the one with a recovery of about 70~120% with a relative standard deviation (RSD) ≤ 20% at both concentrations.

Sample (5.0 g) in a 50 mL PP disposal tube
Add 20 mL of ethyl acetate and 3 g of $MgSO_4$
Homogenize (1 min) and centrifuge
Ethyl acetate layer 8.0 mL (equivalent to 2.0 g sample)
Evaporate and reconstituted in 10 mL of hexane in 50 mL PP tube
Add 20 mL of acetonitrile saturated with hexane
Shake (1 min) and centrifuge
Acetonitrile layer Hexane layer
Add 20 mL of acetonitrile
Shake (1 min) and centrifuge
Acetonitrile layer
Load on a GCB/PSA column
Elute with 30 mL of acetonitrile-toluene (3+1)
Collect loading and eluting fractions in a round bottom flask
Evaporate and reconstituted in appropriate solvent
GC-MS, GC-MS/MS or LC-MS/MS analysis

Scheme III. Procedure of sample preparation for the determination of pesticides residues in processed foods categorized as the high-lipid group

4.1.2.1 Analysis of 250 pesticides via GC-MS/MS (agricultural products)

We spiked the 250 pesticides for 3 agricultural products (bananas (Ba), carrots (Ca), and grapefruits (Gf)) at concentrations of 0.02 and 0.10 ppm. We conducted 3 trials for each test. These pesticides were classified into 3 groups as per the number of foods with acceptable results at both concentrations: A, 3; B, 2; and C, 1. Details are described below.

Group A (207 pesticides with the acceptable results in 3 agricultural products at the both concentrations):

2-Phenylphenol, Acetochlor, Aldrin, Allidochlor, Ametryn, Anilofos, Atrazine, Azinphos-methyl, Azoxystrobin, Benalaxyl, Bendiocarb, Benfluralin, Benfuresate, Benoxacor, BHC (total), Bifenthrin, Bitertanol, Bromacil, Bromophos, Bromopropylate, Bupirimate, Buprofezin, Butachlor, Butafenacil, Butamifos, Cadusafos, Cafenstrole, Carbaryl, Carbofuran, Carfentrazone-ethyl, Chlorfenvinphos, Chlorobenzilate, Chlorpropham, Chlorpyrifos, Chlorpyrifos-methyl, Chlorthal-dimethyl, Clomazone, Cloquintocet-1-methylhexyl, Cyanazine, Cyanophos, Cyflufenamid, Cyfluthrin, Cyhalofop-butyl, Cyhalothrin, Cypermethrin, Cyproconazole, Cyprodinil, DDT (total), Diazinon, Dichlofenthion, Diclobutrazol, Diclofop-methyl, Dicloran, Diethofencarb, Difenoconazole, Diflufenican, Dimepiperate, Dimethametryn, Dimethenamid, Dimethipin, Dimethylvinphos, Diofenolan, Dioxabenzofos, Diphenamid, Disulfoton, Dithiopyr, Edifenphos, Esprocarb, Ethalfluralin, Ethiofencarb, Ethion, Ethofumesate, Ethoprophos, Etobenzanid, Etoxazole, Etrimfos, Fenamiphos, Fenarimol, Fenbuconazole, Fenchlorphos, Fenitrothion, Fenobucarb, Fenothiocarb, Fenoxanil, Fenoxycarb, Fenpropimorph,

Fensulfothion, Fenthion, Fenvalerate, Fipronil, Flamprop-methyl, Fluacrypyrim, Flucythrinate, Fludioxonil, Fluquinconazole, Flusilazole, Flutolanil, Flutriafol, Fosthiazate, Fthalide, Furathiocarb, Furilazole, Halfenprox, Heptachlor, Hexazinone, Indoxacarb-MP, Iprobenfos, Iprodione, Iprovalicarb, Isazofos, Isofenphos, Isoprothiolane, Isoxathion, Kresoxim-methyl, Lactofen, Lenacil, Malathion, Mefenacet, Mepronil, Metalaxyl, Methacrifos, Methidathion, Methiocarb, Methoxychlor, Metolachlor, Metolcarb, Metominostrobin, Metribuzin, Mevinphos, Molinate, Myclobutanil, Napropamide, Nitrothal-isopropyl, Oxadiazon, Oxadixyl, Oxyfluorfen, Paclobutrazol, Parathion, Parathion-methyl, Penconazole, Pencycuron, Pendimethalin, Permethrin, Phenothrin, Phenthoate, Phorate, Phosalone, Phosphamidon, Picolinafen, Piperophos, Pirimicarb, Pirimiphos-ethyl, Pirimiphos-methyl, Pretilachlor, Procymidone, Profenofos, Promecarb, Prometryn, Propachlor, Propanil, Propaphos, Propham, Propiconazole, Propoxur, Propyzamide, Prothiofos, Pyraclofos, Pyraflufen-ethyl, Pyrazophos, Pyributicarb, Pyridaphenthion, Pyrimethanil, Pyrimidifen, Pyriminobac-methyl, Pyriproxyfen, Quinalphos, Quinoxyfen, Quintozene, Silafluofen, Simazine, Simeconazole, Simetryn, Sulprofos, Tebuconazole, Tebufenpyrad, Tecnazene, Tefluthrin, Terbacil, Terbufos, Terbutryn, Tetrachlorvinphos, Tetradifon, Thenylchlor, Thifluzamide, Thiobencarb, Thiometon, Tolclofos-methyl, Tralomethrin, Tri-allate, Triadimefon, Triazophos, Tribuphos, Triflumizole, Trifluralin, Uniconazole P, Vinclozolin, XMC

Group B (36 pesticides with the acceptable results in 2 agricultural products at the both concentrations of 0.02 and 0.1 ppm except for the agricultural product exhibited in parentheses):

Acephate (Gf), Bifenox (Gf), Bioallethrin (Gf), Bromobutide (Gf), Chlorfenapyr (Gf), Clodinafop-propargyl (Ca), Clomeprop (Gf), Cyanofenphos (Gf), Deltamethrin (Ba), Dieldrin (Ca), Dimethoate (Gf), Diphenylamine (Ba), Endrin (Gf), EPN (Ba), Fenpropathrin (Gf), Flumioxazin (Gf), Fluvalinate (Gf), Furametpyr (Gf), Heptachlor-epoxide (Ca), Hexaconazole (Gf), Isoprocarb (Ca), Methamidophos (Gf), Monocrotophos (Gf), Norflurazon (Gf), Omethoate (Gf), Phosmet (Ba), Prochloraz (Gf), Propargite (Ba), Pyridaben (Gf), Pyrifenox (Gf), Quinoclamine (Ba), Tetraconazole (Gf), Thiazopyr (Ca), Triadimenol (Gf), Trifloxystrobin (Gf), Xylylcarb (Ba)

Group C (7 pesticides with the acceptable results in 1 agricultural products at the both concentrations of 0.02 and 0.1 ppm exhibited in square brackets):

Acrinathrin [Ca], Dichlorvos [Ca], Dicofol-metabolite (4,4'-Dichlorobenzophenone) [Gf], Diphenyl [Gf], Endosulfan (α+β) [Ba], Flumiclorac-pentyl [Ca], Hexythiazox [Gf]

4.1.2.2 Analysis of 99 pesticides via LC-MS/MS (agricultural products)

We spiked the 99 pesticides for 7 agricultural products (cabbage (Cb), potatoes (Po), spinach (Sp), apples (Ap), oranges (Or), brown rice (Br), and soybeans (Sy)) at concentrations of 0.02 and 0.1 ppm. We conducted 5 trials for each test. These pesticides were classified into 6 groups as per the number of foods with acceptable results: A, 7; B, 6; C, 5; D, 4; E, 3; and F, 2. Details are described below.

Group A (47 pesticides with the acceptable results in all the 7 agricultural products):

Acetamiprid, Acetochlor, Alachlor, Atrazine, Bensulide, Bitertanol, Bromobutide, Bupirimate, Clomeprop, Cumyluron, Diethofencarb, Difenoconazole, Diflufenican, Dimethametryn, Dimethoate, Dimethomorph, Esprocarb, Fenbuconazole, Fenoxycarb, Flusilazole, Hexaconazole, Imazalil, Indanofan, Iprovalicarb, Isoxathion, Mepanipyrim, Methabenzthiazuron, Methomyl, Monocrotophos, Napropamide, Paclobutrazol,

Penconazole, Pirimicarb, Pretilachlor, Prochloraz, Propoxur, Propyzamide, Pyroquilon, Tebuconazole, Tebufenozide, Teflubenzuron, Thenylchlor, Thiacloprid, Thiobencarb, Triadimefon, Triadimenol, Triflumizole

Group B (29 pesticides with acceptable results in 6 agricultural products except for the agricultural product exhibited in parentheses):

Acephate (Or), Allethrin (Or), Azoxystrobin (Or), Buprofezin (Sy), Carbaryl (Sy), Chlorpropham (Sy), Cyanazine (Po), Cyflufenamid (Cb), Cyhalofop-butyl (Po), Daimuron (Sy), Diflubenzuron (Sy), Dimepiperate (Br), Diphenamid (Sp), Ethofumesate (Ap), Etobenzanid (Po), Fenobucarb (Po), Flufenoxuron (Sp), Hexaflumuron (Sp), Imibenconazole (Sp), Isoprocarb (Sy), Isoprothiolane (Or), Mefenacet (Sy), Metalaxyl (Cb), Methamidophos (Po), Omethoate (Po), Oxamyl (Br), Pencycuron (Or), Quizalofop-ethyl (Po), Tebufenpyrad (Sy)

Group C (10 pesticides with acceptable results in 5 agricultural products except for the agricultural products exhibited in parentheses):

Carbofuran (Sp, Ap), Fenoxaprop-ethyl (Po, Sy), Fenpropimorph (Cb, Ap), Furathiocarb (Sp, Sy), Lufenuron (Sp, Br), Pentoxazone (Br, Sy), Pyriproxyfen (Cb, Sy), Quinoclamine (Cb, Sy), Tri-allate (Cb, Sy), Trichlamide (Cb, Sy)

Group D (11 pesticides with acceptable results in 4 agricultural products except for the agricultural products exhibited in parentheses):

Bendiocarb (Ap, Br, Sy), Benfuresate (Po, Sp, Br), Cafenstrole (Or, Br, Sy), Carfentrazone-ethyl (Po, Br, Sy), Ethiofencarb (Po, Ap, Sy), Fenarimol (Cb, Or, Br), Inabenfide (Sp, Ap, Sy), Metolcarb (Cb, Po, Sy), Phenmedipham (Po, Br, Sy), Phoxim (Cb, Po, Sy), Propamocarb (Po, Br, Sy)

Group E (1 pesticide with acceptable results in 3 agricultural products exhibited in square brackets):

Molinate [Cb, Sp, Or]

Group F (1 pesticide with acceptable results in 2 agricultural products exhibited in square brackets):

Propiconazole [Cb, Sy]

4.2 Method for processed foods in high lipid group

4.2.1 Rapidity of the method

In this procedure, we used ethyl acetate for the first extraction step given its lipid solubility. The time for one chemist to prepare test solutions for 12 samples was approximately 5~6 h. For rapidity, we adopted the acetonitrile-hexane partition to remove lipids from the extract in this procedure. A chemist can conduct the procedure simultaneously for up to 12 samples. Furthermore, the collected acetonitrile layer can be applied onto the GCB/PSA column directly. Gel permeation column chromatography is an established technique to remove lipid from the extract in pesticide analysis (Gilbert-Lopez, B., et al., 2009, Sannino, A., et al., 1999). However, this technique cannot be used to analyze many samples simultaneously; for more than 12 samples, the collected fraction should be concentrated and reconstituted in an appropriate solvent for further purification. To confirm the effectiveness of the acetonitrile-hexane partition, we recorded the weight of the residues in the analysis of pesticides in Chinese dumplings. The remaining residue prior to the acetonitrile-hexane partition was 8.9%, corresponding to the original sample weight in the aliquot (2.0 g). After the acetonitrile-hexane partition, the remaining residue was less than 0.1%. Thus, the acetonitrile-hexane partition would be one of the most efficient and suitable techniques for removing lipids.

4.2.2 Recovery tests

We performed recovery tests of pesticides sensitively detectable with GC-MS or GC-MS/MS via fortification of the pesticide mixtures of the 5 processed foods (Chinese dumplings, curry, French fries, fried chicken, and fried fish) at the final concentrations of 0.02 and 0.10 ppm, respectively. We conducted 3 trials for each test and defined an acceptable result as the one with a recovery of 70~120% with a RSD ≤ 20% for both concentrations.

4.2.2.1 Analysis of 225 pesticides via GC-MS (processed foods in high lipid group)

We conducted recovery tests of 225 pesticides detectable at a concentration of 0.01 ppm by GC-MS. These pesticides were classified into 6 groups as per the number of processed foods with acceptable results: A, 5; B, 4; C, 3; D, 2; E, 1; and F, 0. Details are described below. Notations of the processed foods are as follows: D, Chinese dumplings; C, curry; P, French fries; Ck, fried chicken; and F, fried fish.

Group A (99 pesticides with the acceptable results in all the 5 processed foods):
Acetochlor, Alachlor, Ametryn, Anilofos, Atrazine, Azinphos-methyl, Azoxystrobin, Benfluralin, Benfuresate, Benoxacor, Bromacil, Bupirimate, Butafenacil, Chlorfenvinphos-*E*, Clodinafop-propargyl, Cyflufenamid, Cyhalofop-butyl, Cyprodinil, Dichlofenthion, Diflufenican, Dimethamethryn, Dioxabenzofos, Diphenylamine, Dithiopyr, Edifenphos, Esprocarb, Ethalfluralin, Ethion, Ethoprophos, Etofenprox, Etoxazole, Etrimfos, Fenoxanil, Fenthion, Flamprop-methyl, Fluacrypyrim, Fludioxonil, Flumiclorac-pentyl, Fluquinconazole, Flusilazole, Flutolanil, Fluvalinate, Furathiocarb, Hexaconazole, Iprobenfos, Isazofos, Isofenphos, Isoprocarb, Kresoxim-methyl, Mefenacet, Mepronil, Metolachlor, Metolcarb, Metribuzin, Mevinphos, Nitrothal-isopropyl, Oxadiazon, Parathion, Parathion-methyl, Penconazole, Phenthoate, Phorate, Picolinafen, Piperophos, Pirimiphos-methyl, Pretilachlor, Prometryn, Propachlor, Propanil, Propaphos, Propiconazole, Prothiofos, Pyraclofos, Pyrazophos, Pyridaben, Pyridaphenthion, Pyrimethanil, Pyrimidifen, Pyriminobac-methyl-*E*, Pyriminobac-methyl-Z, Qunalphos, Simazine, Simetryn, Sulprofos, Tebuconazole, Tebufenpyrad, Terbufos, Terbutryn, Tetraconazole, Thenylchlor, Thiobencarb, Thiazopyr, Tolclofos-methyl, Triadimefon, Triadimenol, Triazophos, Trifluralin, Uniconazole P, Vinclozolin, Xylylcarb

Group B (65 pesticides with the acceptable results in the 4 processed foods except for the processed food exhibited in parentheses):
Acrinathrin (D), Bendiocarb (C), β-BHC (C), γ-BHC (D), Butachlor (C), Butamifos (Ck), Cafenstrole (Ck), Carbofuran (F), Carfentrazone-ethyl, (Ck) Chlorfenvinphos-Z (C), Chlorobenzilate (C), Chlorpropham (Ck), Clomeprop (C), Cyanophos (Ck), p,p'-DDD (C), Diazinon (D), Diclobutrazol (Ck), Diclofop-methyl (C), Diethofencarb (C), Dimethenamid (Ck), Dimethoate (D), Dimethylvinphos (C), Diofenolan (P), EPN (F), Ethofumesate (P), Etobenzanid (P), Fenamiphos (Ck), Fenbuconazole (D), Fenitrothion (Ck), Fenobucarb (C), Flucythrinate (F), Flumioxazin (D), Furametpyr (P), Furilazole (C), Halfenprox (C), Hexazinone (P), Isoprothiolane (C), Malathion (C), Methacrifos (P), Methiocarb (C), Metominostrobin-*E* (C), Metominostrobin-Z (Ck), Monocrotophos (C), Myclobutanil (P), Napropamide (F), Norflurazon (P), Oxyfluorfen (Ck), Pendimethalin (Ck), 2-Phenylphenol (Ck), Phosalone (C), Primiphos-ethyl (C), Promecarb (C), Propham (C), Propoxur, (C) Pyributicarb (F), Pyriproxyfen (C), Silafluofen (Ck), Simeconazole (Ck), Tefluthrin (C), Terbacil (Ck), Tetradifon (C), Thiabendazole (Ck), Thifluzamide (D), XMC (Ck)

Group C (36 pesticides with the acceptable results in the 3 processed foods except for the processed foods exhibited in parentheses):
Acephate (P, F), δ-BHC (D, Ck), Bifenthrin (C, Ck), Bromobutide (D, C), Chlorpyrifos (C, P), Chlorpyrifos-methyl (C, P), Cyanazine (C, P), Cyanofenphos (C, Ck), Cyhalothrin (D, C), Cyproconazole (D, P), o,p'-DDT (C, Ck), p,p'-DDT (C, Ck), Dimepiperate (C, P), Diphenamid (P, Ck), Fenarimol (Ck, F), Fenchlorphos (C, P), Fenothiocarb(P, Ck), Fenpropathrin (D, F), Fensulfothion (C, P), Fipronil (C, P), Flutriafol (C, P), Iprovalicarb (D, C), Methidathion (Ck, F), Molinate (C, P), Omethoate (C, Ck), Phenothrin (C ,Ck), Primicarb (C, Ck), Procymidone (C, F), Propyzamide (D, Ck), Quinoxyfen (C, Ck), Tecnazene (C, P), Tetrachlorvinphos (C, P), Tri-allate (C, P), Tribufos (P, Ck)
Group D (18 pesticides with the acceptable results in the 2 processed foods exhibited in parentheses):
Benalaxyl [D, F], Cadusafos [P, F], Carbaryl [D, P], Clomazone [C, F], Cloquintocet-1-methylhexyl [C, F], p,p'-DDE [D, F], Dichlorvos [Ck, F], Diphenyl [Ck, F], Fthalide [Ck, F], Metalaxyl [D, F], Oxadixyl [D, P], Paclobutrazol [D, C], Pencycuron [Ck, F], Phosmet [P, F], Phosphamidon, [D, P] Profenofos [Ck, F], Pyraflufen-ethyl [Ck, F]
Group E (6 pesticides with the acceptable results in the 1 processed foods exhibited in square brackets):
Allidochlor [D], α-BHC [F], Bromophos [F], Chlorthal-dimethy,l [F], Fenoxycarb [D], Methamidophos [P]
Group F (1 pesticide without the acceptable results in all the 5 processed foods):
Fenpropimorph

4.2.2.2 Analysis of 258 pesticides via GC-MS/MS (processed foods in high lipid group)

On the chromatograms obtained via GC-MS analysis, some pesticides were interfered from the matrix derived from the foods. A GC-MS/MS is one of the most useful tools to overcome the interference on these chromatograms. As a relevant example, Figure 1 shows the chromatograms of methidathion fortified in the fried fish at a concentration of 0.02 ppm obtained via GC-MS and GC-MS/MS (Kitagawa, Y., et al., 2009, 2009). On the GC-MS chromatogram, methidathion could not be detected with either of the selected ion monitoring channels because of the interference. On the other hand, methidathion could be clearly detected with quantitative accuracy on the GC-MS/MS chromatogram. The improvement in the signal to noise ratio on the chromatogram (i.e. sensitivity) was due to the use of GC-MS/MS, given its high selectivity in monitoring pesticides. The GC-MS/MS also expanded the pesticides detectable at a concentration of 0.01 ppm. We conducted recovery tests of 258 pesticides detectable at this concentration via GC-MS/MS. The pesticides with asterisks were examined only with GC-MS/MS in this section. These pesticides were classified into 6 groups (A~F) as described above. The percentage of pesticides classified in group "A" increased from 44.0% with GC-MS to 71.3% using GC-MS/MS. The sensitivity and selectivity of GC-MS/MS would be helpful for the determination of pesticides in foods with interference, such as processed foods classified in the high lipid group. The relevant details are as follows.
Group A (184 pesticides with the acceptable results in all the 5 processed foods):
Acetochlor, Alachlor, Ametryn, Anilofos, Azinphos-methyl, Azoxystrobin, Benalaxyl, Benfluralin, Benfuresate, Benoxacor, β-BHC, δ-BHC, γ-BHC, Bifenox, Bifenthrin, Bitertanol, Bromacil, Bromobutide, Bromophos, Bromopropylate, Bupirimate, Buprofezin, Butafenacil,

Butamifos, Cadusafos, Cafenstrole, Carbaryl, Carbofuran, Carfentrazone-ethyl, Chlorfenapyr, α-Chlorfenvinphos, β-Chlorfenvinphos, Chlorobenzilate, Chlorpropham, Chlorpyrifos, Chlorpyrifos-methyl, Chlorthal-dimethyl, Clomazone, Clomeprop, Cloquintocet-1-methylhexyl, Cyanazine, Cyanophos, Cyflufenamid, Cyfluthrin, Cyhalofop-butyl, Cyhalothrin, Cypermethrin, Cyproconazole, Cyprodinil, p,p'-DDD, p,p'-DDT, Diazinon, Dichlofenthion, Diclobutrazol, Dicloran, Dieldrin, Diethofencarb, Diflufenican, Dimethametryn, Dimethenamid, Dimethylvinphos, Diofenolan, Dioxabenzofos, Diphenamid, Diphenylamine, Dithiopyr, Edifenphos, α-Endosulfan, β-Endosulfan, EPN, Esprocarb, Ethalfluralin, Ethion, Ethofumesate, Ethoprophos, Etobenzanid, Etofenprox, Etoxazole, Etrimfos, Fenarimol, Fenbuconazole, Fenchlorphos, Fenitrothion, Fenothiocarb, Fenoxanil, Fenpropathrin, Fenvalerate, Fipronil, Flamprop-methyl, Fluacrypyrim, Flucythrinate, Fludioxonil, Flumioxazin, Fluquinconazole, Flusilazole, Flutolanil, Fluvalinate, Fosthiazate, Fthalide, Furametpyr, Furathiocarb, Halfenprox, Iprobenfos, Iprovalicarb, Isofenphos, Mefenacet, Mepronil, Metalaxyl, Methidathion, Methiocarb, Methoxychlor, Metolachlor, Metominostrobin-E, Metominostrobin-Z, Metribuzin, Monocrotophos, Myclobutanil, Napropamide, Nitrothal-isopropyl, Omethoate, Oxadiazon, Oxadixyl, Oxyfluorfen, Paclobutrazol, Parathion, Parathion-methyl, Penconazole, Pencycuron, Pendimethalin, Permethrin, Phenothrin, Phenthoate, 2-Phenylphenol, Phosalone, Phosphamidon, Piperophos, Pirimicarb, Pirimiphos-ethyl, Pirimiphos-methyl, Pretilachlor, Procymidone, Profenofos, Promecarb, Prometryn, Propachlor, Propanil, Propoxur, Propyzamide, Prothiofos, Pyraclofos, Pyraflufen-ethyl, Pyrazophos, Pyributicarb, Pyridaphenthion, Pyrifenox-E, Pyrimethanil, Pyriminobac-methyl-E, Pyriminobac-methyl-Z, Quinalphos, Quinoxyfen, Silafluofen, Simazine, Simeconazole, Simetryn, Tebuconazole, Tebufenpyrad, Tefluthrin, Terbutryn, Tetrachlorvinphos, Tetraconazole, Thenylchlor, Thifluzamide, Thiobencarb, Tolclofos-methyl, Triadimefon, Triadimenol, Tri-allate, Triazophos, Tribufos, Trifloxystrobin, Triflumizole, Trifluralin, Uniconazole P, XMC, Xylylcarb

Group B (39 pesticides with the acceptable results in the 4 processed foods except for the processed food exhibited in parentheses):

Bendiocarb (C), α-BHC (C), Butachlor (D), Clodinafop-propargyl (C), p,p'-DDE (C), o,p'-DDT (C), Diclofop-methyl (D), Difenoconazole (C), Dimethoate (D), Fenobucarb (C), Fensulfothion (C), Flumiclorac-pentyl (C), Furilazole (Ck), Heptachlor (C), Heptachlor-epoxide (D), Hexaconazole (Ck), Indoxacarb-MP (C), Iprodione (P), Isazofos (C), Isoprocarb (C), Isoprothiolane (D), Isoxathion (D), Kresoxim-methyl (C), Lactofen (D), Lenacil (P), Malathion (D), Metolcarb (P), Mevinphos (P), Norflurazon (P), Picolinafen (C), Prochloraz (D), Propiconazole (F), Pyridaben (Ck), Pyrimidifen (C), Quintozene (C), Terbacil (C), Terbufos (P), Vinclozolin (C)

Group C (18 pesticides with the acceptable results in the 3 processed foods except for the processed foods exhibited in parentheses):

Atrazine (D, C), Bioallethrin (C, Ck), Cyanofenphos (D, F), Deltamethrin (C, Ck), Dimepiperate (C, P), Endrin (C, P), Fenoxycarb (D, Ck), Fenthion (P, Ck), Flutriafol (P, F), Hexazinone (P, F), Methacrifos (C, P), Molinate (C, P), Propargite (D, Ck), Pyrifenox-Z (C, P), Pyriproxyfen (D, F), Tecnazene (C, P), Tetradifon (D, Ck), Tralomethrin (D, Ck)

Group D (7 pesticides with the acceptable results in the 2 processed foods exhibited in square brackets):

Acrinathrin [D, P], Dicofol [D, F], Fenamiphos [D, Ck], Propaphos [D, F], Propham [P, Ck], Sulprofos [D, C], Thiazopyr [C, Ck]

Group E (6 pesticides with the acceptable results in the 1 processed foods exhibited in square brackets):
Acephate [Ck], Allidochlor [F], Methamidophos [F], Phorate [D], Phosmet [F], Thiabendazole [D]
Group F (4 pesticides without the acceptable results in all the 5 processed foods):
Aldrin, Dichlorvos, Diphenyl, Fenpropimorph

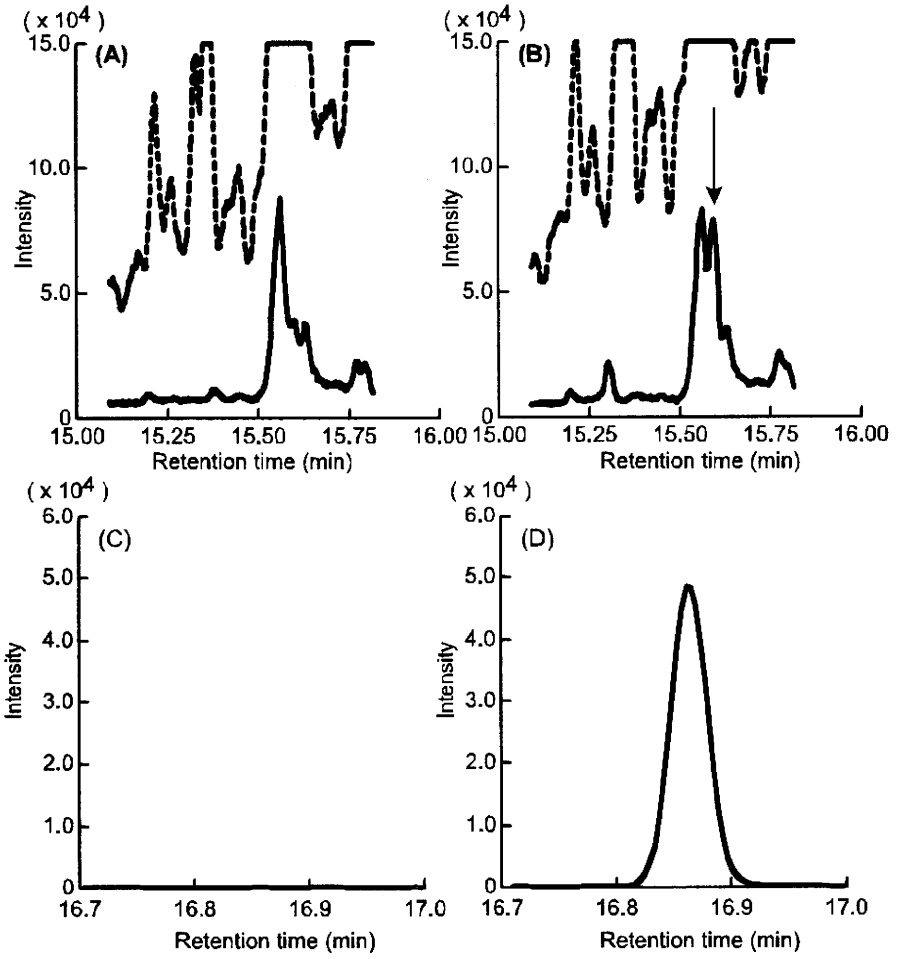

Fig. 1. SIM [(A) and (B)] and SRM [(C) and (D)] chromatograms of methidathion (0.02 ppm) in the test solution obtained from the recovery test of fried fish. (A) and (C), non-fortified fried fish; (B) and (D), fortified fried fish. The broken and solid lines in (A) and (B) are monitoring at m/z's of 145 and 85, respectively. The solid lines in (C) and (D) are monitoring the transition from 145 to 85. An arrow in (B) indicates the retention time of methidathion. The retention time of methidathion in SIM and SRM were 15.60 and 16.87, respectively. (The time programs of the GC oven temperature were not the same in these experiments)

4.2.2.3 Analysis of 99 pesticides via LC-MS/MS (Chinese dumplings)

The 99 pesticides detectable via LC-MS/MS were fortified to the Chinese dumplings at the concentrations of 0.02 and 0.10 ppm, respectively. We conducted 5 trials for each test. The pesticides were categorized into 4 groups on the basis of the results.

Group A (72 pesticides exhibiting the acceptable results at the both concentrations):
Acetochlor, Allethrin, Atrazine, Azoxystrobin, Benfuresate, Bensulide, Bitertanol, Bromobutide, Bupirimate, Buprofezin, Cafenstrole, Carfentrazone-ethyl, Carbaryl, Carbofuran, Chlorpropham, Cyanazine, Cyhalofop-butyl, Daimuron, Diethofencarb, Difenoconazole, Diflubenzuron, Diflufenican, Dimepiperate, Dimethametryn, Dimethoate, Dimethomorph, Diphenamid, Ethofumesate, Etobenzanid, Fenarimol, Fenbuconazole, Fenoxycarb, Fenoxaprop-ethyl, Flusilazole, Furathiocarb, Hexaconazole, Hexaflumuron, Imibenconazole, Inabenfide, Indanofan, Iprovalicarb, Isoprocarb, Isoprothiolane, Isoxathion, Mefenacet, Mepanipyrim, Metalaxyl, Methabenzthiazuron, Methomyl, Metolcarb, Monocrotophos, Napropamide, Omethoate, Paclobutrazol, Penconazole, Pencycuron, Pirimicarb, Pretilachlor, Prochloraz, Propoxur, Pyriproxyfen, Pyroquilon, Quizalofop-ethyl, Tebuconazole, Tebufenozide, Teflubenzuron, Thenylchlor, Thiacloprid, Thiobencarb, Tri-allate, Triadimefon, Triadimenol

Group B (15 pesticides exhibiting the acceptable results at only 0.10 ppm):
Acetamiprid, Acephate, Alachlor, Clomeprop, Cyflufenamid, Fenobucarb, Flufenoxuron, Imazalil, Lufenuron, Pentoxazone, Phoxim, Propyzamide, Quinoclamine, Tebufenpyrad, Triflumizole

Group C (4 pesticides exhibiting the semi-acceptable recovery results, 50~69% or 120~150%, at least at the concentrations 0.10 ppm with RSD ≤ 20%):
Cumyluron, Esprocarb, Methamidophos, Oxamyl

Group D (8 pesticides could not be categorized as A~C):
Bendiocarb, Ethiofencarb, Fenpropimorph, Molinate, Phenmedipham, Propamocarb, Propiconazole, Trichlamide

4.2.3 Case of a pesticide detected with our method

The method should be useful for analyzing pesticide residues in foods with complaints, such as odors derived from uncertain chemicals. As an example, we present a case in which we successfully detected the pesticide, phenothrin, from a suspected consumers' food (omelets in catering lunch boxes) at a concentration of 0.06 ppm in a half-day period. In this case, the pesticide, which was used to sanitize the catering kitchen, migrated into the refrigerator due to a faulty door and contaminated the omelets inside. To analyze the pesticide residues in foods in cases such as this, rapidity is one of the most pivotal aspects. Thus, governments and food industries should develop rapid methods to analyze pesticide residues in foods as a part of crisis management.

4.3 Method for processed foods in non- and low lipid groups (under study)

We are currently studying methods to determine the presence and quantities of pesticide residues in processed food in the low and non-lipid groups, such as dried fruits, marmalade, pickles (including soured vegetables and fruits), and seasonings. In this method, the process for removing lipids is not necessary. For the sake of efficiency, a method should be developed with minimum modification of the method described in 3.4.1 to determine pesticides in agricultural products. We conducted pilot studies for the development of such

a method and found that dried fruits and marmalades were not miscible with acetonitrile in the extraction step. This problem was successfully overcome by adding an equal weight of water to the sample and allowing it to stand for 30 min prior to the extraction with acetonitrile. We are currently validating the method in our laboratory. Details will be published later.

Homogenized sample 5.0 g in a 50 mL PP disposal tube
- Add 5 mL of water and stand for 30 min
- Add 20 mL of acetonitrile
- Homogenize (1 min)
- Add 4 g of $MgSO_4$ and 1 g of NaCl
- Shake (1 min) and centrifuge

Acetonitrile layer 16 mL (equivalent to 4.0 g sample)
- Load on a GCB/PSA column
- Elute with 30 mL acetonitrile-toluene (3+1)
- Collect loading and eluting fractions in a round bottom flask
- Evaporate and reconstituted in appropriate solvent

GC-MS (/MS) or LC-MS/MS analysis

Scheme IV. Procedure of sample preparation for the determination of pesticides residues in processed foods categorized as the non or low-lipid group.

	Foods			
Category	Agricultural Products		Processed Food	
Group	Vegetables and fruits	Cereals	High-lipid	Non or Low-lipid
Water #	No	Yes	No	Yes
Extraction ##	AcCN	AcCN	EtOAc	AcCN
Cleanup $	GCB/PSA	C18 GCB/PSA	AcCN/Hex GCB/PSA	GCB/PSA
Time (h) $$	3~4	3.5~4.5	5~6	3.5~4.5
Analysis	GC-MS, GC-MS/MS and LC-MS/MS			
Scheme	I	II	III	IV

#: Add water before extraction.
##: Extraction solvents; acetonitrile and ethyl acetate are abbreviated to AcCN and EtOAc, respectively.
$: Using columns and extraction procedure; GCB/PSA, GCB, and PSA double layer column; C18, octadecylsilyl column; AcCN/Hex, acetonitrile-hexane partition.
$$: Time for preparation of 12 samples by a chemist.

Table 1. Summary of the analytical methods of pesticides in foods.

5. Conclusion

"Rapid and easy" multiresidue methods for the determination of pesticide residues in foods have been developed. Table 1 summarizes these methods. The methods are based on a simple extraction with organic solvents, a purification with SPE cleanup, and determinations with GC-MS, GC-MS/MS, and LC-MS/MS. The proposed methods

exhibited good sensitivity and recovery and allowed for rapid analysis. For agricultural products, a single chemist could prepare test solutions from 12 corresponding homogenized samples within 4.5 h. For processed foods, a single chemist could prepare test solutions for 12 corresponding homogenized samples within 6 h. Our method does not require special techniques in sample preparation. The characteristic points of the methods, "rapid and easy," would induce substantial benefits: (a) reduction of time and costs for sample preparation, (b) reduction of time for mastering the operations, and (c) reduction of the errors within the procedures. These reductions would produce more time and money to simultaneously analyze more pesticides with better performance and to test the adaptation of new pesticides to this method. The methods described here have a high potential covering a wide range of pesticides. Thus, they would be applicable to various foods and ideally suited for use in regulatory laboratories.

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Note

Determination Method for Ractopamine in Swine and Cattle Tissues Using LC/MS

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Simple and reliable methods using LC/MS have been developed for the determination of the β-agonist ractopamine in swine and cattle tissues. Ractopamine was extracted with ethyl acetate from muscle and liver, and the ethyl acetate layer was evaporated to dryness. The residue was purified by partition with acetonitrile/*n*-hexane. In the case of fat, ractopamine was extracted and purified by partition with acetonitrile/*n*-hexane. The resulting acetonitrile solutions were evaporated to dryness. The residue was dissolved in methanol, and subjected to LC/MS. The LC separation was performed on a Wakosil-II 3C18HG column (150×3 mm i.d.) in isocratic mode with 0.05% trifluoroacetic acid-acetonitrile (80 : 20) as a mobile phase at a flow rate of 0.4 mL/min. The MS detection was performed in the selected ion recording (SIR) mode, with detection of the $M+H^+$ ion of ractopamine (*m/z* 302) produced by electrospray ionization (ESI). The mean recoveries of the drug from swine muscle (0.01 μg/g fortified), fat (0.01 μg/g fortified) and liver (0.04 μg/g fortified) were 99.7%, 99.5% and 100.8%, and those from cattle samples were 108.3%, 97.0% and 109.4%, respectively. The relative standard deviations (RSDs) ranged from 0.1% to 9.5%. The limit of quantification (LOQ) of the drug was 1 ng/g.

Key words: ractopamine; β-agonist; swine; cattle; LC/MS

Introduction

β-Adrenergic agonists (β-agonists) are widely used as bronchodilators, tocolytics and heart tonics in clinical and veterinary medicine[1]. Ractopamine (Fig. 1) is a β-agonist, and the impact of this drug on growth performance and carcass quality has been clearly demonstrated in pig, mainly in terms of increased weight gain and lean tissue accretion, as well as improved feed conversion ratio[2-4]. The advantages of feeding pigs with this drug have been reported as increased daily weight gain, improved feed efficiency, saving on feed, increased nitrogen retention and shortened breeding period[5,6].

Recently, veterinary drug residues have become a matter of public concern because of possible adverse effects on human health owing to carryover from drug-treated animals to the human diet. Ractopamine has been approved as a feed supplement in the U.S.A. and Australia. On the other hand, the EU has officially banned the use of such adrenergic drugs as growth-promoting agents and the import of ractopamine-treated meat. In Japan, the use of this drug has also been banned and maximum residue limits (MRLs) have been set for the drug as shown in Table 1.

Several screening methods for ractopamine have been reported based on enzyme immunoassay (EIA) or enzyme-linked immunosorbent assay (ELISA)[7-9]. The detection limits of these methods were in the 1-50 ng/mL (ppb) range, and the cross-reactivities for other β-agonists were generally less than 0.5%. These methods are able to detect ractopamine without complicated purification, due to the specificity of antibody used, though matrix effects often occur. Other screening methods using HPLC with electrochemical detection[10,11], UV detection[12], and fluorescence

Table 1. Maximum residue limits (MRLs) for ractopamine in Japan

Animals	Tissues	MRL (ppm)
Cattle and Swine	Muscle	0.01
	Fat	0.01
	Liver	0.04
	Kidney	0.09
	Other edible parts	0.04

detection[5] have also been reported. These methods are sensitive, but generally require complicated pretreatments and are also time-consuming for detection of ppb levels of ractopamine (Table 1). The methods using HPLC with tandem mass spectrometry (LC/MS/MS) are highly sensitive[8,13,14], but these instruments are expensive.

On the other hand, LC/MS systems are widely available, and are sensitive enough to detect ppb levels of ractopamine as listed in Table 1. So, if a simple and reliable method using LC/MS is developed, more inspection agencies will be able to perform residue analysis of ractopamine.

The aim of this study was to develop a simple and reliable method for the quantification and confirmation of trace amounts of ractopamine in swine and cattle tissues using LC/MS. The procedure was developed for use in routine monitoring of ractopamine residues, as mandated by the inspection agencies.

Materials and Methods

Samples, chemicals and materials

Swine and cattle tissues (muscle, fat and liver) were purchased from a market in Tokyo, Japan. These samples were stored at −40℃ until analysis. Standard ractopamine hydrochloride was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Ractopamine standard solutions were prepared as described below. A standard stock solution containing 100 μg/mL of ractopamine was prepared in methanol, and the solution was diluted successively with methanol. These standard solutions were stored at −20℃ until analysis. All the solvents used were of LC grade and other chemicals used were of analytical grade unless otherwise stated.

Liquid chromatograph and mass spectrometer

Alliance 2695 separation module liquid chromatograph (Waters, Co., Milford, MA, USA) coupled with a micromass ZQ 2000 mass spectrometer (Waters).

LC/MS conditions

Column: Wakosil-II 3C18HG (150×3 mm i.d., 3 μm, Wako Pure Chemical Industries, Ltd.)
Column oven temperature: 40℃
Mobile phase composition: 0.05% trifluoroacetic acid–acetonitrile (80 : 20)
Flow rate: 0.4 mL/min
Source temperature: 100℃
Desolvation temperature: 350℃
Gas: nitrogen, flow rate: *ca.* 360 L/hr
Capillary voltage: 3.5 kV
Ionization mode: ESI, positive ion mode
Measuring mode: SIR
Monitoring ion: *m/z* 302
Cone voltage: 20 V

Sample preparation

For meat and liver, a finely chopped sample (5.0 g) was weighed into a 50 mL centrifuge tube. Then 20 mL of ethyl acetate and 1 mL of 4 mol/L potassium carbonate solution were added to the tube, and the sample was homogenized for 2 min and centrifuged for 10 min at 3000 rpm. The ethyl acetate layer was transferred to a recovery flask, and 20 mL of ethyl acetate was added to the pellet in the tube. The mixture was homogenized and centrifuged. The resulting ethyl acetate solutions were combined and evaporated to dryness below 40℃. The residue was dissolved in 30 mL of acetonitrile, and the acetonitrile solution was vigorously shaken twice with 30 mL of *n*-hexane saturated with acetonitrile. The resulting acetonitrile solution was evaporated to dryness, and the residue was redissolved in 1.0 mL of methanol.

For fat samples, preparation of the test solution was performed as described below. A sample (5.0 g) was weighed into a 250 mL glass tube. Then 30 mL of acetonitrile and an equivalent volume of *n*-hexane saturated with acetonitrile were added, and the sample was homogenized for 2 min and centrifuged for 10 min at 3,000 rpm. The acetonitrile layer was transferred to a recovery flask, and 30 mL of acetonitrile was added to the glass tube. The mixture was homogenized and centrifuged. The two acetonitrile layers were combined and defatted with 30 mL of *n*-hexane saturated with acetonitrile. The acetonitrile layer was evaporated to dryness, and the residue was redissolved in 1.0 mL of methanol.

Determination of ractopamine

Determination of ractopamine was performed by LC/MS. Aliquots of 2 μL of the ractopamine standard solutions and the test solutions were injected into the LC/MS system. The quantification was performed by calculating the peak areas. Detection was performed by SIR at *m/z* 302.

Results and Discussion

MS conditions

Ractopamine is a basic compound possessing an amino group (Fig. 1). Therefore, the positive ESI mode was applied for the analysis of this drug. In the MS spectrum of ractopamine, the proton adduct of the ractopamine molecule $(M+H)^+$ (*m/z* 302) was observed as a base peak. Therefore, the *m/z* 302 ion was selected as the monitor ion for SIR. The cone voltage was set to 20 V, since the monitoring ion (*m/z* 302) was observed most strongly.

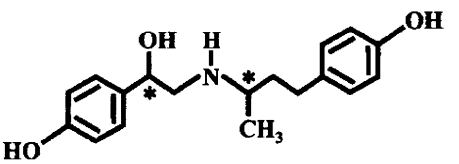

Fig. 1. Structure of the β-agonist ractopamine
The asterisks indicate asymmetric carbon atoms.

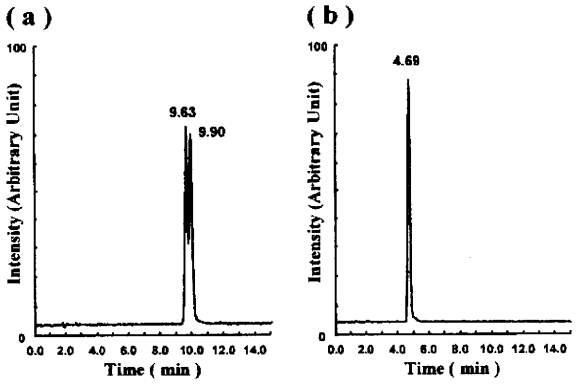

Fig. 2. Chromatograms of ractopamine standard solutions with two different mobile phases

0.2 μg/mL of ractopamine standard solution was injected into the LC/MS with the mobile phase of 0.05% trifluoroacetic acid–acetonitrile (85 : 15) (a) or (80 : 20) (b).

HPLC conditions

A standard C18 column, which is generally used for the analysis of veterinary drugs, was selected as the analytical HPLC column.

Acetonitrile and 0.05% trifluoroacetic acid were selected as the mobile phase and the flow rate was set to 0.4 mL/min, based on the Multiresidue Method I for Veterinary Drugs, etc. by HPLC (Ministry of Health, Labour and Walfare, Japan).

The standard solution of ractopamine was subjected to LC/MS using 2 different mobile phases (0.05% trifluoroacetic acid–acetonitrile (80 : 20 and 85 : 15)). The chromatograms obtained are shown in Fig. 2. The peak obtained was very sharp with 0.05% trifluoroacetic acid–acetonitrile (80 : 20), whereas it was slightly broadened with 0.05% trifluoroacetic acid–acetonitrile (85 : 15). It was considered that the isomers of ractopamine were partially separated in the latter mobile phase.

The MRLs for ractopamine are set as the total concentration of these isomers. Therefore the mobile phase of 0.05% trifluoroacetic acid–acetonitrile (80 : 20) was selected in this study and the ractopamine was determined based on the one peak.

Extraction and clean-up

First, solvents for the extraction of ractopamine were investigated. Almost all added ractopamine was extracted by using methanol/acetic acid[1], methanol[5] or acetonitrile, but materials which interfered with the determination of ractopamine were also extracted. Therefore, we used ethyl acetate as a solvent for extraction, and transferred un-ionized ractopamine to the ethyl acetate layer by adding 4 mol/L potassium carbonate. This method could extract ractopamine almost entirely, without extracting any interfering material. In the case of fat, ractopamine was extracted and purified by partition with acetonitrile/*n*-hexane, because no interfering material was extracted.

Table 2. Results of recovery tests for ractopamine

	Fortified (ppm)	Swine		Cattle	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Muscle	0.01	99.7	6.6	108.3	0.1
Fat	0.01	99.5	9.5	97.0	0.1
Liver	0.04	100.8	5.7	109.4	1.9

$n=3$

Next, clean-up procedures were investigated using several cartridge columns (polymer, C18 reverse phase and cation-exchange type). The retention and elution of ractopamine were good in the case of the standard solution, but less good in the case of food matrices, and the recoveries varied widely. So, the chosen clean-up procedure consisted of only partition with acetonitrile/*n*-hexane, without using the cartridge columns.

With such a simple method, the extraction of ractopamine was achieved with high recovery and high reproducibility, without interfering peaks on the chromatograms.

Recovery test and precision of analysis

The results of recovery tests are shown in Table 2. Ractopamine was added to muscle and fat at the level of 0.01 μg/g, and added to liver at the level of 0.04 μg/g. These samples were analyzed by the proposed methods. Mean recoveries of ractopamine from swine tissues were 99.5–100.8%, and those from cattle tissues were 97.0–109.4%. The RSDs ($n=3$) ranged from 5.7% to 9.5% for swine samples, and from 0.1% to 1.9% for cattle samples. The LOQ was 1 ng/g ($S/N=10$). These results satisfied the standards for residual analysis of veterinary drugs in foods established by the Codex Alimentarius Commission (CAC). Therefore the method proposed in this study offers good performance for the analysis of ractopamine residues in foods.

Conclusion

A simple and reliable method using LC/MS has been developed for the determination of ractopamine in swine and cattle tissues. The method consists of extraction with ethyl acetate for muscle and liver and with acetonitrile/*n*-hexane for fat, clean-up by extraction with acetonitrile/*n*-hexane saturated with acetonitrile, and determination using LC/MS (SIR mode).

The mean recoveries of ractopamine from swine samples were 99.5–100.8%, and those from cattle samples were 97.0–109.4%. The RSDs ranged from 0.1% to 9.5% ($n=3$). The LOQ was 1 ng/g ($S/N=10$). These results meet the standards for residual analysis of veterinary drugs in foods established by CAC. Therefore, the simple method developed in the present study is suitable for routine monitoring of ractopamine.

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報 文

LC-MS/MSによる畜水産食品中のビコザマイシンの定量

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Determination of Bicozamycin in Livestock Products and Seafoods by Liquid Chromatography-Tandem Mass Spectrometry

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A novel liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed for trace residue determination of bicozamycin (BZM) in livestock products and seafoods. BZM was extracted from a sample with acetonitrile–water (4 : 1), followed by a two-stage SPE enrichment and cleanup. The first stage involved a styrene–divinylbenzene copolymer cartridge (GL-Pak PLS-2), and the second stage involved a divinylbenzene–*N*-vinylpyrrolidone copolymer cartridge (Oasis HLB). The LC separation was performed on a C18 column using 0.01% formic acid–methanol (8 : 2) as the mobile phase and MS detection with negative ion electrospray ionization. The mean recoveries from swine muscle, liver, yellowtail, and milk fortified at the minimum residue limit (MRL) levels and 0.01 μg/g were >70%, and the relative standard deviations (RSDs) were <20%. Limits of quantitation (LOQs) ranged from 0.002 to 0.005 μg/g.

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Key words: ビコザマイシン bicozamycin; 畜産食品 livestock product; 水産食品 seafood; 液体クロマトグラフィー/タンデム質量分析法 LC-MS/MS

緒 言

ビコザマイシン(BZM)は，*Streptomyces sapporonensis* から産生される抗生物質で，Fig. 1 に示す構造を有し，*Escherichia coli*, *Salmonella* などのグラム陰性菌に対して抗菌作用を有している．わが国においては，飼料添加物として鶏用および豚用飼料への使用が認められており，また，安息香酸塩は水産用医薬品としてスズキ目魚類への使用が認められている．これらのことから，薬剤が不適切な使用をされた場合，家畜などへの残留が懸念される．

BZMの化学的な残留分析法としては，高性能薄層クロマトグラフィー(HPTLC)により分離し，デンシトメトリーで蛍光検出する方法[1]とUV検出器を用いた高速液体クロマトグラフィー(HPLC)[2]による方法が報告されているが，ポジティブリスト制度において設定された暫定基準値をクリアできる検出感度を有していない．

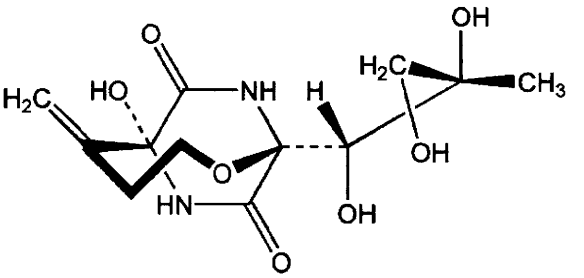

Fig. 1. Chemical structure of BZM.

そこで著者らは，種々のポリマー系カートリッジに対するBZMの挙動を確認した後，2種類のカートリッジを用いて濃縮・精製した後，液体クロマトグラフィー/タンデム質量分析法(LC-MS/MS)による方法を検討したところ，良好な結果が得られたので報告する．

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実験方法

1. 試　　料

試料は大阪府内で市販されていた豚筋肉，豚肝臓，ブリおよび牛乳を使用した．

2. 試 薬 等

標準品：BZM は独立行政法人農林水産消費安全技術センター検定品（力価 916.8 μg/mg）を用いた．

標準溶液：BZM 標準品 20 mg（力価として）を精密に量り，メタノール 20 mL に溶解して標準原液 (1,000 μg/mL) とし，標準溶液は用時適宜 0.01% ギ酸で希釈して調製した．なお，標準原液は −25℃ で保存した．

GL-Pak PLS-2 カートリッジ (500 mg)：ジーエルサイエンス社製，カートリッジはあらかじめメタノール 10 mL および水 10 mL でコンディショニングした後，使用した．

Oasis HLB カートリッジ (60 mg)：Waters 社製，カートリッジはあらかじめメタノール 5 mL および水 5 mL でコンディショニングした後，使用した．

試薬：メタノールおよびギ酸は LC/MS 用を，その他の試薬は市販特級品を，水は Millipore 社製 Milli-Q 超純水製造装置で精製した水を使用した．

メンブランフィルター：Millipore 社製 Millex-LG 0.20 μm

3. 装置および測定条件

高速液体クロマトグラフは，島津製作所社製 LC-20 シリーズ，タンデム質量分析装置は Applied Biosystems 社製 4000Qtrap を用い，Table 1 および 2 に示した条件で測定した．

4. 検 量 線

BZM 標準原液を 0.01% ギ酸で希釈し，0.5〜25 ng/mL の標準溶液を調製し，その 5 μL を LC-MS/MS に注入した．検出には MRM (Multiple Reaction Monitoring) 法を採用し，得られたクロマトグラムよりピーク面積を求め，絶対検量線法により検量線を作成した．

Table 1. Operating conditions of MS/MS

Ionization	ESI, negative
Ion spray voltage	−4,500 V
Ion spray temp.	450℃
Declustering potential	−40 V
Dwell time	150 ms
Curtain gas	20 psi
Collision gas	6 psi
Nebulizer gas	60 psi

MRM Transition (*m/z*)	CE[*1] (eV)	CXP[*2] (V)
301→209[*3]	−22	−3
301→184	−18	−5
301→124	−34	−7

[*1] Collision energy.
[*2] Collision exit potential.
[*3] Daughter ion for quantitation.

Table 2. Operating conditions of LC

Column	Hydrosphere C18 HS-301-3, (3 μm, 100×4.6 mm i.d.)		
Flow rate	0.2 mL/min		
Oven temp.	40℃		
Injection volume	5 μL		
Eluent	A=0.01% Formic acid, B=Methanol		
	Time (min)	A (%)	B (%)
	0.00→10.00	100	0
	10.01→24.00	80	20
	24.01→34.00	100	0

5. 試験溶液の調製

試料 5.0 g を採取し，アセトニトリル−水 (4 : 1) 40 mL を加えてホモジナイズ（30 秒間）した後，遠心分離 (3,500 rpm, 20℃, 5 分間）し，上澄み液を分取した．残留物にさらにアセトニトリル−水 (4 : 1) 40 mL を加えて 5 分間振とう抽出した後，同様に遠心分離した．上澄み液を先のものと合わせ，アセトニトリル−水 (4 : 1) で 100 mL に定容し，その 20 mL を分取した後，1-プロパノール 5 mL を加えて減圧下濃縮乾固した．残留物に 2% 塩化ナトリウム溶液 30 mL およびジエチルエーテル 30 mL を加えて 5 分間振とうした後，遠心分離 (3,500 rpm, 20℃, 5 分間）して水層を分取し，1-プロパノール 10 mL を加えて再度濃縮乾固した．残留物を水 10 mL (5 mL×2 回）に溶解して，GL-Pak PLS-2 カートリッジに負荷し，カートリッジを水 5 mL で洗浄後，メタノール−水 (3 : 2) 10 mL で溶出した．溶出液を減圧下濃縮乾固した後，残留物を 0.01% ギ酸 10 mL に溶解した後，Oasis HLB カートリッジに負荷した．通過液の最初の 4 mL を廃棄し，残りの 6 mL を分取してメンブランフィルターでろ過して試験溶液とし，この 5 μL を LC-MS/MS に注入した．

結果および考察

1. LC-MS/MS 測定条件の検討

1.1 MS/MS 条件の検討

BZM は，極性が高い化合物であることから，イオン化のインターフェイスとして ESI を選択した．標準溶液 (1 μg/mL) をインフュージョン法により直接 MS 部に導入してイオン化条件を検討した．その結果，Fig. 2 に示すように，脱プロトン化分子 $[M-H]^-$ (*m/z* 301) を高感度に検出することができたため，このイオンをプレカーサーイオンとして選択し，イオン化電圧ならびにコリジョンエネルギーなどの最適化を行った．Fig. 3 にプロダクトイオンのマススペクトルを示した．この結果から，プロダクトイオンには，*m/z* 301 > 209, *m/z* 301 > 184, *m/z* 301 > 124 の 3 イオンを採用し，最も高感度な *m/z* 301 > 209 を定量用イオンとし，MRM モードで測定することとした (Table 1).

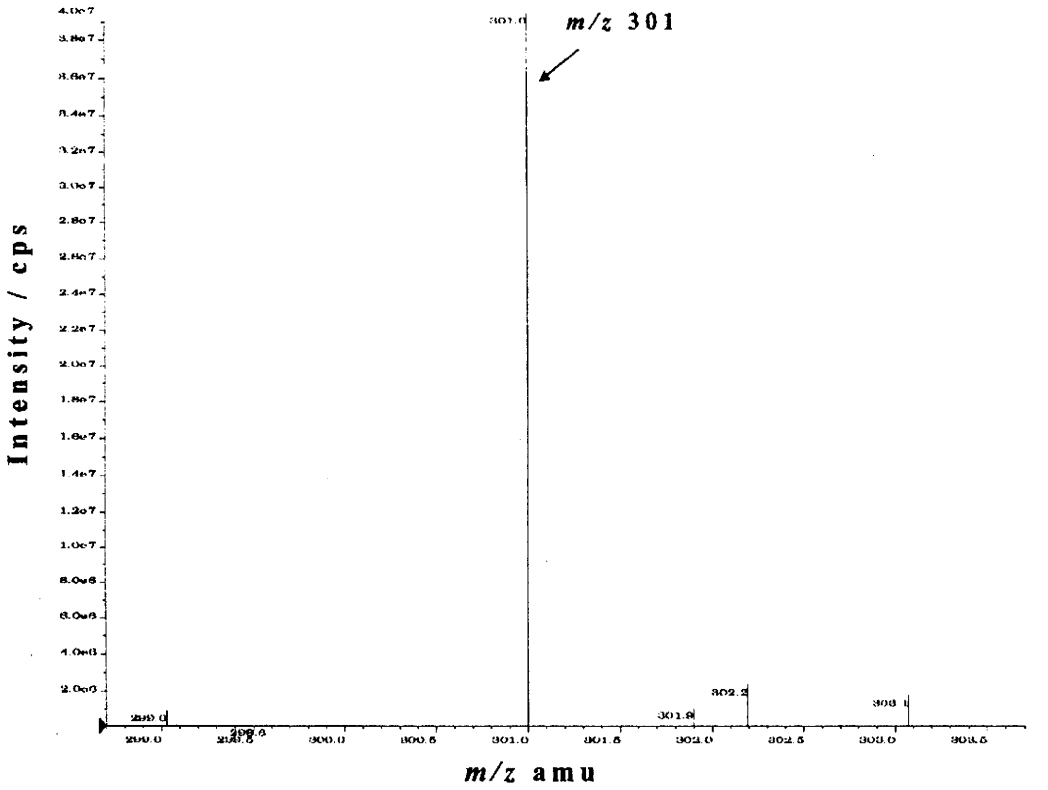

Fig. 2. MS spectrum of BZM obtained by direct infusion of the standard solution (1 μg/mL).

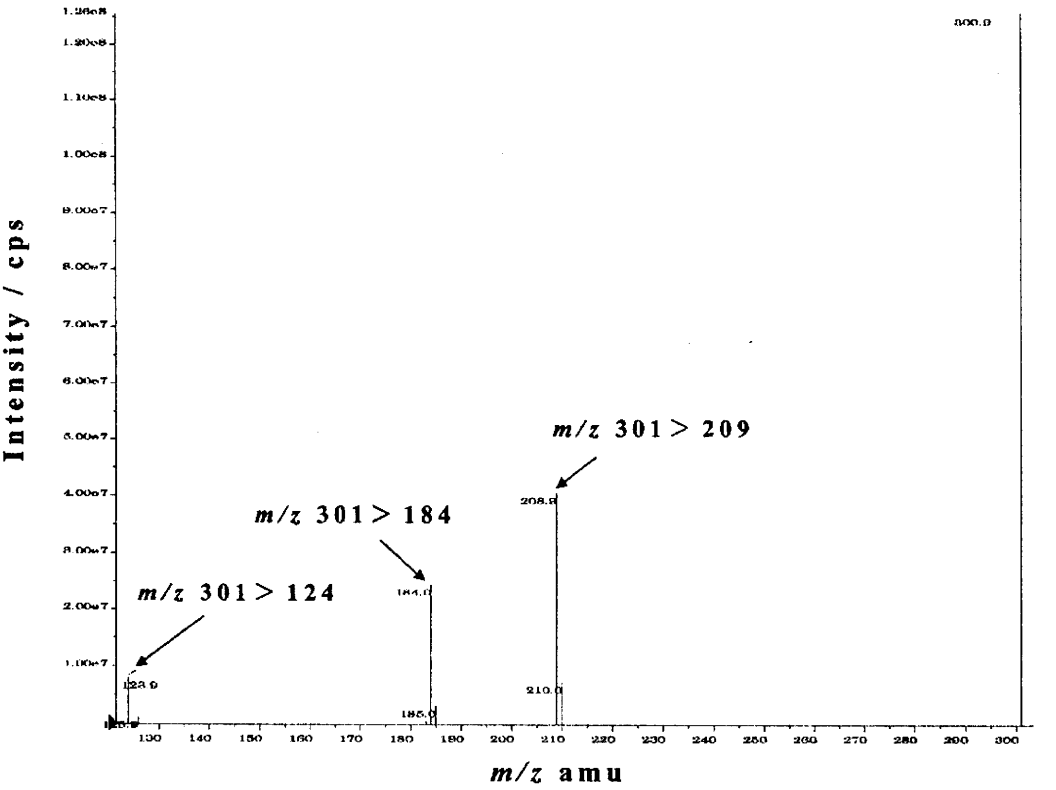

Fig. 3. Product ion scan of BZM, *m/z* 209, showing the most intense fragment ions at *m/z* 184 and 124.

1.2 LC 条件の検討

BZM は非常に極性が高く，ODS 系カラムへの保持が弱い．そのため，Ise らは，過塩素酸溶液やリン酸二水素カリウム溶液にほとんどメタノールなどの有機溶媒を加えない移動相を使用している[2]．ODS 系以外のカラムとして，極性物質に対して保持能力が良いとされる HILIC 系カラムの一種である Inertsil Diol について検討したが，良好な結果が得られなかった．そこで ODS 系カラムでの測定条件を検討した．

分析カラムとして，水の割合が多い移動相において耐久性が高い，Hydrosphere C18 H-301-3 を使用し，BZM の保持を向上させるため，内径 4.6 mm，長さ 100 mm の通常の HPLC で汎用されるカラムサイズとした．また，移動相に用いる有機溶媒として，保持が比較的良好であったメタノールを選択した．

次に，移動相に加える揮発性の酸として酢酸，ギ酸を，添加剤として酢酸アンモニウム，ギ酸アンモニウムを用いて，ピーク形状，検出感度に及ぼす影響を調べた．その結果，0.01% のギ酸を加えることにより，最も良好な感度が得られ，ピーク形状も良好であった．したがって，移動相には 0.01% ギ酸-メタノール系を用いることとした．BZM は，0.01% ギ酸-メタノール (80 : 20) でピーク形状，感度とも良好に測定可能であったが，試料が豚肝臓の場合，マトリックスの影響によるイオン化抑制が残留基準値濃度 (0.2 μg/g) の添加回収実験の回収率として，30% 程度認められた．通常，イオン化抑制を回避する方法として，グラジエント溶出が用いられるが，BZM は ODS 系カラムへの保持が弱いため，グラジエント溶出の条件設定が困難であった．そこで，最初の数分間 0.01% ギ酸のみを通液し，その後に 0.01% ギ酸-メタノール (80 : 20) に切り替えて通液して BZM を溶出させる手法を検討した．その結果，10 分間 0.01% ギ酸を通液させてから BZM を溶出させた場合，前述の添加回収実験の回収率として，約 20% のイオン化率の改善が見られた．よって，本条件で測定を行うこととした (Table 2)．また，定量用イオンを用いて，本条件で検量線を作成したところ，0.5～25 ng/mL の範囲で良好な直線性 ($R^2 = 0.999$) が得られた．なお，確認イオンとして用いた m/z 301 > 184 および m/z 301 > 124 においても同様の濃度範囲で良好な直線性 ($R^2 = 0.999$) が得られた．

2. 前処理法の検討

BZM の残留分析の前処理法としては，斉藤らは，各種の固相抽出カートリッジ，吸着樹脂を検討した結果，XAD-2 樹脂および XAD-4 樹脂にのみ BZM が吸着し，より吸着能が高かった XAD-4 樹脂を採用している[1]．しかしながら，当時は XAD-4 に該当するスチレンジビニルベンゼン共重合体などのポリマー系カートリッジが存在しなかったことから，本研究においては，操作性を考慮して，現在汎用されている，ポリマー系カートリッジについて BZM の挙動を確認することとした．

Table 3. Comparison of recovery of BZM from various SPE cartridges

Cartridge	Recovery* (%)
Oasis HLB (60 mg)	< 5.0
Oasis HLB (200 mg)	9.2
GL-Pak PLS-2 (270 mg)	39.0
GL-Pak PLS-2 (500 mg)	84.0
Aqusis PLS-3 (200 mg)	11.6
InertSep Pharma-1 (200 mg)	4.8
InertSep Pharma-2 (500 mg)	46.7

* 10 mL of standard solution of BZM (0.025 μg/mL in water) was applied to the cartridge in duplicate.

Table 4. Effect of volume of water used for washing the GL-Pak PLS-2 (500 mg) cartridge on the recovery of BZM

Volume of water (mL)	Recovery* (%)
5	89.3
10	81.9
20	63.5
50	4.0

* 10 mL of standard solution of BZM (25 ng/mL in water) was applied to the cartridge in duplicate.

Table 5. Elution fraction of BZM from the Oasis HLB cartridge

Elution fraction (mL)	Recovery* (%)
0～2	< 5.0
2～4	32.3
4～6	93.5
6～8	103.8
8～10	104.8

* 10 mL of Standard solution of BZM (50 ng/mL in 0.01% formic acid) was applied to the cartridge in duplicate.

25 ng/mL の標準溶液 10 mL (250 ng) を負荷して検討した結果，含窒素ポリマー系カートリッジである Oasis HLB, Aqusis PLS-3, Inertsil Pharma は，BZM の吸着は弱く，XAD-4 と同様のスチレンジビニルベンゼン共重合体カートリッジである GL-Pak PLS-2 の方が BZM は強く吸着されていた (Table 3)．しかしながら，充填量の差が大きく回収率に影響していることから，GL-Pak PLS-2 も水溶液中で不可逆的な吸着をしているものとは考えられなかった．そこで次に，上記と同様に GL-Pak PLS-2 (500 mg) を用いて，水洗の容量の違いによる BZM の回収率を調べた．その結果，水洗の容量が増すにつれて回収率が低下していることから，BZM の十分な回収を得るには，5 mL の水洗で行う必要があるものと示唆された (Table 4)．

これらの結果に基づいて，豚肝臓を用いて精製効果を確認した．なお，抽出は，カートリッジに負荷する際に濃縮する必要性があることから，Ise らの方法[2] に準拠して含水アセトニトリルを用いることとした．その結果，**1.2 LC 条件の検討**において記述したように，移動相条件を検