was next investigated in reactions carried out at 30°C by adding 5 mL of buffers of different pH, 1 mL of 1% sodium tetraphenylborate solution, and 2.5 mL of *n*-heptane to 1 mL of 100 ng/mL methylmercury solution. The following buffers were evaluated: 0.2 mol/L phosphate buffer at pH 2, 3, 4, 6, 7, 8, 11, and 12; 0.2 mol/L acetate buffer at pH 4, 5, and 6; and 0.2 mol/L Tris buffer at pH 7, 8, and 9. The effect of reaction time was investigated at reaction times of 0, 10, 20, and 30 min. For 0 time reaction, centrifugation was performed immediately after mixing of all solutions and solvent. Figure 1 shows the peak areas for methyl phenyl mercury and biphenyl generated by phenyl derivatization under various conditions. With increasing pH, the methyl phenyl mercury peak area decreased. However, the degree of the change was small, and the peak area for reaction at pH 7 was about 90% of that at pH 2. In contrast, the biphenyl peak area was greatest at pH 2, declined steeply up to pH 5, and was negligibly small at higher pHs. The methyl phenyl mercury peak area remained relatively constant regardless of the reaction time, and the reaction was considered to proceed quickly. In contrast, the biphenyl peak area increased as the reaction time increased. No difference in the methyl phenyl mercury peak area was observed at reaction temperatures of 25, 30, and 40°C. Based on these results, phenyl derivatization reactions were carried out with 1% sodium tetraphenylborate solution in 0.2 mol/L phosphate buffer (pH 7.0) with shaking for 10 min at room temperature.

Subsequently, it was confirmed that under the above reaction conditions, methylmercury can be phenylated even in organic solvent, similarly to aqueous solutions. Toluene was selected as the organic solvent because it permits easy transfer of methylmercury. To 2.5 mL of a 40 ng/mL solution of methylmercury in toluene, 5 mL of 0.2 mol/L phosphate buffer (pH 7.0) and 1 mL of 1% sodium tetraphenylborate solution were added and reacted by shaking for 10 min, after which the toluene layer was collected (condition A). At the same time, to 2.5 mL of 40 ng/mL methylmercury in 0.2 mol/L phosphate buffer (pH 7.0), 2.5 mL of 0.2 mol/L phosphate buffer (pH 7.0), 1 mL of 1% sodium tetraphenylborate solution, and 2.5 mL of toluene were added and reacted by shaking for 10 min, after which the toluene layer was collected (con-

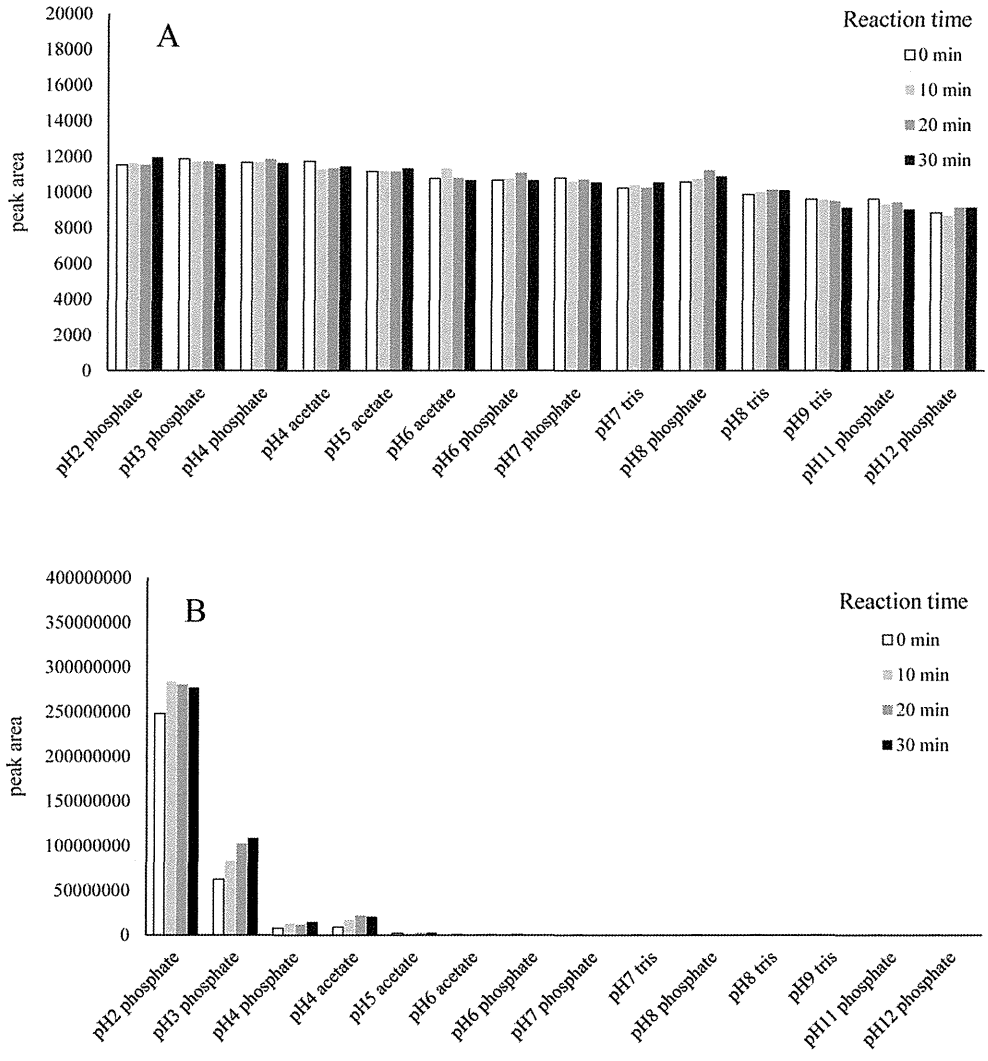


**Fig. 1.** Effects of pH and reaction time on (A) methyl phenyl mercury and (B) biphenyl GC-MS peak areas

dition B). The methyl phenyl mercury peak area obtained was 11,134±160 under condition A and was 11,311±87 under condition B ($n$=3).

The transfer conditions of methylmercury to toluene from acidified L-cysteine solution were also investigated. L-cysteine solution was acidified with 30 mL of 6 mol/L hydrochloric acid, and methylmercury was extracted with 30 mL of toluene three times. The transfer rate was evaluated based on the amount of methylmercury in toluene layers obtained in three steps. The transfer rate at the first procedure was 74.1%. At the second and third times, the rate was 19.0% and 5.1%, respectively. Thus, it was concluded that efficient derivatization can be achieved by using 1% sodium tetraphenylborate solution for the reaction after transfer of methylmercury in 1% L-cysteine solution to toluene by extraction three times.

*Selection of PEG amount co-injected*

In GC-MS calibration curves constructed from data obtained by direct injection of methyl phenyl mercury solutions prepared by the derivatization of methylmercury, the intercept was always negative. It was suspected that a portion of the injected methyl phenyl mercury was lost by adsorption in the GC-MS instrument. Therefore, the effect of co-injecting PEG to suppress adsorption of methyl phenyl mercury was investigated. As a preliminary experiment, 1 mL of measurement solution prepared from 20 ng/mL methyl mercury solution was mixed with 0.5 mL of 1.5 mg/mL PEG200 solution or 0.5 mL of toluene and both mixtures were injected into the GC-MS. The peak area of the solution containing PEG was 3884±49, while that of the solution not containing PEG was 2550±190 ($n$=3). These results suggested that PEG worked as anticipated. Then, the conditions of PEG co-injection were optimized. Based on the molecular weight of methyl phenyl mercury, PEG200 and PEG300 were evaluated.

Measurement solutions containing 250, 500, or 750 ng of PEG200 or PEG300 and methyl phenyl mercury were prepared, and 1 μL of each solution was injected into the GC-MS. Although, co-injection with PEG increased the peak area about 1.5 times as described above, increasing the amount of PEG injected from 250 to 750 ng produced only a slight increase in the methyl phenyl mercury peak area. In addition, no difference in peak area was observed between PEG200 and PEG300 (Table 1). Because the boiling point of PEG200 is lower than that of PEG300, so that the final GC column temperature can be set lower, PEG200 was selected for co-injection. In order to minimize contamination of the GC-MS instrument, the minimum effective amount of PEG200 was co-injected, which was 500 ng. No extra peaks was found on the chromatogram of methyl phenyl mercury solution (100 ng/mL as methyl mercury) co-injected with 500 ng of PEG200 (Fig. 2)

*Elimination of ethylenediamine-N-propyl silane column (PSA column) clean-up from the original method*

In the original method, the orange pigment thought to be a reaction by-product of the phenyl derivatization of methylmercury was removed using a PSA column. In the present method, this pigment was not observed, presumably due to the improvements in the phenyl derivatization method. Therefore, we considered that removal of the PSA column purification step from the analytical procedure would be desirable from the viewpoint of labor reduction. However, it was unclear whether or not the matrix derived from the sample and any remaining derivatization reagent need to be removed. To address this issue, measurement solutions prepared using various fish samples (cod, tuna, mackerel, and bonito) were analyzed after purification using a PSA column and the results were compared with those of measurement solutions not subjected to PSA purification. A measurement solution prepared from methylmercury standard solution was similarly measured as a control. No difference was observed between the chromatograms of the methylmercury standard solution and those of the fish samples. The ratios of the methyl phenyl mercury peak areas for the measurement solutions prepared with and without PSA column purification from methylmercury standard, cod, tuna, mackerel, and bonito were in

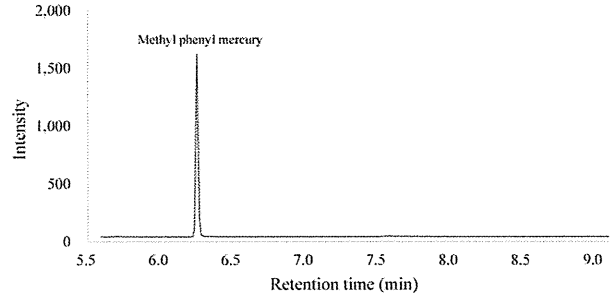


**Fig. 2.** GC chromatogram obtained from methyl phenyl mercury co-injected with 500 ng of PEG200

**Table 1.** Comparison of GC-MS peak areas for methyl phenyl mercury co-injected with different amounts of PEG200 or 300

| Methcyl mercury (ng/mL) | Peak area of methyl phenyl mercury | | | | | |
|---|---|---|---|---|---|---|
| | Amount of PEG200 co-injected (ng) | | | Amount of PEG300 co-injected (ng) | | |
| | 250 | 500 | 750 | 250 | 500 | 750 |
| 0 | — | — | — | — | — | — |
| 2.5 | 460 | 480 | 513 | 452 | 459 | 507 |
| 100 | 18,597 | 18,754 | 19,269 | 18,642 | 18,976 | 19,389 |

—: signal not detected

the range of 1.00–1.01 in all cases. Thus, the use of a PSA column to remove the pigment had only a minimal effect on both the chromatograms and the peak areas, and as the degree of purification afforded by the column was considered to be small, its use was omitted.

*Validation of the analytical method*

The trueness, repeatability, and intralaboratory reproducibility estimated based on the analytical results for four different CRMs are shown in Table 2. The same parameters estimated based on the analytical results for two different spiked samples are shown in Table 3. Trueness is the closeness of agreement between the average of the analytical results and the reference quantity value (certified level for CRMs or the spiked level) in this study. Repeatability is estimated as the standard deviation of the analytical results obtained in repeated determinations. Intralaboratory reproducibility is one kind of estimate of intermediate precision, and is estimated as the standard deviation of the analytical results under intralaboratory condition in this study. The trueness estimated from analysis of the certified reference

**Table 2.** Trueness, repeatability and intralaboratory reproducibility estimated by analysis of certified reference materials

| Sample | Certified level (mg/kg) | Analyst | | Result (mg/kg) 1st | 2nd | 3rd | 4th | 5th | average | Trueness (%) | Repeatability (RSD%) | Intralaboratory reproducibility (RSD%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CRM 7402-a | 0.58±0.02 (as Hg) | A | Portion 1 | 0.5261 | 0.4763 | 0.5013 | 0.5072 | 0.4982 | 0.504 | 87 | 0.8 | 3.9 |
| | | | Portion 2 | 0.5347 | 0.4771 | 0.5036 | 0.5108 | 0.5074 | | | | |
| | | B | Portion 1 | 0.5685 | 0.5397 | 0.5518 | 0.5102 | 0.5339 | 0.544 | 94 | 1.4 | 4.3 |
| | | | Portion 2 | 0.5756 | 0.5595 | 0.5568 | 0.5165 | 0.5251 | | | | |
| | | C | Portion 1 | 0.5200 | 0.4816 | 0.5201 | 0.4413 | 0.4420 | 0.493 | 85 | 5.5 | 7.3 |
| | | | Portion 2 | 0.5017 | 0.5085 | 0.5374 | 0.4574 | 0.5184 | | | | |
| BCR-463 | 3.04±0.16 (as MeHg) | A | Portion 1 | 3.078 | 2.952 | 3.030 | 3.091 | 3.000 | 2.984 | 98 | 4.6 | 4.6 |
| | | | Portion 2 | 3.056 | 3.137 | 2.904 | 2.910 | 2.677 | | | | |
| | | B | Portion 1 | 2.997 | 2.896 | 2.841 | 2.924 | 2.865 | 2.944 | 97 | 3.0 | 3.0 |
| | | | Portion 2 | 2.913 | 3.079 | 2.865 | 3.055 | 3.008 | | | | |
| | | C | Portion 1 | 2.959 | 2.752 | 2.788 | 2.742 | 2.677 | 2.801 | 92 | 6.7 | 6.8 |
| | | | Portion 2 | 2.700 | 2.664 | 2.857 | 3.263 | 2.607 | | | | |
| ERM-CE464 | 5.50±0.17 (as MeHg) | A | Portion 1 | 5.432 | 6.032 | 5.078 | 4.942 | 5.321 | 5.225 | 95 | 6.0 | 6.5 |
| | | | Portion 2 | 5.316 | 5.086 | 4.866 | 4.976 | 5.205 | | | | |
| | | B | Portion 1 | 5.332 | 5.287 | 4.830 | 5.150 | 5.367 | 5.245 | 95 | 2.6 | 3.8 |
| | | | Portion 2 | 5.456 | 5.150 | 5.093 | 5.436 | 5.349 | | | | |
| | | C | Portion 1 | 5.607 | 4.805 | 4.739 | 5.085 | 4.664 | 5.013 | 91 | 3.6 | 6.1 |
| | | | Portion 2 | 5.341 | 4.854 | 5.180 | 4.986 | 4.872 | | | | |
| CRM 7403-a | 5.00±0.22 (as Hg) | A | Portion 1 | 4.562 | 4.620 | 4.419 | 4.457 | 4.433 | 4.619 | 92 | 6.0 | 6.0 |
| | | | Portion 2 | 5.297 | 4.497 | 4.853 | 4.478 | 4.575 | | | | |
| | | B | Portion 1 | 4.752 | 4.724 | 4.701 | 4.776 | 4.839 | 4.750 | 95 | 0.9 | 1.6 |
| | | | Portion 2 | 4.819 | 4.689 | 4.619 | 4.718 | 4.861 | | | | |
| | | C | Portion 1 | 5.177 | 4.185 | 4.331 | 4.762 | 4.406 | 4.476 | 90 | 4.9 | 6.9 |
| | | | Portion 2 | 4.600 | 4.225 | 4.285 | 4.370 | 4.416 | | | | |

**Table 3.** Trueness, repeatability and intralaboratory reproducibility estimated by analysis of spiked samples

| Sample | Spiked concentration (mg/kg) | Analyst | | Result (mg/kg) 1st | 2nd | 3rd | 4th | 5th | average | Trueness (%) | Repeatability (RSD%) | Intralaboratory reproducibility (RSD%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cod | 0.3 | A | Portion 1 | 0.2449 | 0.2616 | 0.2726 | 0.2766 | 0.2692 | 0.266 | 89 | 1.2 | 5.0 |
| | | | Portion 2 | 0.2486 | 0.2590 | 0.2812 | 0.2791 | 0.2709 | | | | |
| | | B | Portion 1 | 0.3012 | 0.2879 | 0.2750 | 0.2814 | 0.2868 | 0.285 | 95 | 4.4 | 4.4 |
| | | | Portion 2 | 0.2781 | 0.2855 | 0.2710 | 0.3111 | 0.2739 | | | | |
| Tuna | 0.3 | A | Portion 1 | 0.2613 | 0.2569 | 0.2435 | 0.2352 | 0.2913 | 0.265 | 88 | 6.7 | 8.1 |
| | | | Portion 2 | 0.2554 | 0.2819 | 0.2917 | 0.2481 | 0.2866 | | | | |
| | | B | Portion 1 | 0.2672 | 0.3318 | 0.2663 | 0.3181 | 0.3058 | 0.294 | 98 | 6.3 | 7.6 |
| | | | Portion 2 | 0.3004 | 0.2952 | 0.2694 | 0.2874 | 0.2991 | | | | |

materials was 87–98% for analyst A, 94–97% for analyst B, and 85–92% for analyst C. The repeatability, expressed as RSD%, was 0.8–6.0% for analyst A, 0.9–3.0% for analyst B, and 3.6–6.7% for analyst C. The intralaboratory reproducibility was 3.9–6.5% for analyst A, 1.6–4.3% for analyst B, and 6.1–7.3% for analyst C. With respect to analyses of the spiked samples, the estimated trueness was 89 and 88% for analyst A and 95 and 98% for analyst B. The repeatability was 1.2 and 6.7% for analyst A and 4.4 and 6.3% for analyst B. The intralaboratory reproducibility was 5.0 and 8.1% for analyst A and 4.4 and 7.6% for analyst B.

## Discussion

*Phenyl derivatization of methylmercury*

In the original method, the conditions for the reaction of tetraphenylborate and methylmercury were investigated in the presence of L-cysteine. When the pH exceeded 2, the amount of methyl phenyl mercury formed decreased. Therefore, the reaction was carried out at pH 0.5–1.5 at 30°C for 1 hr. In this study, the effect of omitting L-cysteine from the reaction conditions was investigated. In the absence of L-cysteine, no decrease in the amount of methyl phenyl mercury formed was observed in the range of pH 2–9. Accordingly, the reported decrease in the amount of methyl phenyl mercury formed at pH≥2 with the original method was considered to be due to the formation of a complex between methylmercury and L-cysteine, and the optimum pH of the derivatization reaction itself was considered to be in the above-mentioned range. When the derivatization reaction was carried out in the neutral pH range, the amount of phenyl compounds formed, including the sodium tetraphenylborate degradation product biphenyl, decreased, and unlike the case of the original method, no formation of orange pigment was observed. Therefore, performing the derivatization reaction in the neutral pH range reduces the amount of undesired by-products formed without decreasing the concentration of methyl phenyl mercury in the measurement solution, thus lessening the potential for contamination of the GC instrument.

When methylmercury was extracted from a sample into the toluene layer and then transferred into water, 1% L-cysteine solution was used to increase the percentage of transfer. Under neutral pH conditions, L-cysteine and methylmercury form a complex, and the methylmercury contained in this complex cannot be phenylated by sodium tetraphenylborate. Therefore, phenylation of methylmercury with sodium tetraphenylborate in toluene in the absence of aqueous L-cysteine was investigated. In this case, the amount of methyl phenyl mercury formed in toluene was the same as that formed when methylmercury was phenylated in aqueous solution. In addition, the reaction proceeded easily upon shaking for 10 min at room temperature. It is not known whether this phenyl derivatization reaction takes place in the water layer, in the toluene layer, or at the interface between these layers.

In order to phenylate methylmercury in toluene solution, a step was added to the analytical procedure, in which methylmercury is again transferred from the L-cysteine solution to toluene. By adding this step, the need to transfer methyl phenyl mercury from the reaction solution to *n*-heptane, as was required in the original method, was eliminated.

*Method validation*

The following fish-based reference materials containing certified levels of methylmercury were selected for method validation: CRM 7402-a (cod fish powder), BCR-463 (tuna fish powder), ERM-CE464 (tuna fish powder), and CRM 7403-a (swordfish powder). The certified levels of methylmercury in the dry state were 0.58–5.00 mg/kg, concentrations close to the provisional regulation value of 0.3 mg/kg (wet weight).

The trueness, repeatability and intralaboratory reproducibility estimated by using independent analytical results obtained by three analysts (analyst A, B, and C) under various conditions were in agreement with the target values shown in the validation guidelines for test methods for metals (*i.e.*, trueness, 80–110%; repeatability, <10% [RSD%]; and intralaboratory reproducibility, <15% [RSD%]). The trueness estimate for the lowest-concentration of CRM7402-a sample was slightly lower than that for the other CRMs for all three analysts; but no systematic difference was observed with respect to precision.

The number of available reference materials containing certified levels of methylmercury is limited. The concentration to be confirmed by the analytical method used for the determination of compliance with the provisional regulation value may not necessarily be in agreement with the certified value. In addition, CRMs are usually expensive; thus, they are not easy to procure. Furthermore, in order to ensure test reliability, the analytical method must be validated using actual samples (wet samples of targeted fish species) before it is used to determine regulatory compliance. Therefore, the performance of the improved method was also evaluated using spiked samples. Samples usable as blanks were selected by analyzing the methylmercury content of several species of fresh fish purchased at a market. Fish found to contain less than 0.15 mg/kg, which corresponds to one-half the provisional regulation concentration, were selected for use as blanks. In the general preparation of spiked samples, it is desirable to use a solvent that is miscible with the sample; thus, an aqueous solution of methylmercury was used for this purpose.

Because some methylmercury is contained in the blank sample, variations in the concentration of mercury in the blank sample and variations arising from analysis contribute to the variations in the measured concentration of methylmercury. In order to reduce the first variation as much as possible, the blank sample was mixed to be as homogeneous as possible. The SD for the results of 10 analyses of the methylmercury concentration in the cod blank sample was 0.0028–0.007 mg/kg. The SD for

the results of 10 analyses of the methylmercury concentration in the tuna blank sample was 0.0068–0.011 mg/kg, slightly larger than that of the cod blank sample. The SD for the analytical results of the spiked samples was ≥2 times the SD of the results for the blank samples. Thus, the contribution made by non-homogeneity of the blank sample to the precision estimates was not large.

The trueness, repeatability and intralaboratory reproducibility estimated based on the analytical results obtained by analysts A and B were similar to the values estimated from analyses of certified reference materials, in agreement with the target values specified in the guidelines. Thus, we concluded that validation of an analytical method is possible in circumstances where only samples containing the analyte are available.

## Conclusion

In the present study, we improved the original method for the analysis of methylmercury in fish *via* phenyl derivatization and subsequent GC-MS measurement. By optimizing the phenyl derivatization reaction conditions, the formation of degradation products from the phenyl derivatization reagent could be successfully reduced. In addition, undesirable adsorption of methyl phenyl mercury within the GC-MS instrument was suppressed by the co-injection of PEG200.

The performance of the improved method was evaluated by three independent analysts using CRMs, and the practicability of validation using spiked samples was also demonstrated. The results of these analyses were in agreement with the target values specified in the guidelines for the validation of test methods for metals. Thus, the present improved analytical method is valid for determining compliance with the provisional regulation value for methylmercury in fish.

## Acknowledgments

This study was supported by a Health and Labour Sciences Research Grant for "Research Concerning Evaluation of the Intake of Toxic Substances such as Dioxins through Food and Associated Method Development" from the Ministry of Health, Labour, and Welfare of Japan.

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# 食品に含まれる有害物質
## トランス脂肪酸と多環芳香族炭化水素類

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つつみ ともあき　同 第二室長

食品に本来含まれている化学物質が，加熱といった加工によって変化し有害物質となることが知られている．この事実は「焼き魚の焦げた部分は食べないように」といった食生活の知恵に古くから反映されてきている．現在では，食品に由来し加工を原因として生成する有害物質には複数が知られており，それらを総称して製造副生成物と呼んでいる．本稿では，複数が知られる製造副生成物中，トランス脂肪酸と多環芳香族炭化水素類について概説する．

## トランス脂肪酸

情報伝達の不十分さもありトランス脂肪酸に対する一般の認識に混乱があると感じている．この混乱解消の一助となることを期待し，本稿では詳細を記載するのではなく俯瞰することで，トランス脂肪酸と今後どのようにつきあえばよいのかを考えてみたい．なお記述のもととした情報や知見の詳細は，食品安全委員会が2011年に取りまとめた評価書をもとに，適宜入手し確認していただければ幸いである．

### 1．トランス脂肪酸とは何か？

言うまでもなく，脂質はヒトのエネルギー源であり栄養として不可欠である．栄養学的に重要な脂質には，脂肪酸と中性脂肪が含まれる．脂肪酸とは，炭化水素鎖の末端にカルボキシル基を有する総炭素数が4～36の分子である．また中性脂肪とは脂肪酸とグリセロールがエステル結合した化合物である．食品に主として含まれているのは，3分子の脂肪酸がグリセロールにエステル結合したトリアシルグリセロールであり，これを摂取し代謝することでヒトは活動に必要なエネルギーを抽出している．その名称の一部であることからも分かるとおり，トランス脂肪酸は脂肪酸の一種である．食品国際規格の策定等を主務とする国際政府間組織であるコーデックス(Codex)委員会では，トランス脂肪酸を「少なくとも1つ以上のメチレン基で隔てられたトランス型の非共役炭素―炭素二重結合を持つ一価不飽和脂肪酸及び多価不飽和脂肪酸のすべての幾何異性体」と定義している．総炭素数並びに分子内の二重結合の位置および数の違いに応じ，たくさんの種類の脂肪酸(脂肪酸分子種)が存在する．そのため，理論上はそれら脂肪酸分子種の数だけトランス脂肪酸分子種が存在すると言っても過言ではない．

トランス脂肪酸が話題に取りあげられる際，特定の1つの物質が想起されているように感じることが多いが，共通の特徴を有する多数の脂肪酸分子種が総じてトランス脂肪酸であることを明確に理解することが，トランス脂肪酸に関

連する諸問題を考えるための出発点になるだろう．食品には脂肪酸でなく脂肪が主として含まれることから，トランス脂肪酸ではなくトランス脂肪として扱われる場合もある．なお，現在Codex委員会によるトランス脂肪酸の定義は世界保健機関(WHO)が設置する食事と健康に関する栄養ガイダンス専門家諮問グループ(NUGAG)において見直しが検討されている．

## 2. トランス脂肪酸分析の限界とそれに伴う科学的知見の制約

流通する個々の食品や1回の食事に含まれるトランス脂肪酸の量は，濃度実態の把握や健康リスク評価の基礎となる科学的知見である．濃度を知るために必要な分析の基本は，試料からの抽出(精製)，分離，検出(同定)，そして定量である．また分析するためには，はじめに分析対象を決めなければならない．ここでいう分析対象とは，特定のトランス脂肪酸分子種の1つ1つを指す．理論上存在し得るトランス脂肪酸分子種のうち，どの分子種が食品に含まれているかも十分に知られていない．科学的知見が不足する状況で，やみくもに不特定多数のトランス脂肪酸分子種を分析対象とすることは非効率的であり，分離の観点からは実現不可能である．さらに同定と定量には，試料と標準品との間での結果比較が不可欠である．そのため，標準品の存在しない分子種は分析対象になり得ない．しかし，トランス脂肪酸標準品の入手はごく一部の分子種に限られているのが現状である．分析対象の選択と標準品の制限の他，Codex委員会分析・サンプリング法部会(CCMAS)では，現在妥当性確認中の新規トランス脂肪酸分析法の審議に関連して，試料となる食品の種類に応じた抽出の問題点や，分析可能な濃度の下限に関する懸念が情報として共有されている．分析上の工夫はあり得るが，これがトランス脂肪酸分析の限界である．つまり，これまでに報告されているトランス脂肪酸の濃度等は，分析可能な範囲で得られた制約のある知見であることに留意すべきである．ただし，同定されたトランス脂肪酸分子種の一定以上濃度の値は信頼できるといって良いだろう．分析の限界にも強く関係するが，トランス脂肪酸分子種別のリスク評価は十分でなく，総じてトランス脂肪酸としてのリスクが評価されている．

## 3. トランス脂肪酸を含む食品

トランス脂肪酸を含む代表的な食品には，硬化油と反芻動物(ヤギやヒツジ，ウシ)由来の畜肉および乳製品が挙げられる．ただし，硬化油と畜肉乳製品のそれぞれに主として含まれるトランス脂肪酸分子種は異なる．硬化油ではエライジン酸が主であり，畜肉乳製品ではバクセン酸が主である．硬化油は，部分水素添加の手法によりシス型不飽和脂肪酸を飽和脂肪酸に変化させることで製造された工業製品である．代表的な硬化油であるマーガリンやファットスプレッドを想起すると分かりやすい．硬化油を原料とするペストリー等の各種食品にも必然的にエライジン酸が含まれる．エライジン酸は，部分水素添加つまりは製造方法を原因として副次的に生成することが知られている．エライジン酸生成の原因が明らかになって以降，事業者らの取り組みにより製造方法は改善され，現在の硬化油のエライジン酸濃度は劇的に低下したといわれている．一方，反芻動物由来の畜肉乳製品に含まれるバクセン酸は，当該動物の胃に生息する微生物により天然に生成することが知られている．努力によって改善できる製造方法と違い，天然に存在する畜肉乳製品のバクセン酸濃度を低減させることは極めて困難である．

## 4. トランス脂肪酸によりもたらされる可能性のある健康危害とそのリスク

トランス脂肪酸の過剰摂取によりもたらされる可能性のある健康危害として冠動脈疾患が挙げられる．冠動脈疾患に関係する因子として他にも喫煙や高血圧，糖尿病，加齢が挙げられる

が，それら因子によるリスクに比べるとトランス脂肪酸のリスクは低い．またトランス脂肪酸を摂取後直ちに冠動脈疾患を患うことになるわけではない．継続的な摂取が問題になり得る．さらに，摂取量によってはもとより低いリスクがさらに低くなる．トランス脂肪酸のリスクを考える上で，WHOが示している「総エネルギー摂取量の1%未満」という値が摂取量の指標となる．集団の平均摂取量を見ると，欧米の一部の国の調査では1%を超えることが報告されている．一方，わが国で実施された調査では，平均摂取量として1%を超える報告はない．この違いは国レベルでの食習慣の違い(あるいは食文化の違い)によるものと推測される．なお，後述する行政施策や前述の硬化油製造方法の改善が効果を上げ，過去には高かった国の摂取量も近年では減少している．

## 5. トランス脂肪酸をめぐる各国の対応

トランス脂肪酸摂取量の低減を目的とし，これまでにも多くの国が，食品製造事業者また飲食事業者と協調して，食品に含まれるトランス脂肪酸量の低減や消費者への情報提供に取り組んでいる．わが国でも2011年に消費者庁が「トランス脂肪酸の情報開示に関する指針について」を公開し，食品製造事業者による自主的な表示を促している．最近では2015年6月に，トランス脂肪酸を含む硬化油の使用規制を米国食品医薬品局(FDA)が決定し，国内でも多数報道がされた．余談だが，これら報道の多くで「食品へのトランス脂肪酸の添加が禁止された」と説明されていた．この説明は明らかな間違いである．硬化油製造方法の改善について述べたが，人の活動の結果意図せず生成した物質は，その活動の見直しにより低減させるあるいは完全になくすことも可能であろう．また，人の活動が原因なのだから，人の努力によってなくそうと考えるのも自然かと思う．米国FDAは今回の発表を行う数年前から食品製造事業者に改善策の実行可能性についてヒアリングを行った．その結果から実行可能と判断し，「トランス脂肪酸を含む硬化油を，その他の一般に安全と認識されている(GRAS; Generally Recognized As Safe)食品と同じように使用すること」を禁止した．全食品におけるトランス脂肪酸含有が禁止されたのでも，天然由来のトランス脂肪酸の含有が禁止されたのでも，ましてやトランス脂肪酸の添加が禁止されたのでもない(語弊のないよう補足しておくと，トランス脂肪酸にはベネフィットが認められていないので，そもそも添加されない)．規制対象となった硬化油であっても，使用の目的と量やリスクに関する科学的根拠を提出し当局により妥当と判断されれば，使用される可能性はある．なお，天然に存在する有害物質については，合理的に可能な限りの低減策を講じるのが国際的なリスク管理の基本ではある．しかし，天然由来のトランス脂肪酸の場合にはそのような低減策がないのが現実である．無くすことができない有害物質によるリスクもまた，無くしようがない．そのような有害物質とは，あえてリスクを高めないように，個々人が自分の食生活を律しながらつきあっていくことが必要になる．先人の知恵あるいは常識に学び，バランスの良い食生活を営み，時に望むままに食の楽しみを享受するぐらいが丁度良いのだろうと考える．

## 6. トランス脂肪酸に関するまとめ

本稿で概説したとおり，トランス脂肪酸を特定の健康危害をもたらす因子(ハザード)の一つであると考えることは妥当であろう．ただし同じ健康危害をもたらす他のハザードに比べるとリスクは小さい．特に摂取量の少ない日本人にとってのリスクは，摂取量の多い諸外国の人々に比べると，さらに小さい．その背景には食習慣の違いがある．一般論として，限られた資源には優先順位をつけ，小さなリスクの回避よりは大きなリスクの回避あるいは，知られていな

いハザードの発見とリスク評価に費やすことで，トータルとして健康の維持・向上への効果は大きくなる．「リスク」という言葉を「やばい」という言葉に換えて考えると理解しやすいかもしれない．トランス脂肪酸によるリスクは，「やばさがないとは言い切れない」といった程度かと思う．例えば，コメを主食とする日本人にとっては，コメに含まれる無機ヒ素のほうがトランス脂肪酸に比べてよほどやばい．ただし，やばいといってむやみに恐れるのではなく，どのくらいやばいのかの程度を落ち着いて考え，「偏食や過食をしなければ特に問題なし」といった程度のやばさだと個人が納得できることが大事になるだろう．そのためには，難しいと敬遠されがちな科学的根拠に基づく説明を，聞く側の立場に立って正しく理解してもらえるようにする，そのためのコミュニケーションのあり方がますます重要になるのかもしれない．

## 多環芳香族炭化水素類

多環芳香族炭化水素類(PAHs)については古くから食品中に含まれることが知られている．最近では2012年に韓国において，韓国内の基準値を超えるBenzo[a]pyrene(BaP)が検出された鰹節をスープに使用したとして，日本でも該当製品の回収が行われたことは記憶に新しい．ここでは，食品に含まれるPAHsに関して最近の情報も含めて述べる．

### 1. PAHsとは

PAHsとはベンゼン環を2つ以上持つ炭化水素化合物の総称であり，ベンゼン環の数や位置により多くのPAHsが存在する．PAHsは化学的安定性が比較的高く，脂溶性が高い性質を有する．PAHsは食品の燻製や乾燥，加熱(直火調理)などの過程で生成されることが知られており，これらの加工処理をした食品ではPAHsの含有が懸念されている．この他，PAHsは化石燃料である石炭や石油の燃焼に伴っても生成するため環境中に広く存在している．一般的には食品がPAHsの主要な摂取源であると考えられており，最も代表的なPAHsとしてBaPが知られている．

### 2. PAHsの毒性とリスク評価方法

PAHsの急性毒性はそれほど強くなく，もっぱら発がん性が問題になる．EUの食品科学委員会(SCF)やFAO/WHO合同食品添加物専門家会議(JECFA)により，遺伝毒性や発がん性があると評価されたPAHs16種を**表1**に示した．なお，Benzo[c]fluoreneについては，JECFAの評価で発がんに関与している可能性が示唆されていることから**表1**に含めている．このような背景から，食品中のPAHs分析ではPAHs16種を分析対象とすることが多くなっている．

また，**表1**には国際がん研究機関(IARC)による各PAHsの発がん性評価についても示した．BaPはグループ1(ヒトに対して発がん性がある)に分類されており，他にも3種のPAHsがグループ2A(ヒトに対しておそらく発がん性がある)，9種のPAHsがグループ2B(ヒトに対して発がん性がある可能性がある)に分類されている．PAHsは遺伝毒性発がん物質であることから，ゼロでなければリスクはゼロではないとみなしてALARA(合理的に達成可能な限り低く抑える)の原則が適用されてきた．しかし，近年になり遺伝子発がん物質の定量的なリスク評価の手法の一つとして，曝露マージン(MOE)による評価が利用されるようになってきた．MOEは動物試験で得られたベンチマーク用量信頼下限値(BMDL)などの毒性影響に関する指標値をヒトの推定摂取量で割った値である．この値が10,000以上であれば健康への懸念が低くリスク管理の優先度が低いと判断される．代表的なPAHsであるBaPについてリスク評価されることが多いが，食品中の

表1　EUでモニタリングが推奨されているPAHs 16種

| 化合物名 | | PAHs 4種 | PAHs 8種 | 分子式 | 分子量 | IARCの分類 |
|---|---|---|---|---|---|---|
| Benzo[c]fluorene | ベンゾ[c]フルオレン | | | $C_{17}H_{12}$ | 216 | Group 3 |
| Benzo[a]anthracene | ベンゾ[a]アントラセン | ✓ | ✓ | $C_{18}H_{12}$ | 228 | Group 2B |
| Cyclopenta[c, d]pyrene | シクロペンタ[c, d]ピレン | | | $C_{18}H_{10}$ | 226 | Group 2A |
| Chrysene | クリセン | ✓ | ✓ | $C_{18}H_{12}$ | 228 | Group 2B |
| 5-methylchrysene | 5メチルクリセン | | | $C_{19}H_{14}$ | 242 | Group 2B |
| Benzo[b]fluoranthene | ベンゾ[b]フルオランテン | ✓ | ✓ | $C_{20}H_{12}$ | 252 | Group 2B |
| Benzo[k]fluoranthene | ベンゾ[k]フルオランテン | | ✓ | $C_{20}H_{12}$ | 252 | Group 2B |
| Benzo[j]fluoranthene | ベンゾ[j]フルオランテン | | | $C_{20}H_{12}$ | 252 | Group 2B |
| Benzo[a]pyrene | ベンゾ[a]ピレン | ✓ | ✓ | $C_{20}H_{12}$ | 252 | Group 1 |
| Indeno[1, 2, 3-c, d]pyrene | インデノ[1, 2, 3-c, d]ピレン | | ✓ | $C_{22}H_{12}$ | 276 | Group 2B |
| Dibenzo[a, h]anthracene | ジベンゾ[a, h]アントラセン | | ✓ | $C_{22}H_{14}$ | 278 | Group 2A |
| Benzo[g, h, i]perylene | ベンゾ[g, h, i]ペリレン | | ✓ | $C_{22}H_{12}$ | 276 | Group 3 |
| Dibenzo[a, l]pyrene | ジベンゾ[a, l]ピレン | | | $C_{24}H_{14}$ | 302 | Group 2A |
| Dibenzo[a, e]pyrene | ジベンゾ[a, e]ピレン | | | $C_{24}H_{14}$ | 302 | Group 3 |
| Dibenzo[a, i]pyrene | ジベンゾ[a, i]ピレン | | | $C_{24}H_{14}$ | 302 | Group 2B |
| Dibenzo[a, h]pyrene | ジベンゾ[a, h]ピレン | | | $C_{24}H_{14}$ | 302 | Group 2B |

PAHsの指標としてはPAHs4種やPAHs8種が適当であるとされている．

### 3. 食品中のPAHsとリスク評価

PAHsを含有する可能性が高いと考えられる燻製や乾燥・加熱などの加工処理をした食品を中心にPAHs含有量の調査が実施されている．例えば，よく焼いたり，バーベキューされた肉類ではBaP濃度が高くなり，最大5 μg/kg程度になることが報告されている[1]．また，欧州食品安全機関(EFSA)ではEU内の約1万検体の種々の食品のPAHs濃度データを収集し，PAHs濃度が比較的高かった食品は燻製魚やココアバターおよび栄養補助食品等であり，全体の2.3%にあたる検体のBaP濃度が10 μg/kgを超えたことを報告している[2]．さらに，EFSAでは食品からのPAHs摂取量を推定しMOEによるリスク評価を行っている．平均的摂取者のMOEは，BaPで17,900，PAHs4種で17,500，PAHs8種で17,000，高摂取者のMOEは同じ順で，それぞれ10,800，9,900，9,600と試算された．平均的摂取者においては，MOEは10,000を大きく上回ることから，健康リスクへの懸念は小さいとされている．一方で，高摂取者では10,000をわずかではあるが下回る場合もあることから，リスク管理措置が必要になる可能性が示されている．その他，JECFAにおいても食品からのBaP摂取量を推定しMOEによるリスク評価を行っており，平均的摂取者のMOEが25,000，高摂取者のMOEが10,000と試算されたことから，健康リスクへの懸念は小さいと結論している[3]．

日本国内でも食品中のPAHs含有量が調査されており，特に鰹節および鰹節加工品が比較的高いPAHsを含有することが報告されている[4,5]．鰹節は製造時に焙乾工程において繰り返し燻されるため，PAHsが比較的高い濃度で含まれてくる．また，鰹節は主にダシに使用されるため，水でダシを取った際の鰹節からダシへのPAHsの移行率についても調査されている．PAHsの移行率は5%以下であり，PAHsは脂溶性が高い性質を有することから，水でダシを取る際にはダシに移行しにくいと考えられる[5,6]．その他，国内では，マーケットバスケット方式や陰膳方式によるトータルダイエット

調査が実施されており，食品からのBaPの摂取量は1.4～2.4 ng/kg体重/日(体重当たりの表記が無い場合は，日本人の平均体重を50 kgとして計算)と推定されている[7,8]．JECFAのBaPのBMDL(100,000 ng/kg体重/日)を使用してMOEを試算すると40,000以上となる．

## 4. 食品中のPAHsに関する各国の対応

食品中のPAHsについては，EUが食用油脂，燻製肉類，燻製魚介，乳幼児用食品などにBaPの基準値(1.0～5.0 μg/kg)を設けている．基準値が設けられている食品は，PAHsを含有する蓋然性が高い食品や，有害化学物質に対する感受性の高い乳幼児が食する食品が対象となっている．また，これらの基準値はALARAの原則に従い設定されたものであり，当初はBaPのみに基準値が設けられていた．その後，2012年より食品中のPAHsの指標としてPAHs4種の基準値(1.0～35.0 μg/kg)も追加された．その他，韓国では食用油脂，燻製魚介類等にBaPの基準値(1.0～10.0 μg/kg)が，中国では食用油脂等にBaPの基準値(5～10 μg/kg)が，カナダではオリーブポーマスオイルにBaPの基準値(3 μg/kg)が設けられている．

一方で，日本では食品衛生法に基づくPAHsの基準値は設定されていない．国内のトータルダイエット調査の結果から，通常の食生活ではBaPによる健康リスクへの懸念は小さいと考えられる．しかし，鰹節および鰹節加工品の中にはPAHsを高い濃度含むものがあることから，鰹節の製造時におけるPAHsの低減対策が検討されている[9]．また，Codex委員会においても，燻製などの工程における食品中のPAHsの低減に関する実施規範が採択されている[10]．こういった低減化対策は製造工程で生成するPAHsなどの有害化学物質の低減には有効な手段であると考えられる．しかし，燻製という技術は食品を保存する方法の一つとして用いられてきたことから，仮にPAHsを低減させる燻製条件が食品の安全性や保存期間に影響を与えた場合，別のリスクが上昇する可能性に注意が必要である．

## 5. PAHsに関するまとめ

PAHsは近年に新たに発生した有害化学物質ではなく，人類が加熱調理を覚えたはるか昔から食品に存在しており，人は食品からPAHsを長年摂取してきた．現在までの科学的知見によると，通常の食生活においては食品からのPAHs摂取がヒトの健康リスクに及ぼす影響は小さいと考えられる．また，PAHs濃度が比較的高い食品を中心に製造工程におけるPAHs低減化の取り組みも始まっている．これらの状況を踏まえると，食品中のPAHsについて過度に心配する必要は無く，偏食に注意しバランスの良い食生活を送ることが重要と考える．

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# DIETARY INTAKE OF HEXABROMOCYCLODODECANE IN JAPAN

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## Introduction

Hexabromocyclododecane (HBCD) is a brominated flame retardant (BFR) that has been used for many years in plastics and textile coatings around the world. In Japan, the domestic use of HBCD has recently become more common, as it has increasingly replaced or supplemented other BFRs. A total of 2,600 t of HBCD was produced in or imported into Japan in 2011.

HBCD's toxicity and the environmental threat it poses are subjects of current discussion. HBCD has been identified in environmental samples from birds, mammals, fish and other aquatic organisms, as well as in soil and sediment. In May 2013, the Stockholm Convention on Persistent Organic Pollutants (POPs) added HBCD to the Convention's Annex A for elimination. In May 2014, Japan added HBCD to its list of class I specified substances of the Chemical Substances Control Law to enact a ban on the import and production of HBCD. However, HBCD is still diffusing into the environment from waste materials that contain HBCD.

It is important to estimate humans' dietary intakes of HBCD. A total diet (TD) study is a useful method of estimating the average dietary intake of contaminants. We reported that the Japanese populace is exposed to HBCD mostly via fish and shellfish among the TD samples studied[1,2]. The dietary intake of HBCD can thus be estimated in an analysis of TD samples consist of fish and shellfish. In the present study, we analyzed HBCD in the TD samples and estimated the daily intake of HBCD across Japan.

## Materials and methods

*Chemicals*

Non-labeled and $^{13}C_{12}$-labeled α-, β-and γ-HBCD analytical standards were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Dichloromethane, n-hexane, acetone, cyclohexane, methanol and distilled water (washed with hexane) of dioxin or pesticide analysis grade were purchased from Kanto Chemical (Tokyo, Japan). The 44% sulfuric acid-impregnated silica gel was purchased from Wako Pure Chemical Industries (Osaka, Japan).

*Samples*

TD samples were prepared at 10 locations in seven regions across Japan in 2012. The constituents of the TD samples were designed based on the official food classification and consumption data obtained by the National Health and Nutrition Survey in Japan. The collected various kinds of fish and shellfish were cooked or prepared in typical ways for consumption. The samples were then blended for TD samples. The TD samples were maintained at temperatures below –20°C until analysis.

*Sample Preparation*

The protocol for the HBCD analysis is illustrated in Fig. 1. Each 5 g of TD sample was mixed with 5 g of diatomaceous earth powder. After mixing, the sample was spiked with $^{13}C_{12}$-labeled α-, β- and γ-HBCD, and was extracted using a Dionex ASE-350 Accelerated Solvent Extractor (Thermo Scientific, Sunnyvale, CA, USA) under conditions of 1,500 psi, with acetone/hexane (1:3) as an extraction solvent. The extracts were shaken with 5% sodium chloride solution, and the organic layer was dried over anhydrous sodium sulfate and concentrated to dryness in order to determine the lipid content gravimetrically. The residue was dissolved in 10 mL of acetone/cyclohexane (3:7), and 2 mL of this solution was subjected to gel permeation chromatography. HBCD was fractionated over 12.5–16.5 min after large molecules such as crude fatty acids eluted in 10–12 min. The fraction was concentrated and dissolved in 0.3 mL of hexane, re-purified with a 1 g of 44% sulfuric acid-impregnated silica gel-packed mini-column, and then reconstituted to 50 μL using methanol.

*Analytical Methods and Instrumentation*

The HBCD concentrations were determined using liquid chromatography/mass spectrometry (LC/MS) performed on a Quattro Ultima Pt mass spectrometer (Waters, Milford, MA, USA) connected to a 2695 liquid

chromatography system (Waters). The HPLC column was a 150 mm × 2.1 mm i.d. 5-μm Inertsil ODS-3 (GL-Science, Tokyo, Japan). The detection limit of both α- and γ-HBCD was 0.02 ng/g wet weight (ww); that for β-HBCD was 0.01 ng/g ww.

**Results and discussion**

Table 1 presents the results of the HBCD analysis of the TD samples (aggregated sample of cooked fish and shellfish following those consumption data). HBCD was detected in all samples prepared at all 10 locations. The concentrations of detected total HBCD were 0.17–1.24 ng/g ww when ND was assumed to be zero. The mean concentration of total HBCD was 0.68 ng/g ww. The concentrations of the isomers of all samples were in the order: α-> γ- > β-HBCD. We observed no correlation between the HBCD concentration and the fat content.

The estimates of HBCD daily intake basing on the concentration and consumption data are summarized in Table 2. As mentioned earlier, the Japanese populace is exposed to HBCD mostly via fish and shellfish, so that the daily intakes estimated basing on the concentrations of the TD sample consist of various kinds of fish and shellfish can be considered the entire dietary intake. The daily intake of HBCD was estimated to be 13.1–86.9 ng/day. In the case of 50 kg of body weight (bw), the daily intake was calculated as 0.26–1.74 ng/kg bw/day. In Japan, it was reported that the no observable adverse effect level (NOAEL) for HBCD was 10.2 mg/kg bw/day. Using an uncertain (safety) factor of 100, this suggests that the total daily intake of HBCD should be less than 0.102 mg/kg bw/day. Compared with this value, the levels of the HBCD obtained in this study are not considered a serious problem.

**Acknowledgements**

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Health, Labor, and Welfare, Japan.

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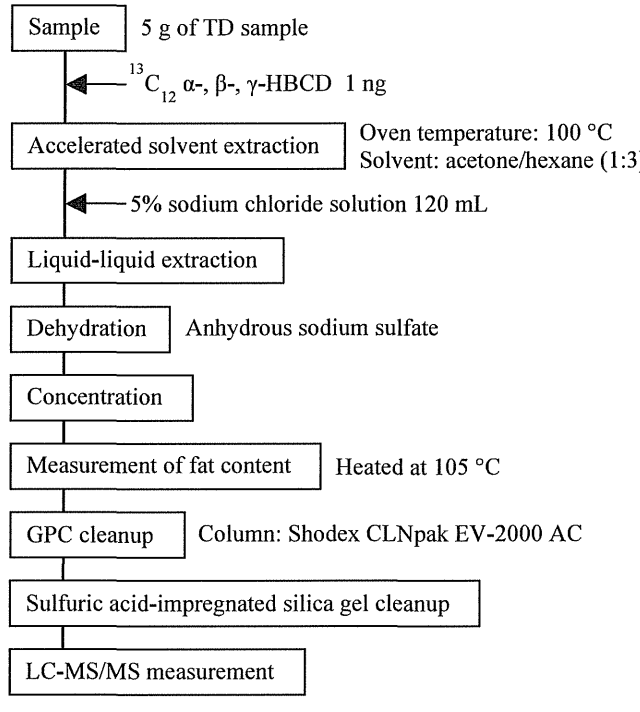


Fig.1 Protocol for HBCD analysis

Table 1 Concentrations of HBCD in the TD samples

| No. | Location | Fat content (%) | HBCD concentration (ng/g ww) | | | |
|---|---|---|---|---|---|---|
| | | | α-HBCD | β-HBCD | γ-HBCD | Total HBCD |
| 1 | A | 7.15 | 0.71 | ND | 0.08 | 0.79 |
| 2 | B | 6.27 | 0.66 | ND | 0.09 | 0.75 |
| 3 | C | 5.13 | 1.14 | ND | 0.10 | 1.24 |
| 4 | D | 5.98 | 0.70 | ND | 0.12 | 0.82 |
| 5 | E | 5.38 | 0.62 | ND | 0.10 | 0.72 |
| 6 | F | 4.76 | 0.43 | ND | 0.08 | 0.51 |
| 7 | G | 5.04 | 0.17 | ND | ND | 0.17 |
| 8 | H | 3.38 | 0.30 | ND | 0.04 | 0.34 |
| 9 | I | 3.90 | 1.14 | ND | 0.08 | 1.22 |
| 10 | J | 5.31 | 0.22 | ND | 0.03 | 0.25 |
| Mean | | | | | | 0.68 |

Table 2 Daily intake of HBCD in 10 locations in Japan

| No. | Location | Daily intake (ng/day) |
|---|---|---|
| 1 | A | 65.9 |
| 2 | B | 49.5 |
| 3 | C | 81.8 |
| 4 | D | 67.1 |
| 5 | E | 58.9 |
| 6 | F | 32.8 |
| 7 | G | 13.1 |
| 8 | H | 22.5 |
| 9 | I | 86.9 |
| 10 | J | 17.8 |
| Mean | | 49.6 |

# CONCENTRATION OF POLYCHLORINATED BIPHENYLS (PCBs) AND HYDROXYLATED PCBs IN SEAFOOD SAMPLES COLLECTED IN KYUSHU DISTRICT, JAPAN

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## Introduction

The hydroxylated polychlorinated biphenyls (OH-PCBs) are well known as metabolites of polychlorinated biphenyls (PCBs). The major route of OH-PCB formation in biota is oxidation via the cytochrome P450 enzyme system.[1] OH-PCBs of para- and meta-substituted OH with an adjacent chlorine atom have relatively high affinity for the thyroid hormone transport proteins.[2] The concentrations of OH-PCBs and their distribution in human serum and other biological and environmental media have been reported recently.[3–5]

The thorough elucidation of OH-PCBs in foods is very important to our understanding of both the metabolism of OH-PCBs in biota and the persistence of OH-PCBs in human tissues, neither of which has been determined.

## Materials and Methods

### *Chemicals and reagents*

Non-labeled OH-PCBs and PCBs, $^{13}C_{12}$-labeled OH-PCBs and PCBs were purchased from Wellington Laboratories (Guelph, ON, Canada) and AccuStandard (New Haven, CT, USA). Solvents such as acetonitrile, n-hexane and methanol of dioxin-analysis grade were purchased from Kanto Chemical (Tokyo).

### Samples

The seafood samples for the OH-PCBs and PCBs analysis were purchased from fish markets in the Kyushu district, Japan during the period 2013–2014 (Table 1). Each edible parts were collected, cut into small pieces and blended. The samples were maintained at temperatures below −20°C until analysis.

Table 1. The seafood samples used in this study

| No | Sample | Production regions | Fat content (%) |
|---|---|---|---|
| 1 | Sardine | Chugoku-Shikoku | 1.3 |
| 2 | Mackerel-1 | Kyushu | 3.8 |
| 3 | Mackerel-2 | Kyushu | 4.1 |
| 4 | Yellowtail | Kyushu | 3.5 |
| 5 | Japanese seabass-1 | Kyushu | 0.54 |
| 6 | Japanese seabass-2 | Kyushu | 0.41 |
| 7 | Sea bream-1 | Chugoku-Shikoku | 5.0 |
| 8 | Sea bream-2 | Kyushu | 0.96 |
| 9 | Tuna-1 | Kyushu | 18 |
| 10 | Tuna-2 | Kyushu | 2.8 |
| 11 | Horse mackerel-1 | Kyushu | 0.39 |
| 12 | Horse mackerel-2 | Kyushu | 0.11 |
| 13 | Horse mackerel-3 | Kyushu | 0.32 |
| 14 | Horse mackerel-4 | Kyushu | 1.4 |
| 15 | Cod | Tohoku | 0.078 |
| 16 | Largehead hairtail | Kyushu | 2.9 |

### *Sample preparation for OH-PCBs*

The protocol for the OH-PCBs analysis is illustrated in Figure 1.[4] First, $^{13}C_{12}$-labeled OH-PCBs (mono- to heptachlorinated 11 congeners) were spiked as surrogate standards in the samples. The samples were homogenized twice in acetonitrile, added to 10% NaCl(aq.) and extracted two times with n-hexane. The extracts were fractionated using a Florisil cartridge column (Sep-Pak Florisil Plus, Waters, Milford, MA). Non-polarity compounds were removed with 0.5% diethyl ether/n-hexane, and then the OH-PCBs were eluted with 50% acetone/methanol. OH-PCBs were

derivatized to methoxylated PCBs (OMe-PCBs) by reaction with dimethyl sulfate and 3N KOH/ethanol (70°C for 1 h). OMe-PCBs were extracted twice with n-hexane and then purified using a Florisil cartridge column. The fractions were concentrated to 0.05mL and spiked with $^{13}C_{12}$-labeled PCB111 as an injection standard.

***Sample preparation for PCBs***

The protocol for the PCB analysis is illustrated in Figure 2.[6] The sample was loaded into the extraction cell filled with Isolute. $^{13}C_{12}$-labeled PCBs (tri- to decachlorinated 20 congeners) were spiked as surrogate standards in the extraction cells. N-hexane was used as the extraction solvent of an accelerated solvent extractor. The extract was cleaned up using 1N KOH/ethanol, concentrated sulfuric acid, and a multilayer silica gel column. The clean-up solution was concentrated to 0.05 mL and spiked with $^{13}C_{12}$-labeled PCB111 as an injection standard.

***Analytical methods and instrumentation***

We identified and quantified the OH-PCBs (OMe-PCBs) and PCBs by gas chromatograph (GC; 6890GC, Agilent, Santa Clara, CA)/high-resolution mass spectrometer (Autospec Premier, Waters) at the resolution of R > 10,000 (10% valley). The injector temperature was 280°C, and 2 μL samples were injected in the splitless mode. The HT8-PCB capillary column (60 m × 0.25 mm i.d., Kanto Chemical) was used for the GC. Tri- to decachlorinated congeners of PCBs and mono- to heptachlorinated congeners of OH-PCB were determined.

**Results and Discussion:**

***The concentrations of OH-PCBs in the seafood samples***

Table 2 shows the concentrations of OH-PCBs in the seafood samples. OH-PCBs were detected in all of the seafood samples collected in this study. The concentrations of ΣOH-PCBs were 0.014–0.98 ng/g wet weight (ww); the mean concentration was 0.18 ng/g ww. The ΣOH-PCBs in the seafood samples were unrelated to the fish species or the fat content of the fish. The dominant congeners were classified as those in the fish species that exhibited OH-MonoCBs and OH-DiCBs and those that exhibited OH-PentaCBs and OH-HexaCBs.

***The concentration of PCBs in the seafood samples***

Table 3 shows the concentrations of PCBs in the seafood samples. PCBs were detected in all of the seafood samples. The concentrations of ΣPCBs were 0.20–50 ng/g ww, and the mean concentration was 9.2 ng/g ww. The ΣPCBs in the seafood samples were related to the fat content of the fish samples. The dominant congeners were pentaCBs and hexaCBs.

***Comparison of the persistence of OH-PCBs and PCBs***

Table 4 summarizes the presence of ΣOH-PCBs/ΣPCBs in the seafood samples. The concentrations of ΣOH-PCBs/ΣPCBs were 0.00094–0.25, and the mean was 0.051. The ΣOH-PCBs/ΣPCBs in the fish samples were not related to the fish species or the fat content. The levels of ΣOH-PCBs were low compared with those of ΣPCBs in all of the seafood samples. Our study findings demonstrate that OH-PCBs are accumulated in the human body by the intake of fish.

**Acknowledgements:**

This work was supported in part by a Health and Labour Sciences Research Grant from the Ministry of Health, Labour and Welfare, Japan.

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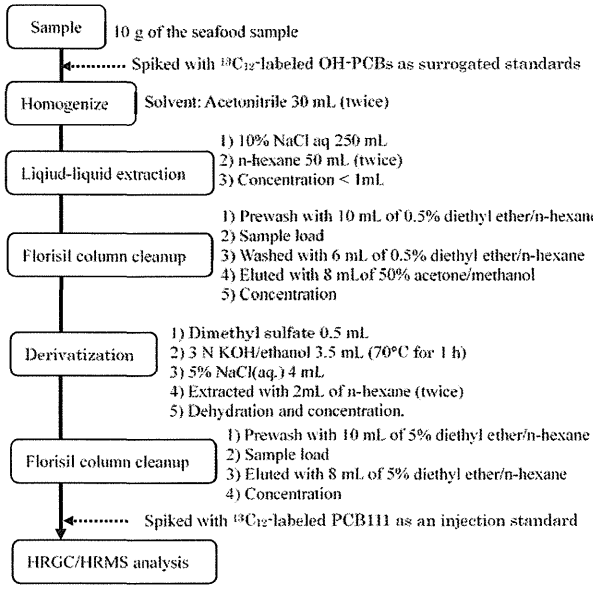


Figure 1. The protcol for OH-PCBs analysis.

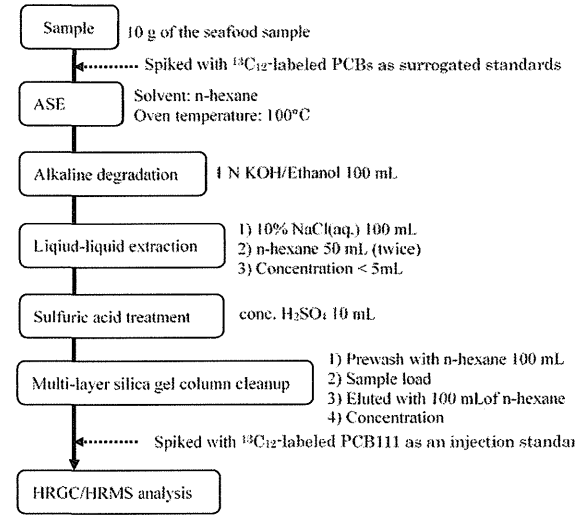


Figure 2. The protcol for PCBs analysis.

Table 2. The concentrations of OH-PCBs in the seafood samples.

(ng/g ww)

| | OH-MoCBs | OH-DiCBs | OH-TriCBs | OH-TeCBs | OH-PeCBs | OH-HxCBs | OH-HpCBs | ΣOH-PCBs |
|---|---|---|---|---|---|---|---|---|
| Sardine | 0.14 | 0.12 | 0.010 | 0.023 | 0.0058 | 0.0032 | 0.0034 | 0.31 |
| Mackerel-1 | 0.016 | 0.084 | 0.0035 | 0.010 | 0.0063 | 0.0029 | 0.0029 | 0.12 |
| Mackerel-2 | 0.038 | 0.19 | ND | 0.032 | 0.010 | 0.0040 | 0.0031 | 0.28 |
| Yellowtail | 0.016 | 0.074 | 0.00071 | 0.0097 | 0.0080 | 0.0084 | 0.0040 | 0.12 |
| Japanese seabass-1 | 0.037 | ND | 0.0031 | 0.013 | 0.0015 | 0.0064 | 0.0032 | 0.064 |
| Japanese seabass-2 | 0.0038 | 0.0026 | 0.0010 | 0.0019 | 0.0021 | 0.0033 | 0.0037 | 0.018 |
| Sea bream-1 | 0.011 | 0.020 | 0.0016 | 0.0077 | 0.0042 | 0.0031 | 0.0028 | 0.050 |
| Sea bream-2 | 0.0010 | 0.0020 | 0.0010 | 0.0023 | 0.0020 | 0.0035 | 0.0031 | 0.015 |
| Tuna-1 | 0.27 | 0.44 | 0.0018 | 0.039 | 0.21 | 0.0082 | 0.0083 | 0.98 |
| Tuna-2 | 0.066 | 0.097 | 0.00023 | 0.011 | 0.058 | 0.0079 | 0.0051 | 0.24 |
| Horse mackerel-1 | 0.17 | 0.16 | 0.014 | 0.026 | 0.0049 | 0.0042 | 0.0039 | 0.38 |
| Horse mackerel-2 | 0.011 | 0.0088 | 0.0013 | 0.0040 | 0.014 | 0.0035 | 0.0058 | 0.049 |
| Horse mackerel-3 | 0.013 | 0.016 | 0.0090 | 0.026 | 0.024 | 0.012 | 0.0062 | 0.11 |
| Horse mackerel-4 | 0.0058 | 0.013 | ND | 0.0015 | 0.0044 | 0.0043 | 0.00012 | 0.029 |
| Cod | ND | 0.00098 | 0.00059 | 0.0018 | 0.0016 | 0.0044 | 0.0051 | 0.014 |
| Largehead hairtail | 0.013 | 0.036 | 0.0013 | 0.0020 | 0.0053 | 0.0037 | 0.0046 | 0.066 |
| Mean | 0.051 | 0.079 | 0.0031 | 0.013 | 0.023 | 0.0052 | 0.0041 | 0.18 |
| Min. | ND | ND | ND | 0.0015 | 0.0015 | 0.0029 | 0.00012 | 0.014 |
| Max. | 0.27 | 0.44 | 0.014 | 0.039 | 0.21 | 0.012 | 0.0083 | 0.98 |

Table 3. The concentration of PCBs in the seafood samples.

(ng/g ww)

| | TrCBs | TeCBs | PeCBs | HxCBs | HpCBs | OcCBs | NoCBs | DeCB | ΣPCBs |
|---|---|---|---|---|---|---|---|---|---|
| Sardine | 0.053 | 0.22 | 0.41 | 0.75 | 0.38 | 0.066 | 0.0094 | 0.0074 | 1.9 |
| Mackerel-1 | 0.062 | 0.18 | 0.43 | 0.76 | 0.41 | 0.049 | 0.014 | 0.012 | 1.9 |
| Mackerel-2 | 0.19 | 0.96 | 2.4 | 3.3 | 1.4 | 0.19 | 0.038 | 0.028 | 8.5 |
| Yellowtail | 0.26 | 1.6 | 5.4 | 10 | 4.9 | 0.66 | 0.093 | 0.086 | 23 |
| Japanese seabass-1 | 0.14 | 0.65 | 1.2 | 1.5 | 0.47 | 0.072 | 0.0053 | 0.0019 | 4.1 |
| Japanese seabass-2 | 0.34 | 1.3 | 2.5 | 2.7 | 0.90 | 0.14 | 0.012 | 0.0046 | 7.8 |
| Sea bream-1 | 0.35 | 1.1 | 1.9 | 2.4 | 0.99 | 0.12 | 0.015 | 0.011 | 6.9 |
| Sea bream-2 | 0.94 | 2.6 | 4.5 | 5.6 | 2.4 | 0.31 | 0.038 | 0.023 | 16 |
| Tuna-1 | 0.79 | 5.1 | 15 | 20 | 8.0 | 1.1 | 0.20 | 0.12 | 50 |
| Tuna-2 | 0.16 | 0.82 | 2.5 | 3.3 | 1.4 | 0.22 | 0.043 | 0.027 | 8.5 |
| Horse mackerel-1 | 0.030 | 0.12 | 0.35 | 0.62 | 0.34 | 0.046 | 0.0028 | 0.0011 | 1.5 |
| Horse mackerel-2 | 0.047 | 0.23 | 0.58 | 1.1 | 0.77 | 0.12 | 0.0083 | 0.0047 | 2.8 |
| Horse mackerel-3 | 0.039 | 0.11 | 0.20 | 0.35 | 0.22 | 0.038 | 0.0049 | 0.0042 | 0.96 |
| Horse mackerel-4 | 0.065 | 0.20 | 0.44 | 0.71 | 0.43 | 0.071 | 0.012 | 0.014 | 1.9 |
| Cod | 0.029 | 0.061 | 0.064 | 0.040 | 0.0093 | 0.0010 | ND | 0.00037 | 0.20 |
| Largehead hairtail | 0.18 | 0.80 | 1.9 | 4.4 | 2.5 | 0.32 | 0.018 | 0.0047 | 10 |
| Mean | 0.23 | 1.0 | 2.5 | 3.6 | 1.6 | 0.22 | 0.034 | 0.022 | 9.2 |
| Min. | 0.029 | 0.061 | 0.064 | 0.040 | 0.0093 | 0.0010 | ND | 0.00037 | 0.20 |
| Max. | 0.94 | 5.1 | 15 | 20 | 8.0 | 1.1 | 0.20 | 0.12 | 50 |

Table 4. The presence of ΣOH-PCBs/ΣPCBs in the seafood samples.

| | Fat content ( % ) | ΣPCBs ( ng/g ww ) | ΣOH-PCBs ( ng/g ww ) | ΣOH-PCBs/ΣPCBs |
|---|---|---|---|---|
| Sardine | 1.3 | 1.9 | 0.31 | 0.16 |
| Mackerel-1 | 3.8 | 1.9 | 0.12 | 0.063 |
| Mackerel-2 | 4.1 | 8.5 | 0.28 | 0.033 |
| Yellowtail | 3.5 | 23 | 0.12 | 0.052 |
| Japanese seabass-1 | 0.54 | 4.1 | 0.064 | 0.016 |
| Japanese seabass-2 | 0.41 | 7.8 | 0.018 | 0.0023 |
| Sea bream-1 | 5.0 | 6.9 | 0.050 | 0.0072 |
| Sea bream-2 | 0.96 | 16 | 0.015 | 0.00094 |
| Tuna-1 | 18 | 50 | 0.98 | 0.020 |
| Tuna-2 | 2.8 | 8.5 | 0.24 | 0.028 |
| Horse mackerel-1 | 0.39 | 1.5 | 0.38 | 0.25 |
| Horse mackerel-2 | 0.11 | 2.8 | 0.049 | 0.018 |
| Horse mackerel-3 | 0.32 | 0.96 | 0.11 | 0.11 |
| Horse mackerel-4 | 1.4 | 1.9 | 0.029 | 0.015 |
| Cod | 0.078 | 0.20 | 0.014 | 0.070 |
| Largehead hairtail | 2.9 | 10 | 0.066 | 0.0066 |
| Mean | 2.9 | 9.2 | 0.18 | 0.051 |
| Min. | 0.078 | 0.20 | 0.014 | 0.00094 |
| Max. | 18 | 50 | 0.98 | 0.25 |

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Article

# Detection of Aryl Hydrocarbon Receptor Activation by Some Chemicals in Food Using a Reporter Gene Assay

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Academic Editor: Andrea Buettner
Received: 20 November 2015; Accepted: 22 February 2016; Published: 25 February 2016

**Abstract:** The purpose of this study was to examine whether a simple bioassay used for the detection of dioxins (DXNs) could be applied to detect trace amounts of harmful DXN-like substances in food products. To identify substances with possible DXN-like activity, we assessed the ability of various compounds in the environment to bind the aryl hydrocarbon receptor (AhR) that binds specifically to DXNs. The compounds tested included 19 polycyclic aromatic hydrocarbons (PAHs), 20 PAH derivatives (nitrated, halogenated, and aminated derivatives), 23 pesticides, six amino acids, and eight amino acid metabolites. The AhR binding activities (AhR activity) of these compounds were measured using the chemical activated luciferase gene expression (CALUX) reporter gene assay system. The majority of the PAHs exhibited marked AhR activity that increased in a concentration-dependent manner. Furthermore, there was a positive link between AhR activity and the number of aromatic rings in the PAH derivatives. Conversely, there appeared to be a negative correlation between AhR activity and the number of chlorine residues present on halogenated PAH derivatives. However, there was no correlation between AhR activity and the number and position of substituents among nitrated and aminated derivatives. Among the pesticides tested, the indole-type compounds carbendazim and thiabendazole showed high levels of activity. Similarly, the indole compound tryptamine was the only amino acid metabolite to induce AhR activity. The results are useful in understanding the identification and characterization of AhR ligands in the CALUX assay.

**Keywords:** aryl hydrocarbon receptor; reporter gene assay; food hygiene; polycyclic aromatic hydrocarbon; pesticide; amino acid

## 1. Introduction

Food products can be contaminated by a number of harmful substances, including dioxins (DXNs) and polycyclic aromatic hydrocarbons (PAHs); previous studies have focused on evaluating the health risks associated with these substances as well as ways to manage these health risks [1,2]. Human exposure to DXNs mainly occurs through foods, such as fish, meat, and dairy products. PAHs are formed mainly during cooking and during processes, such as smoking. There are currently 16 PAHs that are considered worthy of health concern by the EU (EU priority PAHs); the EU has set maximum

limits of PAHs in foodstuffs. Additionally, some pesticides can be detected at relatively high rates in foodstuffs. These findings prompted us to investigate the aryl hydrocarbon receptor (AhR) activities of DXNs, PAHs, and pesticides. Although trace amounts of these contaminants pose a low health risk, the risk management of the maximum limits of their residual levels is crucial to food safety.

The DXN and PAH families are comprised of various analogs that have similar chemical structures. Detection and quantification of these compounds in food products are currently achieved using a GC-MS approach, wherein the levels of DXNs and PAHs are determined using standardized methods as indicators [3,4]. However, because this approach is both complicated and costly, various simplified analytical methods have been proposed. For example, bioassays using AhR are commonly performed to measure levels of environmental pollutants, including DXNs [5]. Indeed, these assays led to the discovery that AhR is a dioxin receptor. It has, therefore, been suggested that AhR binding could be associated with the biological toxicity of these pollutants. In addition, AhR was reported to be activated by binding of PAHs [6]. The AhR-binding properties of PAHs could enable bioassay screening of foodstuffs for both DXNs and PAHs. Furthermore, because AhR bioassays are rapid and inexpensive, they could provide a convenient alternative to the current screening methods for toxic compounds, as well as other aforementioned substances.

To assess the efficacy of such an assay for detecting food contaminants, we developed a simple AhR-based bioassay technique to detect potentially hazardous substances with DXN-like activity, and determined whether there was a correlation between chemical structure and AhR activity. For this assay, AhR activity was evaluated using the chemical activated luciferase gene expression (CALUX) system, which was originally designed to detect DXNs [7]. The CALUX assay has been widely used for screening for dioxins in food and feed [8,9]. It is important to know the dioxin-like activity for various compounds in food to understand the possible influence of these compounds on our screening assay. The compounds examined included 39 PAHs (including nitrated derivatives, halogenated derivatives, and aminated derivatives), 23 pesticides, and 14 amino acids or amino acid metabolites.

## 2. Experimental Section

### *2.1. Chemicals and Reagents*

The following 39 PAHs (including nitrated, halogenated, and aminated derivatives) were used in this study: benzo(*c*)fluorene, 1,2-benzanthracene (benzo(*a*)anthracene), cyclopenta(*c,d*)pyrene, chrysene, 5-methylchrysene, benzo(*b*)fluoranthene, benzo(*k*)fluoranthene, benzo(*j*)fluoranthene, benzo(*a*)pyrene, indeno(1,2,3-*c,d*)pyrene, dibenzo(*a,h*)anthracene, benzo(*g,h,i*)perylene, dibenzo(*a,l*)pyrene, dibenzo(*a,e*)pyrene, dibenzo(*a,i*)pyrene, dibenzo(*a,h*)pyrene, 1-amino-4-nitronaphthalene, 9,10-dinitroanthracene, 1,3-dinitronapthalene, 1,5-dinitronaphthalene, 1,8-dinitronaphtalene, 2-nitroanthracene, 9-nitroanthracene, 7-nitrobenzo(*a*)anthracene, 6-nitrobenzo(*a*)pyrene, 1-nitronaphthalene, 2-nitronaphthalene, 1-chloronaphthalene, 2-chloronaphthalene, 1,4-dichloronaphthalene, octachloronaphthalene, 1,2,3,4-tetrachloronaphthalene, 1-aminoanthracene, 2-aminoanthracene, 1-aminonaphthalene, 1,8-diaminonaphthalene, naphthalene, anthracene, and fluorene were purchased from Kanto Chemical (Tokyo, Japan). The following 23 pesticide residues were also used: malathion, chlorpyrifos, diazinon, prothiofos, pirimiphos-methyl, fenitrothion, ethyl-*p*-nitrophenyl phenylthiophosphonothiate (EPN), tolclofos-methyl, parathion-methyl, phenthoate, chlorpyrifos-methyl, methidathion, imazalil, carbendazim, leucomalachite green, imidacloprid, acetamiprid, thiabendazole, azoxystrobin, tribenuron-methyl, flufenoxuron, pyraclostrobin, and kresoxim-methyl (Kanto Chemical). The amino acids L-tryptophan, L-glutamic acid, L-tyrosine, L-lysine, L-arginine, and histidine, as well as the amino acid metabolites tryptamine, 4-aminobutanoic acid, tyramine, putrescine, cadaverine, and histamine were purchased from Wako Pure Chemical Industries (Osaka, Japan). L-Ornithine and agmatine were obtained from Tokyo Chemical Industry (Tokyo, Japan) (Table 1, Figure 1). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and dimethyl sulfoxide (DMSO) were purchased from Wako Pure Chemical Industries. All chemicals were of analytical grade.