された新たな定量法として，一次標準測定法の資格を原理的に有する核磁気共鳴装置（NMR）を用いた定量分析法（quantitative NMR: qNMR）を開発してきた[12-19]。本研究では，qNMRの測定対象として，過去の全国水道事業体の農薬実態調査において，浄水での検出率や個別評価値が高い農薬等を30種選択し，標準物質として使用される可能性の高いこれら市販試薬の純度を測定し，同製品のラベルや成績書に記載されている純度値が分析精度に及ぼす影響について検討した。

2. 方法

2. 1 対象化合物

Table 1に測定に供した市販試薬30種41製品を示した。その化合物名，化学構造による分類，分子式，分子量，試薬メーカーを示す。

2. 2 試薬および試液

高純度hexadimethyldisilane（HMD）は和光純薬工業株式会社特注品，重溶媒はacetone-d_6（99.9 atom % D）およびmethanol-d_4（99.8 atom % D）（Isotec），diethyl phthalate（DEP）は，独立行政法人 産業技術総合研究所の認証標準物質（NMIJ CRM 4022-b：純度99.98 ± 0.01 w/w%（99.74 ± 0.09 mol/mol %））を用いた。

2. 3 装置

ウルトラミクロ天秤はXP2U（メトラートレド株式会社）を使用した。試料の秤量値は，最小目盛0.0001 mgまで読み取った値を用いた。

核磁気共鳴装置（NMR）はオートサンプラー付きJNM-ECA（600 MHz，日本電子株式会社（現：株式会社JEOL RESONANCE））を使用した。qNMRのケミカルシフト値は，HMDを基準シグナル（0 ppm）とし，δ値をppm単位で表した。NMR測定条件はTable 2に示す。

なお，qNMR用標準液および試料溶液の調製には，化学用体積計（50，100 mLメスフラスコ）または電動オートピペッター（マルチピペットXstream（エッペンドルフ製），10 mL（不確かさ ± 0.4 %），3～5 mL（不確かさ ± 0.5 %））を用いた。

2. 4 qNMR用標準液の調製およびHMDの濃度校正

HMD約20 mgを精密に量り取り，acetone-d_6 100 mLに定容した。もしくは，HMD約100 mgを精密に量り取り，methanol-d_4 50 mLに定容し，この溶液をmethanol-d_4で25倍希釈した。これらHMDのacetone-d_6溶液またはmethanol-d_4溶液をqNMR用標準液とした。qNMR用標準液中のHMDの濃度を下記に従い，DEPを内標準物質として校正して求めた。すなわち，*CRM*であるDEP約5-10 mgを精密に量り取り，qNMR用標準液1.0 mLに溶解した。この溶液0.6 mLをNMR試験管（5 mm φ × 200 mm，日本電子株式会社）に封入したものをHMD濃度校正用試料溶液とした。この溶液をqNMRに付し，DEPのCH_2 × 2およびHMDのCH_3 × 6に由来するシグナル面積，分子量，濃度等を式（1）に代入し，qNMR用標準液中のHMDの濃度を校正した。

$$W_{HMD} = \frac{M_{HMD} \times I_{HMD}}{H_{HMD}} / \frac{M_{DEP} \times I_{DEP}}{H_{DEP} \times W_{DEP}} \times \frac{P_{DEP}}{100} \quad (1)$$

ただし，W_{HMD}, W_{DEP} = HMDおよびDEPの濃度（mg/mL），M_{HMD}, M_{DEP} = HMDおよびDEPの分子量（146.3781および222.2337，分子量はIUPAC 2007年発表の原子量表[20]を用いて算出），H_{HMD}, H_{DEP} = HMDのCH_3 × 6およびDEPのCH_2 × 2のプロトン数，I_{HMD}, I_{DEP} = HMDのCH_3 × 6およびDEPのCH_2 × 2のシグナル面積，P_{DEP} = DEPの純度（99.98 w/w%）を示す。

2. 5 qNMRによる市販試薬の純度測定

市販試薬を約5-10 mg精密に量り取り，予め調製したqNMR用標準液1.0 mLに溶解した。qNMR用標準液として，benzofenapおよびdalaponはmethanol-d_4溶液，その他はacetone-d_6溶液を使用した。これらの溶液0.6 mLをNMR試験管に封入したものを試料溶液とした。この試料溶液をqNMRに付し，HMDのシグナル強度面積，化合物に由来するそれぞれの特定シグナルの相対面積，分子量，濃度等を式（2）に代入し，農薬等の対象化合物の純度を算出した。

$$P_{target} = \frac{I_{target} / H_{target}}{I_{HMD} / H_{HMD}} \times \frac{M_{target} / W_{target}}{M_{HMD} / W_{HMD}} \times 100 \quad (2)$$

ただし，W_{HMD}, W_{target} = HMDおよび対象化合物の濃度（mg/mL），M_{HMD}, M_{target} = HMDおよび対象化合物の分子量[20]（146.3781およびTable 1），I_{HMD}, I_{target} = HMDおよび対象化合物の特定基のシグナル強度面積，H_{HMD}, H_{target} = HMDおよび対象化合物の特定基のプロトン数，P_{target} = 対象化合物の純度（%）を示す。

2. 6 qNMR測定条件および解析処理

市販試薬41製品の純度は，既報のqNMR[16-19]により計量学的に精確に決定した。qNMRのケミカルシフト値は，HMDを基準シグナル（0 ppm）とし，δ値をppm単位で表した。qNMRデータ解析は，得られたFree Induction Decay（FID）信号データを定量解析ソフトウェア（Alice2 for qNMR，日本電子株式会社（現：株式会社JEOL RESONANCE））に導入して自動処理した。すなわち，

Table 1 Information of commercial reagent and reference material products

Compound	Class [a]	Formula	Molecular mass	Manufacturer [b]	Sample No.
Acetamiprid	NN	$C_{10}H_{11}ClN_4$	222.67414	Wako	1
Benzofenap	PZ	$C_{22}H_{20}Cl_2N_2O_3$	431.31180	Hayashi	2
Bromobutide	AA	$C_{15}H_{22}BrNO$	312.24528	Wako	3
Butamifos	OP	$C_{13}H_{21}N_2O_4PS$	332.35560	Wako, Kanto, Hayashi	4, 5, 6
Cafenstrole	AA	$C_{16}H_{22}N_4O_3S$	340.43588	Wako	7
Carbofuran	CM	$C_{12}H_{15}NO_3$	221.25240	Sigma	8
Dalapon	FA	$C_3H_4Cl_2O_2$	142.96866	Kanto	9
Dichlorvos (DDVP)	OP	$C_4H_7Cl_2O_4P$	220.97574	Wako	10, 11
Disulfoton	OP	$C_8H_{19}O_2PS_3$	274.40402	Wako, Kanto, Sigma	12, 13, 14
Diuron	U	$C_9H_{10}Cl_2N_2O$	233.09450	Wako	15
Fenobucarb	CM	$C_{12}H_{17}NO_2$	207.26888	Wako	16
Fipronil	PZ	$C_{12}H_4Cl_2F_6N_4OS$	437.14778	Wako	17
Fipronil sulfone	PZ	$C_{12}H_4Cl_2F_6N_4O_2S$	453.14718	Wako	18
Flutolanil	AA	$C_{17}H_{16}F_3NO_2$	323.30965	Wako	19
Iprobenfos (IBP)	OP	$C_{13}H_{21}O_3PS$	288.34280	Wako	20
Imidacloprid	NN	$C_9H_{10}ClN_5O_2$	255.66100	Wako	21
Isoxathion	OP	$C_{13}H_{16}NO_4PS$	313.30920	Wako, Kanto, Hayashi	22, 23, 24
MCPA	PA	$C_9H_9ClO_3$	200.61896	GL Sciences	25
Mecoprop	PA	$C_{10}H_{11}ClO_3$	214.64554	GL Sciences	26
Mefenaset	AA	$C_{16}H_{14}N_2O_2S$	298.35956	Wako	27
Fenthion (MPP)	OP	$C_{10}H_{15}O_3PS_2$	278.32806	Wako	28
MPP sulfoxide	OP	$C_{10}H_{15}O_4PS_2$	294.32746	Wako	29
MPP sulfone	OP	$C_{10}H_{15}O_5PS_2$	310.32686	Wako	30
MPP oxon	OP	$C_{10}H_{15}O_4PS$	262.26246	Wako	31
MPP oxon sulfoxide	OP	$C_{10}H_{15}O_5PS$	278.26186	Wako	32
MPP oxon sulfone	OP	$C_{10}H_{15}O_6PS$	294.26126	Wako	33
Pirimiphos-methyl	OP	$C_{11}H_{20}N_3O_3PS$	305.33356	Wako	34
Pretilachlor	AA	$C_{17}H_{26}ClNO$	311.84684	Wako, Kanto, Sigma	35, 36, 37
Pyroquilon	HF	$C_{11}H_{11}NO$	173.21114	Wako, Kanto, Sigma	38, 39, 40
Tricyclazole	HF	$C_9H_7N_3S$	189.23698	Wako	41

[a] The analyte compounds were classed by the chemical structure: neonicotinoid, NN; pyrazole, PZ; acid amido, AA; organophosphorus, OP; carbamate, CM; fatty acid, FA; urea, U; phenoxyalkanoic acid, PA; heterocyclic fungicide, HF.

[b] The compounds were purchased from five manufacturers: Wako Pure Chemical Industries, Ltd., Wako; Hayashi Pure Chemical Ind., Ltd., Hayashi; Kanto Chemical Co., Inc., Kanto; Sigma-Aldrich Inc., Sigma; GL Sciences Inc., GL Sciences.

Table 2 Instruments and acquisition parameters

Spectrometer	JNM-ECA600 (JEOL)
Probe	5 mm broadband autotune probe
^{13}C decoupling	Multi pulse decoupling with Phase and Frequency switching (MPF-8)
Spectral width	-5 ~15 ppm
Data points	64000
Auto filter	on (8 times)
Flip angle	90°
Pulse delay	60 s (>5*T_1)
Scan times	8
Sample spin	no spin
Probe temperature	22-25℃
Sample solvent	Acetone-d_6 or Methanol-d_4
qNMR reference material	HMD
Primary standard material	DEP
Window function	—

このソフトウェア上で，qNMR データをフーリエ変換および自動位相調整を行い，HMD および特定シグナルの積分範囲設定等を設定後，予め入力した HMD および対象化合物の濃度，分子量，特定基のプロトン数等の化合物情報から，純度を式 (2) に従い算出した。

3. 結果および考察

3. 1 qNMR の原理

^1H-NMR は，測定対象化合物の分子構造にかかわらず，すべての水素原子が個々に定量的な信号として観測され，スペクトル上に観察される異なる化合物のシグナル強度の比は化合物に寄与する水素のモル比に対応する特徴を有する。このことから，2 つのシグナルが異なる化合物 (A, B) に由来する場合には，個々のシグナル面積と化合物の濃度は関係式 (3) で表すことができる。言いかえれば，一方の化合物の純度が明らかで定量的に混合すれば，それぞれのシグナル面積と寄与する水素原子を勘案することで，両者のモル比の関係から測定対象の化合物の純度を決定する関係式 (4) が成り立つ。したがって，qNMR は，測定対象の化合物以外の標準物質を上位標準として定量分析値を得ることが可能であり，一次標

準測定法のうち，一次比率法すなわち「物質量の基準となる別の化学物質を用い，それとの比較において目的の化学物質の物質量を測定する方法」の資格を原理的に有する。故に本法は，国家標準物質や認証標準物質（CRM）を上位標準とすることで，SIにトレーサブルな定量分析値（純度）を得ることが可能である。

$$\frac{I_A}{I_B}=\frac{H_A m_A}{H_B m_B}=\frac{H_A W_A / M_A}{H_B W_B / M_B} \quad (3)$$

$$P_{sample}=\frac{I_{sample}/H_{sample}}{I_{std}/H_{std}}\times\frac{M_{sample}/W_{sample}}{M_{std}/W_{std}}\times P_{std} \quad (4)$$

ただし，I＝シグナル面積，H＝特定基のプロトン数，m＝モル濃度，W＝重量，M＝分子量，P＝純度（%），*sample*＝試料，*std*＝基準物質を示す。

本報告では，qNMR基準物質としてHMDを用いた。しかしながら，SIにトレーサブルな純度が証明されたHMDが流通していないことから，qNMRによる定量分析値のSIトレーサビリティの確保には，SIにトレーサブルなCRMであるDEPを一次標準として用い，qNMR標準液中のHMDの濃度を校正した後に，HMDを二次標準として測定対象化合物のqNMR測定を行う二段階方式を採用した。すなわち，HMDをqNMR基準物質として用いた際の測定対象化合物の定量分析値のSIトレーサビリティは，CRMのDEPを介して実現した（Fig. 1）。また，qNMRは1測定当たりの所要時間が約10-20分であり，得られた測定値は高い再現性を示し，測定結果の不確かさは概ね1％以内が達成されている。

3. 2 市販試薬製品の純度

市販試薬は，新品23製品，開封後冷蔵保存約2～5年の18製品の計41製品について，qNMRにより計量計測トレーサビリティの確保された純度を測定した。試薬メーカー3社から購入した農薬はそれぞれの試薬メーカーの頭文字で表した。また，同一製品の開封後冷蔵保存約5年と新品（未開封）の2製品を測定したDDVPはDDVP-old，DDVP-newと表記した。qNMRスペクトル上に観察された基準物質HMDおよび対象化合物に由来する各シグナル面積，水素数，濃度等を関係式（2）に代入し，それぞれの純度を算出した。なお，観察されたシグナルのうち，OHやNH基由来のプロトンは重水素置換が考えられるため，定量には用いなかった。また，低分子化合物のNMRスペクトルは，低磁場側より高磁場側のシグナルに予想される不純物のシグナルが重なる危険性が高い。qNMRの定量用シグナルは，不純物のシグナルと十分に分離していることが理想であるため，明らかに不純物のシグナルと重なっているシグナルを除外し，それぞれのシグナルより算出された平均値を対象化合物の純度とした。定量に用いたシグナルと算出された純度をTable 3に示す。qNMRを用いて得られた純度（± n＝3 RSD％）は47.9 ± 2.5～100.9 ± 0.2％であった。純度の低い製品については，NMRスペクトル上の化合物と基準物質のHMDに由来するシグナル以外に不純物のシグナルが観察された。DDVP-oldに関しては，各シグナルから得られた純度のRSDが15.0％と大きく，不純物を含んでいる可能性が高いと考えられた。従ってDDVP-oldは，すべてのシグナルによる定量値を用いず，最も低い定量値47.9％を純度値とした。

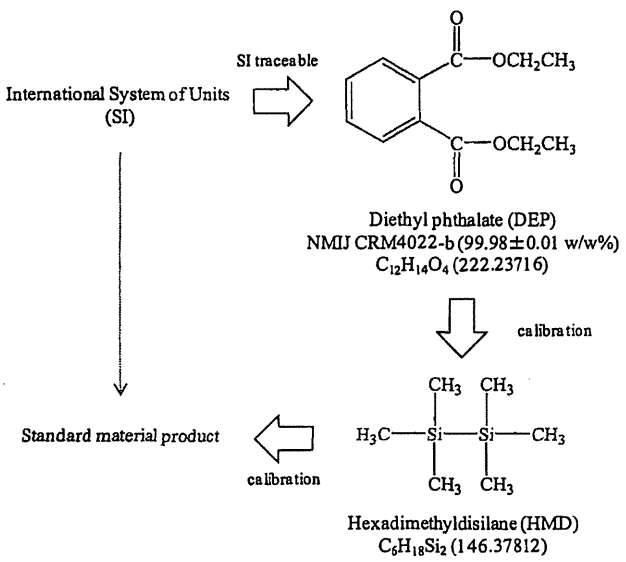

Fig. 1 Strategy of building up SI traceability of qNMR analysis
The concentration of HMD in qNMR solvent was corrected by DEP.

3. 3 純度が定量分析値の精度に及ぼす影響

対象化合物の計量計測トレーサビリティの確保された純度と成績書記載の純度値と比較した（Table 4）。その結果，市販試薬製品に記載の面積百分率による純度値は，95.3～100％であり，41製品のうち73.2％に相当する30製品（新品16製品，開封済み14製品）のqNMRによる純度は95％以上で，それぞれの成績書記載の純度値と総じてほぼ等しい値を示した（Fig. 2）。一方，41製品のうち，新品のbutamifos-W, K, H，pretilachlor-W, K, Sの6製品，開封済み製品のacetamiprid，dalapon，DDVP-old，fipronilの4製品計10製品はqNMRによる純度と成績書記載の純度値との差が5％以上あった。これらの10製品について，qNMRによる純度と成績書記載の純度値との比較をFig. 3に示す。開封済み製品では5.1～50.8％，新品では5.2～22.8％の差があった。

現状では，市販試薬製品の純度値は精確に値付けされていると前提して，定量用標準物質として使用することが多い。仮に市販試薬製品の成績書記載の純度値を信用してクロマトグラフィーにより定量分析を行ったとすると，qNMRによる純度と成績書記載の純度値に差があった10製品の得られる定量分析値は真値より5.1～50.8％の誤差を生じることになると言える。

3. 4 純度評価の問題点

新品のpretilachlor 3製品はqNMRによる純度が76.9～77.1％となり，添付の成績書に記載の面積百分率による純度値97.7～99.8％と大きな開きがあった。得られたNMRスペクトルをFig. 4に示す。pretilachlorに由来するシグナルと基準物質のHMDに由来するシグナル以外に，分解物または製造原料に由来すると思われるシグナル（Fig. 4中*印のシグナル：0.89，1.12，1.55，3.38，3.78，4.55，7.12，7.20 ppm）が観察され，明らかに純度が低いことが予想された。この市販試薬製品をガスクロマトグラフ／質量分析計（GC/MS）により測定し，不純物のピークを観察した結果，クロマトグラム上にはpretilachlor以外のピークは検出されなかった（データ未収載）。つまり，qNMRスペクトル上には不純物のシグナルが明瞭に観察されていることを考慮すると，添付の成績書に記載されているクロマトグラフィーから得られた純度値を質量％純度として扱うことは不適切であると考えられる。

クロマトグラフィーによる面積百分率を用いて値付けられた試薬メーカーの成績書記載の純度値が，あらゆる不純物のレスポンス

Table 3 Calculated purities of samples from listed proton signals
The signals of OH, NH and overlapped protons with the impurities were not used for the quantification.

Sample No.	Compound	Signal	1	2	3	4	5	6	7	8	9	Average (%, RSD%)
		a)	3H,s	3H,s	2H,s	1H,d						
		b)	2.45	3.20	4.79	7.40	—	—	—	—	—	
1	Acetamiprid	c)	74.6	75.1	75.0	74.8						74.9 (0.3)
			3H,s	6H,2s	3H,s	2H,s	1H,d	2H,d	1H,d	2H,d		
			1.81	2.35	3.72	5.64	7.08	7.27	7.34	7.71	—	
2	Benzofenap		99.8	99.0	99.3	94.1	103.7	99.8	100.5	97.5		99.2 (2.7)
			9H,s	3H,s	3H,s	1H,s	1H,t	2H,t	2H,d			
			1.06	1.56	1.63	4.29	7.13	7.24	7.37	—	—	
3	Bromobutide		99.7	100.0	99.5	99.4	101.0	99.9	100.4			100.0 (0.6)
			1H,d	1H,s	1H,d							
			7.09	7.42	7.74	—	—	—	—	—	—	
4	Butamifos-W		94.4	95.0	94.7							94.7 (0.3)
5	Butamifos-K		90.1	90.4	90.5							90.3 (0.2)
6	Butamifos-H		94.5	94.9	94.9							94.8 (0.2)
			6H,s	3H,s	6H,s	4H,q	2H,s	1H,s				
			1.15	2.25	2.61	3.47	7.03	8.96	—	—	—	
7	Cafenstrole		98.5	97.7	97.9	98.2	97.9	97.4				97.9 (0.4)
			6H,s	2H,s	1H,t	1H,d	1H,d					
			1.37	2.99	6.68	6.80	6.92	—	—	—	—	
8	Carbofuran		100.2	100.3	100.8	100.4	100.1					100.3 (0.3)
			3H,s									
			2.16	—	—	—	—	—	—	—	—	
9	Dalapon		71.8									71.8 (—)
			3H,s	3H,s	1H,d							
			3.80	3.81	7.16	—	—	—	—	—	—	
10	DDVP-old		64.9	47.9	57.9							56.9 (15.0)
11	DDVP-new		96.6	93.4	93.5							94.5 (1.9)
			3H,t	6H,t	2H,q	2H,m	4H,m					
			1.18	1.28	2.55	3.04	4.11	—	—	—	—	
12	Disulfoton-W		99.6	99.9	99.9	99.4	99.7					99.7 (0.2)
13	Disulfoton-K		102.0	97.7	102.2	96.9	97.4					99.3 (2.6)
14	Disulfoton-S		100.6	99.3	100.1	98.4	99.2					99.5 (0.9)
			6H,s	1H,d	1H,d	1H,s						
			2.94	7.32	7.43	7.89	—	—	—	—	—	
15	Diuron		99.2	98.9	98.9	99.6						99.1 (0.3)
			2H,m	1H,m	2H,m	1H,m						
			1.51	7.11	7.21	6.97	—	—	—	—	—	
16	Fenobucarb		100.0	100.1	99.6	100.0						99.9 (0.2)
			2H,s									
			8.09	—	—	—	—	—	—	—	—	
17	Fipronil		92.9									92.9 (—)
			2H,s									
			8.13	—	—	—	—	—	—	—	—	
18	Fipronil sulfone		99.9									99.9 (—)
			6H,d	1H,m	1H,d	2H,m	1H,s	2H,2t	1H,t	1H,d		
			1.26	4.55	6.64	7.19	7.46	7.65	7.70	7.76	—	
19	Flutolanil		100.6	99.7	99.7	99.5	99.9	100.4	98.9	100.7		99.9 (0.6)
			12H,m	1H,s	1H,s	2H,m	1H,t	2H,t	2H,d			
			1.21	4.01	4.03	4.55	7.22	7.28	7.35	—	—	
20	IBP		99.8	100.3	98.0	100.2	99.8	99.7	98.8			99.5 (0.9)
			2H,t	2H,t	2H,s	1H,d	1H,d	1H,s				
			3.61	3.78	4.52	7.41	7.80	8.36	—	—	—	
21	Imidacloprid		99.3	99.2	98.4	98.9	99.0	99.6				99.1 (0.4)
			6H,t	4H,m	1H,s	3H,m	2H,d					
			1.33	4.28	6.82	7.50	7.83	—	—	—	—	
22	Isoxathion-W		98.7	98.5	98.0	99.2	98.0					98.5 (0.5)
23	Isoxathion-K		99.9	99.8	99.4	99.9	99.8					99.7 (0.2)
24	Isoxathion-H		98.8	98.7	98.3	99.2	98.3					98.6 (0.4)

Table 3 Continued

Sample No.	Compound	Signal 1	2	3	4	5	6	7	8	9	Average (%, RSD%)
		3H,s	2H,s	1H,d	1H,d	1H,s					
		2.18	4.71	6.83	7.08	7.12	—	—	—	—	
25	MCPA	100.1	100.2	99.2	100.4	99.1					99.8 (0.6)
		3H,d	3H,s	1H,q	1H,d	1H,d	1H,s				
		1.56	2.17	4.81	6.77	7.06	7.12	—	—	—	
26	Mecoprop	100.2	99.7	99.5	99.5	99.9	99.0				99.6 (0.4)
		3H,s	2H,s	1H,t	1H,t	5H,m	1H,d	1H,d			
		3.20	4.87	7.21	7.40	7.45	7.57	7.75	—	—	
27	Mefenaset	95.3	90.1	101.9	107.3	95.5	106.7	100.7			99.6 (6.4)
		3H,s	3H,s	3H,s	3H,s	2H,s+d	1H,d				
		2.24	2.42	3.79	3.80	6.98	7.17	—	—	—	
28	MPP	101.1	100.9	100.4	99.3	100.1	100.5				100.4 (0.6)
		3H,s	3H,s	3H,s	3H,s	1H,s	1H,d	1H,d			
		2.34	2.61	3.81	3.84	7.06	7.25	7.82	—	—	
29	MPP sulfoxide	100.8	100.7	100.1	98.8	100.2	99.8	99.8			100.0 (0.6)
		3H,s	3H,s	3H,s	3H,s	2H,s+d	1H,d				
		2.65	3.11	3.83	3.85	7.22	7.95	—	—	—	
30	MPP sulfone	99.3	99.4	99.8	98.9	99.3	99.8				99.4 (0.3)
		3H,s	3H,s	3H,s	3H,s	2H,s+d	1H,d				
		2.24	2.41	3.76	3.78	7.04	7.17	—	—	—	
31	MPP oxon	97.7	97.3	98.5	96.7	97.3	97.6				97.5 (0.6)
		3H,s	3H,s	3H,s	3H,s	1H,s	1H,d	1H,d			
		2.34	2.61	3.80	3.82	7.11	7.24	7.81	—	—	
32	MPP oxon sulfoxide	99.4	99.9	98.6	97.5	98.2	98.0	97.9			98.5 (0.9)
		3H,s	3H,s	3H,s	3H,s	2H,s+d	1H,d				
		2.65	3.10	3.82	3.84	7.26	7.95	—	—	—	
33	MPP oxon sulfone	99.6	99.8	97.7	96.9	99.0	99.6				98.8 (1.2)
		6H,t	3H,s	4H,s	3H,s	3H,s	1H,s				
		1.11	2.23	3.55	3.85	3.85	6.02	—	—	—	
34	Pirimiphos-methyl	99.2	99.6	97.2	99.8	99.7	98.7				99.0 (1.0)
		3H,t	6H,t	2H,m	2H,t	2H,t	4H,s+t	2H,d	1H,t		
		0.79	1.20	1.42	3.25	3.51	3.69	7.24	7.32	—	
35	Pretilachlor-W	79.1	79.5	73.4	79.2	79.1	79.0	78.5	78.3		77.1 (0.4)
36	Pretilachlor-K	78.6	80.2	71.6	79.3	79.9	78.7	78.2	79.8		77.0 (1.2)
37	Pretilachlor-S	78.9	79.6	72.8	78.9	78.9	78.4	78.1	78.3		76.9 (0.3)
		2H,t	2H,t	2H,t	2H,t	1H,t	1H,d	1H,d			
		2.49	2.89	3.11	3.92	6.82	6.93	7.01	—	—	
38	Pyroquilon-W	101.1	100.8	100.7	100.8	101.0	100.9	100.9			100.9 (0.1)
39	Pyroquilon-K	100.6	100.2	100.3	100.2	100.4	100.3	100.3			100.3 (0.1)
40	Pyroquilon-S	101.1	100.8	100.8	100.8	101.0	100.9	100.8			100.9 (0.1)
		2H,m	1H,d	1H,s							
		7.36	7.65	9.27	—	—	—	—	—	—	
41	Tricyclazole	99.6	99.8	98.8							99.4 (0.5)

a) Upper column shows a number of proton with the spin-spin coupling (s: singlet, d: doublet, t: triplet, q: quartet, m: multiplet).
b) Middle column shows the signal region (ppm).
c) Lower column shows the purity of each signal (%).

ファクターが主成分と同じであると仮定して主成分の相対比を示すもので，絶対量を示しているわけではないことは明らかであるが，問題視されることはなかった。今回，qNMR を用いた純度測定により，市販試薬製品に記載の純度値が絶対量と異なることがあり得ることが証明された。なお，qNMR による純度試験に供した化合物は概ね 1 製品 1 ロットについてであり，市販試薬製品の純度について正確な情報を収集するためには，ロット間差についても今後検討する必要があると考えられる。

3. 5 開封済み製品の品質管理

市販試薬製品の純度の誤差が定量分析値の精度に及ぼす要因として，開封済の製品における品質管理について着目した。

DDVP は未開封の新品 DDVP-new の qNMR による純度が 94.5 % であるのに対し，開封後冷蔵保存約 5 年の製品 DDVP-old は 47.9 % であった。DDVP は熱には安定であるとされているが，長期保存により加水分解や酸化による純度値の低下が生じたと考えられた。化合物の安定性は化合物毎に異なり，保存期間や保存状態によっては

Table 4 Summary of commercial reagent products purities calculated by qNMR and labeled percentage of peak area on chromatogram by manufacturer

Sample No.	Compound		Purity (%) qNMR (n=3 average, RSD %)		Manufacturer[a]	Sample No.	Compound		Purity (%) qNMR (n=3 average, RSD %)		Manufacturer[a]
1	Acetamiprid	*	74.9	0.4	98.8 (GC/FID)	21	Imidacloprid	*	99.1	0.6	100 (LC/UV)
2	Benzofenap		99.2	1.9	99.6	22	Isoxathion-W		98.5	0.5	98.9 (GC/FID)
3	Bromobutide	*	100.0	0.2	—[b]	23	Isoxathion-K		99.7	0.3	99.1 (GC/FID)
4	Butamifos-W		94.7	0.8	99.8 (GC/FID)	24	Isoxathion-H		98.6	0.1	99.9 (GC/FID)
5	Butamifos-K		90.3	0.6	99.2 (GC/FID)	25	MCPA	*	99.8	0.2	—[b]
6	Butamifos-H		94.8	0.7	99.9 (GC/FID)	26	Mecoprop		99.6	0.7	—[b]
7	Cafenstrole	*	97.9	0.8	99.8 (HPLC/UV)	27	Mefenaset	*	99.6	0.2	99.9 (GC/FID)
8	Carbofuran		100.3	0.4	99.9 (HPLC)	28	MPP	*	100.4	0.4	99.9 (GC/FID)
9	Dalapon	*	71.8	0.8	99 (GC/FID)	29	MPP sulfoxide		100.0	0.3	99.8 (LC/UV)
10	DDVP-old	*	47.9	2.5	98.7 (GC/FID)	30	MPP sulfone	*	99.4	0.4	99.3 (GC/FID)
11	DDVP-new		94.5	0.4	99 (GC/FID)	31	MPP oxon	*	97.5	0.3	100 (GC/FID)
12	Disulfoton-W		99.7	0.4	98.6 (GC/FID)	32	MPP oxon sulfoxide		98.5	0.5	99.4 (GC/FID)
13	Disulfoton-K		99.3	0.2	95.3 (GC/MS, LC/DAD)	33	MPP oxon sulfone		98.8	0.5	99.8 (GC/FID)
						34	Pirimiphos-methyl		99.0	0.1	98.9 (GC/FID)
14	Disulfoton-S		99.5	0.3	98.6 (HPLC/UV)	35	Pretilachlor-W		77.1	0.5	99.2 (GC/FID)
15	Diuron	*	99.1	0.01	100 (LC/UV)	36	Pretilachlor-K		77.0	1.6	99.8 (GC/FID)
16	Fenobucarb	*	99.9	0.5	—[b]	37	Pretilachlor-S		76.9	0.4	97.7 (HPLC/UV)
17	Fipronil	*	92.9	0.2	98.1 (GC/FID)	38	Pyroquilon-W		100.9	0.2	100 (GC/FID)
18	Fipronil sulfone	*	99.9	0.4	—[b]	39	Pyroquilon-K		100.3	1.1	99 (HPLC/DAD)
19	Flutolanil	*	99.9	0.7	100 (GC/FID)	40	Pyroquilon-S		100.8	0.5	99.9 (GC)
20	IBP	*	99.5	0.3	100 (GC/FID)	41	Tricyclazole	*	99.4	0.1	100 (LC/UV)

[a] The purity means the area percentage of main peak on chromatogram.
[b] There was no mention of the purity in the certificate.
* Eighteen products are stored in 4℃ for about 2-5 years after first opening the pack.

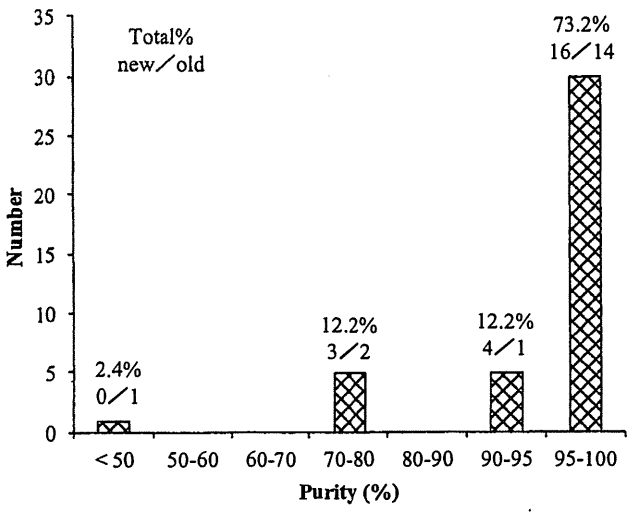

Fig. 2 Distribution of absolute purities of commercial reagent material products measured by qNMR

The 41 products were classified according to the absolute purities. The proportions and the number of new and old products were shown.

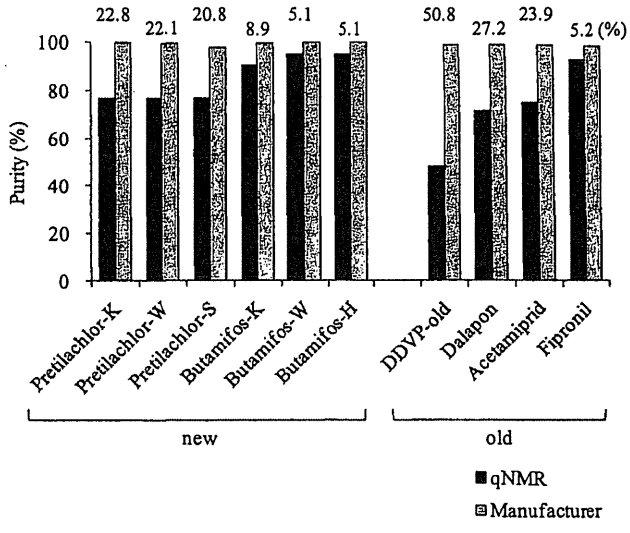

Fig. 3 Differences between manufacturer's labeled purities and the absolute purities measured by qNMR

Ten pesticide reagent products, which had difference more than 5% between the labeled purities and absolute purities, were shown.

分解し，純度値が低下していく可能性を否定できない。この純度の低下が，結果として定量分析値の精度を悪くする恐れがあることが明らかとなった。

農薬等の試薬に記載されている有効期限は，開封前のメーカーの保証期間であり，開封後についてメーカーは保証していない。また，開封後の使用期限を設定しても，管理を徹底しなければ意味をなさない。つまり，現状では試薬の保管状態，開封試薬の廃棄期限等研究者自身が把握する必要がある。これらのことから，qNMRによる純度試験は，開封後の試薬の純度変化または有効期限の記載がない研究用試薬が，試験を行うことができる許容範囲にあるかを評価する手段としても有用である。さらに，市販製品の純度が低下していても，分析時に純度を測定することで，計量計測トレーサビリティの確保された純度値が得られるという利点がある。

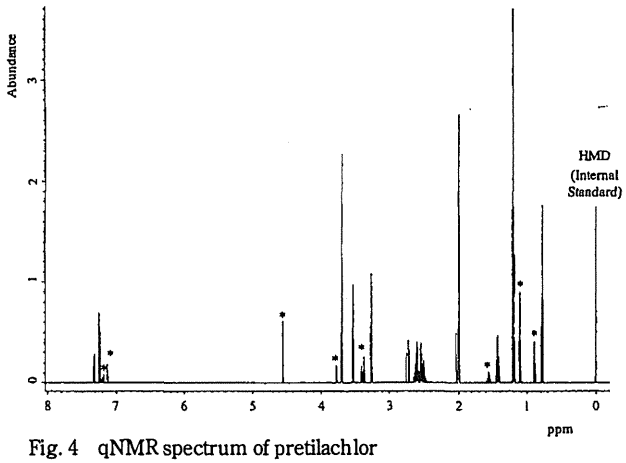

Fig. 4 qNMR spectrum of pretilachlor

HMD was used as a reference at δ 0 ppm and an internal standard for quantitative analysis. * Signals were impurities.

4. まとめ

環境分析に用いられるクロマトグラフィーは，測定対象の標準物質が必要であり，測定対象と同一の基準となる物質のピーク面積の比から求められる相対的な値のため，定量分析値の信頼性は標準物質の質に依存する。科学的な根拠に基づく定量分析値の信頼性確保には，市販試薬製品それぞれについて計量計測トレーサビリティの確保された純度の決定が最重要であると考えられる。そこで本研究では，qNMRの高い定量精度を応用し，市販試薬41製品について純度評価を行った。その結果，約75％が成績書記載の純度値と総じてほぼ等しい値を与えた。しかし，それ以外は成績書記載の純度値より5％以上低く，その純度の低下が定量分析値に誤差を与える要因となることが示唆された。本研究により，定量分析値の実験室間の相互比較には，純度が計量学的に証明された標準物質の精確な使用が重要であり，純度に対してSIトレーサビリティを確保しておけば，定量分析値の信頼性が飛躍的に向上すると考えられた。近年では，第三者の認定を受けた標準物質生産者が供給する認証標準物質や計量標準供給制度（JCSS）の体系内で扱われている認証標準物質の販売を行うようになってきている。しかし，環境分析において測定対象となる化合物は多種多様であるため，市販されている認証標準物質の数は未だ少ない。これらのことから，分析技術者が精確に値付けされた標準品を標準物質として使用する重要性を認識し，qNMRによる計量計測トレーサビリティの確保された定量法が標準品の純度や濃度の値付けや品質の保証を行う体制の整備に重要なツールとなることが期待される。

要　約

環境分野で広く用いられているクロマトグラフィーは測定対象化合物の標準物質が不可欠であるが，国際単位系（SI）への計量計測トレーサビリティが保証された市販の標準物質は少ない。SIトレーサビリティが確保された標準物質を用いない以上，標準物質の純度が定量分析値の信頼性を大きく損なっている可能性を否定できない。本研究では，核磁気共鳴装置を用いたSIトレーサブルな定量分析法を応用して，市販試薬41製品の純度を測定した。41製品のうち26.8％に相当する10製品の純度は，成績書記載の純度値との差が5％以上の47.9～94.8％であった。新品の純度の結果から，成績書記載の純度値を質量％純度として扱うことは不適切であると考えられた。また，市販試薬製品の純度および開封後の品質管理が定量分析値の室間精度に大きく影響を及ぼすことが示唆され，成績書記載の純度値を信用して使用すると，得られる定量分析値は真値より5.1～50.8％の誤差を生じることが明らかとなった。定量分析値の信頼性確保には，計量計測トレーサビリティの確保された純度が精確な標準物質を使用していくことで，飛躍的に向上すると考えられた。

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Personal and Atmospheric Concentrations of Ozone in Southeastern Hyogo Prefecture, Japan

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Twenty-one data sets composed of readings collected by atmospheric ozone monitors worn by individuals on their clothing and installed outside their home or office were collected using Ogawa passive ozone samplers in southeastern Hyogo prefecture, Japan from September 12 to 13, 2011. The concentrations of personal and outdoor ozone ranged from not detectable to 23.2 ppb and from 4.7 to 38.3 ppb, respectively. The mean concentration of personal exposure to ozone was 3.7 ppb and was significantly lower than that of outdoor ozone (18.5 ppb). This suggests that the concentrations of outdoor ozone affect personal ozone exposure. However, in this study, we found no correlation between the concentrations of personal ozone and the total time spent outdoors or the time of day the individual was outside. In contrast, the mean concentrations of outdoor ozone were similar to those of ozone measured at the 12 nearest Ambient Monitoring Stations (AMSs). However, when the AMS was situated near a main road, the regional ozone levels were underestimated.

Key words ozone; air pollution; personal exposure; nitrogen dioxide; Ogawa sampler

Ozone is a strong oxidant and is mainly formed by photochemical reactions from nitrogen oxides and/or reactive volatile organic compounds in the atmosphere. Acute and chronic effects of ozone exposure on human respiratory system have been investigated by many epidemiological studies.[1–3] Some researchers have also reported that ozone had negative effects on cardiovascular and respiratory mortality.[4–6] A strong relationship has been noted between measured outdoor ozone levels and ambient ozone levels measured by the nearest Ambient Monitoring Station (AMS).[7] Ozone concentrations were usually the highest in outdoor and lowest in indoor environment.[8,9] Therefore, in the past, these AMS data were sometimes used to evaluate the personal health risk of ozone.[5,6] However, the specific location of the AMS site and wind direction around the AMS affect its value for evaluating ozone levels at specific localities. The passive ozone sampler, named Harvard or Ogawa passive sampler, developed by Koutrakis *et al.*[10] is a useful tool to accurately measure ozone concentrations in a much smaller area.[8,9,11,12] The passive ozone sampler is able to provide a precise measure of the ozone to which a single person may be exposed, which we call the "personal ozone" in this paper. These samplers can measure personal ozone exposure with a precision of ±4 ppb and relative error of ±10%.[13]

In this study, passive ozone samplers were used to evaluate the ozone exposure levels of selected personnel from Hyogo College of Medicine and of their outdoors. This is the first study to determine the ozone exposure levels for people living in Japan. This study also investigates the relationship between personal outdoor ozone exposure levels and the AMS ozone data recorded by the nearest outdoor sampler.

Experimental

Sampling and Pretreatment Procedures Twenty-one employees of Hyogo College of Medicine were selected as study participants. This study was approved by the ethics committee of Hyogo College of Medicine, and informed consent was obtained from each participant before the study. All participants live in five cities of southeastern Hyogo prefecture and their houses are located there (Fig. 1). Each person and house was monitored by a set of personal/outdoor passive ozone samplers (Ogawa sampler manufactured by Ogawa and Co., Ltd., Kobe, Japan). The personal sampler was pinned to the top front side of participant's clothes; the outdoor sampler was placed in a well-ventilated area outside the participant's house. Sampling was performed simultaneously starting at 07:00 September 12, 2011. A total of forty-two 24-h samples were collected and stored in a refrigerator (4°C) until they were analyzed. The study participants were also instructed to record their daily activities from 7:00 to 12:00, from 12:00 to 18:00, and from 18:00 to 24:00, so that the amount of time spent outdoors could be estimated (Fig. 2).

The passive ozone sampler consists of two glass fiber filters coated with sodium nitrite and potassium carbonate.[14] The mechanism is based on the oxidation reaction of nitrite by ozone to produce nitrate. In this study, the mean concentrations of ozone during the sampling period are estimated by the amount of produced nitrate and collection rate (21.8 mL/min).[14] The filters were treated according to the Ozone Passive Sampler Protocol published by Ogawa & Co., Ltd.[14] Two glass fiber filters were placed in a vial and 5 mL ultrapure water (Milli-Q) was poured in the vial. After shaking gently, the vial was left to stand for approximately 30 min. About 1 mL of supernatant solution was filtered (Cosmonice Filter (W), pore size 0.45 μm, φ13 mm, Nacalai Tesque, Kyoto, Japan) and 100 μL of filtrate was injected into the HPLC system described below.

HPLC System and Chemicals Nitrate was analyzed by using an HPLC system with an UV detector. The HPLC system consisted of a mobile phase pump (SI-1/2001, Shiseido, Tokyo, Japan), an UV-visible detector (SI-1/2002, Shiseido, Tokyo, Japan), a chromatopac integrator (C-R7A, Shimadzu, Kyoto, Japan), a degasser (SI-1/2009, Shiseido, Tokyo, Japan), a column oven with a manual injector (SI-1/2004,

The authors declare no conflict of interest.

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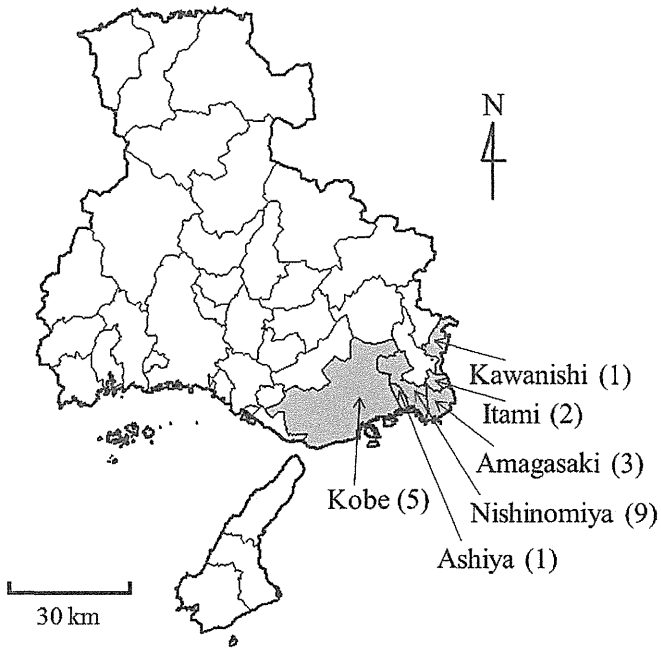


Fig. 1. Cities in Southeastern Hyogo Prefecture in Which Samples Were Collected

Numbers in parentheses indicate the number of samples collected in each city.

Shiseido, Tokyo, Japan), a guard column (Shim-pack IC-GA2, 4.6 i.d.×10 mm, Shimadzu, Kyoto, Japan), and a non-suppressor type analytical column (Shim-pack IC-A3, 4.6 i.d.×150 mm, Shimadzu, Kyoto, Japan). The mobile phase was 0.4 mM disodium phthalate (Tokyo Kasei, Tokyo, Japan) and the flow rate was 1.0 mL/min. Both the guard column and the analytical column were maintained at 40°C in the column oven. Standard nitrate (sodium nitrate) was purchased from Kishida Chemical Co., Ltd. (Osaka, Japan).

Quality Control Indirect photometric detection ion chromatography was used for nitrate analysis. A standard solution of nitrate (100 μL) was injected into the analysis system to verify the method. The calibration curve showed good linearity (r=0.9933) from 0.1 to 1.0 μg/mL. The detection limit (S/N=3) was 0.1 μg/mL and the relative standard deviation (n=3) was 4%.

In order to assess the accuracy of the passive samplers, data from collocated passive samplers were compared to those obtained from a UV Ozone Monitor (EG-700, EBARAJITSU-GYO Co., Ltd., Kanagawa, Japan) at the roof of No. 9 building of Hyogo College of Medicine. The mean (±S.D.) ozone concentrations of five 24-h data sets obtained from passive samplers and Ozone Monitor were 23.5 (±2.96) ppb and 23.9 (±2.14) ppb, respectively. In addition, the Ozone Monitor-passive sampler/Ozone Monitor ratio of each data set was less than 10% (in the range from 0.9 to 8.4%). Approximately 10% of the samplers from the same batch were used as field blanks. The mean (±S.D.) concentrations of four field blanks were 11 (±1.1) ppb. The values from the field blanks were subtracted from the ozone measurement for calculation of the concentrations of nitrate collected by the passive samplers.

Data Analysis For statistical treatment, correlation analysis and multiple regression analysis were performed by using a statistical analysis program (PASW Statistic 18, IBM, U.S.A.). For the multiple regression analysis, the following form of the model was used:

$$C_o = \beta_1 C_a + \beta_2 P + \beta_3 D + \varepsilon \quad (1)$$

where C_o and C_a are the outdoor and the AMS ozone concentrations, respectively. P is the relative position of the outdoor sampler with respect to the nearest AMS (based on the wind direction including leeward, windward, and parallelism); D is the distance between the outdoor sampler and its nearest AMS and ε is the error term. The distance between the outdoor samplers and the nearest AMS was between 0.5 and 4.4 km.

Results and Discussion

Outdoor Ozone A total of 21 outdoor ozone samples were collected for this study. The mean and median ozone concentrations in these sample were 18.5 and 17.0 ppb, respectively, the same as the concentration levels recorded from the 12 AMS sites nearest to the outdoor samplers (mean, 16.2 ppb; median, 15.5 ppb, Fig. 3).[15] However, the concentrations of outdoor ozone measured at study participants' houses ranged from 4.7 to 38.3 ppb, while those of ozone measured by the AMS ranged from 9.5 to 20.2 ppb. The difference between the two concentration ranges was considerably large (Fig. 3). No clear relationship was observed between the outdoor and AMS ozone concentrations (Fig. 4, solid line; n=21, r=0.161, p=0.481). However, because ozone reacts with nitric oxide in the atmosphere, local variations in nitric oxide concentrations may affect at atmospheric ozone levels.[16] When nine data points for the outdoor samplers and the AMS's located within 100 m of main road were deleted, a significantly positive correlation between the outdoor ozone concentrations and the AMS ozone concentrations exists (Fig. 4, dotted line; n=12, r=0.711, p=0.001). Therefore, these results suggest that the ozone concentrations recorded by the AMS, which keeps is placed away from the main road, could represent the mean levels of outdoor ozone concentrations at the home around the AMS.

In order to further evaluate the effects of wind direction and distance on the ozone concentrations determined from outdoor samplers with respect to its nearest AMS, a multiple regression analysis was performed. As shown in Eq. 2, no relationship was observed between the outdoor ozone concentrations and the relative position of the AMS (p=0.89) or the distance (p=0.11).

$$C_o = 42.6 - 1.02 C_a - 0.46 P - 3.79 D \quad (2)$$

Similarly, if the same nine values for the outdoor samplers and the AMS mentioned above were ignored (samples collected within 100 m of a main road), the analysis result was

$$C_o = 0.26 + 1.08 C_a - 0.80 P - 0.58 D \quad (3)$$

In this model, only the coefficients of outdoor ozone concentrations and the AMS ozone concentrations were significant (p=0.05). The relative position and the distance are less important for the outdoor ozone concentrations measured by the Ogawa samplers.

Personal Ozone This is the first study to report the personal exposure levels of ozone in Japan. A total of 21 personal ozone samples were obtained for this study. The mean and the median concentrations of personal ozone samples were 3.7 and 2.7 ppb, respectively, and these concentrations were significantly lower than outdoor ozone (Fig. 3). The range of

Questionnaire on Ozone Exposure Investigation

1. ID _____

2. Address _____

3. Start Time

( )-1 Sep. 12 2011 _____ H. _____ M. (personal)

( )-2 Sep. 12 2011 _____ H. _____ M. (outdoor)

4. Stop Time

( )-1 Sep. 13 2011 _____ H. _____ M. (personal)

( )-2 Sep. 13 2012 _____ H. _____ M. (outdoor)

5. Time in outdoor

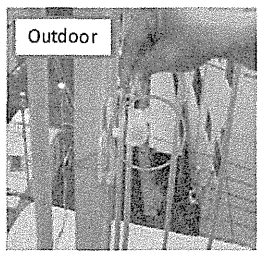
06:00 - 12:00 _____ H. _____ M.

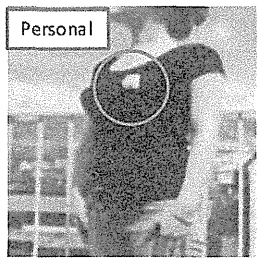
12:00 - 18:00 _____ H. _____ M.

18:00 - 24:00 _____ H. _____ M.

6. Sampling Method

* Open the container and remove the sampling badge from the resealable bag.
* Check that the ID label on the bottle; (ID)-1 use for personal ozone and (ID)-2 use for outdoor ozone.
* Place sampling badge as same as the following Figures.





* After sampling, replace the re-sealable bag in the brown storage bottle and tighten cap securely.
* Record daily activities during sampling period.

Noto

Thank you very much for your collaboration!

Department of Public Health,
Hyogo College of Medicine

Fig. 2. Questionnaire on Ozone Exposure Investigation

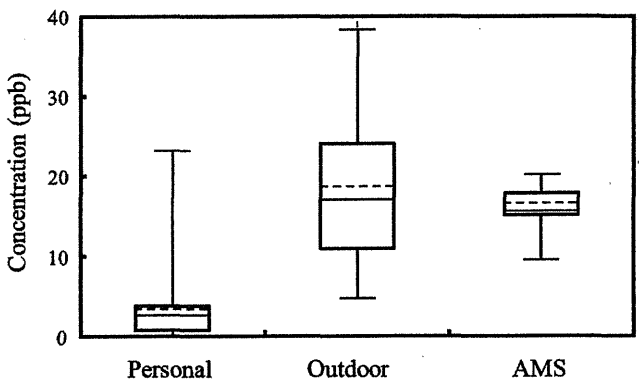


Fig. 3. Ozone Concentrations Found in Personal and Outdoor Samples and Ozone Levels Recorded by the Ambient Monitoring Stations (AMS)

Error bars indicate the range from minimum to maximum concentrations of each sample set. The upper and lower edges of each box represent the 75th and 25th percentile concentration ratios, respectively. Within each box, mean and median concentrations are indicated by the dashed and solid lines, respectively.

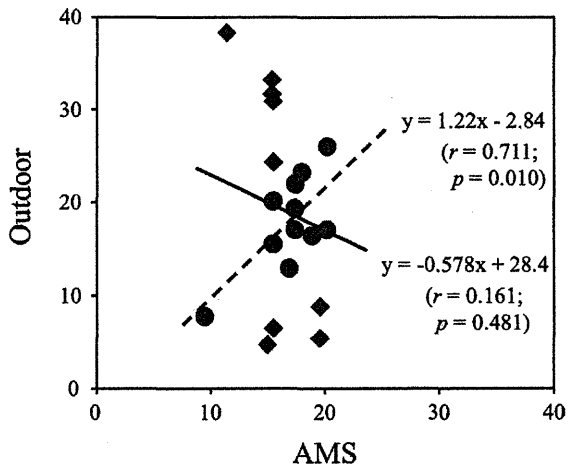


Fig. 4. Correlation between Outdoor Ozone Concentrations and the AMS Ozone Concentrations

Solid line: all data (n=21), Dotted line: nine data points for the outdoor samplers and the AMS's located within 100m of main road were deleted (n=12).

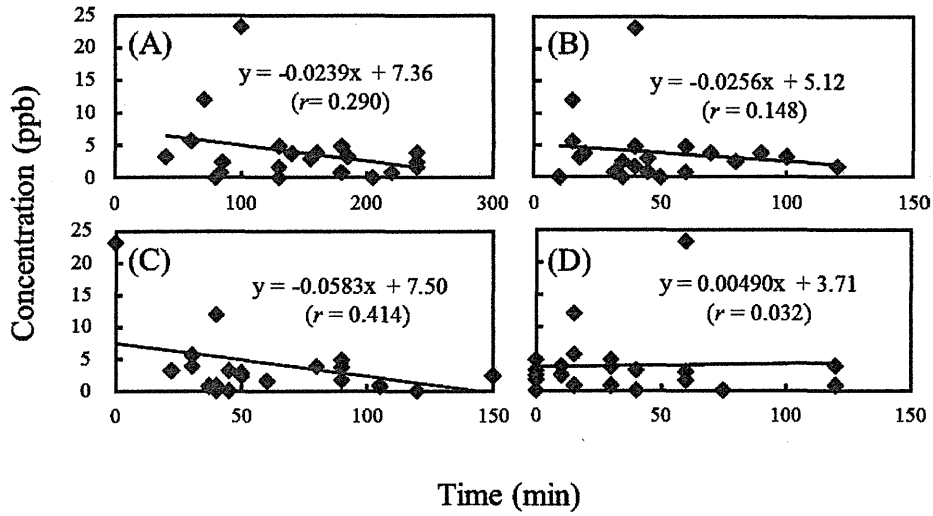


Fig. 5. Distribution of Data Points Showing the Lack of Correlation between Personal Ozone Concentrations and the Total Amount of Time Spent Outdoors

(A) 07:00–24:00; (B) 07:00–12:00; (C) 12:00–18:00; (D) 18:00–24:00.

concentrations for personal ozone was from not detectible to 23.2 ppb. Compared with other studies using the same sampling methodology, the mean concentrations of personal ozone found in this study were similar to levels as in Nashville (3.5 ppb)[9] and lower than those in Mexico City (7.8 ppb).[16]

Atmospheric ozone is formed during daylight from complex mixtures of nitrogen oxides and reactive volatile organic compounds by photochemical reactions.[17] At night, however, ozone reacts with nitrogen monoxide and disappears slowly.[18] Therefore, ozone concentrations are usually the highest in the afternoon and low in the morning. Lee *et al.* reported that the concentrations of personal ozone were affected not only by the total amount of time spent outdoors but also the time of day the personal was outside.[9] In this study, we plotted the concentrations of personal ozone and personal daily activities, as shown in Figs. 5A through D. We, however, found no correlation between the concentrations of personal ozone and the total time spent outdoors or the time of day the individual was outside. Furthermore, we also found no correlation between the concentrations of personal ozone and the concentrations of outdoor ozone at the same locality (r=−0.208, p=0.352). We cannot explain these results, perhaps because our questionnaire did not include questions about the office or house construction, equipment, and outdoor environment that they spent during sampling period. Items such as a window fan,[9] a laser printer,[19] or an air cleaner[20] might increase ozone levels, and the close proximity of a main road might be decrease the ozone level.[9] An improved questionnaire will be used in our next study.

In conclusion, the concentrations of personal ozone and outdoor ozone in southeastern Hyogo were investigated by using the Ogawa sampler. The concentrations of personal ozone were significantly lower than that of outdoor ozone. This suggests that the concentrations of outdoor ozone are important for personal ozone exposure. However, our data found no correlation between the concentrations of personal ozone and the total time spent outdoors or the time of day the individual was outside. In contrast, according to our data, the regional ozone levels may be underestimating by installation feature of the AMS, such as near the main roads.

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Gene Expression Changes of Phases I and II Metabolizing Enzymes Induced by PAH Derivatives

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Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants, and generate various types of PAH derivatives, such as nitrated PAHs, hydroxylated PAHs, and PAH quinones through chemical reactions in the atmosphere. PAHs are well known to activate the aryl hydrocarbon receptor (AhR), followed by the induction of metabolizing enzymes mainly in the liver, while biological responses to PAH derivatives are not understood well. In this study, we investigated the induction patterns of gene expression of CYP1 family and Phase II metabolizing enzymes in rat H4IIE cells exposed to PAH quinones and their parent PAHs for 24 h.

Dibenz[*a,h*]anthracene and benzo[*a*]pyrene dramatically induced mRNA expression of CYP1 family, such as cytochrome P450 (*Cyp*) *1a1*, *Cyp1a2*, and *Cyp1b1*, and PAH quinones, especially 1,4-chrysenequinone, possess a high potential to induce CYP1 family. As for Phase II enzymes, PAHs induced NAD(P)H: quinone oxidoreductase 1 (*Nqo1*) and UDP-glucuronosyltransferase (*Ugt*)*1a6*, and their induction potencies by PAHs were similar to those of CYP1 family. On the other hand, expression of sulfotransferase (*Sult*)*1a1*, heme oxygenase-1 (*Hmox1*), and *Ugt2b1* were augmented mainly by PAH derivatives. Finally, we examined gene expression changes of metabolizing enzymes by the airborne particles. Their organic extracts significantly up-regulated the

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expression of *Cyp1a1*, *Cyp1a2*, *Cyp1b1*, *Ugt1a6*, and *Nqo1*, but not *Hmox1*, *Ugt2b1*, and *Sult1a1*.

These results suggest that PAHs mainly induce the expression of genes encoding CYP1 family while PAH derivatives, especially quinones, induce the expression of genes encoding both CYP1 family and Phase II enzymes. Furthermore, our results show the organic chemicals which adsorb on the airborne particles exert biological effects in the similar manner of PAHs, suggesting the involvement of mainly AhR activation.

Key Words: AhR, metabolizing enzyme, PAH, PAH derivatives

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs), which generated from the combustion of fossil fuels and biomass burnings, cause various health effects through the diverse changes of gene expression and signal transduction. In the atmosphere, PAHs generate their derivatives, such as nitro PAHs (NPAHs) (1), hydroxylated PAHs (OHPAHs), and PAH quinones (PAHQs) through the chemical reactions of nitrogen radicals (·NO^3), hydroxide radicals (·OH), and ultraviolet light (2).

Many studies have shown that PAHs and PAH derivatives have various physiologic actions *in vivo* and *in vitro*. For instance, PAHs activate the aryl hydrocarbon receptor (AhR), which is a transcriptional factor and play a pivotal role in the PAH toxicity (3). On the other hand, the derivatization of PAHs gives PAHs different functions from those of the PAHs. It has been reported that PAHQs have toxicities related to oxidative stress-producing reactive oxygen species (ROS) (4). OHPAHs have estrogenic/antiestrogenic activity, suggesting that they act as endocrine disruptors (5). In most cases, these biological effects of PAHs and PAH derivatives are caused concomitantly with gene expression changes, since PAHs and PAH derivatives activate various transcriptional factors. It is anticipated that such gene expression changes may be useful for understanding the biological effects of PAHs and their derivatives, and may also be useful as biomarkers.

When PAHs and PAH derivatives enter into animal bodies, they induce the expression of genes encoding metabolizing enzymes, such as CYP1 family (e.g., cytochrome P450) and Phase II enzymes (e.g., (NAD(P)H: quinone oxidoreductase 1 and heme oxygenase-1), via transcriptional regulation in the liver (6–8). Since PAHs and PAH derivatives have different physiological effects, it is speculated that numerous kinds of atmospheric pollutants show various regulation of genes, such as metabolizing enzymes. However, it has not been fully understood how the derivatization of PAHs changes the physiological effects under gene expression level.

Although derivatization of 4-rings PAH, especially quinones, gives biological effects such as ROS generation (4) and estrogenic/antiestrogenic activities (5), there are few studies about comparison of cytochromes P-450 between

parent PAHs and their derivatives (9). In this study, we investigated the induction potency of genes encoding CYP1 family and Phase II metabolizing enzymes by PAHs and PAH derivatives having 4-rings. In addition, we examined gene expression changes by 5-rings PAHs, which have strong activities of AhR activation, as a substance strongly inducing the genes of CYP1 family. Four-rings and 5-rings PAHs and their derivatives are the main components adsorbed in airborne particles, and are thought to be involved in most of toxicities caused by airborne particles. Therefore, in order to explore mechanisms under which PAHs and their derivatives adsorbed in airborne particles cause biological effects, we also examined the expression changes of CYP1 family and Phase II genes by exposure to the extracts of airborne particles.

MATERIALS AND METHODS

Chemicals

We used four PAHs and four PAH derivatives (Figure 1), which show AhR activation by dioxin-responsive chemical-activated luciferase gene expression (DR-CALUX) assay according to the previous report (10,11). 3,4-Dihydrobenz[*a*]anthracen-1(2H)-one (3,4-OH-B[*a*]A) was purchased from Sigma-Aldrich Corp. (Tokyo, Japan). Dibenz[*a, h*]anthracene (DBA) was

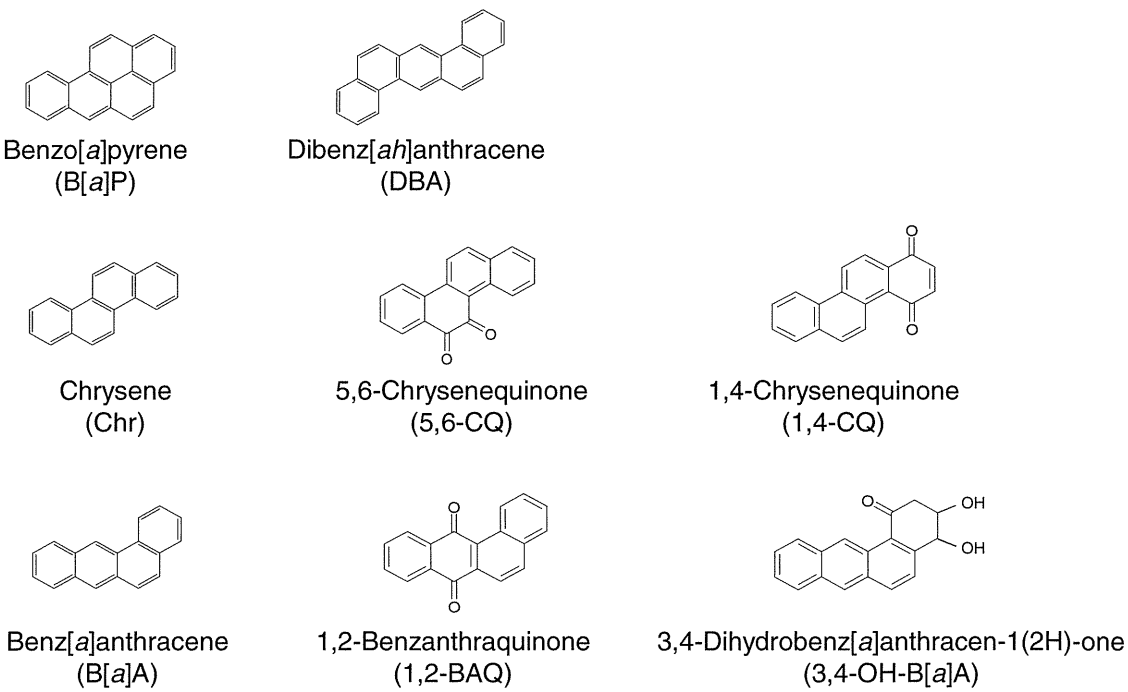

Figure 1: Structures of PAHs and PAH derivatives tested in this study PAH: B(a)P, DBA, Chr, B(a)A, PAH derivatives: 5,6-CQ, 1,4-CQ, 1,2-BAQ, 3,4-OH-B(a)A.

purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). 5,6-Chrysenequinone (5,6-CQ) and 1,4-chrysenequinone (1,4-CQ) were purchased from Chiron AS (Trondheim, Norway). Benz[*a*]anthracene (B[*a*]A), chrysene (Chr), benz[*a*]pyrene (B[*a*]P) and 1,2-benzanthraquinone (1,2-BAQ) were purchased from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). These chemicals were dissolved in dimethyl sulfoxide (DMSO).

Airborne Particle Extracts

Airborne particles were collected on the roof of the 5th floor building of the research center for eco-environmental science from the Chinese Academy of Science in Beijing, China for 10 days in March and April in 2008 using high-volume air samplers (Kimoto Electric Company Limited, Osaka, Japan) at a flow rate of 700 l/min on the quartz fiber filters (2500QAT-UP, Pallflex Products) (Putnam, CT, USA). Every 24 h, the filter was changed. The amount of volume of air that passed through each filter was 16,800 m^3. After being dried in the desiccator in the dark, the filters were weighed and then stored in a refrigerator (−20°C) until use. The average weight of airborne particles of each filter was 4.8 g and the particle concentration was calculated to be approximately 300 $\mu g/m^3$ on average. Extraction was conducted from these filters with benzene/ethanol (3:1, v/v), and then the extract solution was filtered by ADVANTEC filter paper (125 mm in diameter) (Toyo Roshi, Tokyo, Japan). The extracts were concentrated by evaporation and residue was dissolved in DMSO. Among ten day samples we chose three which show relativity high AhR agonist activity by DR-CALUX.

H4IIE Cell Culture and RNA Isolation

H4IIE cell line rat liver cancer cells were obtained from BioDetection Systems B.V. (Amsterdam, The Netherlands). The cells were cultured in alpha-minimal essential medium (α-MEM) (Invitrogen Corp., NY, USA) supplemented with 10% fetal bovine serum (FBS) at 37°C under 5% CO_2. The cells were seeded into a 6-well plate, and grown up to 75% confluence. Then, the cells were exposed to each PAH and PAH derivative in triplicate in a final concentration of 4 μM. These concentration was in the range from 1–10 μM which didi not show any cytotoxicities in our previous study (10). DMSO alone (0.4%) was used as a control group. In the experiment of exposure to the airborne particles, cells were exposed to the extracts of airborne particles (corresponding to 3.2 mg particles/well). These concentrations showed no cytotoxicity to the cells in our previous study (10) and thus selected as testing concentraions. After 24 h exposure, the medium was removed, and then total RNA was isolated from the cells using RNeasy Mini Kit (Qiagen, Valencia). The total RNA concentration was determined spectrometrically, and quickly frozen at −80°C until use.

Quantification of mRNA Encoding Metabolizing Enzymes by Real Time PCR

First-strand cDNA of each gene was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Tokyo, Japan). 1 μg of total RNA was added to 2 μl random hexamer primer (600 pmol/μl), and then the mixture was heated at 65°C for 10 min. Four μl of transcriptor RT reaction buffer, 0.5 μl of protector RNase inhibitor (40 U/μl), 2 μl of deoxynucleotide mix (10 mM each) and 0.5 μl of transcriptor reverse transcriptase were added to the reaction mixture, and they were heated at 55°C for 30 min, followed by reaction at 85 °C for 5 min to inactivate the enzyme. Expression of genes encoding CYP1 family (*Cyp1a1*, *Cyp1a2*, and *Cyp1b1*), Phase II (*Nqo1*, *Ugt1a6*, *Ugt2b1*, *Sult1a1,* and *Hmox1*) metabolizing enzymes and β-actin (housekeeping) were evaluated using TaqMan master (Roche, Tokyo, Japan) and a LightCycler® 480 system (Roche Applied Science). Primers and hybridization probes were designed using the LightCycler® Probe Design Software 2.0. Oligonucleotides used for RT-PCR were commercially synthesized by Japan Gene Research Laboratories Inc. (Sendai, Japan) (Table 1).

The relative changes in gene expression were calculated by the ddCt method (12.13) for the mRNA expression of CYP1 family and Phase II metabolizing enzymes, using the following formula: $ddCt = [C_{t\text{-gene}} - C_{t\text{-}\beta\text{-actin}}]_{Sample} - [C_{t\text{-gene}} - C_{t\text{-}\beta\text{-actin}}]_{Control}$. The expression was normalized with β-actin, and the relative gene expression was calculated by the equation 2^{-ddCt}.

Table 1: List of primers used for quantitative RT-PCR

Primer	Sequence (5′-3′)
Phase I	
Cyp1a1	gagaaagatccaggaggagttaga
	gggacaaaggatgaatgtcg
Cyp1a2	tcctacaactctgccagtctcc
	cctctcaacacccagaacact
Cyp1b1	ctgctctacaccgctggaa
	tcagctgctgtggactgtct
Phase II	
Nqo1	agcgcttgacactacgatcc
	caatcagggctcttctcacc
Ugt1a6	aagcgatggaaattgctgag
	cgatggtctagttccggtgt
Ugt2b1	tttggtttcaaccatttaaagaga
	gccttccccatcatctcag
Sult1a1	acacatctgcccctgtcct
	gcatttcgggcaatgtaga
Hmox1	gtcaggtgtccagggaagg
	ctcttccagggccgtataga

RESULTS

Expression Changes of Genes Encoding CYP1 Family Metabolizing Enzymes in PAH and PAH Derivative-exposed Cells

We investigated expression changes of *Cyp1a1*, *Cyp1a2*, and *Cyp1b1* genes, which encode cytochrome P450 1a1, 1a2, and 1b1, respectively, when cells were exposed to each PAH and PAH derivative (Figure 2). Because B[*a*]P and DBA are well known as strong AhR agonists (14), we used them to show gene expression changes elicited by pathways through AhR activation. Predictably, both B[*a*]P and DBA induced the expression of *Cyp1a1*, *Cyp1a2*, and *Cyp1b1* (Figure 2A). Four-rings PAHs, Chr and B[*a*]A, significantly induced the expression of *Cyp1a1*, although their effects were less than those of B[*a*]P and DBA (Figure 2B). In addition, Chr and B[*a*]A induced the same expression level of *Cyp1a2* and *Cyp1b1*, while B[*a*]P and DBA exerted a greater effect on the expression of *Cyp1b1* than that of *Cyp1a2* (Figure 2A). On the other hand, quinone derivatives of Chr and B[*a*]A also induced the expression of *Cyp1a1*, *Cyp1a2* and *Cyp1b1* (Figure 2B). 1,2-BAQ significantly induced the genes of CYP1 family, and was comparable in degree to the parent PAH, while 3,4-OH-B[*a*]A showed no ability to induce the genes of CYP1 family. Surprisingly, 1,4-CQ more strongly induced the expression of genes of CYP1 family than its parent PAH (Chr), although 5,6-CQ more weakly induced the expression of genes of CYP1 family than Chr.

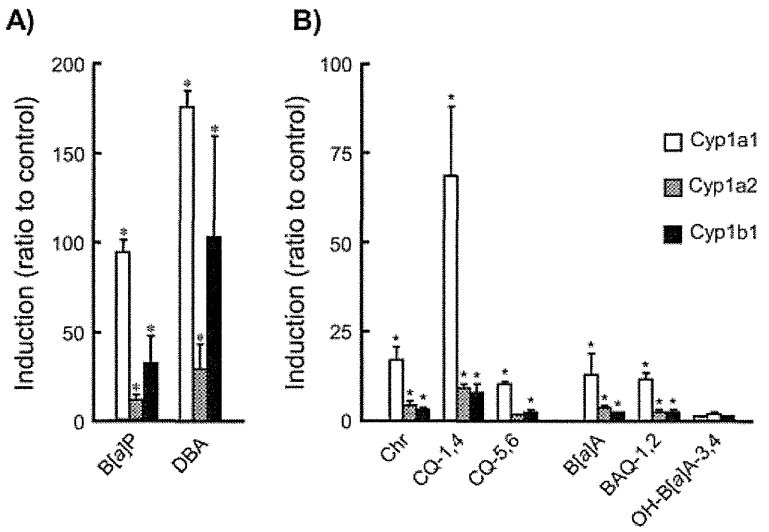

Figure 2: mRNA expression of CYP1 family metabolizing enzymes by PAHs and PAH derivatives. The cells were exposed to PAHs and PAH derivatives at concentration of 4 μM for 24 h. Symbols and vertical bars respectively represent the mean and ±S. D. ($n = 3$). Asterisk (*) shows significant difference ($p < 0.05$).