THE HIGHLY EFFECTIVE METHYLATION METHOD FOR HYDROXYLATED POLYCHLORINATED BIPHENYLS

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Introduction

Polychlorinated biphenyls (PCBs) have been still detected in diverse species such as fish and birds[1], although it has been passed 40 years after the production and the use banned. PCBs are converted to the metabolites such as hydroxylated PCBs (OH-PCBs) in organisms. OH-PCBs have been also identified in fish[2], birds[3], mammals[4], and human[5, 6].

Various analytical methods for OH-PCBs using GC/MS, GC/ECD[7], LC/TOF-MS[8], or LC/ESI-MS-MS[9] have been reported. Capillary column was used to separate target chemicals in GC methods described above. OH-PCBs are hardly volatile because of their polar functional group. Therefore on the analysis of OH-PCBs requires the derivatization of phenolic hydroxyl group such as methylation or ethylation, to to make these compounds be volatile. Most common derivatization methods for OH-PCBs used are methylation[6] of phenolic hydroxyl group. There are two methylation methods for OH-PCBs such as ion-pair alkylation with methyl iodide[10] and the other is used with diazomethane[6]. However, diazomethane method is the most popular for OH-PCBs. Dimethyl sulfate and trimethylsilyldiazomethane[11] (TMS-diazomethane) is also used as the derivatization method for phenolic compounds.

However, the evaluation of the derivatization efficiency for OH-PCBs using these methods, has been qute few in spite that there are 837 congeners of OH-PCBs. The confirmation of these derivatization efficiency might be useful for the surveillance of the accumulation of OH-PCBs in biological organisms. On this study, derivatization efficiency of four methods for OH-PCBs were evaluated and highly effective derivatization method were established.

Materials and Methods

1. *Chemicals and reagents:*

1.1. *OH-PCBs:* OH-PCBs were obtained from AccuStandard, Inc. (New Haven, CT, USA), consist of OH-CBs, OH-DiCBs, OH-TriCBs, OH-TeCBs, OH-PnCBs, and OH-HxCB. 2',3,4',5,5'-pentachloro-4-[$^{13}C_{12}$] biphenylol ([$^{13}C_{12}$] 4-OH-2',3,4',5,5'-PnCB) was used for surrogate standard (SS) purchased from Wellington Laboratories Inc. (Ontario, Canada). Fluoranthene-d_{10} was used for internal standard (IS) purchased from Kanto Chemical Co., Inc. (Tokyo, Japan).

1.2. *Reagents:* Dimethyl sulfate, dimethyl carbonate, diazomethane, and TMS-diazomethane were used to methoxylate PCBs (MeO-PCBs). Dimethyl sulfate (extra pure), dimethyl carbonate 99%, and TMS-diazomethane (2.0 M solution in hexane) were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan), Across Organics (New Jersey, USA), and Sigma-Aldrich, Inc. (St. Louis, MO, USA), respectively. Diazomethane was generated in diethyl ether from N-Methyl-N-Nitro-N-Nitrosoguanidine (MNNG) purchased from GL science Inc. (Tokyo, Japan).

2. *Derivatization procedure:*

Five orders (0.1ng, 0.5ng, 2ng, 10ng, 25ng) of OH-PCBs mixture contain SS (2ng) were derivatized and subsequently 2ng of IS were added in all OH-PCBs mixtures for GC/MS analysis.

2.1. *Dimethyl sulfate and dimethyl carbonate:* Five orders of OH-PCBs mixture contained SS standard were putted in each centrifugation tube. These solutions were evaporated at gentle stream under nitrogen until several μL. 3M

potassium hydroxide in ethanol (KOH/EtOH, 0.2 mL) was added for dimethyl sulfate but 1M KOH/EtOH (0.2mL) for dimethyl carbonate. 0.5mL of dimethyl sulfate or dimethyl carbonate was added in these OH-PCBs mixtures after stirred slightly. 0.5mL of 3M KOH/EtOH was sequentially added until the reaction is end. Equal volume with KOH/EtOH of hexane-rinsed water was added in each the solution after the derivation, was extracted by 2mL of hexane. Each hexane extract was separated by centrifuge at 3,000rpm x 10minutes. The extraction procedure was repeated three times in all tested OH-PCB mixture. 2ng of IS was added for GC/MS analysis and these solutions were subsequently evaporated at gentle stream under nitrogen until 100µL.

2.2. *Diazomethane:* OH-PCBs mixtures contained SS were measured up to 1mL with hexane in centrifugation tube. 0.6mL of diazomethane in diethyl ether was added in each standard solution. After stirred slightly, these solutions were reacted for 12 hours in centrifugation tubes capped. Conclusively, each solution was evaporated at gentle stream under nitrogen until 100µL and IS was subsequently added.

2.3. *TMS-diazomethane:* 0.1mL of TMS-diazomethane was added in each 1mL of OH-PCBs mixture contained SS in hexane/methanol (1:1). After stirred slightly, these solutions were reacted for 12 hours in centrifugation tubes capped. Conclusively, these solutions were evaporate at gentle stream under nitrogen until 100µL and then IS were added.

3. *GC/MS analysis*

GC/MS analysis for MeO-PCBs was performed using JMS-700 (JEOL, Ltd, Japan) equipped with DB-5MS capillary column at resolution less than 10,000. Additionally, methylated PCBs (Me-PCBs) and ethoxylated PCBs (EtO-PCBs) were analyzed only for diazomethane derivatization.

Results and Discussion

Simple linear regression lines and their coefficients of determination (R^2) were calculated from calibration curve of MeO-PCBs concentrations in the mixtures (5 orders, replication) reacted using three derivatization methods (Fig.1, Table 1). Coefficient of variation (CV) of relative response factor (RRF) for MeO-PCBs to SS (RRF_{SS}) or to IS (RRF*is*) were also estimated (Table 1). In dimethyl carbonate derivatization, no methoxylation of OH-PCBs were observed at all concentrations in this study condition.

1. *Efficiency*

From slop of linear regression lines, we assessed derivatization efficiency in three methods for OH-PCBs. Derivatization efficiency for OH-PCBs except OH-HxCB was high in order of dimethyl sulfate > TMS-diazomethane > diazomethane. Furthermore, the efficiency for two PnCBs , 4-OH-2',3,4',5,6'-PnCB and 4-OH-2',3,4',5,5'-PnC[$^{13}C_{12}$]B, at 2ng were significantly different among three methods (Scheffe's multiple comparison test, $p<0.0001$), regardless of both compounds is the same chemical structure except one chlorine substituted position (5' or 6', Fig.2). These results show that derivatization efficiency was remarkably different depend on the reagents and target OH-PCBs.

2. *Stability*

From R^2 of linear regression lines, derivatization stability was good in order to TMS-diazomethane (0.9944 to 0.9993) > dimethyl sulfates (0.9047 to 0.9975) > diazomethane (0.2822 to 0.9765, Table1). CV of 2-OH-2',3',4',5,5'-PnCB and 4-MeO-2',3,3',5,5',6'-HxCB at 250ng, were 27 (SS) and 33 (IS) in dimethyl sulfate but was 1.37 (SS) and 3.2 (IS) in TMS-diazomethane (data not shown). CV as well as R^2 in diazomethane remarkably varied among compounds (CV (RRF*is*): 18-100, Table 1). Sandau *et al.*[10] have reported diethyl ether may reduce derivatization efficiency of OH-PCB with diazomethane in order to produce EtO-PCB. In results on diazomethane methods in this study, EtO-PCBs could generate by the side reaction because the about 1/3 of derivatization solvent used is diethyl ether. In fact, EtO-PCBs were detected in test solution by diazomethane (data not shown). Therefore, diethyl ether may be a part of the reason that derivatization efficiency in diazomethane method was low in this study.

These results show that derivatization with TMS-diazomethane was more stable than that with dimethyl sulfate that is the strongest derivatization reagent in three methods and was considered with the most useful in derivatization reagents for OH-PCBs.

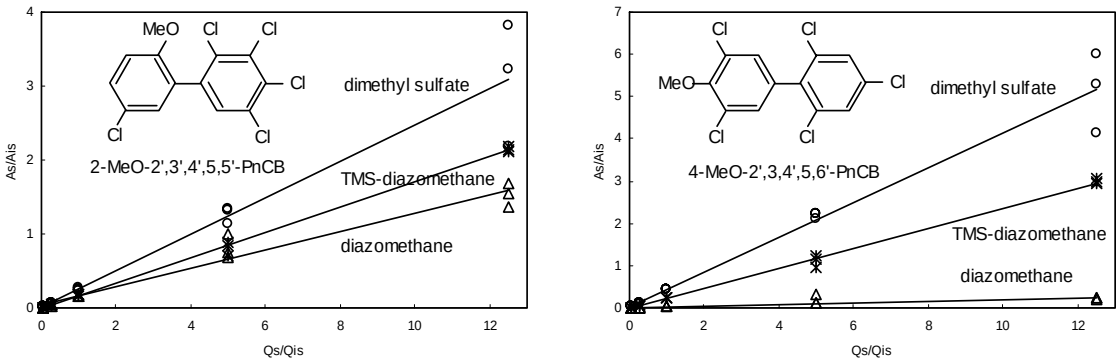

Figure1. Calibration curve for each derivatization reagents about 2-MeO-2',3',4',5,5'-PnCB and 4-MeO-2',3,4',5,6'-PnCB.

Table1. Liner regression lines, coefficient of determination (R^2), coefficient of variation (CV) of RRF*ss* and RRF*is* for compounds relevant to each derivatization reagents.

Compound	dimethyl sulfate				diazomethane				TMS-diazometane			
	Liner regression line	R^2	CV		Liner regression line	R^2	CV		Liner regression line	R^2	CV	
			RRF*ss*	RRF*is*			RRF*ss*	RRF*is*			RRF*ss*	RRF*is*
2-MeO-5-CB	y = 0.5424x - 0.0102	0.9975	18	4.5	y = 0.0089x + 0.0083	0.8227	34*	44*	y = 0.3600x + 0.0394	0.9960	18	7.5
4-MeO-4'-CB	y = 0.6091x - 0.0613	0.9968	20	7.7	y = 0.0149x + 0.0365	0.2822	82*	100*	y = 0.4578x + 0.0434	0.9980	16	13
2-MeO-2',5'-DiCB	y = 0.4453x + 0.0087	0.9955	15	6.0	y = 0.0030x + 0.0042	0.8604	29*	37*	y = 0.2963x + 0.0281	0.9978	12	7.7
4-MeO-2',5'-DiCB	y = 0.4266x - 0.0047	0.9962	16	5.9	y = 0.0044x + 0.0052	0.8834	23*	33*	y = 0.2851x + 0.0318	0.9967	14	5.8
2-MeO-2',3'-DiCB	y = 0.7605x + 0.0050	0.9956	15	4.6	y = 0.0160x + 0.0180	0.7618	33*	46*	y = 0.5575x + 0.0178	0.9993	13	4.7
2-MeO-2',4',6'-TriCB	y = 0.3756x + 0.0065	0.9939	15	6.6	y = 0.0020x + 0.0029	0.8322	25*	36*	y = 0.2233x + 0.0195	0.9989	11	6.7
2-MeO-2',5,5'-TriCB	y = 0.4435x + 0.0049	0.9925	14	5.3	y = 0.0061x + 0.0086	0.7894	20*	34*	y = 0.2469x + 0.0057	0.9967	10	7.6
4-MeO-2,2',5'-TriCB	y = 0.5810x - 0.0135	0.9915	15	5.0	y = 0.0232x + 0.0386	0.5552	46*	63*	y = 0.3639x - 0.0018	0.9988	9.6	6.7
2-MeO-2',3',4',5'-TeCB	y = 0.4846x + 0.0212	0.9817	12	7.4	y = 0.0051x + 0.0096	0.6911	31*	44*	y = 0.2930x - 0.0043	0.9988	8.5	7.2
2-MeO-2',4',5,6'-TeCB	y = 0.6222x + 0.0331	0.9775	10	7.7	y = 0.0259x + 0.0267	0.6536	38	54	y = 0.3756x + 0.0029	0.9988	7.5	11
3-MeO-2',3',5',6'-TeCB	y = 0.5832x + 0.0044	0.9836	13	7.2	y = 0.0081x + 0.0086	0.8583	31*	41*	y = 0.3811x - 0.0072	0.9983	7.0	8.9
4-MeO-2,2',4',6'-TeCB	y = 0.3299x + 0.0022	0.9872	13	6.7	y = 0.0021x + 0.0026	0.8903	25*	33*	y = 0.2000x - 0.0071	0.9970	11	7.9
4-MeO-2',3',5',6'-TeCB	y = 0.5832x - 0.0053	0.9836	14	7.1	y = 0.0113x + 0.0102	0.8475	30*	40*	y = 0.3675x - 0.0035	0.9944	8.3	9.7
2-MeO-2',3',4',5,5'-PnCB	y = 0.2464x + 0.0048	0.9362	7.6	16	y = 0.1237x + 0.0421	0.9645	9.1	18	y = 0.1711x - 0.0076	0.9975	6.7	14
4-MeO-2,2',3',4',5'-PnCB	y = 0.3824x + 0.0160	0.9685	11	9.3	y = 0.0149x + 0.0237	0.6166	42*	58*	y = 0.2230x + 0.0002	0.9975	6.8	13
4-MeO-2,2',3',5',6'-PnCB	y = 0.2731x + 0.0132	0.9743	11	12	y = 0.0027x + 0.0048	0.7998	31*	41*	y = 0.1424x + 0.0026	0.9980	10	16
4-MeO-2',3,4',5,6'-PnCB	y = 0.4131x + 0.0237	0.9697	9.1	11	y = 0.0181x + 0.0181	0.6880	38	53	y = 0.2376x - 0.0110	0.9969	6.6	14
4-MeO-2',3,3',5,5',6'-HxCB	y = 0.1263x - 0.0070	0.9047	21	28	y = 0.0720x + 0.0122	0.9765	14	19	y = 0.0555x - 0.0057	0.9945	12	18

*: Results of n=12 except 0.1ng (n=3) because MeO-PCBs were not detected in this concentration.

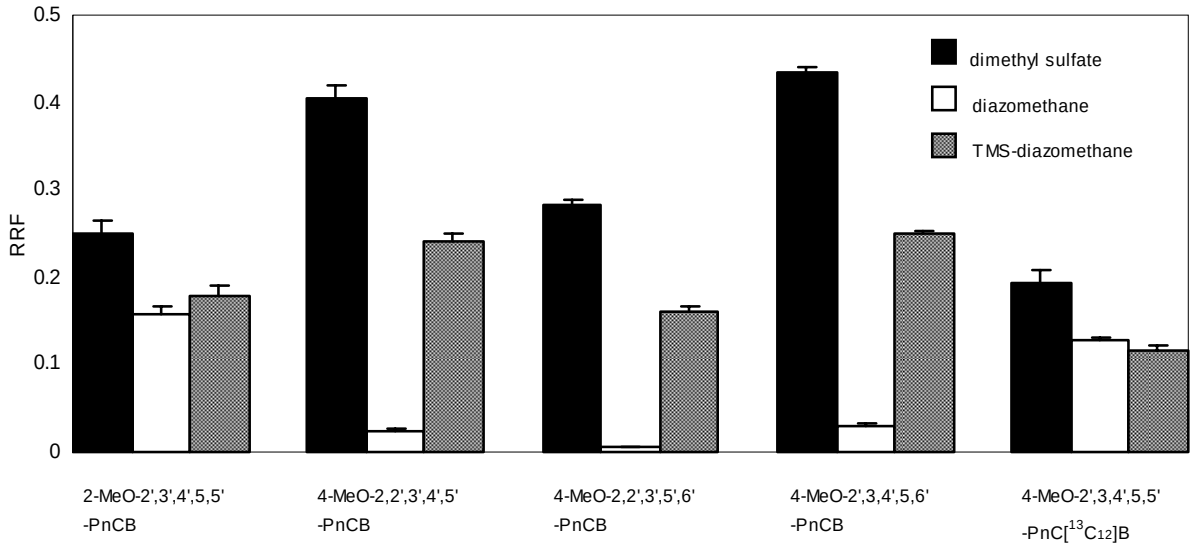

Figure2. Comparison of RRF for internal standard of MeO-PnCBs about each derivatization reagents at 2ng.

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