摂取量推定を目的とした分析法開発研究

研究目的

健康への影響が懸念され，今後摂取量を評価すべきと考えられる化学物質が選択された場合，TD 試料あるいは広範囲の食品に適用可能な分析法を確立することは，化学物質の健康影響評価における基本的な段階である．本研究分野では，食品からの摂取量評価が必要と考えられる有害物質の分析法開発を目的として，ダイオキシン類の迅速分析法開発とそれを用いた食品由来ダイオキシン様物質の探索，食品中 PCB 代謝物の分析法開発，食品中の多環芳香族炭化水素(PAHs)分析法の開発，有機臭素化合物分析法開発を行った．

研究方法及び研究結果

ダイオキシン類の迅速分析法開発

スクリーニング法の実用化をめざし，高感度 CALUX アッセイの開発を行い，高感度 CALUX アッセイの魚試料への適用性について詳細に検討し，スクリーニング法としての利用について考察した．

平成 22 年は，高感度 CALUX アッセイの基本性能を評価するために，種々のダイオキシン類に対する応答性を求め，さらに魚試料のマトリックスの影響の評価を目的として，希釈直線性試験を実施した．WHO-TEF が定められている 29 種の異性体に対する応答性は，多くの異性体で WHO-TEF の数倍以内の比率に収まっていた．特に，魚の毒性等量濃度において占める割合の高い異性体である，2,3,7,8-TCDD，1,2,3,7,8-PeCDD，2,3,7,8-TCDF，1,2,3,7,8-PeCDF，2,3,4,7,8-Pe CDF 及び 3,3',4,4',5-PeCB における，本法の応答性は良好であった．魚試料から調製した試験液を段階希釈し，希釈直線性試験を行った結果，PCDD/Fs 測定及び Co-PCBs 測定の両者において，希釈操作により定量値が 2 倍程度増加する場合が認められ，マトリックスの影響が疑われた．魚試料 7 試料の高感度 CALUX アッセイによる分析結果と，HRGC-HRMS 分析によるダイオキシン毒性等量濃度を比較した．PCDD/Fs 測定における両者の相関係数は 0.93，Co-PCBs 測定における相関係数は 0.99 であった．高感度 CALUX アッセイ結果と HRGC-HRMS 分析結果の比は，PCDD/Fs で約 4 倍，Co-PCBs で約 0.8 倍であった．

平成 23 年は，牛乳，鶏卵および豚肉を用いて添加回収試験を行った．ダイオキシン類の回収率の変動は大きく，回収率が低い場合が多かった．試験溶液に含まれる夾雑物の影響を評価するため，豚肉の試験溶液への添加回収試験を実施したところ，回収率は 100%となった．以上の結果から，前処理のいずれかの操作でダイオキシン類が損失したため，回収率の低下が生じたと考えられた．脂肪抽出量の変動は小さく，精製操作が低回収率の原因と考えられたため，活性炭カラムの影響を検討した．回収率は大きく変動し，低回収率になる場合が認められ，活性炭カラムの精製操作について再検討する必要があった．

平成 24 年は，試料マトリックスの影響を検討するため，試験溶液にダイオキシン類を添加して，回収試験を実施した．PCDD/Fs の回収率は 90～110%，Co-PCBs の回収率は 93～105%であった．魚試料への添加回収試験での PCDD/Fs の

回収率は 74～95%，PCB 126 の回収率は 76～95%であったことから，試験液の調製の際にダイオキシン類の大きな損失はないと考えられた．また，5 日間のくり返し測定の変動係数は，PCDD/Fs が 17～21%，Co-PCBs は 12～23%であった．スクリーニング法としては許容範囲内の変動であると考えられるが，顕著に低い測定値が得られることがあったため，魚試料を 2 試行で測定し，その平均値を試料濃度とすることとした．魚試料の HRGC/HRMS 分析によるダイオキシン類毒性等量と本アッセイ法結果の間には，良好な相関が認められ，本アッセイは市販魚中のダイオキシン類毒性等量濃度のスクリーニング法として有用であると考えられた．これらの結果から得られた回帰直線の 95%予測区間の下限値を，カットオフ値に設定することで，2.0 pg-TEQ/g 以上のダイオキシン類を含む魚試料のスクリーニングに利用することが可能であると考えられた．

ダイオキシン類の迅速分析法を用いた食品由来ダイオキシン様物質の探索研究

CALUX アッセイはアリル炭化水素レセプター（AhR）とダイオキシン等の結合を利用した方法である．この方法を使用する際には，ダイオキシン以外のダイオキシン様物質の影響をいかに取り除くかが課題となる．しかし食品中のダイオキシン様物質に関する情報は少なく，バイオアッセイによる迅速測定法の信頼性確保のためにはより多くの基礎データの集積が必要不可欠となる．またそれらデータは，天然のダイオキシン様活性物質として，健康影響の観点からも調査すべき課題であると考えられ，AhR の機能解明への応用も期待される．

本研究では，CALUX アッセイを用いて，植物エキス等の AhR 活性を評価した．平成 22 年には，野菜，果物，およびハーブ，健康食品エキス原料の試料 30 種類について，CALUX アッセイによる AhR 活性を測定し，ホウレンソウ，ブロッコリー，ジャガイモ，セロリ，レモン，グレープフルーツ，ローズマリー，セージに高濃度域で AhR 活性が認められた．

平成 23 年は，ローズマリーの 80%エタノール抽出物を分画精製し，2 種の化合物を単離した．これらの化合物は Rosmaninic acid 及び Nepitrin (6-Methoxyluteolin 7- glucoside)と同定され，AhR 活性が認められた．ローズマリー中の AhR 活性因子が天然由来成分であることを確認するため，酢酸エチル分画物のダイオキシン類含有量を HR-GC/MS により測定した．ダイオキシン含有量は検出限界近くの低濃度であり，天然アゴニストの存在が確認された．

平成 24 年は，ローズマリーの AhR 活性画分から，6 種の化合物を単離し，構造解析を行った．単離した 6 種の化合物中 4 化合物から AhR 活性が認められた．

主要な生薬エキス 30 種について AhR 活性を測定したところ，オウゴン，カンゾウ，カッコン，次いでクジン，チョウトウコウに強い AhR 活性が認められた．活性の強かったオウゴン由来の 5 成分が AhR 活性を示した．天然由来成分 38 種の AhR 活性を評価した結果，インドール骨格及びフラノクマリン骨格を持つ成分に AhR 活性が認められた．そこで，インドール骨格を有する化合物 17 種の AhR 活性を評価したところ，大半の化合物に活性が認められた．

食品中 PCB 代謝物の分析法開発

食品中の PCB 代謝物分析法の確立を目的とし，OH-PCBs を誘導体化せずに測定する方法（非誘導体化法）とメトキシ体（OMe-PCBs）に誘導体化して測定する方法（メチル化法）を

検討した.

平成22年は,非誘導体化法に使用可能なキャピラリーカラムを選択し，VF5MS（長さ 30m, 内径 0.25mm, 膜厚 0.1 μm）を HRGC/HRMS に装着して行う GC 条件を確立した．メチル化法開発研究では，誘導体化条件及び GC 条件を検討した．非誘導体化法とメチル化法を比較すると,装置感度はメチル化法がやや優れているが,反応の安定性に問題があり，非誘導体化法を採用することとした.

平成 23-24 年は抽出・精製法を検討した．フロリジル固相カラムを用いた魚抽出物の精製では不十分であり，追加精製法として C18 カラムを用いたところ良好な回収率が得られた．この結果より，ASE で抽出し，硫酸処理を行った後フロリジルカラム精製を行い，さらに C18 カラム精製を行う方法を確立した.

魚試料に ^{13}C-OH-PCBs 標準品を添加した試料からの回収率を評価したところ，7 塩素化物では当該ピークの定量性が悪く，評価が困難であったが，5 塩素化物は 72%と 78%，6 塩素化物が 90%と 80%となった．PCB の濃度が明らかなブリ試料の OH-PCBs 分析を行った結果，一部の OH-PCBs 異性体が検出され，定量値（湿重量あたり）は 4-OH-CB120 が 18 pg/g, 4-OH-CB159 が 2.5 pg/g, 4-OH-CB172 が 1.1 pg/g, 4-OH-CB187 が 2.5 pg/g と見積もられた.

<u>食品中の多環芳香族炭化水素(PAHs)分析法の開発</u>

PAHsについては種々の化合物が存在するが,欧州食品科学委員会(SCF)や食品添加物専門家会議（JECFA）を中心にリスク評価が行われ,モニタリングすべき16種のPAHs（以下, PAHs16種と表記）が提案されている．また，日本では食品中のPAHsの基準値は設定されていないが,現在，EU，カナダ，中国及び韓国で食品中のBAPに基準値が設定されている．さらに，EUでは Benzo[a]pyrene(BAP)， Benzo[a]anthracene (BAA)， Chrysene(CHR)， Benzo[b]fluoranthene(BBF)を含めたPAHs4種の合計値について2012年9月より基準値が施行されている.

本研究では, PAHsの含有が懸念される燻製食品を対象に，PAHs16種を分析するGC/MS/MS法を検討した．平成22年は，GC注入口温度,GC-MS/MS条件を検討し,プリカーサーイオン,プロダクトイオン，コリジョンエネルギーを最適化した．平成23年は内標準物質の選択を行った．13C標識体は測定対象物質のモニターイオンに^{13}C標識体由来のフラグメントピークが出現する現象が認められた．一方，D標識化合物からは測定対象物質の溶出位置にピークは確認されず，内標準物質としてD標識体が適していると結論した．また検量線の範囲を決定した.前処理に使用する固相ミニカラムを検討し,PSAミニカラムではPAHs16種の回収率は80～122%となり，DIP及びDHPも良好な回収率が得られた．以上の分析条件で，ウィスキーに1.0 μg/kgを添加し，添加回収試験を行った．PAHs16種の真度は80%以上，併行精度は2.4%以下であった．魚燻製食品に2.0 μg/kgを添加した添加回収試験では，4化合物で真度が120%を超え，その他のPAHsも真度が100%をこえる場合が多かった．基準値が設定されたPAHs4種の真度は96.0 ～113.5%，併行精度は0.3～1.7%で,比較的良好な結果であった．内標準物質の回収率は概ね80～120%の範囲であったが, D12-BAPの回収率が120%を超過するケースが認められた.

平成24年には，魚介類や畜肉類などの燻製食品を対象にした分析法の確立を目指し，PAHs抽出法及び精製方法について追加検討した．ま

た，BAP等の基準値濃度の適合判定への利用も検討し，PAHs含有実態調査を目的とした性能評価に加えて，BAP等の基準値濃度への適合判定を目的とした性能評価についても実施した．さらに，本分析法により認証標準試料や市販の燻製食品を分析し，その適用性を検証した．

GPC精製前の脱脂方法として，アセトニトリルーヘキサン分配を検討し，脂肪の98%を除去できた．GPC精製後の精製操作として，PSAミニカラムとシリカゲルミニカラムを検討したところ，無色の試験溶液が得られた．アセトニトリルーヘキサン分配，PSAミニカラム及びシリカゲルミニカラムにおけるPAHs16種の回収率は，80～107%，80～108%及び91～117%であった．

燻製サケ，燻製ソーセージ，燻製卵，及びかつお削り節の各ブランク試料より調製した試験溶液に検量線用標準溶液を添加したマトリックス標準溶液を用い，マトリクスの影響を評価した．燻製サケ，燻製ソーセージ及び燻製卵では，試料マトリックスの大きな影響を受けることなくPAHs16種の定量が可能であった．かつお削り節では，PAHs16種は試料マトリックスの大きな影響を受けることなく定量可能であったが，サロゲートの定量値は試料量が5 g以上であると120%を超える場合があった．そこで，かつお削り節については，測定対象物及びサロゲートの定量に，試料マトリックスが大きく影響しない試料量として2 gを設定した．

食品試料からのPAHsの抽出法としては，アルカリ分解抽出やソックスレー抽出が汎用されているが，危険性が高いこと，長時間を要すること等の問題点があるため，抽出操作の迅速化を目的として，ポリトロンによるPAHs抽出効率を評価した．燻製サバ及びかつお削り節を使用して，ポリトロン抽出とソックスレー抽出の結果を比較したところ，ポリトロン抽出により得られたPAHs濃度は，ソックスレー抽出の濃度の94～108%であり，両抽出法のPAHs抽出効率には大差が無かった．乾燥品である，かつお削り節及び貝凍結乾燥品を用いた比較でも，ポリトロン抽出のPAHs濃度はアルカリ分解抽出の96～104%，ソックスレー抽出の濃度の94～112%であった．この結果から，ポリトロン抽出のPAHs抽出効率は，アルカリ分解抽出及びソックスレー抽出とほぼ同等であると考えられた．

かつお削り節を用い，膨潤時間がPAHs抽出効率に与える影響を調べたところ，0～120minの範囲で大きな差はなかったが，予防的な観点から1時間とした．

燻製サケおよび燻製ソーセージでは0.5 μg/kg，燻製卵では1.0 μg/kg，かつお削り節では10 μg/kgを添加濃度として，PAHs含有実態調査を目的とした性能評価を実施した．燻製サケ及び燻製ソーセージでは，PAHs16種の真度は104～117%及び102～119%，併行精度は0.5～3.8%及び0.3～2.8%であった．燻製卵では，Dibenzo[a,h]pyrene (DHP)の真度が80%を僅かに下回ったが，それ以外は真度が90～104%，併行精度は0.5～4.1%であった．かつお削り節では，Dibenzo[a,l] pyrene (DLP)，Dibenzo[a,e]pyrene (DEP)及びDHPの真度が100%より20%以上乖離したが，それ以外のPAHsの真度は103～116%，併行精度は0.2%～3.1%と良好であった．

BAPの基準値への適合判定を目的とした性能評価も実施した．燻製魚および燻製畜肉におけるBAPの基準値を参考に，PAHs16種について2.0 及び5.0 μg/kgを添加濃度とした．燻製サケでは，添加した2濃度において，PAHs16種の真度は86～105%，併行精度は0.6～7.1%，及び室内精度は0.8～7.1%であった．サロゲートの回収率は87～109%であった．また，燻製ソーセージ

では，添加した2濃度において，PAHs16種の真度は77～116%，併行精度は0.4～5.9%，及び室内精度は1.0～12.7%であった．DHPにおいて，真度がやや低く，室内精度がやや大きい傾向が認められた．多くの国で基準値が定められているBAPについては，良好な真度及び精度が得られており，適合判定が可能であると考えられた．

認証標準試料（SRM2974a）を用い，実試料への適用検証を行った．CHRについては参考値と比較して2倍以上高い値が得られた．この試料にはTriphenyleneが含まれており，使用したカラムではTriphenylene とCHRの分離が困難であることから，CHRの分析値が高くなったと考えられる．EUで設定されているCHRを含むPAHs4種の基準値濃度への適合判定の目的には，本分析法の性能は適当ではなく，別途，CHRとTriphenyleneが分離できるような GCキャピラリーカラムを使用した分析が望まれる．

本法により，燻製魚19試料，燻製畜肉類10試料，燻製卵5試料，かつお削り節8試料を分析し，適用性を検証した．PAHs濃度は食品種により大きく異なった．燻製卵では，全てのPAHsが定量下限未満であった．燻製魚と燻製畜肉類では，BCL，BAA，CPP，及びCHR等が定量下限値以上となる頻度が高かった．かつお削り節では，他の試料よりも定量下限値が高いにもかかわらず，多くのPAHsが定量下限値以上となった．諸外国で基準値が設けられていることの多いBAPは，8試料中7試料で定量下限以上となり，その濃度は10 ～32 μg/kgであった．

有機臭素化合物分析法開発研究

平成23年は，DBDPEのHRGC-HRMSによる測定条件を検討するとともに，DBDPE及びその他の臭素系難燃剤について，より簡便で高感度な測定法を確立するため，LC-MS/MS及びGC-MS/MSを用いた測定も検討した．LC-MS/MS測定はDeBDEのみのモニタリングに有用であると考えられた．シリカゲルカラム，フロリジルカラム，液液分配，スルホキシドカラムによる精製を検討した．1-7群のTD試料及び魚試料について40～70%の範囲の回収率が得られた．第4群と第7群についてはDMSO/ヘキサン分配の替わりにスルホキシドカラムを用いて精製を行った結果も，ほぼ同等の回収率が得られた．

平成23年は，魚介類個別食品試料中の臭素系難燃剤の分析を行った．12検体中4検体の魚介類からDBDPEが検出され，検出濃度は5.86～8.08 pg/gであった．

C. 結論

ダイオキシン類の迅速分析法開発からは以下の結論が得られた．

高感度 CALUX アッセイの WHO-TEF が定められている 29 種の異性体に対する応答性は，多くの異性体で WHO-TEF の数倍以内の比率に収まっていた．魚試料の高感度 CALUX アッセイによる分析結果と，HRGC-HRMS 分析によるダイオキシン毒性等量濃度の相関係数は PCDD/Fs では 0.93，Co-PCBs では 0.99 であった．高感度 CALUX アッセイ結果と HRGC-HRMS 分析結果の比は，PCDD/Fs で約 4 倍，Co-PCBs で約 0.8 倍であった．5 日間のくり返し測定の変動係数は，PCDD/Fs が 17～21%，Co-PCBs は 12～23%で，スクリーニング法としては許容範囲内の変動であると考えられた．魚試料の HRGC/HRMS 分析によるダイオキシン類毒性等量と本アッセイ法結果の間には，良好な相関が認められ，本アッセイは市販魚中のダイオキシン類毒性等量濃度のスクリーニング法として有用であると考えられた．

ダイオキシン類の迅速分析法を用いた食品由

来ダイオキシン様物質の探索研究からは以下の結論が得られた.

CALUX アッセイによりホウレンソウ，ブロッコリー，ジャガイモ，セロリ，レモン，グレープフルーツ，ローズマリー，セージに高濃度域で AhR 活性が認められた. ローズマリーから AhR 活性を有する 6 種の化合物を単離した.

主要な生薬エキス 30 種中，オウゴン，カンゾウ，カッコン，次いでクジン，チョウトウコウに強い AhR 活性が認められた. 活性の強かったオウゴン由来の 5 成分が AhR 活性を示した. 天然由来成分 38 種の AhR 活性を評価した結果，インドール骨格及びフラノクマリン骨格を持つ成分に AhR 活性が認められた. そこで，インドール骨格を有する化合物 17 種の AhR 活性を評価したところ，大半の化合物に活性が認められた.

食品中 PCB 代謝物の分析法開発からは以下の結論が得られた.

OH-PCBs を誘導体化せずに測定する方法（非誘導体化法）とメトキシ体（OMe-PCBs）に誘導体化して測定する方法（メチル化法）を検討し，比較の結果非誘導体化法を採用した. 非誘導体化法における抽出・精製法を確立した. 回収率は，7 塩素化物では評価が困難であったが，5 塩素化物は 72%と 78%，6 塩素化物が 90%と 80%となった. PCB の濃度が明らかなブリ試料の OH-PCBs 分析を行った結果，一部の OH-PCBs 異性体が検出され，定量値（湿重量あたり）は 4-OH-CB120 が 18 pg/g，4-OH-CB159 が 2.5 pg/g, 4-OH-CB172 が 1.1 pg/g, 4-OH-CB187 が 2.5 pg/g と見積もられた.

食品中の多環芳香族炭化水素(PAHs)分析法の開発からは以下の結論が得られた.

PAHsの含有が懸念される燻製食品を対象に，PAHs16種を分析するGC/MS/MS法を検討した. GC-MS/MS条件を最適化した. 内標準物質としてD標識体が適していると結論した. ポリトロン抽出法，精製法，脱脂方法等を検討し，方法を確立した. PAHs含有実態調査を目的とした性能評価及び基準値への適合判定を目的とした性能評価を実施した. BAPについては良好な真度及び精度が得られており，適合判定が可能であると考えられた. 実試料への適用検証を行った. CHRでは高い値が得られ， 試料中に存在するTriphenyleneの影響と考えられた. 本法により，燻製魚19試料, 燻製畜肉類10試料, 燻製卵5試料, かつお削り節8試料を分析し, 適用性を検証した.

有機臭素化合物分析法開発研究からは以下の結論が得られた.

DBDPEのHRGC-HRMSによる測定条件を検討するとともに，LC-MS/MS及びGC-MS/MSを用いた測定も検討した. LC-MS/ MS測定はDeBDEのみのモニタリングに有用であると考えられた. シリカゲルカラム，フロリジルカラム，液液分配，スルホキシドカラムによる精製を検討した. 1-7群のTD試料及び魚試料について40～70%の範囲の回収率が得られた. 第4群と第7群についてはDMSO/ヘキサン分配の替わりにスルホキシドカラムを用いて精製を行った結果も，ほぼ同等の回収率が得られた.

魚介類 12 試料中臭素系難燃剤の分析を行った. 4 試料から DBDPE が検出され，検出濃度は 5.86～8.08 pg/g であった.

Ⅱ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル	発表誌名	巻号	ページ	出版年
1. Amakura, Y., Tsutsumi, T., Nakamura, M., Handa, H., Yoshimura, M., Matsuda, R., Yoshida, T.	Tectochrysin in propolis is a potent natural aryl hydrocarbon receptor ligand	*Planta Medica,*	76	1380	2010
2. Amakura, Y., Tsutsumi, T., Nakamura, M., Handa, H., Yoshimura, M., Matsuda, R., Yoshida, T.	Aryl hydrocarbon receptor ligand activity of commercial health foods	*Food Chemistry*	126	1515-1520	2010
3. Ashizuka, Y. , Nakagawa, R. , Yasutake, D. , Shintani, Y. , Hori, T. , Horie, M. , Tanaka, Y. , Tsutsumi, T.	Daily intake of brominated dioxins, Co-PCBs and brominated flame retardants estimated from market basket study	BFR2010			2010
4. Nakagawa, R. , Murata, S., Ashizuka, Y., Shintani, Y., Hori, T., Tsutsumi, T.	Hexabromocyclododecane determination in seafood samples collected from Japanese coastal areas.	Chemosphere	81	445-452	2010
5. 坂本智徳，赤木浩一，渡邉敬浩，松田りえ子，樋脇弘	食品中メチル水銀の定量分析のためのフェニル誘導体化 GC-MS 法の開発	*BUNSEKI KAGAKU*	61	327-333	2012
6. Amakura Y, Tsutsumi T, Nakamura M, Handa H, Yoshimura M, Matsuda R, Yoshida T	Characterization of natural AhR ligands in health foods estimated by in vitro reporter gene assay	*Organohalogen Compd.*	73	738-741	2011
7. Yasutake, D., Hori, T., Kurokawa, Y., Kajiwara, J., Tsutsumi, T., Amakura, Y.	The Measurement of Hydroxylated Polychlorinated Biphenyls without Derivatization using a high-resolution gas chromatograph / high- resolution	*Organohalogen Compd.*	73	625-628	2011

	mass spectrometer				
8. Ashizuka, Y., Yasutake, D., Nakagawa, R., Shintani, Y., Hori, T., Tsutsumi, T., Matsuda, R	Improvement of methods for analyzing brominated flame retardant in food.	*Organohalogen Compd.*	73		2011
9. 松田りえ子，渡邉敬浩	食品からの有害物質摂取量推定とその意義	*ファルマシア*	49	17-21	2013
10. 松田りえ子	食品からの有害物質摂取量推定	*食品衛生研究*	63	9-19	2013
11. Nakagawa R, Ashizuka Y, Hori T, Kajiwara J, Takahashi K, Tsutsumi T, Matsuda R	Dietary exposure to hexabromo cyclododecanes in Japan.	*Organohalogen Compounds*	74	819-822	2012
12. Ashizuka Y, Takahashi K, Yasutake D, Nakagawa R, Shintani Y, Hori T, Kajiwara J, Tsutsumi T, Matsuda R	Determination of brominated flame retardants in food from Japanese markets	*Organohalogen Compounds*	74	851-854	2012

Ⅲ．研究成果の刊行物別刷

separate cages throughout the gestational period and were acclimatized and fed in the same conditions. Animals in all group received tape water in whole gestational period. In 20th day of pregnancy, animals were anesthetized and their foetuses were extracted through a cesarean section. The placenta was excised, weighed, and the number and placement of implantation sites and other parameters were recorded. Mean number of live, dead or resorbed foetuses in animals receiving saffron extracts before the mating were dose dependently less than in the control group. A maximum decrease was observed in animals receiving 0.8% saffron solution. Saffron and its components may affect embryonic implantation and may result in contraceptive-like effects. References: 1. Rios J.L. et al. (1996) Phytother Res 10: 189 – 193.

P676 Tectochrysin in propolis is a potent natural aryl hydrocarbon receptor ligand

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The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that mediates the toxic and biological actions of many aromatic environmental pollutants such as dioxins. As part of an investigation to clarify the interaction of foods with AhR, we previously reported that excessive intake of foods containing AhR-activators may be conductive to promote dioxin-like toxicity though there would not be a problem following normal intake. Additionally, it is discussed that the signal transduction of natural AhR ligands, which occurs after AhR activation, should differ from that of dioxins [1]. In this study, we examined the binding ability of 50 extracts prepared from kinds of commercial supplements and health foods to the AhR using reporter gene assay. Though most tested samples did not show any luciferase induction at a high concentration level, propolis product showed activation of luciferase at high concentration range. To characterize the AhR-activating substances in the propolis product, its extract was subjected to fractionation with *n*-hexane, ethyl acetate, and water followed by estimation their AhR activities. HPLC analysis of the *n*-hexane fraction which showed AhR activity detected eight compounds including flavonoids such as tectochrysin and pinocembrin. Among their compounds, tectochrysin showed remarkable AhR activation. Recently, several papers reported that AhR activation may be involved in various immune system [2 – 4]. Therefore it is suggested that natural AhR ligands characterized in the present study may play some beneficial regulatory role in human. Acknowledgements: This work was supported by a Health and Labour Sciences Research Grant from the Ministry of Health, Labour and Welfare of Japan. References: 1. Amakura, Y et al. (2008) Phytochemistry. 69: 3117 – 3130. 2. Quintana, FJ et al. (2008) Nature. 453: 65 – 71. 3. Veldhoen, M et al. (2008), Nature. 453: 106 – 110. 4. Kimura, A et al. (2007), Proc. Natl. Acd. Sci. USA. 105: 9721 – 9726.

P677 Effects of polyphenols: Resveratrol and its natural analogues and tannic acid on DNA oxidative damage and apoptosis in human neutrophils

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Our earlier studies (1,2) showed that tannic acid and naturally occurring stilbenes resveratrol and its analogue pterostilbene, the common ingredients of berries fruits, are involved in the key events of the initiation and promotion stages of carcinogenesis. The latter included the inhibition of transcription factors responsible for the inflammatory response. The aim of this study was to evaluate the effect of these compounds on DNA oxidative damage and apoptosis in human polymorphonuclear neutrophils (PMN). In order to induce oxidative burst PMN were stimulated with 12Otetradecanoylphorbol13acetate. Treatment of PMN with the tested polyphenols at the concentration range which did not show cytotoxicity resulted in the reduced production of reactive oxygen species and subsequently DNA oxidative damage assessed by Single Cell Gel Electrophoresis (comet assay). Tannic acid caused the 50% decrease in DNA oxidative decomposition at the concentration as low as 1 µmol. Resveratrol caused a similar effect at a concentration 100 times higher. All tested polyphenols induced apoptosis by increasing the activity of procaspase3, phosphatidylserine translocation and loss of mitochondrial membrane potential. The highest proapoptotic activity was demonstrated by 3'5,4'trimethoxystilbene and tannic acid. This effect however was dependent on the dose. At the higher concentrations (50 µmol) an antiapoptotic effect was observed. Collectively ROS production in activated PMN seems to influence their lifespan and can be modulated by stilbenes and phenolic acids. Such activity might be useful in adjuvant therapy of inflammatory diseases. References: 1. Cichocki, M. et al. (2008) Mol.Nutr.Food Res. 52: S62-S70. 2. Cichocki, M. et al. (2010) Toxicology 268: 118 – 124.

Poster Young Researcher

P678 *In Vitro* multiplication used in preserving the *Arnica montana* L. species in the Romanian Bistrita Mountains

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Arnica montana is considered a rare and vulnerable species and is intensively harvested in Romania for medicinal aims. In the natural areas of the Romanian Bistrita Mountains, the preservation of the species is important by extending in this zone the results of the Conservation of Estern European Medicinal Plants – *Arnica montana* in Romania [3]. In the present paper we intend the possibility of using *in vitro* multiplication as a complementary component of the strategy to initiate and develop the species preservation in the mentioned area. Being an ever rarer species, we studied and elaborated diverse micropropagation techniques [1 – 2]. The final aim of the study is the reintroduction of the species in the montane grasslands where the species has been fully disappeared, and the introduction of new crops (genes) in small, endangered populations (to increase the heterozys), especially because the micropropagation techniques provides the advantage of a better control of the genetic material, and a relative large number of crops in a short period of time. For the *in vitro* regeneration, the biological material used to initiate the experimental cultures, originates from *Arnica montana* plantlets resulted after the germination of seeds from a natural population in the aimed area. Shoot formation was induced from excised shoot tip explants, on the Murashige and Skoog (MS, 1962) multiplication medium, supplemented with BA (1 mg/l) and ANA (0.1 – 0.3 mg/l) and solidified with 0.8% agar, we obtained a number of 2 – 3 neoplantlets/explant. The multiplication of the buds was followed by the rooting phase, the neoplantlets being transferred for *ex vitro* adaptation, 75 – 80% being the survival ratio. The plants obtained will be transferred, after invigoration, to the experimental field from the natural habitat. The succession of the *in situ* preservation techniques and the *ex situ* ones will be accompanied by comparative phytochemical screening of the generatively obtained variants as well as by *in vitro* multiplication. Acknowledgements: The work is sustained in the PNCDI-2 program financed by the Romanian Government – National R&D Agency References: 1. Butiuc-Keul A.L., Deliu C., Clonal propagation of *Arnica montana* L., a medicinal plant, *In Vitro* Cell Dev. Biol. – Plant (2001), 37, 581 – 585. 2. Cassells A.C., Walsh C., Belin M., Cambornac M., Robin J.R., Lubrano C., Establishment of a plantation from micropropagated *Arnica chamissonis* a pharmaceutical substitute from the endangered A. montana, Plant Cell, Tissue and Organ Culture (1999) 56, 139 – 144. 3. Wolfgang Kathe, Conservation of Estern – European Medicinal Plants – *Arnica montana* in Romania, Medicinal and Aromatic Plants (2006), 203 – 211.

P679 Inhibitory effects of *Cissus barbeyana* leaf extracts on α-amylase and α-glucosidase *in vitro*

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α-amylase and glucosidase inhibitors are used in the therapeutic management of type II diabetes mellitus. Inhibition of pancreatic α-amylase

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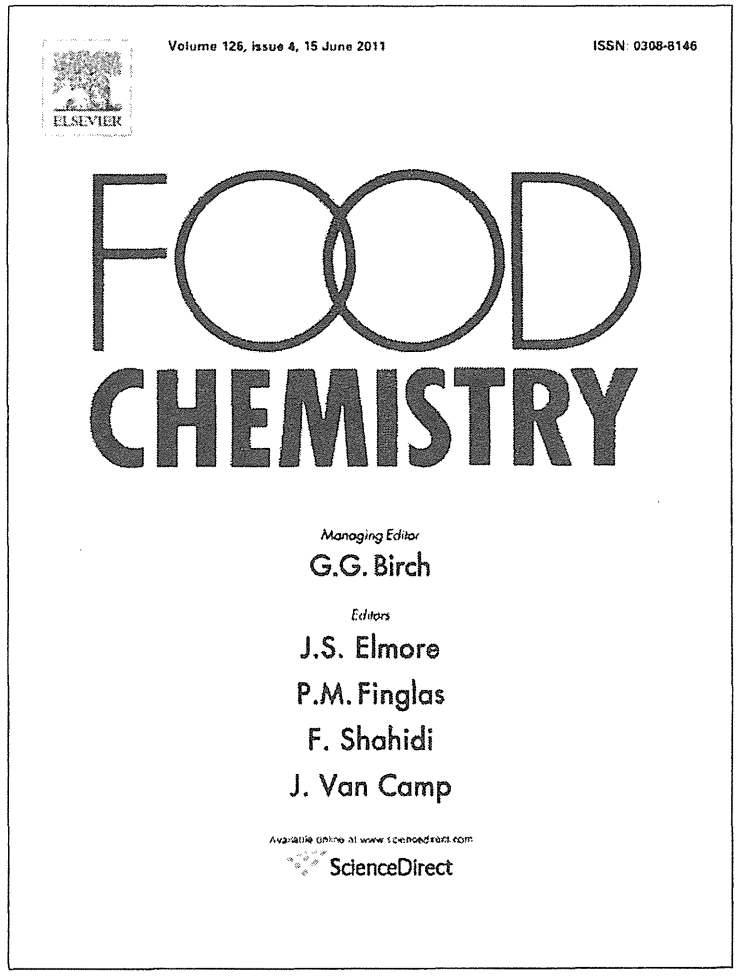

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Rapid Communication

Aryl hydrocarbon receptor ligand activity of commercial health foods

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ABSTRACT

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that mediates toxicological effects by binding to agonists such as dioxins. We previously reported the presence of natural dioxin-like ligands in foods. To further characterise natural ligands with dioxin-like activity, we examined the influence of 50 kinds of commercial supplement and health food on the AhR, using a reporter gene assay. Some samples, prepared using soybean, sesame, or propolis as an ingredient, were revealed to show AhR-binding activity, similar to that of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), at high concentrations. To characterise the AhR-activating substances in eight active samples, the respective extracts were subjected to fractionation with *n*-hexane, ethyl acetate, and water, followed by estimating their AhR activities. The *n*-hexane fraction of the propolis extract sample, and the ethyl acetate fractions of the other samples, showed AhR activity similar to that of TCDD, at a high concentration range. HPLC analysis of the active fractions identified isoflavones, such as daidzein and glycitein, and flavones, such as tectochrysin and chrysin, in the samples. Among these compounds, tectochrysin exhibited marked AhR activation. Flavonoids, which are characterised as natural AhR ligands, are known to have representative beneficial effects on human health. The natural AhR ligands identified in this study are known to be useful for human health. Therefore, it is considered that AhR may play a beneficial regulatory role in humans.
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1. Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that is present in mammalian cells or tissues. This receptor is also called a dioxin receptor because it binds to environmental pollutants (e.g., dioxins) and is involved in the manifestation of biotoxicity linked to xenobiotic AhR ligand exposure in animals, including cancers, reproductive impairment, and immunological impairment (Fujii-Kuriyama & Mimura, 2005; Machala, Vondráček, Bláha, Ciganek, & Neča, 2001; Nebert & Dalton, 2006). Although numerous xenobiotic ligands for AhR, such as dioxins, have been identified, the essential functions of AhR are largely unknown; therefore, AhR is still regarded as an orphan receptor. Natural AhR agonists and antagonists have been suggested to exist in foods in small amounts (Ashida, Fukuda, Yamashita, & Kanazawa, 2000; Jeuken et al., 2003). Moreover, we previously reported the presence of natural dioxin-like ligands in foods (Amakura et al., 2003, 2004, 2008). These agonists have been demonstrated to upregulate an AhR reporter gene at high concentrations compared with dioxins. Although normal meals are unlikely to represent a problem, the influence on health of natural AhR ligands in foods containing concentrated extracts, such as supplements, is of concern. Therefore, it is necessary to elucidate the actual characteristics of supplements in relation to AhR.

A simple technique to measure dioxins, using a biological assay based on the toxicity mechanism of dioxin (AhR-binding activity), has been established and recognised as the standard method for measuring environmental samples in Japan (JIS K0463, 2009). This biological assay provides a rapid and cost-effective method for screening. In contrast to instrumental analysis by conventional high-resolution GC/MS, which analyses each isomer of dioxins separately and integrates data, the biological assays provide only a comprehensive value. Compared with environmental samples, dioxins can be detected only in small amounts in ordinary food samples (Toyoda et al., 1999; Tsutsumi et al., 2001). Thus, when a biological assay is applied to the analysis of dioxins in foods, reliable data can be ensured by identifying and removing impurities exhibiting dioxin-like activity. However, few substances with such activity have been identified in foods, and further information is required on natural AhR-ligand-containing foods. Therefore,

Abbreviations: AhR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; CALUX, chemical activated luciferase gene expression.

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Table 1
List of supplements and health foods used for the estimation of AhR binding activity.

No.	Main materials	Type	No.	Main materials	Type
1	Agaricus mushroom	T	26	Propolis extract	C (S)
2	Amino acid/vitamin/mineral	T	27	Royal jelly extract	C
3	Astaxanthin	C (S)	28	Saw palmetto extract	C (S)
4	Barley grass (A)	P	29	Sesame	C (S)
5	Barley grass (B)	P	30	Soy isoflavone (A)	C (S)
6	Bilberry extract	C (S)	31	Soy isoflavone (B)	T
7	β-Carotene	T	32	Soy isoflavone (C)	T
8	Cassis extract	T	33	Soy isoflavone (D)	T
9	Chitosan	T	34	Soybean extract (A)	C (S)
10	Coenzyme Q10	C (S)	35	Soybean extract (B)	T
11	Fish oil (A)	C (S)	36	Tea (Chinese sweet tea)	TM
12	Fish oil (B)	C (S)	37	Tea (Echinacea)	TM
13	Garcinia extract	T	38	Tea (Eucommia)	TM
14	Ginkgo leaf extracts	T	39	Tea (Guava)	TM
15	Gymnema extract	T	40	Tea (Japanese mugwort)	TM
16	*Gymnema sylvestre* leaf extract	T	41	Tea (Loquat leaf)	TM
17	Haematococcus color	C (S)	42	Tea (Perilla herb)	TM
18	Kidachi aloe (pulp of *Aloe arborescens*)	T	43	Tea (Persimmon leaf)	TM
19	Lutein (A)	C (S)	44	Tea (Rooibos)	TM
20	Lutein (B)	C (S)	45	Turmeric	T
21	Maca extract	C	46	Turmeric extract (A)	C (S)
22	Onion extract	T	47	Turmeric extract (B)	T
23	Plant steroid	C (S)	48	Urazirogashi (leaf of *Quercus salicina*)	T
24	Processed vegetable (A)	T	49	Vitamin C	T
25	Processed vegetable (B)	T	50	Vitamins	C (S)

T, Tablet; C, Capsule; C (S), Soft Capsule; P, Powder; TM, Tea material.

accumulation of basic data is indispensable for ensuring the reliability of rapid measurements employing a biological assay.

In order to further characterise AhR ligands present in foods, we herein examined AhR activity, using an *in vitro* reporter gene assay called the chemical activated luciferase gene expression (CALUX) assay (Misaki et al., 2007; Overmeire et al., 2001), which has been applied for the rapid measurement of dioxins in food samples (Tsutsumi et al., 2003, 2006, 2008), for 50 kinds of supplement and health foods containing a high concentrations of their respective ingredients. Active sample extracts were subsequently fractionated, and reversed-phase HPLC analysis was conducted to characterise the AhR active fractions.

Table 2
Relative responses of the CALUX assay for selected supplements and health food samples.

Sample No.	$TCDD_{0.5}$[a] (mg/mL)
26	2.3
29	7.6
30	5.6
31	1.8
32	19.0
33	1.4
34	28.0
35	16.5
41	56.6
42	54.5

Each value is the mean of at least three replicates.
[a] Concentration producing luciferase activity equal to 0.5 ng/mL of TCDD. Calculated from the slope of the linear portion of each dose–response curve near the origin.

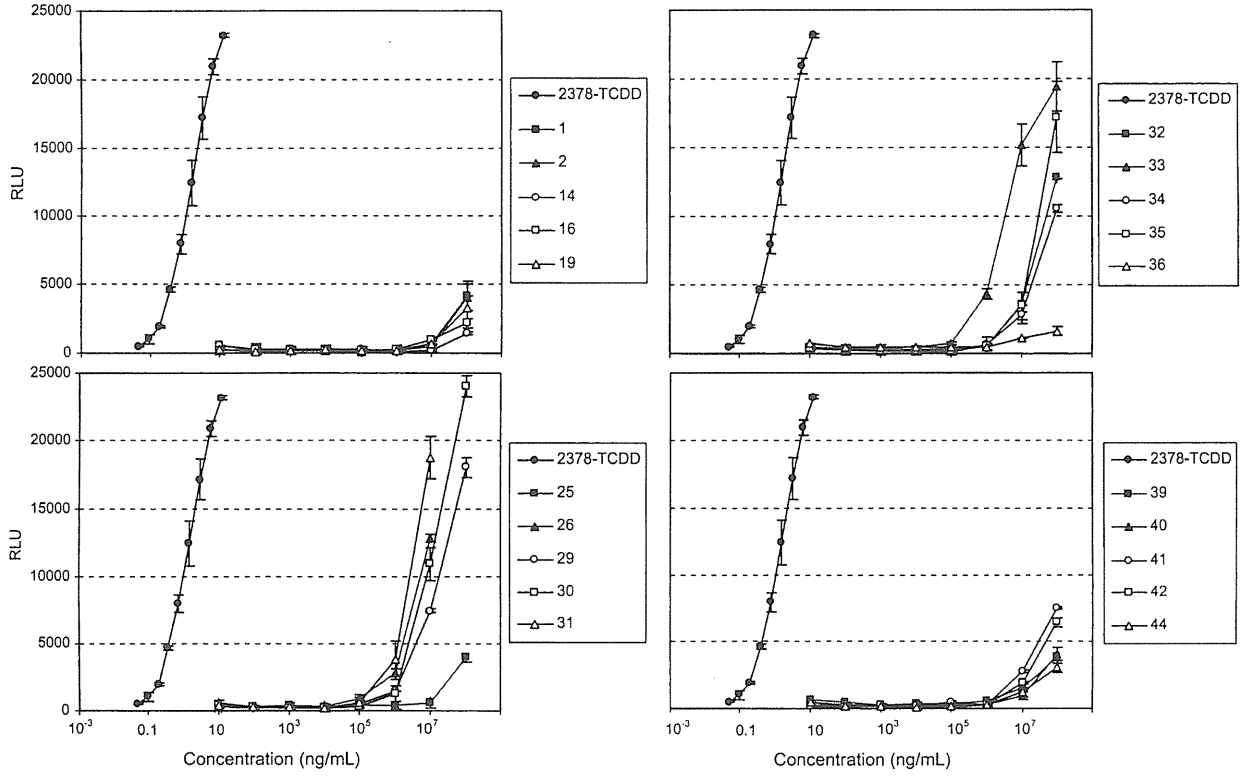

Fig. 1. Concentration–response curve of selected supplement and health food samples and TCDD for the induction of luciferase activity in the CALUX assay. Each point represents the mean of at least three replicate analyses. Results are expressed as means ± SD. All samples showed at several concentrations a statistically increased response in comparison with the blank ($p < 0.05$).

2. Materials and methods

2.1. Samples and reagents

TCDD and reagents used in the present study were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), Funakoshi (Tokyo, Japan), and Tokyo Kasei (Tokyo, Japan), and 50 supplements and health foods, as shown in Table 1, were from drug stores in Japan (in 2007).

2.2. Extraction and isolation

The samples were prepared as follows: Tablets were powdered and the contents of capsules and soft capsules were used for sam-

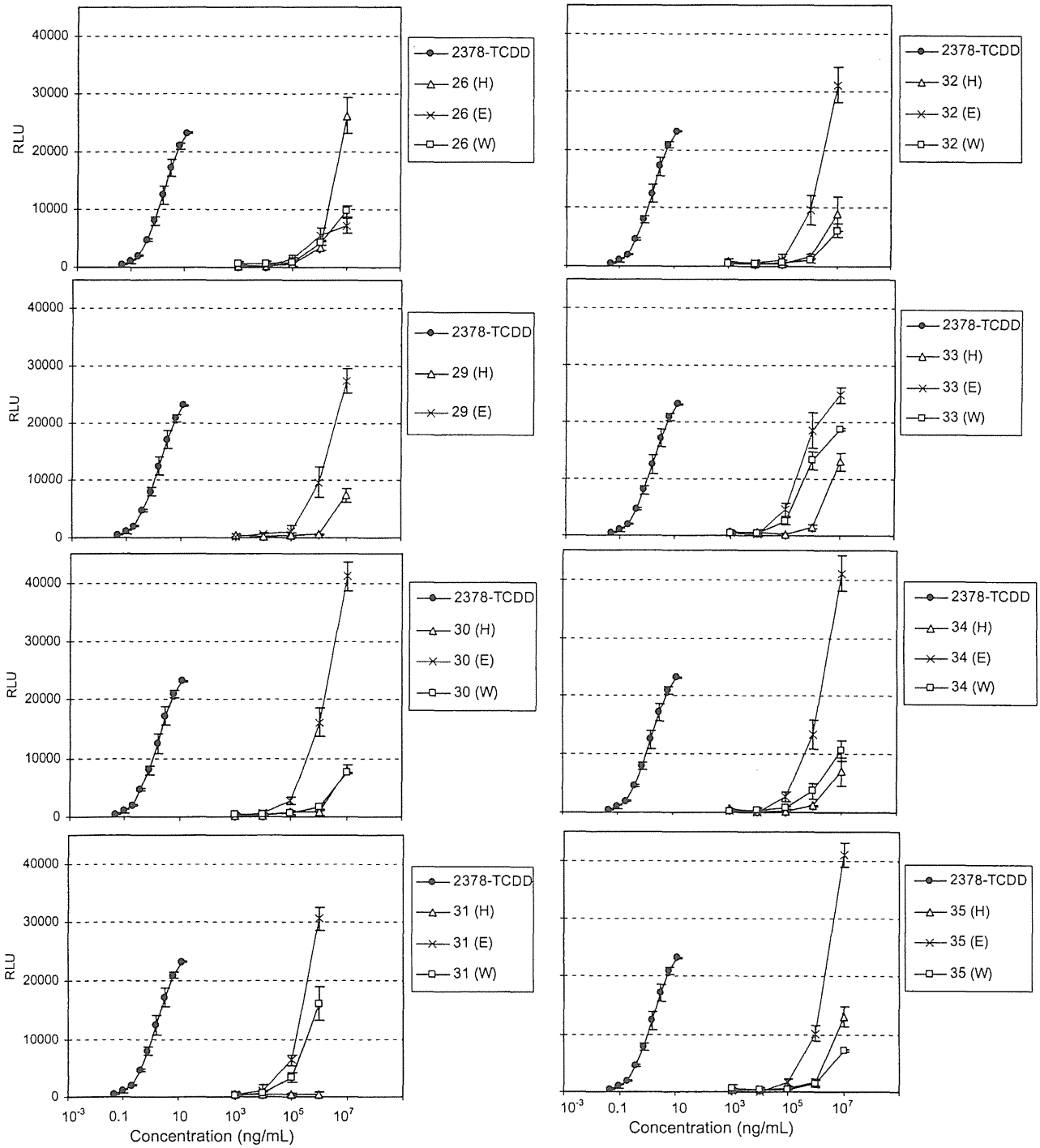

Fig. 2. Concentration–response curve of fractions of active samples and TCDD for the induction of luciferase activity in the CALUX assay. H, *n*-hexane fraction; E, ethyl acetate fraction; W, aqueous fraction. Each point represents the mean of at least two replicate analyses. Results are expressed as means ± SD. Sample 29 did not provide as aqueous fraction for analysis. All samples except 31 (H) showed at several concentrations a statistically increased response in comparison with the blank ($p < 0.1$).

ple preparation. The materials (1 g) were homogenised in aqueous ethanol [ethanol/water (4:1)] (30 ml) for 10 min and filtered. The filtrates were concentrated under reduced pressure and freeze-dried (total extract). Total extracts were added to water (10 ml), and these solutions were subjected to liquid–liquid partitioning (each 30 ml) to give three extracts: *n*-hexane-, ethyl acetate-, and water-soluble portions. Column chromatography for the identification of compounds was conducted using MCI Gel CHP-20P (75–150 μm) (Mitsubishi Chemical, Tokyo, Japan), YMC GEL ODS-AQ (AQ12S50) (YMC, Kyoto, Japan), and Silica Gel 60 (Nacalai Tesque, Kyoto, Japan). Compounds were identified by direct comparison with valid standards or by comparison of their spectral data with those reported in the literature. The extracts were dissolved in dimethyl sulfoxide and evaluated for AhR-binding activity, using a luciferase assay.

2.3. HPLC conditions

HPLC analysis was carried out using a Shimadzu Prominence system (Shimadzu, Kyoto, Japan). Reversed-phase HPLC conditions were as follows: column, L-column ODS (5 μm, 150 × 2.1 mm i.d.; Chemicals Evaluation and Research Institute, Tokyo, Japan); mobile phase, solvent A was 3% acetic acid and solvent B was acetonitrile (0–30 min, 0–50% B in A; 30–35 min, 50–85% B in A; 35–40 min, 85–85% B in A); injection volume, 5 μl; column temperature, 40 °C; flow-rate, 0.3 ml/min; detection, 200–400 nm.

2.4. Estimation of AhR ligand activity

For the identification of AhR-activating materials, a CALUX assay was used. When mouse hepatoma (H1L6.1c2) cells are exposed to environmental ligands, such as dioxins, luciferase protein synthesis is induced. The amount of light emitted by the luciferase protein is directly correlated with the dioxin level, and this system is used as a simple dioxin monitoring method. The CALUX assay for AhR ligand activity was carried out as follows: mouse hepatoma H1L1 cells (*ca.* 1.5×10^5 cells/well) were cultured in 96-well culture plates, and the samples dissolved in DMSO were added at a concentration range of 1×10^{-5}–1×10^2 mg/ml (final concentrations of 1×10^{-4}–1×10^3 μg/ml) in 8-steps (5-steps in fractions). The concentration data express the additional level not the final concentration. The final DMSO concentration was 1% in the cell culture medium. The plates were incubated at 37C in 5% CO_2 for 24 h to produce the optimal expression of luciferase activity. After incubation, cell viability was confirmed using a microscope. Subsequently, the medium was removed and the cells were lysed. After

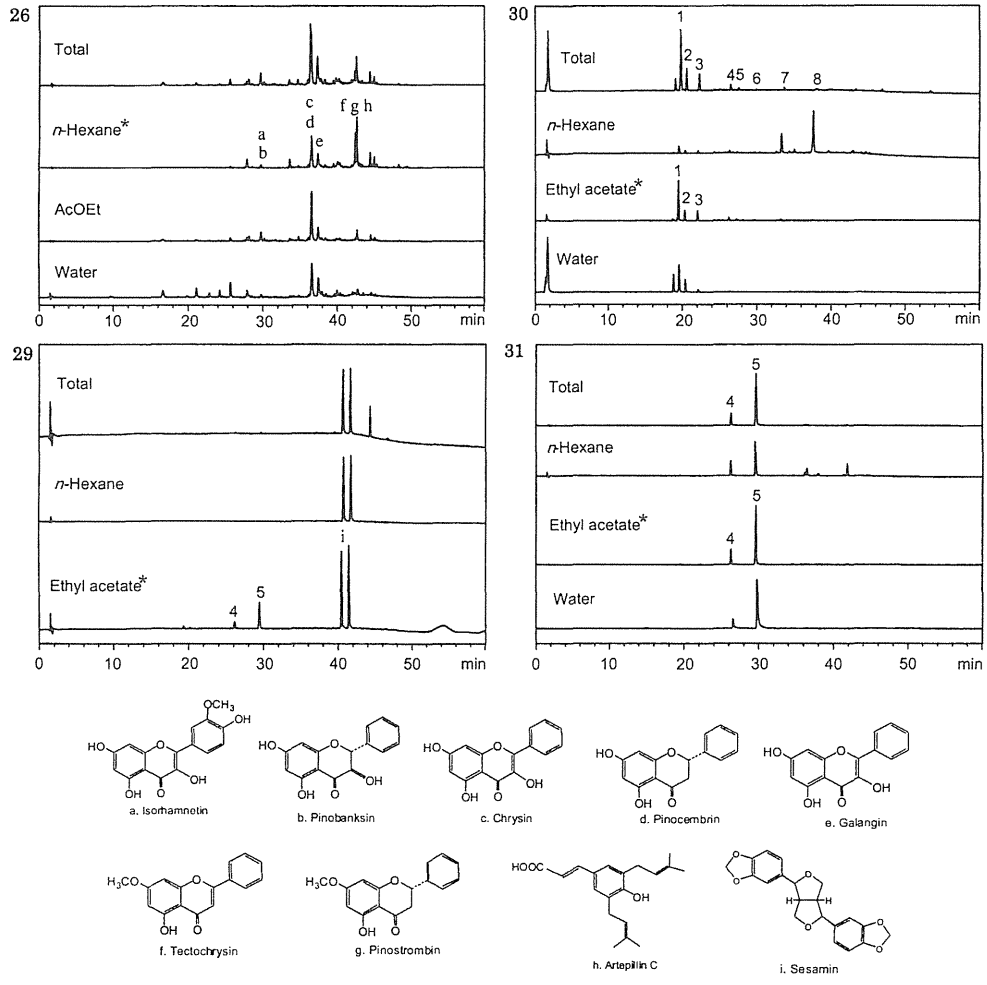

Fig. 3. RP-HPLC profiles of *n*-hexane, ethyl acetate, and aqueous fractions of AhR-activated samples and chemical structures of identified constituents. a, Isorhamnetin; b, pinobanksin; c, chrysin; d, pinocembrin; e, galangin; f, tectochrysin; g, pinostrombin; h, artepillin C; i, sesamin. 1, daidzin; 2, glycitin; 3, genistin; 4, daidzein; 5, glycitein; 6, genistein; 7, formononetin; 8, biochain A. Signals were detected at 254 nm except sample 26 (at 280 nm). Sample 29 did not provide an aqueous fraction for analysis. *AhR activated fraction.

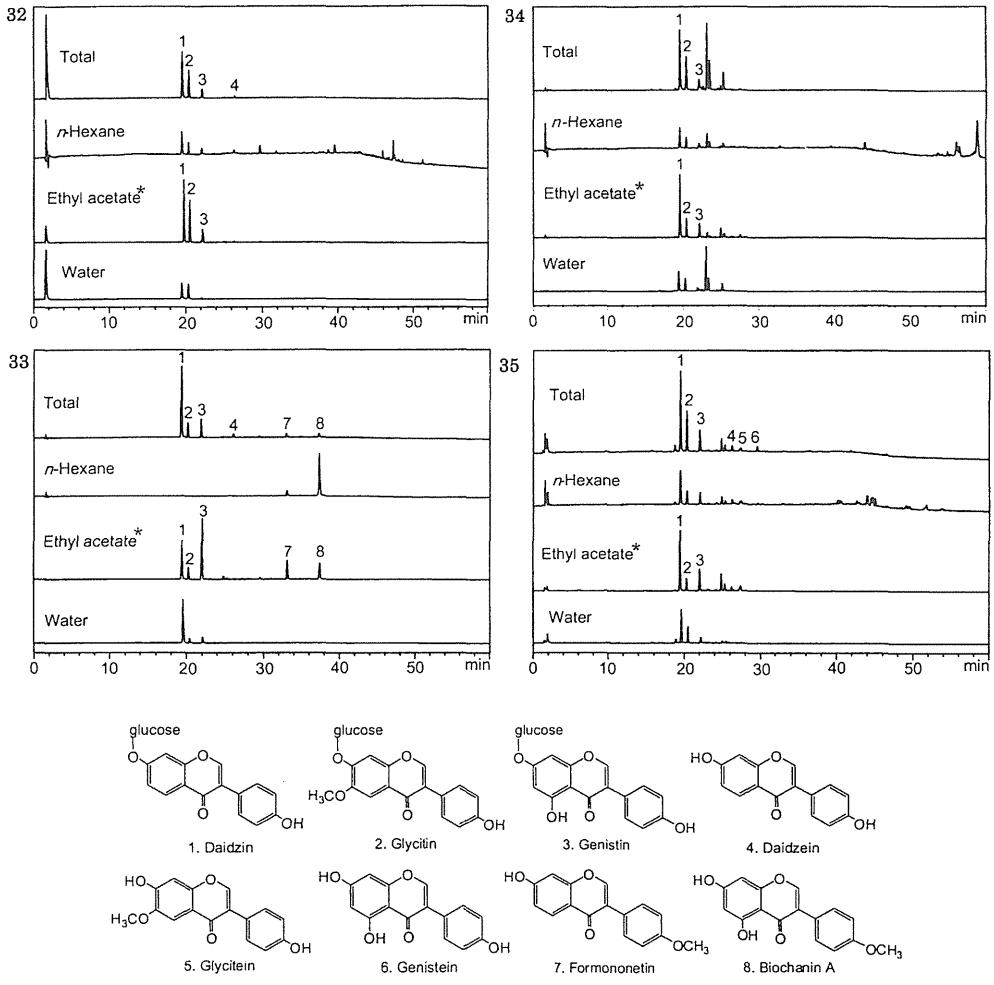

Fig. 3 (*continued*)

the addition of luciferin as the substrate, the luciferase activity was determined using a luminometer (Centro LB960, BERTHOLD, Bad Wildbad, Germany) and reported as relative light units (RLU). Values represent the means ± SD of at least two or three independent determinations for each experiment. Statistical significance was analysed using the Student's *t* test.

3. Results and discussion

AhR activity was estimated for the 50 samples listed in Table 1 using the CALUX assay. Although most of the samples showed no dioxin-like activity even at a high concentration, some samples shown in Fig. 1 exhibited activity at high concentration in a dose-dependent manner. Notably, the active samples included most of the soybean-related foods (samples 30–35). The active samples showed dioxin-like activity similar to that of TCDD at 10–100 mg/ml. Sesame processed food (sample 29) and propolis extract (sample 26) and some teas [loquat leaf (sample 41) and perilla herb (sample 42)] also exhibited an activity similar to that of the soybean extract foods at high concentration. To compare the dioxin-like activity of individual samples with that of TCDD, their relative concentration ($TCDD_{0.5}$) for AhR activity induced by 0.5 ng/ml of TCDD was calculated and the results are given in Table 2. Foods prepared from soybean (samples 30, 31, and 33), sesame (sample 29), and propolis (sample 26), exhibited dioxin-like activity similar to that of TCDD at about a 10^6-fold concentration of TCDD. The following samples showed slight dioxin-like activity of less than the $TCDD_{0.5}$ level at high concentrations: agaricus mushroom processed food (sample 1), amino acid/vitamin/mineral food (sample 2), ginkgo leaf extract (sample 14), *Gymnema sylvestre* extract (sample 16), lutein (sample 19), processed vegetable food (sample 25), and teas [Chinese sweet tea (sample 36), guava (sample 39), Japanese mugwort (sample 40), loquat leaf (sample 41), perilla herb (sample 42), and rooibos (sample 44)].

In order to characterise the active components of the samples from soybean-related samples (30–35), sesame (29), and propolis (26), AhR activity was measured for the respective *n*-hexane, ethyl acetate, and water fractions. The results are given in Fig. 2. The *n*-hexane fraction of the propolis extract sample exhibited AhR activity similar to that of TCDD at a concentration range of 1–10 mg/ml. In addition, marked AhR activity was noted for the ethyl acetate fractions of the other samples (soybean and sesame extract samples) at 0.1–10 mg/ml.

Previously, soy isoflavones, such as daidzein and glycitein, and so-called vegetable estrogens, such as resveratrol, were shown to exhibit dioxin-like activity (Amakura et al., 2003, 2008). The distribution of soy isoflavones and their glycosides in the active fractions was examined by reverse-phase HPLC. As shown in Fig. 3, some soy isoflavones were detected in various combinations in all six soybean extract samples. According to the combination of

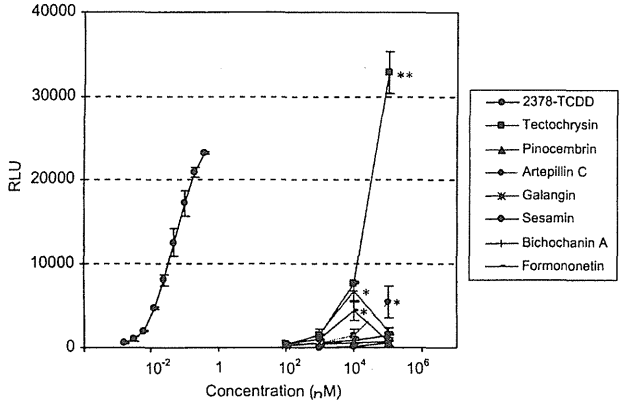

Fig. 4. Concentration–response curve of selected compounds and TCDD for the induction of luciferase activity in the CALUX assay. Each point represents the mean of at least three replicate analyses. Results are expressed as means ± SD, and asterisks indicate statistically significant differences ($^{*}p < 0.05$, $^{**}p < 0.01$).

the six standard isoflavones, they could be roughly classified into three groups [1st group (samples 30, 32, 33, and 35) including both aglycones and glycosides; 2nd group (sample 34) including glycosides; 3rd group (sample 31) including only aglycones]. On the other hand, some of the soybean extract products (samples 30, 31, and 33) also contained other isoflavones, biochanin A, and formononetin, which is also one of components of red clover. Biochanin A and formononetin slightly activated luciferase (Fig. 4). Thus, the methoxy group (–OMe) at C-4 of the B-ring in isoflavones may contribute to weakening the activity (Amakura et al., 2003,2008).

Sesame product (sample 29) was also found to contain daidzin and glycitin, together with sesamin. Sesamin showed no luciferase induction at the level of 10^5 nM (Fig. 4). HPLC analysis of some ethyl acetate fractions (samples 29–34, and 35) suggested the occurrence of components other than the six isoflavones. Thus, the influence of isoflavones and unidentified constituents was also investigated in more detail at the food level, using the biological assay for the determination of dioxins.

The *n*-hexane fraction of the propolis extract product (sample 26) showed the strongest AhR activity, suggesting the existence of dioxin-like active factors other than isoflavones. To clarify the compounds present, the *n*-hexane extract of propolis was subjected to chromatography using MCI-gel CHP 20P and YMC-gel ODS, to give chrysin, pinocembrin, galangin, tectochrysin, pinostrombin, isorhamnetin, pinobanksin, and artepillin C as UV-sensitive constituents. In our previous study, chrysin was reported to show some AhR ligand activity. In addition, the AhR activation of some flavonoids from the propolis extract product was examined by reporter gene assay in the present study. As shown in Fig. 4, tectochrysin showed marked AhR binding activity, followed by artepillin C. These results suggest that tectochrysin might be a natural AhR ligand. Thus, it was suggested that the AhR activity of the propolis extract may be related to flavones.

We herein demonstrated that food isoflavones and flavones are considered to be responsible for the AhR activity observed for several supplements or foods. The determination of dioxins in foods, using biological assays, requires the interpretation of data with regard to the influence of these confounding ingredients. The signal transduction of isoflavones and flavones, which occurs after AhR activation, should differ from that of dioxins. Recently, several papers have reported that the activation of AhR may be involved in various immune system responses; therefore, natural AhR ligands may play some beneficial regulatory role in humans (Kimura, Naka, Nohara, Fujii-Kuriyama, & Kishimoto, 2008; Quintana et al., 2008; Veldhoen et al., 2008). Further studies on the AhR-activating ingredients derived from natural foods might clarify, not only the physiological significance of AhR, but also the risks and benefits from food constituents.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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Daily Intake of Brominated Dioxins, Co-PXBs and Brominated Flame Retardants Estimated from Market Basket Study

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Introduction

Brominated flame retardants (BFRs) such as polybrominated diphenyl ethers (PBDEs) and polybrominated biphenyls (PBBs) have been widely used in plastics and textile coatings throughout the world. The major commercial products made with PBDEs were penta-BDE, octa-BDE and deca-BDE products. In Japan, although the use of low-brominated PBDEs has decreased, deca-BDE is currently in use. PBDEs are additives to polymers such as polystyrene and are not chemically bound to the polymer. Therefore, they are easily released into the environment from waste products. For PBBs, the commercial products are mixtures containing hexa-BB, octa-BB, nona-BB, and deca-BB. Products made with PBBs have not been produced in Japan, but PBBs have been detected in environmental samples in Japan (Ishikawa et al. 2004). It is suspected that the contaminants came from imported products or impurities in other BFRs. Furthermore, *de novo* synthetic compounds related to BFRs, such as polybrominated dibenzo-*p*-dioxins and dibenzofurans (PBDD/DFs) have been found in human samples (Choi et al. 2003) and market fish (Ashizuka et al. 2008). In addition, coplanar polychlorinated / brominated biphenyls (Co-PXBs), which are new contaminants, have been detected (Ohta et al. 2008, Ohta et al. 2007). Although the toxicity of these brominated dioxins is unclear, some studies have shown that the toxicity of 2, 3, 7, 8-TBDD is comparable to that of 2, 3, 7, 8-TCDD (WHO 1998). Co-PXBs may also be formed from BFRs and have toxicities similar to those of Co-PCBs due to the similarity of their structures.

It is important that we investigate levels of these brominated organic compounds in foods and estimate their influence on humans. A market basket study is a useful method for estimating the average intake levels in regions, based on a model of the average domestic diet. In the present study, we analyzed brominated dioxins and PBDEs in food mixtures from each of 13 food groups from 2 regions of Kanto and Kansai in Japan and estimated the daily intake levels of brominated dioxins, Co-PXBs and BFRs. The goal of this research was to evaluate the health risk presented by the daily intake of these brominated compounds in Japan.

Materials and Methods

Sampling.

Table 1 shows the food groups analyzed in this study and their mean daily consumption for 2 regions (A region : Kanto region, B region : Kansai region) as calculated from data from the Japanese Nutrition Survey carried out by the Ministry of Health, Labour and Welfare. For a market basket study, 120-200 kinds of foods were purchased from markets in each of 2 regions in 2007. These foods were divided into 13 food groups, and were weighed and cooked based on the daily consumption data of each region. The foods were then, blended in a food processor. The food mixtures were prepared and analyzed for groups 10, 11, and 12 (n=2) and other groups (n=1). The food mixtures were kept below –20°C until analysis.

Analytical Methods and Instrumentation.

The PBDD/DFs (tetra-octa) and Co-PXBs (4'-Br-2,3',4,5-TeCB, 4'-Br-2,3,3',4-TeCB, 4'-Br-3,3',4,5-TeCB, 4'-Br-2,3,3',4,5-PeCB, 4'-Br-3,3',4,5,5'-PeCB, 3',4',5'-Br-3,4-DiCB) analytical standards were purchased from Cambridge Isotope Laboratories (Cambridge, MA). The PBDEs (tri-deca) analytical standards were purchased from Wellington Laboratories (Guelph, Ontario). The PBBs (tri-deca) analytical standards were purchased from Wellington Laboratories and AccuStandard, Inc. (New Haven, CT). The concentrations of brominated compounds were determined using high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS). Further information about instrumentation can be found in our previous article (Ashizuka et al. 2009).

Sample Preparation.

We analyzed the brominated dioxins, Co-PXBs, PBDEs and PBBs simultaneously using accelerated solvent extraction (ASE). Each 50 g sample was freeze-dried using a model AD 2.0ES-BC (Virtis, Gardiner, NY) freeze dryer, and then dried samples were extracted with 10% (v/v) dichloromethane/*n*-hexane using an accelerated solvent extractor ASE300 (Dionex, Sunnyvale, CA). The extraction temperature was 100°C; the time was 10 min. Extracts were treated with sulfuric acid three times and applied to a silica gel column. The mixture for group 4 was dissolved in 100 mL of *n*-hexane and purified with sulfuric acid and the silica gel column in the same way. The column was prewashed with 100 mL of *n*-hexane, and brominated compounds were eluted with 150 mL of 10% (v/v) dichloromethane/*n*-hexane. The eluate was evaporated and dissolved in *n*-hexane. It was then loaded onto a Florisil (5 g) column. The PBDEs, PBBs and Co-PXBs were obtained by elution with 150 mL of *n*-hexane (fraction 1), and the PBDD/DFs fraction was obtained by elution with 200 mL of 60% (v/v) dichloromethane/*n*-hexane (fraction 2). The fraction 1 was treated with a DMSO/*n*-hexane partition to remove the matrix and then concentrated to a final volume of approximately 25 μL. The fraction 2 was loaded onto an active carbon column, which in advance had been washed with 50 mL of 10% (v/v) dichloromethane/*n*-hexane and eluted with 200 mL of toluene. The fractions were concentrated to a final volume of approximately 15 μL, and these samples were analyzed by HRGC/HRMS.

Table 1 Daily food consumption (13 groups) in 2 regions of Japan

No.	Food group	Daily consumption (g)*	
		A region	B region
1	Rice and rice products	332.8	341.4
2	Cereals seeds and potatoes	175.4	174.2
3	Sugars and confectioneries	32.1	35.1
4	Fats and oils	11.0	10.6
5	Pulses	59.6	57.5
6	Fruits	125.4	120.8
7	Green vegetables	100.3	92.8
8	Other vegetables and sea weeds	209.1	184.1
9	Beverages	540.8	616.4
10	Fish and shellfish	84.8	82.2
11	Meat and eggs	111.3	121.4
12	Milk and dairy products	137.7	142.9
13	Other foods (seasoning)	94.5	92.9
	Total	2014.8	2072.3

*The values obtained from the data of Japanese Nutrition Survey (the Ministry of Health, Labour and Welfare of Japan).

Results and Discussion

We analyzed brominated dioxins (a total of 18 congeners of PBDD/DFs and MoBrPCDD/DFs), Co-PXBs (7 congeners), PBDEs (23 congeners) and PBBs (18 congeners) in food mixtures from each of 13 food groups from 2 regions in Japan. In our study, the limits of detection (LODs) of the