Food Additives and Contaminants, April, 2006; (23)4: 422–430

Taylor & Francis
Taylor & Francis Group

Estrogenic activities of chemicals related to food contact plastics and rubbers tested by the yeast two-hybrid assay

YUKO OGAWA, YOKO KAWAMURA, CHISEKO WAKUI, MOTOH MUTSUGA, TETSUJI NISHIMURA, & KENICHI TANAMOTO

National Institute of Health Sciences, Japan

(Received 3 October 2005; accepted 20 November 2005)

Abstract
Food contact plastics and rubbers possibly contain many kinds of chemicals such as monomers, oligomers, additives, degradation products of polymers and additives, and impurities. Among them, bisphenol A, nonylphenol, benzylbutyl phthalate, styrene oligomers and hydroxylated benzophenones have been reported to possess estrogenic activities. In this study, other chemicals related to food contact plastics and rubbers, and their metabolites induced by the S9-mixture were tested for their estrogenic activities using the yeast two-hybrid assay. Among the 150 chemicals, 10 chemicals such as bis(4-hydroxyphenyl) methane, 4-cyclohexylphenol, 4-phenylphenol, 4,4′-isopropylidenediphenol alkylphosphite, two type of styrenated phenol (including mono type), tris(nonylphenyl) phosphite, 2,2′-dihydroxy-4-methoxybenzophenone, 2-hydroxy-4-methoxybenzophenone and 2,4-diphenyl-4-methyl-1-pentene, their metabolites and the metabolites of 6 other chemicals, such as 2-(phenylmethyl) phenol, styrenated phenol (di and tri type), 1-(*N*-phenylamino)naphthalene, 4-*tert*-butylphenylsalicylate, nonylphenol ethoxylates and 2-methyl-6-*tert*-butylphenol, displayed estrogenic activities. All of them contained a phenol group in their chemical structures or formed one easily by hydrolysis or metabolism. However, most of the chemicals related to food contact plastics and rubbers, and their metabolites did not show any estrogenicity.

Keywords: *Estrogenic activity, food contact plastics, rubbers, yeast two-hybrid assay, 2-(phenylmethyl) phenol, 4,4′-isopropylidenediphenol alkyl(C_{12}-C_{15}) phosphite, 1-(N-phenylamino) naphthalene, styrenated phenol, tris(nonylphenyl) phosphite, 4-tert-butylphenylsalicylate, 2,4-diphenyl-4-methyl-1-pentene, 2-methyl-6-tert-butylphenol*

Introduction

Food contact plastics and rubbers may contain many kinds of chemicals such as free monomers, oligomers, additives, degradation products of polymers and additives, and impurities. Among these substances, several are recognized as endocrine disruptors that have estrogenic activities. Bisphenol A, a monomer for polycarbonate resin and epoxy resin, has been reported to have an estrogenic activity in ovariectomized rats (Dodds and Lawson 1938), in MCF-7 human breast-cancer cells (Krishnan et al. 1993) and in the rat uterine-cytoplasma fraction (Olea et al. 1996), and another report has shown that the prostatic weight of the male offspring was increased 6 months after birth (Nagel et al. 1997). Nonylphenol is well-known for its estrogenic activity in MCF-7 cells and in ovariectomized rats after release from a polystyrene test tube (Soto et al. 1991), and it was also determined in several kinds of food contact plastics which originated from the degradation of tris(nonylphenyl) phosphite or tris(mono and/or dinonylphenyl) phosphite used as an antioxidant (Kawamura et al. 1999, 2000). Benzylbutyl phthalate, a plasticizer used for polyvinyl chloride, has been reported to have a weak binding property against the estrogen receptors in MCF-7 (Jobling et al. 1995). Styrene dimers and trimers have been found in polystyrene products (Kawamura et al. 1998a) and also in instant noodles where they had migrated from the polystyrene cup (Kawamura et al. 1998b). Their estrogenic activity in a yeast two-hybrid assay, MCF-7 assay and competitive binding assay have been reported (Nishihara et al. 2000; Ohyama et al. 2001). UV stabilizers, 2-hydroxy-4-methoxybenzophenone have also been reported

Correspondence: Dr Yoko Kawamura, E-mail: kawamura@nihs.go.jp

ISSN 0265-203X print/ISSN 1464-5122 online © 2006 Taylor & Francis
DOI: 10.1080/02652030500482371

to be positive in the MCF-7 assay and the Uterotropic assay (Schlumpf et al. 2001).

Some chemicals related to food contact plastics and rubbers have similar structures to that of the estrogenic chemicals mentioned above and were suspected to possess estrogenic activities. Therefore, we tried to test these many chemicals for estrogenic activity.

For this purpose, there are many useful *in vitro* screening tests such as the MCF-7 assay, receptor binding assays, reporter gene expression assays and so on. We chose the yeast two-hybrid assay which is a kind of reporter gene expression assays, because it was very simple and highly repeatable (Nishikawa et al. 1998, 1999). The yeast two-hybrid assay is based on the ligand-dependent interaction of the estrogen receptor (ER) α and the coactivator TIF2, and the estrogenic activity was detected as the β-galactosidase activity. Two expression plasmids, pGBT9-ERLBD and pGAD424-TIF2, were introduced into yeast cells (*Saccharomyces cervisiae* Y190), which carry a β-galactosidase reporter gene and require tryptophan and leucine for growth. By this method, more than 500 chemicals have already been tested for estrogenic activity, and the structure-activity relationships have been proposed (Nishihara et al. 2000). We also tested UV stabilizers and benzophenone derivatives by this method (Kawamura et al. 2003), and these results had a good relation with the results tested by a human estrogen receptor mediated mammalian reporter gene assay (Kawamura et al. 2005).

In this study, 150 chemicals were tested which comprised monomers, antioxidants, plasticizers, lubricants, vulcanizing agents, vulcanization accelerators and others, including previously reported UV stabilizers (Kawamura et al. 2003). We also tested all of their metabolites which were prepared with the S9-mixture according to the method by Takatori et al. (2003).

Materials and methods

Reagents

The test chemicals listed in Tables I–VIII were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Sigma-Aldrich Japan Co. (Tokyo, Japan), or obtained from the manufacturers. Zymolyase 20T was purchased from Seikagaku Co. (Tokyo, Japan). *o*-Nitrophenyl-β-D-galactoside (ONPG) was purchased from Sigma-Aldrich Japan Co. and dissolved in 0.1 molel^{-1} phosphate buffer (pH 7.0). The S9-extracts (rat liver 9,000 × g supernatant fraction induced by phenobarbital and 5,6-benzoflavone) and cofactor were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). Cofactor was dissolved in 49 ml of milli-Q water. The S9-mix was prepared with 1 ml of S9-extract and 49 ml of cofactor solution, which contained 20 μl ml^{-1} S9, 0.8 μmol NADPH, 0.8 μmol NADH, 1.0 μmol glucode-6-phosphate (G6P), 0.4 U G6P dehydrogenase, 20 μmol Na_2HPO_4, 20 μmol NaH_2PO_4, 6.6 μmol KCl and 1.6 μmol $MgCl_2$.

Preparation of test chemicals

The test chemicals were dissolved in dimethylsulfoxide (DMSO) at 10^{-1} to 10^{-5} moll^{-1} (final

Table I. Estrogenic activities of monomers and known estrogens.

Compounds (Other name or abbreviated name) [main product]	CAS No.	REC_{10} (moll^{-1})	
		Parent comp.	Metabolite
‡17β-Estradiol (E_2)	87-18-3	3.4×10^{-10}	–
‡Bisphenol A	50-28-2	1.1×10^{-5}	–
‡4-Nonylphenol	80-05-7	4.6×10^{-7}	–
†*trans*-Stylbene	103-30-0	$>1.0 \times 10^{-3}$	1.1×10^{-5}
Acrylonitrile [polyacrylonitrile, AS resin, ABS resin, AB rubber]	107-13-1	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Adipic acid [polyamide, polyurethane]	124-04-9	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
‡Bis(4-glycidyloxyphenyl)methane [epoxy resin]	2095-03-6	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
2,2-Bis(4-glycidyloxyphenyl)propane [epoxy resin]	1675-54-3	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Bis(4-hydroxyphenyl)methane (Bisphenol F) [epoxy resin]	620-92-8	1.3×10^{-5}	2.1×10^{-5}
Cyclohexanone-iso-oxine (ε-Caprolactam) [polyamide]	105-60-2	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
‡4-Cyclohexylphenol [phenolic resin]	1131-60-8	6.2×10^{-7}	2.1×10^{-5}
Diphenylmethane-4,4′-diisocyanate [urethane elastomer]	101-68-8	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
Methylmethacrylate [polymethylmethacrylate]	80-62-6	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
α-Methylstyrene [polystyrene, ABS resin]	98-83-9	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
†2-(Phenylmethyl)phenol (*o*-Benzylphenol) [phenolic resin]	28994-41-4	$>1.0 \times 10^{-3}$	2.5×10^{-5}
‡4-Phenylphenol [phenolic resin]	92-69-3	4.7×10^{-6}	1.3×10^{-5}
Styrene [polystyrene, AS resin, ABS resin]	100-42-5	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Toluene-2,4-diisocyanate [polyurethane]	584-84-9	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$

‡Parent compound was positive, †Only metabolite was positive

Table II. Estrogenic activities of antivities of antioxidants.

Compounds (Other name or composition)	CAS No.	REC_{10} ($mol\,l^{-1}$) Parent comp.	Metabolite
Bis(3,5-di-*tert*-butyl-4-hydroxybenzylphosphoric acid ethyl)calcium	65140-91-2	$>1.0\times10^{-4}$	$>5.0\times10^{-5}$
Bis(2,4-di-*tert*-butylphenyl)pentaerythritol diphosphite	26741-53-7	$>1.0\times10^{-4}$	$>5.0\times10^{-5}$
2,4-Bis-(*n*-octylthio)-6-(4-hydroxy-3,5-di-*tert*-butylanilino)-1,3,5-triazine	991-84-4	$>1.0\times10^{-5}$	$>5.0\times10^{-4}$
4,4′-Butylidenebis(3-methyl-6-*tert*-butylphenol)	85-60-9	$>1.0\times10^{-3}$	$>5.0\times10^{-4}$
4,4′-Butylidenebis(3-methyl-6-*tert*-butylphenyl ditridecyl)phosphite	13003-12-8	$>1.0\times10^{-3}$	$>5.0\times10^{-4}$
2,5-Di-*tert*-amylhydroquinone	79-74-3	$>1.0\times10^{-3}$	$>5.0\times10^{-4}$
2,6-Di-*tert*-butyl-4-ethylphenol	4130-42-1	$>1.0\times10^{-3}$	$>5.0\times10^{-4}$
2,5-Di-*tert*-butylhydroquinone	88-58-4	$>1.0\times10^{-3}$	$>5.0\times10^{-4}$
3-(3′,5′-Di-*tert*-butyl-4′-hydroxyphenyl)propionic acid stearyl ester	2082-79-3	$>1.0\times10^{-3}$	$>5.0\times10^{-4}$
2,6-Di-*tert*-butyl-4-methylphenol	128-37-0	$>1.0\times10^{-3}$	$>5.0\times10^{-4}$
Dimethylsuccinate polymer with tetramethyl hydroxy-1-hydroxyethyl piperidine	65447-77-0	$>1.0\times10^{-4}$	$>5.0\times10^{-5}$
N,N′-Di-2-naphtyl-4-phenylenediamine	93-46-9	$>1.0\times10^{-3}$	$>5.0\times10^{-4}$
Distearyl pentaerythrityl diphosphite	3806-34-6	$>1.0\times10^{-3}$	$>5.0\times10^{-4}$
2,2′-Ethylidenebis(4,6-di-*tert*-butylphenol)	35958-30-6	$>1.0\times10^{-3}$	$>5.0\times10^{-4}$
4-Hydroxy-3-*tert*-butylanisole	25013-16-5	$>1.0\times10^{-3}$	$>5.0\times10^{-4}$
4-Hydroxymethyl-2,6-di-*tert*-butylphenol	88-26-6	$>1.0\times10^{-3}$	$>5.0\times10^{-4}$
‡4,4′-Isopropylidenediphenol alkyl(C_{12}-C_{15})phosphite	3315-29-5	4.4×10^{-5}	2.1×10^{-5}
2,2′-Methylenebis (6-*tert*-butyl-4-methylphenol)	119-47-1	$>1.0\times10^{-3}$	$>5.0\times10^{-4}$
4,4′-Methylenebis (2,6-di-*tert*-butylphenol)	118-82-1	$>1.0\times10^{-4}$	$>5.0\times10^{-5}$
2,2′-Methylenebis(4-ethyl-6-*tert*-butylphenol)	88-24-4	$>1.0\times10^{-3}$	$>5.0\times10^{-4}$
2,2′-Methylenebis(4-methyl-6-methylcyclohexlphenol)	77-62-3	$>1.0\times10^{-3}$	$>5.0\times10^{-4}$
Octadecyl-3,5-di-*tert*-butyl-4-hydroxyhydrocinnamate	2082-79-3	$>1.0\times10^{-3}$	$>5.0\times10^{-4}$
2,2′-Oxamidobis[ethyl-3-(3,5-di-*tert*-butyl-4-hydroxyphenyl) propionate]	70331-94-1	$>1.0\times10^{-3}$	$>5.0\times10^{-4}$
†1-(*N*-phenylamino)naphthalene	90-30-2	$>1.0\times10^{-3}$	1.2×10^{-5}
Poly{[6-[(1,1,3,3-tetramethylbutyl)aminol-*s*-triazine-2,4-diyl][2,2,6,6-tetramethyl-4-piperidyl)imino]hexamethylene[(2,2,6,6-tetramethyl-4-piperidyl)imino]}	71878-19-8	$>1.0\times10^{-4}$	$>5.0\times10^{-5}$
‡Styrenated phenol (mono or di or tri)	61788-44-1	3.4×10^{-5}	4.7×10^{-5}
‡Styrenated phenol (mono 74%, di 24%)	61788-44-1	2.9×10^{-6}	1.3×10^{-5}
†Styrenated phenol (di 93%, tri 7%)	61788-44-1	$>1.0\times10^{-3}$	7.5×10^{-4}
Tetrakis[methylene-3-(3′,5′-di-*tert*-butyl-4′-hydroxyphenyl)-propionate]methane	6683-19-8	$>1.0\times10^{-3}$	$>5.0\times10^{-4}$
4,4′-Thiobis(3-methyl-6-*tert*-butylphenol)	96-69-5	$>1.0\times10^{-3}$	$>5.0\times10^{-4}$
3,3′-Thiodipropionic acid di-*n*-dodecyl ester (Dilauryl thiodipropioate)	123-28-4	$>1.0\times10^{-4}$	$>5.0\times10^{-5}$
3,3′-Thiodipropionic acid di-*n*-octadecyl ester (Distearyl thiodipropioate)	693-36-7	$>1.0\times10^{-3}$	$>5.0\times10^{-4}$
3,3′-Thiodipropionic acid di-*n*-tetradecyl ester (Dimyristyl thiodipropioate)	16545-54-3	$>1.0\times10^{-4}$	$>5.0\times10^{-5}$
Triethyleneglycolbis[3-(3-*tert*-butyl-5-methyl-4-hydroxyphenyl) propionate	36443-68-2	$>1.0\times10^{-3}$	$>5.0\times10^{-4}$
1,3,5-Trimethyl-2,4,6-tris(3,5-di-*tert*-butyl-4-hydroxybenzyl) benzene	1709-70-2	$>1.0\times10^{-4}$	$>5.0\times10^{-5}$
Tris(2,4-di-*tert*-butylphenyl)phosphite	31570-04-4	$>1.0\times10^{-4}$	$>5.0\times10^{-5}$
1,3,5-Tris(3,5-di-*tert*-butyl-4-hydroxybenzyl)-*s*-triazine-2,4,6-(*1H,3H,5H*) trione	27676-62-6	$>1.0\times10^{-4}$	$>5.0\times10^{-5}$
1,3,5-Tris(4-*tert*-butyl-3-hydroxy-2,6-dimethylbenzyl)-1,3,5-triazine-2,4,6-(*1H,3H,5H*) trione	40601-76-1	$>1.0\times10^{-3}$	$>5.0\times10^{-4}$
1,1,3-Tris(2-methyl-4-hydroxy-5-*tert*-butylphenyl)butane	1843-03-4	$>1.0\times10^{-3}$	$>5.0\times10^{-4}$
‡Tris(nonylphenyl)phosphite	26523-78-4	1.0×10^{-5}	4.0×10^{-4}

‡Parent compound was positive, †Only metabolite was positive

concentrations: 10^{-3} to $10^{-7}\,mol\,l^{-1}$). When the chemical could not be dissolved at $10^{-1}\,mol\,l^{-1}$, the concentration was changed to 10^{-2} to $10^{-5}\,mol\,l^{-1}$ (final concentrations: 10^{-4} to $10^{-7}\,mol\,l^{-1}$). The concentration of DMSO was 1% in the assay, which did not inhibit the yeast growth. Each experiment was accompanied by 17β-estradiol (E_2) as the positive control and DMSO as the negative control.

Measurement of estrogenic activity by yeast two-hybrid assay

The yeast two-hybrid cells were preincubated overnight at 30°C with vigorous shaking in a SD medium which was free from tryptophan and leucine. The culture was diluted with 4 volumes of the fresh SD medium and 250 µl of this solution put into a small test tube. The test chemical solution (2.5 µl) was added and incubated for 4 h at 30°C.

After incubation, 150 µl of the culture solution was placed into each of the 96 wells of a microplate and the absorbancy measured at 595 nm. The rest of the culture was centrifuged at 10,000 rpm for 7 min, after which the supernatant was removed. The cells were enzymatically digested by incubation with $1\,mg\,ml^{-1}$ Zymolyase 20T (200 µl) at 30°C for 15 min. The cell lysate was mixed with $4\,mg\,ml^{-1}$ ONPG (40 µl) and incubated at 30°C for exactly 30 min. The reaction was stopped by the addition of $1\,mol\,l^{-1}$

Table III. Estrogenic activities of plasticizers.

Compounds	CAS No.	REC_{10} ($mol\,l^{-1}$) Parent comp.	Metabolite
Bis(2-ethylhexyl)azelate	103-24-2	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
n-butyl benzyl phthalate	85-68-7	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Di(ethylene glycol)dibenzoate	120-55-8	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Di(propylene glycol)dibenzoate	27138-31-4	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Di-2-ethylhexyl adipate	103-23-1	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Di-2-ethylhexyl phthalate	117-81-7	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Diacetyllauroyl glycerol	30899-62-8	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Dibenzyl adipate	2451-84-5	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Dibutyl sebacate	109-43-3	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Dicyclohexyl phthalate	84-61-7	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Diheptyl phthalate	41451-28-9	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Diisobutyl adipate	141-04-8	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Diisobutyl phthalate	84-69-5	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Diisodecyl adipate	27178-16-1	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Diisodecyl phthalate	26761-40-0	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Diisononyl adipate	33703-08-1	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Diisonyl phthalate	28553-12-0	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Diisooctyl phthalate	27554-26-3	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Diisopropyl adipate	6938-94-9	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Dimethyl phthalate	131-11-3	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Di-*n*-alkyl adipate (C = 6, 8, 10)	–	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Di-*n*-butyl adipate	105-99-7	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Dibutyl maleate	105-76-0	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Di-*n*-butyl phthalate	84-74-2	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Dinonyl phthalate	84-76-4	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Di-*n*-octyl adipate	123-79-5	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Di-*n*-octyl phthalate	117-84-0	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Di-*n*-propyl adipate	106-19-4	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Dioctyl sebacate	122-62-3	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Diphenylcresyl phosphate	26444-49-5	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Ditridecyl phthalate	75359-31-8	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Epoxidized soybean oil	8013-07-8	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
Heptylnonyl adipate	68515-75-3	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Phosphoric acid diphenyl 2-ethylhexyl ester	1241-94-7	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Tributyl 2-acetylcitrate	77-90-7	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Tri-*n*-butyl phosphate	126-73-8	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Trimellic acid tris(2-ethylhexyl)ester	3319-31-1	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	6846-50-0	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$

Table IV. Estrogenic activities of lubricants.

Compounds	CAS No.	REC_{10} ($mol\,l^{-1}$) Parent comp.	Metabolite
trans-trans-2,4-Decadienal	25152-84-5	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Docosanoic acid amide	3061-75-4	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
cis-13-Docosenic acid amide	112-84-5	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Hexadecanoic acid amide	629-54-9	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Octadecanoic acid amide	124-26-5	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
9-Octadecenoic acid amide	301-02-0	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Oleic acid	112-80-1	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$

Na_2CO_3 (100 µl). After centrifugation at 10,000 rpm for 5 min, the supernatant (150 µl) was placed into each well of a microplate. The absorbances at 420 and 570 nm were read using a microplate reader. The β-galactosidase activity was calculated using the following equation:

$$U = 1000 \times ([OD_{420}] - [1.75 \times OD_{570}])/([t] \times [v] \times [OD_{595}])$$

where t = time of reaction (min), v = volume of culture used in the assay (ml), OD_{595} = cell density at the start of the assay, OD_{420} = absorbance by *o*-nitrophenol at the end of the reaction, and OD_{570} = light scattering at the end of the reaction.

The β-galactosidase activity was expressed as the mean and standard deviation of the results from three separate test tubes.

Preparation of metabolites and their measurement of estrogenic activity

To a tube containing 990 µl of the S9-mix, 10 µl of the test chemical solution (mainly 10^{-1} to

Table V. Estrogenic activities of UV-stabilizers.

Compounds	CAS No.	REC_{10} ($mol\,l^{-1}$) Parent comp.	Metabolite
2,5-Bis(5′-*tert*-butyl-2′-benzoxazolyl)thiophene	7128-64-5	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
†4-*tert*-Butylphenylsalicylate	87-18-3	$>1.0 \times 10^{-3}$	2.7×10^{-5}
2,4-di-*tert*-Butylphenyl-3,5-di-*tert*-butyl-4-hydroxybenzoate	4221-80-1	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
‡2,2′-Dihydroxy-4-methoxybenzophenone	131-53-3	1.0×10^{-3}	1.3×10^{-4}
2-[2′-Hydroxy-3′,5′-bis(α,α-dimethylbenzyl)phenyl]-2H-benzotriazole	70321-86-7	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
2-(2′-Hydroxy-3′-*tert*-butyl-5′-methylphenyl)-5-chlorobenzotriazole	3896-11-5	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
2-(2′-Hydroxy-3′,5′-di-*tert*-amylphenyl) benzotriazole	25973-55-1	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
‡2-Hydroxy-4-methoxybenzophenone	131-57-7	6.6×10^{-4}	2.0×10^{-5}
2-(2′-Hydroxy-5′-methylphenyl) benzotriazole	2440-22-4	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
2-Hydroxy-4-*n*-octyloxybenzophenone	1843-05-6	$>1.0 \times 10^{-5}$	$>5.0 \times 10^{-4}$

‡Parent compound was positive, †Only metabolite was positive

Table VI. Estrogenic activities of vulcanizing agents and vulcanization accelerators.

Compounds	CAS No.	REC_{10} ($mol\,l^{-1}$) Parent comp.	Metabolite
1,4-Benzoquinone dioxime	105-11-3	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Benzoyl-peroxide	94-36-0	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
n-Cyclohexyl-2-benzothiozolyl sulfenamide	95-33-0	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
2,2′-Dibenzothiazolyl disulfide	120-78-5	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
4,4′-Dibenzoylquinone dioxime	120-52-5	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
1,3-Diphenylguanidine	102-06-7	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
4,4′-Dithiodimorpholine	103-34-4	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Hexamethylenetetramine	100-97-0	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
2-Mercaptobenzothiazole	149-30-4	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
2-(4-Morpholinodithio)benzothiazole	95-32-9	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
2-(Morphilinothio)benzothiazole	102-77-2	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Piperidinium pentamethylenedithiocarbamate	98-77-1	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Sodium dimethyldithiocarbamate dihydrate	72140-17-1	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Tetraethylthiuram disulfide	97-77-8	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Tetra-*n*-butylthiuram disulfide	1634-02-2	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
1-(2-Tolyl)biguanide	93-69-6	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$

Table VII. Estrogenic activities of miscellaneous additives.

Compounds (Other name)	CAS No.	REC_{10} ($mol\,l^{-1}$) Parent comp.	Metabolite
2,5-Bis(*tert*-butylperoxy)-2,5-dimethylhexane	78-63-7	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Di-*tert*-butyl-peroxide	110-05-4	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
‡2,4-Diphenyl-4-methyl-1-pentene	6362-80-7	6.6×10^{-5}	6.6×10^{-5}
Glycerol trilaurate (Trilaurin)	538-24-9	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Isopropyl benzene (Cumene)	98-82-8	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
†Nonylphenol ethoxylate	26027-38-3	$>1.0 \times 10^{-3}$	4.5×10^{-5}
Oleyl alcohol	143-28-2	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$

‡Parent compound was positive, †Only metabolite was positive

$10^{-5}\,mol\,l^{-1}$ which corresponds to 5×10^{-4} to $5 \times 10^{-8}\,mol\,l^{-1}$ of final concentration) was added, incubated at 37°C for 4 h and then stored at −80°C until the yeast two-hybrid test was run as metabolite solution. Each experiment was accompanied by *trans*-stylbene to confirm the metabolic activity.

The yeast two-hybrid cells were pre-incubated overnight at 30°C with vigorous shaking in a SD medium free from tryptophan and leucine, then diluted with 1.5 volumes of fresh 2 × SD medium. In a small test tube, 125 µl of the cell solution and 125 µl of the metabolite solution were mixed and then

Table VIII. Estrogenic activities of other chemicals.

Compounds (Other name)	CAS No.	REC_{10} ($mol l^{-1}$) Parent comp.	Metabolite
Bisphenol A bis(2,3-dihydroxypropyl)ether	5581-32-8	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Bisphenol A bis(3-chloro-2-hydroxyphenol)ether	4809-35-2	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
2-*tert*-Butyl-4-methylphenol	2409-55-4	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
1,3-Diphenylpropane	1081-75-0	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
2,4-Di-*tert*-butylphenol	96-76-4	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
1,3-Dimethylbenzene (*m*-Xylene)	108-38-3	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
1,4-Dimethylbenzene (*p*-Xylene)	106-42-3	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
3,5-Dimethylphenol (3-xylenol)	108-68-9	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Dodecamethylcyclohexanesiloxane	540-97-6	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
1,2-Epoxyethylbenzene (Styrene oxide)	96-09-3	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
†2-Methyl-6-*tert*-butylphenol	2219-82-1	$>1.0 \times 10^{-3}$	3.5×10^{-4}
Phosphoric acid tris(3-methylphenyl)	563-04-2	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Phosphoric acid tris(4-methylphenyl)	78-32-0	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Poly(bisphenol A-co-epichlorohydrin)	25036-25-3	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
Poly(bisphenol A-co-epichlorohydrin)glycidyl end capped	25036-25-3	$>1.0 \times 10^{-3}$	$>5.0 \times 10^{-4}$
β-Sitosterol	83-46-5	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
Stigmasterol	83-48-7	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$
1,3,5-Tri-*tert*-butylbenzene	1460-02-2	$>1.0 \times 10^{-4}$	$>5.0 \times 10^{-5}$

†Only metabolite was positive

incubated at 30°C for 4 h. Thereafter, the same procedure as the *Measurement of estrogenic activity by yeast two-hybrid assay* was carried out.

Data analysis

The results were evaluated on the basis of the relative activity, expressed as 10% relative effective concentration (REC_{10}), which is the concentration of the test chemical showing 10% of the agonist activity of 10^{-6} $mol l^{-1}$ E_2, the highest activity level of E_2. When the activity of the test chemical was higher than the REC_{10} within the concentration range tested, the chemical was judged to be positive. When it was judged to be negative, more than the highest dose tested was indicated.

Results and discussion

Estrogenic activity of monomers

Fourteen kinds of monomers used for food contact plastics and/or rubbers were tested for their estrogenic activity and the results are shown in Table I. This table also shows the activity of E_2, bisphenol A and nonylphenol for comparison which are already known to possess the estrogenic activity as mentioned above. Three chemicals, bis(4-hydroxyphenyl) methane, 4-cyclohexylphenol and 4-phenylphenol, displayed estrogenic activities and their activities were comparable to nonylphenol and bisphenol A. These three chemicals have already been reported to be estrogenic, the bis(4-hydroxyphenyl) methane based on the MCF-7 assay (Pérez et al. 1998), 4-cyclohexylphenol based on the uterotrophic assay (Yamasaki et al. 2003), and 4-phenylphenol based on the recombinant yeast screen assay (Routledge et al. 1997).

Their metabolites also possessed this activity, though they were weaker than the parent compound. The metabolite of 2-(phenylmethyl) phenol also showed a potency. However, the other 10 monomers and their metabolites did not have any estrogenicity.

Estrogenic activity of antioxidants

Forty kinds of antioxidants used for food contact plastics and/or rubbers and their metabolites were tested, and these results are shown in Table II. Four chemicals, 4,4′-isopropylidenediphenol alkyl(C_{12}–C_{15}) phosphite, two type of styrenated phenol, and tris(nonylphenyl)phosphite, displayed estrogenic activities. Their activities were comparable to nonylphenol and bisphenol A. Their metabolites also possessed such an activity and the metabolite of 1-(*N*-phenylamino)naphthalene and one type of styrenated phenol also showed a potency. However, the other 34 antioxidants and their metabolites did not have any estrogenicity.

Regarding three kinds of styrenated phenols, that containing mono-type 74% and di-type 24% showed the strongest activity, and that containing mono, di and/or tri-type (percent compositions were unknown) showed next activity. While, that containing di-type 93% and tri-type 7% did not show the activity, though its metabolite possess a weak activity. It is presumed that mono-type of styrenated phenol plays predominant role in the estrogenic activity.

Estrogenic activity of plasticizers

Thirty-eight kinds of plasticizers used for food contact plastics and rubbers and/or their metabolites were tested, and these results are shown in Table III. All the plasticizers and their metabolites did not display any estrogenicity.

Estrogenic activity of lubricants

Seven kinds of lubricants used for food contact plastics and/or rubbers and their metabolites were tested, and these results are shown in Table IV. All the lubricants and their metabolites did not display any estrogenicity.

Estrogenic activity of UV-stabilizers

Ten kinds of UV-stabilizers used for food contact plastics and/or rubbers and their metabolites were tested, and these results are shown in Table V. Two chemicals, 2,2′-dihydroxy-4-methoxybenzophenone and 2-hydroxy-4-methoxybenzophenone, displayed estrogenic activities based on the same assay (Kawamura et al. 2003) and the latter has been reported based on the MCF-7 cell assay and the Uterotropic assay (Schlumpf et al. 2001). Their metabolites showed such an activity here and already reported on the same assay (Takatori et al. 2003). The metabolite of 4-*tert*-butylphenylsalicylate also showed a potency. However, the other 7 UV-stabilizers and their metabolites did not show any estrogenicity.

Estrogenic activity of vulcanizing agents and vulcanization accelerators

Sixteen kinds of vulcanizing agents and vulcanization accelerators used for food contact rubbers and their metabolites were tested and these results are shown in Table VI. All the vulcanizing agents and vulcanization accelerators and their metabolites did not display any estrogenicity.

Estrogenic activity of miscellaneous additives

Seven kinds of miscellaneous additives used for food contact plastics and/or rubbers and their metabolites were tested, and the results are shown in Table VII. The chain transfer agent for the acrylonitrile butadiene styrene (ABS) plastic and rubber, 2,4-diphenyl-4-methyl-1-pentene, and its metabolites displayed estrogenic activities. The metabolite of an emulsifier, nonylphenol ethoxylate showed an estrogenicity. Nonylphenol ethoxylate is known to be decomposed to nonylphenol, therefore, the estrogenicity of its metabolite was presumed to originate in nonylphenol. However, the other 5 additives and their metabolites did not show any estrogenicity.

Estrogenic activity of other chemicals

Eighteen kinds of oligomers, degradation products and other impurities of plastics or rubbers and their metabolites were tested, and these results are shown in Table VIII. The metabolite of 2-methyl-6-*tert*-butylphenol, which is a material of several antioxidants and also their decomposition products, displayed an estrogenic activity. However, the other 17 compounds and their metabolites did not show any estrogenicity.

Estrogenic chemicals detected in this test

As a result of this test, 10 out of 150 chemicals, bis(4-hydroxyphenyl) methane, 4-cyclohexylphenol, 4-phenylphenol, 4,4′-isopropylidenediphenol alkylphosphite, two type of styrenated

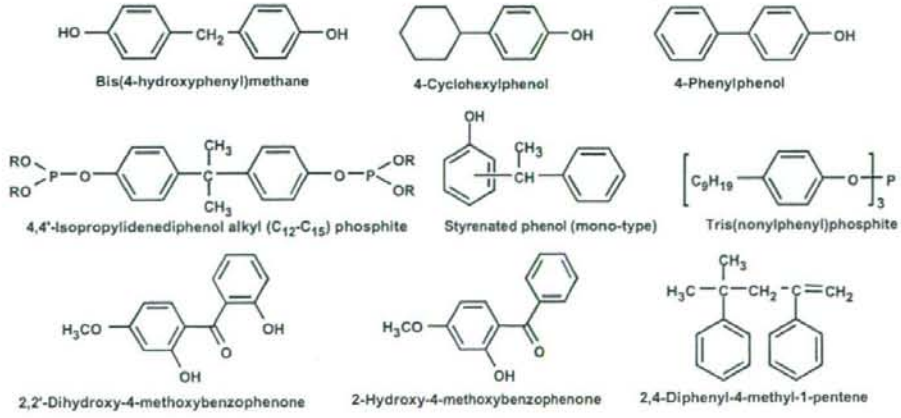


Figure 1. Structures of 9 estrogenic chemicals.

phenol, tris(nonylphenyl) phosphite, 2,2′-dihydroxy-4-methoxybenzophenone, 2-hydroxy-4-methoxybenzophenone and 2,4-diphenyl-4-methyl-1-pentene, displayed estrogenic activities. Their chemical structures are shown in Figure 1. They contained a phenol group or formed one easily by hydrolysis. The dose response curves of their estrogenic activity are shown in Figure 2, together with those of well-known estrogenic chemicals, nonylphenol and bisphenol A. Their activities were almost between nonylphenol and bisphenol A.

Moreover, the metabolites of these 10 chemicals and other 6 chemicals, 2-(phenylmethyl) phenol, 1-(*N*-phenylamino) naphthalene, one type of styrenated phenol, 4-*tert*-butylphenylsalicylate, nonylphenol ethoxylate and 2-methyl-6-*tert*-butylphenol, also displayed the estrogenic activities. The structures of these 5 chemicals except styrenated phenol are shown in Figure 3. They also contained a phenol group or formed one by metabolism. The REC_{10} of the metabolites were between $1.2 \times 10^{-5}\,mol\,l^{-1}$ and $3.5 \times 10^{-4}\,mol\,l^{-1}$.

Among them five chemicals, bis(4-hydroxyphenyl) methane, 4-cyclohexylphenol, 4-phenylphenol, 2-hydroxy-4-methoxybenzophenone and 2,2′-dihydroxy-4-methoxybenzophenone, have already been reported to have estrogenic activities as mentioned above. The other chemicals were newly found to possess an estrogenic activity in the present study.

Conclusions

Our study showed that most of the chemicals related to food contact plastics and rubbers, and their

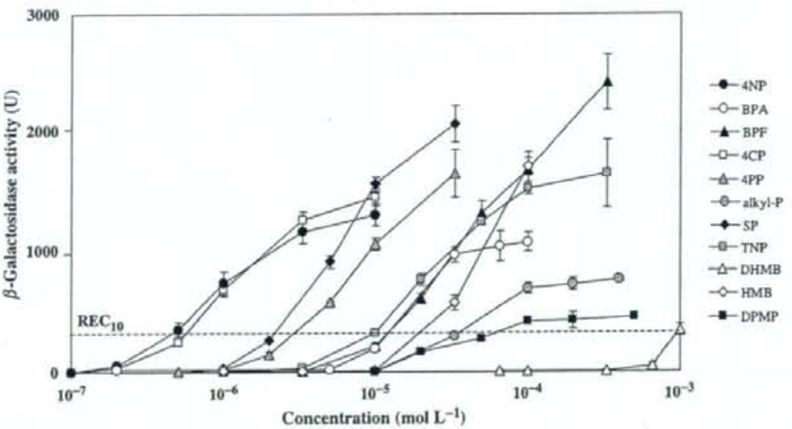


Figure 2. Dose response curves of estrogenic activity of nonylphenol (4NP), bisphenol A (BPA) bis (4-hydroxyphenyl) methane (BPF), 4-cyclohexylphenol (4CP), 4-phenylphenol (4PP), 4,4′-isopropylidenediphenol alkylphosphite (alkyl-p), styrenated phenol (mono 74%, SP), tris (nonylphenyl) phosphite (TNP), 2,2′-dihydroxy-4-methoxybenzophenone (DHMB), 2-hydroxy-4-methoxybenzophenone (HMB) and 2,4-Diphenyl-4-methyl-1-pentene (DPMP).

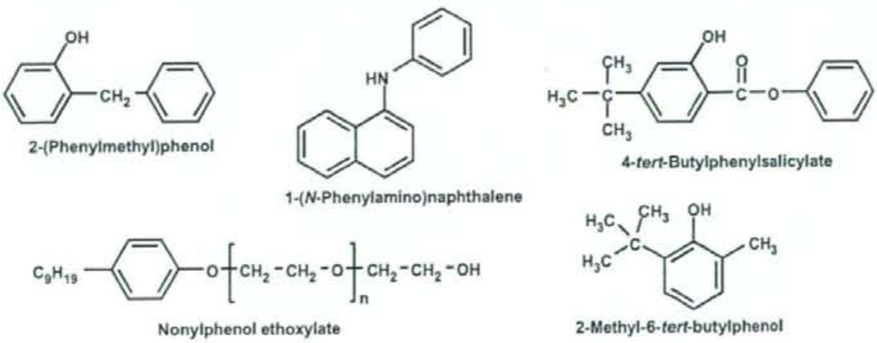


Figure 3. Structures of 5 chemicals which metabolites were estrogenic.

metabolites did not possess any estrogenicity. However, 10 chemicals and 6 other metabolites revealed estrogenic activities based on the yeast two-hybrid assay. These chemicals will need further investigations regarding their toxicity.

Acknowledgements

The authors thank Professor T. Nishihara and J. Nishikawa in Osaka University for providing the yeast two-hybrid cells and useful advice. This research was partially supported by a Grant-in Aid for Scientific Research from the Ministry of Education, Science, Culture, Sports and Technology, Japan.

References

Dodds EC, Lawson W. 1938. Molecular structure in relation to oestrogenic activity – compounds without a phenanthrene nucleus. Proceedings of the Royal Society B 125:222–232.

Jobling S, Reynolds T, White R, Parker MG, Sumpter JP. 1995. A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. Environmental Health Perspectives 103:582–587.

Kawamura Y, Kawamura M, Takeda Y, Yamada T. 1998a. Determination of styrene dimers and trimers in food contact polystyrene. Journal of the Food Hygienic Society of Japan 39:199–205.

Kawamura Y, Nishi A, Maehara T, Yamada T. 1998b. Migration of styrene dimers and trimers from polystyrene containers into instant foods. Journal of the Food Hygienic Society of Japan 39:390–398.

Kawamura Y, Tagai C, Maehara T, Yamada T. 1999. Additives in polyvinyl chloride and polyvinylidene chloride products. Journal of the Food Hygienic Society of Japan 40:274–284.

Kawamura Y, Maehara T, Iijima H, Yamada T. 2000. Nonylphenol in food contact plastics and toys. Journal of the Food Hygienic Society of Japan 41:212–218.

Kawamura Y, Ogawa Y, Nishimura T, Kikuchi Y, Nishikawa J, Nishihara T, Tanamoto K. 2003. Estrogenic activities of UV stabilizers used in food contact plastics and benzophenone derivatives tested by the yeast two-hybrid assay. Journal of Health Science 49:205–212.

Kawamura Y, Mutsuga M, Kato T, Iida M, Tanamoto K. 2005. Estrogenic and anti-androgenic activities of benzophenones in human estrogen and androgen receptor mediated mammalian reporter gene assays. Journal of Health Science 51:48–54.

Krishnan AV, Stathis P, Permuth SF, Tokens L, Feldman D. 1993. Bisphenol A: An estrogenic substance is released from polycarbonate flasks during autoclaving. Endocrinology 132:2279–2286.

Nagel SC, vom Saal FS, Thayer KA, Dhar MG, Boechler M, Welshons WV. 1997. Relative binding affinity-serum modified access (RBA-SMA) assay predicts the relative in vivo bioactivity of the xenoestrogenes bisphenol A and octyl phenol. Environmental Health Perspectives 105:70–76.

Nishikawa J, Goto J, Saito K, Matsuo M, Nishihara T. 1998. Use of yeast two hybrid system to analyze environmental estrogen. Japan Journal of Toxicology and Environmental Health 44: P-32.

Nishikawa J, Saito K, Goto J, Dakeyama F, Matsuo M, Nishihara T. 1999. New screening methods for chemicals with hormonal activities using interaction of nuclear hormone receptor with coactivator. Toxicology Applied Pharmacology 154:76–83.

Nishihara T, Nishikawa J, Kanayama T, Dakeyama F, Saito K, Imagawa M, Takatori S, Kitagawa Y, Hori S, Utsumi H. 2000. Estrogenic activities of 517 chemicals by yeast two-hybrid assay. Journal of Health Science 46:282–298.

Ohyama KI, Nagai F, Tsuchiya Y. 2001. Certain styrene oligomers have proliferative activity on MCF-7 human breast tumor cells and binding affinity for human estrogen receptor. Environmental Health Perspective 109:699–703.

Olea N, Pulgar R, Pérez P, Olea-Serrano F, Rivas A, Novillo-Fertrell A, Pedraza V, Soto AM, Sonnenschein C. 1996. Estrogenicity of resin-based composites and sealants used in dentistry. Environmental Health Perspectives 104:298–305.

Pérez P, Pulgar R, Olea-Serrano F, Villalobos M, Rivas A, Metzler M, Pedraza V, Olea N. 1998. The estrogenicity of bisphenol A-related diphenylalkanes with various substituents at the central carbon and the hydroxy groups. Environmental Health Perspectives 106:167–174.

Routledge EJ, Sumpter JP. 1997. Structural features of alkylphenolic chemicals associated with estrogenic activity. Journal of Biological Chemistry 272:3280–3288.

Schlumpf M, Cotton B, Conscience M, Haller V, Steinmann B, Lichtensteiger W. 2001. In vitro and in vivo estrogenicity of UV screens. Environmental Health Perspectives 109:239–244.

Soto AM, Justicia H, Wray JW, Sonnenschein C. 1991. p-Nonyl-phenol: An estrogenic xenobiotic released from "modified" polystyrene. Environmental Health Perspectives 92:167–173.

Takatori S, Kitagawa Y, Oda H, Miwa G, Nishikawa J, Nishihara T, Nakazawa H, Hori S. 2003. Estrogenicity of metabolites of benzophenone derivatives examined by a yeast two-hybrid assay. Journal of Health Science 49:91–98.

Yamasaki K, Takeyoshi M, Sawaki M, Imatanaka N, Shinoda K, Takatsuki M. 2003. Immature rat uterotrophic assay of 18 chemicals and hershberger assay of 30 chemicals. Toxicology 183:93–115.

Note

Identification of the Main Constituents in Sandarac Resin, a Natural Gum Base

(Received August 18, 2005)

Naoki SUGIMOTO*1,†, Masanori KUROYANAGI*2, Takashi KATO*2, Kyoko SATO*1, Atsuko TADA*1, Takeshi YAMAZAKI*1 and Kenichi TANAMOTO*1

(*1 National Institute of Health Sciences: 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan; *2 School of Bioresources, Hiroshima Prefectural University: 562, Nanatsuka, Shobara, Hiroshima 727-0023, Japan; † Corresponding author)

Sandarac resin, a natural gum base, is described as "a substance composed mainly of sandaracopimaric acid obtained from the secretion of sandarac trees" in the List of Existing Food Additives in Japan. To evaluate its quality as a food additive, the main constituents in a sandarac resin product were investigated. Three constituents were isolated and identified as sandaracopimaric acid, sandaracopimarinol and 4-epidehydroabietic acid by MS and 2D-NMR. Quantification of the main constituent, sandaracopimaric acid, was performed by HPLC and its content in the product was determined to be 11.6%.

Key words: food additive; gum base; sandarac resin; *Tetraclinis articulata*; sandaracopimaric acid

Introduction

Most natural food additives have many constituents, but to date, there have been few investigations on the constituents in most of them. It is necessary for the evaluation of natural food additives to analyze the constituents as completely as possible, since the chemical nature and concentrations of the constituents may differ depending on the extraction and processing methods, and the collection season of the plant of origin. We have been investigating the main and minor constituents in various food additives, for which there are no analytical data and/or reports, in order to develop official analytical methods[1,2].

The List of Existing Food Additives in Japan[3] stipulates that sandarac resin is a natural gum base, which is a substance composed mainly of sandaracopimaric acid (1) obtained from the secretion of sandarac trees. Sandarac tree is *Tetraclinis articulata* (Vahl) Mast., belonging to the Cupressaceae family. It is native to Morocco and is a coniferous tree closely related to arborvitae. The existence of several diterpenoids in the leaves of *T. articulata* has recently been reported[4]. Sandarac resin has been used for many years as a natural resin for artwork. Many reports[5,6] have been published on the characterization and identification of natural resins for painting. It was reported that the dominant component in sandarac resin used for painting is sandaracopimaric acid (1). However, the main constituents of sandarac resin as a food additive have not been clarified. In this study, we identified several constituents of sandarac resin as sandaracopimaric acid (1), sandaracopimarinol (2) and 4-epidehydroabietic acid (3) by MS and 2D-NMR, and quantified the content of the main constituent, sandaracopimaric acid (1), by HPLC.

Materials and Methods

1. *Sample and chemicals*

A sample of sandarac resin product was obtained through the Japan Food Additives Association. Silica gel 60 F_{254} (20 cm×20 cm, Art. 1.05715) (Merck Co., Ltd.) was used for TLC. Silica gel 60N (63-200 μm Cat. No. 37565-79) (Kanto Chemical Co., Inc.) was used for open column chromatography. All chemicals were of reagent grade and were used without further purification.

2. *Spectroscopic analysis*

NMR spectra were recorded on JNM-ECA800 and JNM-ECA500 (800 MHz and 500 MHz) instruments (JEOL Co., Ltd.) with chloroform-*d* ($CDCl_3$) as the solvent. ^{1}H-NMR and ^{13}C-NMR spectra were referenced internally to tetramethylsilane (TMS). Assignments of the proton and carbon signals of all isolated compounds were confirmed by pulse field gradient (PFG) heteronuclear multiple quantum coherence (HMQC), PFG heteronuclear multiple bond connectivity (HMBC), double quantum filtered correlation spectroscopy (DQF-COSY) and nuclear Overhauser effect (NOE) experiments. High-resolution electron impact mass spectrometry (HR-EI-MS) spectra were obtained with a JMS-700 (JEOL) mass spectrometer. Melting points were determined using a MP-S3 apparatus (Yanaco New Science Inc.) without correction.

3. *TLC and HPLC conditions*

Analytical and preparative HPLC conditions: pump, PU-1580 (JASCO); detector, UV-1575 (JASCO); column, Mightysil RP-18 (4.6 mm i.d.×250 mm (for analysis), 20 mm i.d.×250 mm (for preparation), Kanto Chemical); column temp., 30℃; solvent, acetonitrile(CH_3CN)–water (H_2O)–acetic acid(AcOH)=90 : 10 : 0.3; injection volume, 10 μL (for analysis), 100 μL (for preparation); flow rate, 1.0 mL/min (for analysis), 8.0 mL/min (for preparation); detection, UV 210 nm.

TLC conditions: developing solvent, chloroform ($CHCl_3$)–methanol(MeOH)=75 : 1. After development to about 10 cm, the spots of constituents were visualized by spraying the plate with 50% sulfuric acid (H_2SO_4) followed by gentle heating.

4. *Isolation of compounds 1, 2 and 3*

Sandarac resin product (5.0 g) was dissolved in $CHCl_3$. The solution was added to a small amount of silica gel with stirring and then the solvent was evaporated *in vacuo*. The silica gel with absorbed sandarac resin was applied on an open silica gel column and fractionated by eluting with $CHCl_3$–MeOH (100:0→0:100 gradient), with monitoring by TLC. The eluates were concentrated *in vacuo*, affording seven fractions (Frs. 1–7). Fr. 2 (610 mg), including compounds **1**–**3**, was fractionated by preparative HPLC (see preparative HPLC conditions) to give pure compounds **1** (146 mg), **2** (94 mg) and **3** (113 mg), respectively.

Compound **1**: sandaracopimaric acid[7], colorless needles, mp 171–172℃ (from MeOH), $[\alpha]_D^{26}$ −17.7° (*c* 0.13, EtOH) (lit.[8]: mp 171–173℃, $[\alpha]_D^{26}$ −19.8° (*c* 0.2, EtOH)). HR-EI-MS: *m/z* 302.2220 M^+ (Calcd. for $C_{20}H_{30}O_2$ *m/z* 302.2246).

Compound **2**: sandaracopimarinol[9], amorphous solid, $[\alpha]_D^{26}$ −9.5° (*c* 0.092, MeOH) (lit.[9]: $[\alpha]_D^{26}$ −20° (*c* 0.1, MeOH)). HR-EI-MS: *m/z* 288.2440 M^+ (Calcd. for $C_{20}H_{32}O$ *m/z* 288.2453).

Compound **3**: 4-epidehydroabietic acid[10], colorless prisms, mp 149–150℃ (from aq. EtOH), $[\alpha]_D^{26}$ +128° (*c* 0.094, MeOH) (lit.[11]: mp 144–145℃, $[\alpha]_D^{26}$ +106.6° (*c* 0.8, MeOH)). HR-EI-MS: *m/z* 300.2094 M^+ (Calcd. for $C_{20}H_{28}O_2$ *m/z* 300.2089).

^{1}H- and ^{13}C-NMR data of compounds **1**, **2** and **3** are shown in Table 1.

5. *Quantification of sandaracopimaric acid (1)*

Sandarac resin product (50 mg) was dissolved in MeOH (10 mL). The quantity of sandaracopimaric acid (**1**) was determined by using an absolute calibration

Table 1. NMR Data of Compounds Isolated from Sandarac Resin

Position	Sandaracopimaric acid (1)			Sandaracopimarinol (2)			4-Epidehydroabietic acid (3)		
	δ_C[a]	δ_H[b] (*J* in Hz)		δ_C[c]	δ_H[d] (*J* in Hz)		δ_C[c]	δ_H[d] (*J* in Hz)	
1	38.3	1.13	dt, 5.0, 12.8	39.0	1.00	dt, 5.1, 12.4	39.4	1.38	dt, 4.3, 13.2
		1.64	br. d, 12.8		1.71	br. d, 12.4		2.24	m overlapped
2	18.6	1.55	m overlapped	18.4	1.45–1.60	m overlapped	20.0	1.6	m
								1.98	m overlapped
3	37.0	1.31	br. d, 12.8	35.5	1.32–1.47	m overlapped	37.5	1.08	dt, 4.0, 13.2
		1.78	dt, 5.0, 12.8					2.24	m overlapped
4	47.3	—		37.9	—		43.9		
5	48.9	1.91	dd, 2.7, 12.8	47.9	1.34	m overlapped	52.9	1.57	dd, 1.5, 12.1
6	24.9	1.26	m overlapped	22.5	1.30	m overlapped	21.0	2.16	m
		1.45	m overlapped		1.47	m overlapped		2.01	m overlapped
7	35.5	2.11	br. dt, 5.5, 14.2	35.8	2.06	br. t, 11.8	32.1	2.86	br. dd, 4.6, 14.2
		2.21	ddd, 1.8, 4.6, 14.2		2.23	ddd, 1.8, 4.3, 11.8		2.78	dt, 4.6, 14.2
8	136.6	—		137.1	—		135.1	—	
9	50.6	1.80	br. t, 7.8	50.6	1.75	br. t, 7.5	145.5	—	
10	37.8	—		38.2	—		38.4	—	
11	18.2	1.50	m overlapped	18.9	1.44	m overlapped	125.5	7.17	d, 8.0
		1.60	m overlapped		1.58	m overlapped			
12	34.5	1.36	dt, 3.6, 12.3	34.6	1.32–1.47	m overlapped	124.1	6.99	dd, 1.7, 8.0
		1.45	m overlaped						
13	37.4	—		37.5	—		145.8	—	
14	129.2	5.21	br. s	128.8	5.20	br. s	126.9	6.88	d, 1.7
15	148.9	5.76	dd, 10.5, 17.3	149.2	5.77	dd, 10.6, 17.4	33.5	2.81	hep., 6.9
16	110.2	4.87	dd, 1.5, 10.5	110.1	4.87	dd, 1.4, 10.6	24.1	1.21	d, 6.9
		4.90	dd, 1.5, 17.3		4.90	dd, 1.4, 17.4			
17	26.1	1.03	s	26.0	1.03	s	24.1	1.21	d, 6.9
18	184.4	—		72.3	3.12	d, 10.9	183.7	—	
					3.39	d, 10.9			
19	16.8	1.20	s	18.0	0.80	s	28.8	1.32	s
20	15.2	0.83	s	15.7	0.83	s	23.3	1.11	s

All signals were assigned based on DQF-COSY, HMQC, and HMBC experiments.
a) Recorded at 200 MHz. b) Recorded at 800 MHz. c) Recorded at 125 MHz. d) Recorded at 500 MHz.

curve based on peak height at UV 210 nm of compound (1) isolated from sandarac resin product.

Results and Discussion

1. *Identification of the main constituents in sandarac resin*

The silica gel TLC profile of the sandarac resin product is illustrated in Fig. 1. Several spots were observed on the TLC plate along with the tailing spot after spraying the plate with H_2SO_4, followed by gentle heating. The spot at Rf 0.28 was the most intense one. The HPLC profile of the product is illustrated in Fig. 2. Six to seven peaks were observed, and the largest peak (peak B) at T_R 13.0 min corresponded to the most intense spot on the TLC plate. In order to identify the major constituents, the sandarac resin product was fractionated *via* silica gel and preparative HPLC, affording compounds **1** (peak B), **2** (peak C), and **3** (peak A). The structures were elucidated on the basis of the spectral data.

Compound **1** showed a molecular ion peak at m/z 302.2220 in HR-EI-MS. The ion peak indicated that the molecular formula could be represented as $C_{20}H_{30}O_2$. The ^{1}H-NMR spectrum showed three singlet methyl signals [δ 0.83 (3H, s), 1.03 (3H, s), 1.20 (3H, s)], vinyl groups [δ 5.76 (1H, dd, J=17.3, 10.5 Hz), 4.87 (1H, dd, J=10.5, 1.5 Hz), 4.90 (1H, dd, J=17.3, 1.5 Hz)], a detached olefinic group [δ 5.21 (1H, br. s)], and many methylene signals. The ^{13}C-NMR spectrum showed a carboxyl group (δ 184.4), two olefinic signals including an exomethylene group (δ 110.2, 129.2, 136.6, 148.9), and 15 other signals due to methyl, methylene, and methine carbons. All the ^{1}H- and ^{13}C-NMR signals of compound **1** were assigned based on 2D-NMR results (DQF-COSY, HMQC, HMBC). Compound **1** was identified as sandaracopimaric acid[7,8] (Fig. 3). The reported ^{13}C-NMR assignments[5] at C-4, 5 and 18 of **1** should be revised according to our assignment.

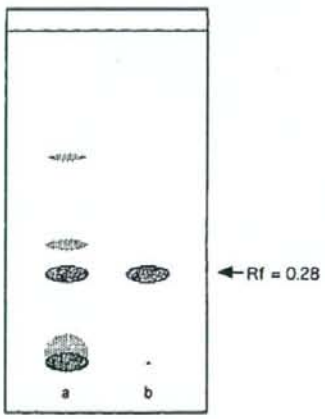


Fig. 1. Silica gel TLC profiles of sandarac resin product and sandaracopimaric acid (1)

a) Sandarac resin product. b) Sandaracopimaric acid (1). Solvent: $CHCl_3$: MeOH = 75 : 1. Spots were visualized with H_2SO_4/heat.

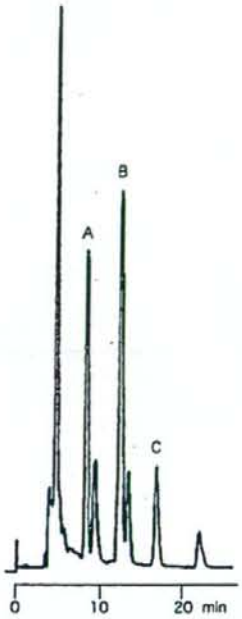


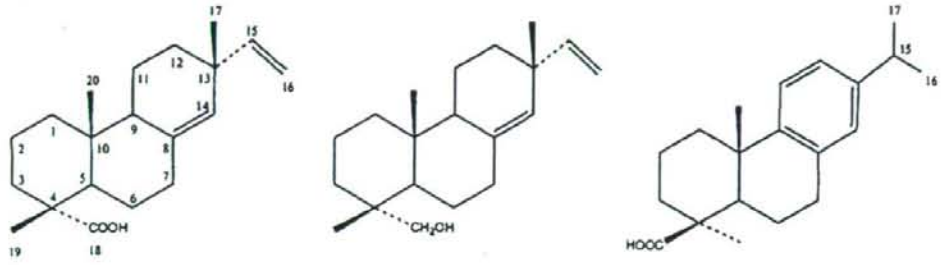
Fig. 2. HPLC profile of sandarac resin product

Peak A=4-epidehydroabietic acid (3). Peak B=sandaracopimaric acid (1). Peak C=sandaracopimarinol (2).

Compound **2** showed a molecular ion peak at m/z 288.2440, indicating that its molecular formula is $C_{20}H_{32}O$. The ^{1}H- and ^{13}C-NMR spectra of compound **2** were very similar to those of sandaracopimaric acid (1). Comparison of compound **2** with sandaracopimaric acid (1) indicated that compound **2** is also an isopimarane derivative having one hydroxyl methyl group [^{1}H: δ 3.12, 3.39 (each ^{1}H, d, =10.9 Hz), ^{13}C: δ 72.3], instead of the carboxyl group at C-18 on sandaracopimaric acid (1). Since the ^{13}C-NMR data of compound **2** were the same as the literature data for sandaracopimarinol, compound **2** was identified as sandaracopimarinol[9] (Fig. 3).

The molecular formula of compound **3**, $C_{20}H_{28}O_2$, was deduced from the molecular ion peak at m/z 300.2094. The ^{1}H-NMR spectrum showed two singlet methyl signals [δ 1.11 (3H, s), 1.32 (3H, s)], a doublet methyl signal [δ 1.21 (6H, d, J=6.9 Hz)], three aromatic signals [δ 6.88 (1H, d, J=1.7 Hz), 6.99 (1H, dd, J=1.7, 8.0 Hz), 7.17 (1H, d, J=8.0 Hz)], and many methylene signals. The ^{13}C-NMR spectrum showed a carboxyl group [δ 183.7] and an aromatic group [δ 124.1, 125.5, 126.9, 135.1, 145.5, 145.8], and 13 other signals derived from methyl, methylene and methine carbons. NOE was observed for the methyl group [δ 1.32 (3H, s)] on C-4 with H-5α [δ 1.57 (1H, dd, J=1.5, 12.1 Hz)]. By comparison of the spectral and physical data with published data[11,12], compound **3** was identified as 4-epidehydroabietic acid[10,11] (Fig. 3).

Other peaks on HPLC were still mixtures of two or



sandaracopimaric acid (1) sandaracopimarinol (2) 4-epidehydroabietic acid (3)

Fig. 3. Structures of sandaracopimaric acid (1), sandaracopimarinol (2) and 4-epidehydroabietic acid (3)

more diterpenoids, and hence could not be identified at this time. The concentrations of the other diterpenoids appear to be lower than that of sandaracopimaric acid (1), because peak **B** due to sandaracopimaric acid (1) was the largest on HPLC. Therefore, the main constituent, sandaracopimaric acid (1), was selected as a characteristic constituent to develop a quality standard and/or verification test of sandarac resin.

2. Quantification of sandaracopimaric acid (1) in sandarac resin product

In order to quantify sandaracopimaric acid (1), a calibration curve based on peak height was prepared within the range of 0.25–2.0 mg/mL of sandaracopimaric acid (1). The concentration of sandaracopimaric acid (1) in the sandarac resin product was found to be 11.6% by HPLC. Since neither other sandarac resin products nor *T. articulata*, the origin of sandarac resin, are available in Japan, comparisons could not be made. However, we concluded that the sandarac resin product used in this research had been derived from *T. articulata*, since sandaracopimaric acid (1), a characteristic constituent in sandarac resin, was detected as the main constituent.

Conclusion

This report is the first investigation of the major constituents of commercial sandarac resin product used as a food additive. The major constituents were isolated from the product and identified as sandaracopimaric acid (1), sandaracopimarinol (2) and 4-epidehydroabietic acid (3) by 2D-NMR. Based on TLC and HPLC analyses, we confirmed that the main constituent was sandaracopimaric acid (1) and its content was found to be 11.6%. This result will be useful for setting official standards for sandarac resin as a natural food additive.

Acknowledgments

This work was supported by a Grant-in-Aid for Research on Food Sanitation from the Ministry of Health, Labor and Welfare. The authors are grateful to the Japan Food Additives Association for providing sandarac resin.

References

1) Sugimoto, N., Sato, K., Yamazaki, T., Tanamoto, K., Analysis of constituents in Jamaica quassia extract, a natural bittering agent. Shokuhin Eiseigaku Zasshi (J. Food Hyg. Soc. Japan), 44, 328–331 (2003).
2) Yashiro, T., Sugimoto, N., Sato, K., Yamazaki, T., Tanamoto, K., Analysis of absinthin in absinth extract bittering agent. Shokuhin Kagaku Gakkaishi (Jpn. J. Food Chem.), 11, 86–90 (2004).
3) Notice No. 210 (Apr. 16, 1996) List of Existing Food Additives. Ministry of Health and Welfare.
4) Barrero, A. F., Quílez del Moral, J. F., Lucas, R., Payá, M., Akssira, M., Akaad, S., Mellouki, F., Diterpenoids from *Tetraclinis articulata* that inhibit various human leukocyte functions. J. Nat. Prod., 66, 844–850 (2003).
5) Osete-Cortina, L., Doménech-Carbó, M. T., Analytical characterization of diterpenoid resins present in pictorial varnishes using pyrolysis-gas chromatography-mass spectrometry with on line trimethylsilylation. J. Chromatogr. A, 1065, 265–278 (2005).
6) Scalarone, D., var der Horst, J., Boon, J. J., Chiantore, O., Direct-temperature mass spectrometric detection of volatile terpenoids and natural terpenoid polymers in fresh and artificially aged resins. J. Mass Spectrom., 38, 607–617 (2003).
7) Sakar, M. K., Feliciano, A. S., Diterpenoids of *Juniperus foetidissima* unripe berries. Fitoterepia, 65, 304–306 (1994).
8) Afonso, A, 4-Carbomethoxy-5α-androstane derivatives. Synthesis of (−)-sandaracopimaric acid. J. Org. Chem., 35, 1,949–1,953 (1970).
9) Morisawa, J., Kim, C.-S., Kashiwagi, T., Tebayashi, S., Horiike, M., Repellents in the Japanese cedar, *Cryptomeria japonica*, against the pill-bug, *Armadillidium vulgare*. Biosci. Biotechnol. Biochem., 66, 2424–2428 (2002).
10) Carman, R. M., Marty, R. A., Diterpenoids, Aust. J. Chem., 23, 1,457 (1970).
11) Lee, C.-K., Fang, J.-M., Cheng, Y.-S., Abietanes from leaves of *Juniperus chinensis*. Phytochemistry, 35, 983–986 (1994).
12) Miguel Del Corral, J. M., Gordaliza, M., Salinero, M. A., San Feliciano, A., ^{13}C NMR data for abieta-8,11,13-triene diterpenoids. Magn. Reson. Chem., 32, 774–781 (1994).

Note

Standard Infrared Absorption Spectrum of Betaine and Optimal Conditions for its Measurement

(Received May 15, 2006)

Youji KITAMURA *1, Toshinobu IWASAKI*2, Madoka SAITO*2, Masaki MIFUNE*1, †, Yutaka SAITO*1, Kyoko SATO*3, Chikako YOMOTA*3 and Kenichi TANAMOTO*3

(*1Department of Pharmaceutical Sciences, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University: Tsushima-Naka, Okayama 700-8530, Japan;
*2Department of Pharmaceutical Chemistry, Graduate School of Natural Science and Technology, Okayama University: Tsushima-Naka, Okayama 700-8530, Japan;
*3National Institute of Health Sciences, Kamiyoga: Setagaya-ku, Tokyo 158-8501, Japan;
† Corresponding author)

The infrared absorption (IR) spectrum is often used as a standard reference in identification tests of food additives in Japan. In the case of betaine, many different IR spectra have been reported and, therefore, it is necessary to establish an IR spectrum that is reproducible and reliable enough to be used as a standard for identification. In the present study, suitable conditions to obtain a standard IR spectrum were examined from various viewpoints, including pretreatment, selection of method, and measuring technique. The KBr disk method, which has generally been used to identify betaine, was found to be humidity-dependent, and there was also an interaction between betaine and KBr. A reproducible IR spectrum suitable as a standard could be obtained by drying betaine at 105℃ for 3 hours over phosphorus pentoxide, and then measuring the IR spectrum by the liquid paraffin (Nujol) paste method.

Key words: betaine; seasoning; infrared spectrum; reference spectrum; anhydride; hydrate; identification

Introduction

Betaine (Fig. 1) occurs widely in nature, especially in beets, and is used as a food additive for seasoning or as a flavor-improving agent in Japan. An identification-test for betaine is described in "Voluntary Specifications of Existing Food Additives: 3rd Ed"[1], which suggests identification of betaine on the basis of the wave numbers of the absorption bands in an infrared absorption (IR) spectrum measured by the KBr method, as well as retention time in HPLC, and the like. However, the criteria for identification employed in the test are not comprehensive; for example, the defined wave numbers do not include that of the absorption band of the COO^- group, which is one of the characteristic bands of betaine.

Apart from their use as food additives, betaine and related compounds have attracted much interest in terms of the relationship between IR spectra and structure, specifically, the influence of hydrogen bonding[2-5]. IR spectra of betaine are available from databases, for example, "The Sigma Library of FT-IR Spectra"[6], "Spectral Database for Organic Compounds, SDBS"*1 of the National Institute of Advanced Industrial Science and Technology, Japan (AIST), and "WebBook"*2 of the National Institute of Standards and Technology, USA (NIST). However, these IR spectra are very different from each other and hardly appear to represent the same compound.

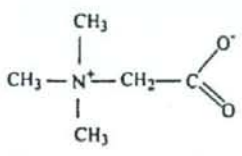


Fig. 1. Structure of betaine

In this study, we examined the influence of pretreatment, measuring method, and measurement conditions on the IR spectrum of betaine in order to establish a method to obtain a reliable and reproducible IR spectrum of betaine, to serve as a standard for an identification test.

Materials and Methods

1. *Samples and reagents*

Betaines (products of Nippon Beet Sugar Manufacturing Co., Ltd., and Danisco Japan Co., Ltd. Tokyo, Japan)

*1 http://www.aist.go.jp/RIODB/SDBS/cgi-bin/direct_frame_top.cgi

*2 http://webbook.nist.gov/cgi/cbook.cgi?ID=C590476&Units=SI&Mask=80

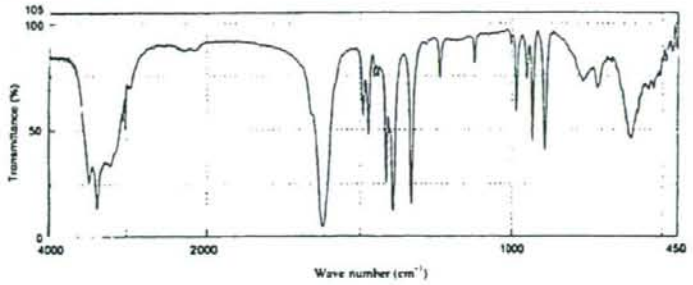


Fig. 2. IR spectrum of betaine dried over phosphorus pentoxide at 105℃ for 3 hours (the KBr method)

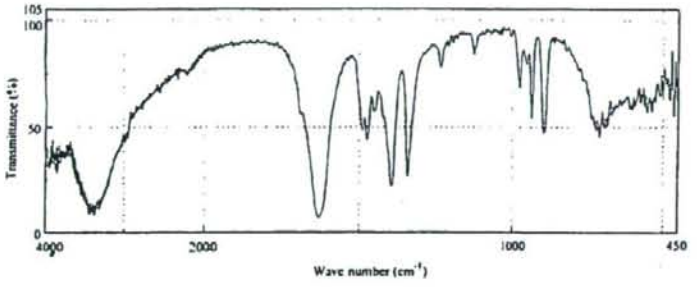


Fig. 3. IR spectrum of betaine dried over phosphorus pentoxide at 105℃ for 3 hours (the KCl method)

were provided by Japan Food Additives Association (Tokyo, Japan) and used as received. Both betaines were in the form of white crystalline powders.

Liquid paraffin (Nujol®) and potassium bromide (KBr) for IR spectroscopy were purchased from Merck AG. (Germany) and JASCO Co., Ltd. (Hachiouji, Japan), respectively. Potassium chloride (KCl) for IR spectroscopy was from JASCO Co., Ltd. (Japan).

2. Measurements of IR spectra

Measurements were carried out by the KBr method and the paste method using liquid paraffin as described in the 7th Ed. of Japan's Specifications and Standards for Food Additives[7] for measuring a solid sample. A KBr disk without any sample and a KBr optical plate were used as references in the KBr method and the paste method, respectively. The resolution was about 4 cm^{-1} (32 or 64 scans). The Fourier transform (FT)-IR device used was an Impact 400 FT-infrared spectrophotometer (Nicolet Co., Madison, Wis., USA) which could nominally measure up to 400 cm^{-1}, though the practical limit was about 450 cm^{-1}. The measurement was conducted in a room used exclusively for IR spectral measurement, where the humidity and temperature were controlled to 30–40% and 23℃, respectively. KBr disks and pastes were usually prepared in the controlled room, but sometimes in an ordinary laboratory. Measurements using the KCl disk or paste method were carried out under the same conditions.

3. Recommended procedure for measurement of a standard IR spectrum of betaine

Betaine as a sample was dried over phosphorus pentoxide at 105℃ for 3 hours. The IR spectrum was measured by using the paste method as described in the General Methods of the 7th Ed. of Japan's Specifications and Standards for Food Additives[7]. As a reference, a KBr optical plate was used.

Results and Discussion

1. Examination by the disk method

In the measurement by the KBr disk method, commercial betaine was used after having been dried at 105℃ for 3 hours according to the "Loss on Drying Test" in the Voluntary Specifications of Existing Food Additives: 3rd Ed.[1] The dried betaine was first subjected to measurement by the KBr method according to the General Methods of the 7th Ed. of Japan's Specifications and Standards for Food Additives[7]. The IR spectrum obtained is shown in Fig. 2. The IR spectrum agreed well with that described in SDBS, but differed from those described in The Sigma Library (the paste method), NIST (the paste method) and reference 2 cited above (the KBr method), suggesting the importance of moisture absorption during disk preparation or an interaction between betaine and KBr. To examine whether or not an interaction between betaine and KBr exists, a betaine disk was prepared using KCl, and the IR spectrum was measured. The result is shown in Fig. 3. It is clear from Figs. 2 and 3 that the spectrum of betaine in the KBr disk is quite different from that in the KCl disk throughout the entire region measured.

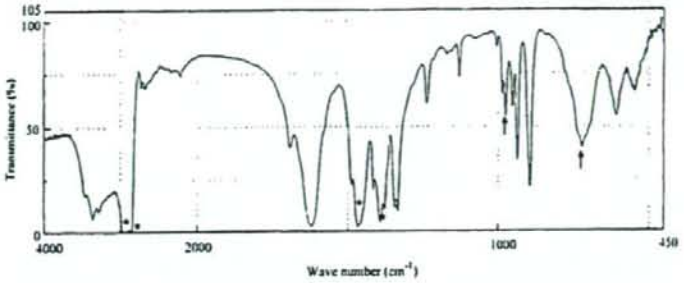


Fig. 4. IR spectrum of a 1 : 10 mixture of dried betaine and KBr (the paste method)
*Bands due to liquid paraffin.
† extra bands observed by the paste method.

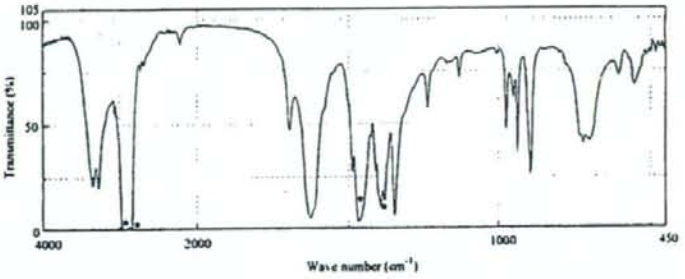


Fig. 5. IR spectrum of a 1 : 10 mixture of dried betaine and KCl (the paste method)
*Bands due to liquid paraffin.

Thus, there was an interaction between betaine and KBr during the procedures of mixing and grinding, or compressing, which resulted in a change of the IR spectrum. In the KBr method, it is well known that a sample may form a solid complex with KBr[8], or may interact with KBr through hydrogen bonding or ion-exchange[9, 10]. To elucidate the causes of the altered spectrum, the IR spectrum of betaine was measured by the paste method using a paste prepared by mixing betaine and alkali metal halide (KBr or KCl) at the mixing ratio of about 1 : 10 (betaine : alkali metal halide), and grinding in agate mortar. The measurement was carried out using a portion of the resultant paste, with a greater thickness than usual. The results are shown in Figs. 4 and 5.

The IR spectrum measured by the paste method in the presence of KBr (Fig. 4) and that of the KBr method (Fig. 2) are in good agreement in the region between 3,500 and 3,000 cm^{-1}, and almost in agreement in the region of 1,000–800 cm^{-1} although extra bands (marked with †) exist. From these results, it was presumed that the spectrum of betaine is easily changed even by very mixing and grinding betaine with KBr. When the KBr disk was prepared by very brief grinding, the IR spectrum of betaine was again different from that shown in Fig. 2.

It has been reported that the spectrum of betaine measured by the KBr method is influenced by the relative humidity in the measuring room[2]. It has also been reported that the IR spectrum of betaine anhydride, which was obtained by crystallization from ethanol, coincided with that of betaine hydrate when measurement was conducted by the KBr method under the condition of 12% or higher relative humidity[2]. We measured the IR spectrum at a humidity level as low as possible (23℃, relative humidity 30%), but could not obtain the IR spectrum of betaine anhydride. The relative humidity varies greatly within Japan, and also among the cold districts (Hokkaido, North Europe, *etc.*) where betaine is mainly produced. Such regional differences in humidity may be linked to the variations observed in the IR spectra provided by the manufacturers in different districts, since test laboratories are not necessarily environmentally controlled. It was therefore considered that the KBr method does not necessarily give an IR spectrum with good reproducibility.

On the other hand, the IR spectrum measured by the paste method in the presence of KCl (Fig. 5) is in agreement with that measured by the KCl disk method (Fig. 3) in the region between 1,000 and 800 cm^{-1}. In addition, these IR spectra are almost in agreement with the IR spectrum of betaine hydrate measured by the paste method described below, and, also are similar to the IR spectrum provided by NIST. These facts indicate that the KCl method is preferable, and that betaine tends to absorb moisture to form a hydrate. Therefore, further investigation into the IR spectrum was conducted by the paste method, which was hardly affected by humidity.

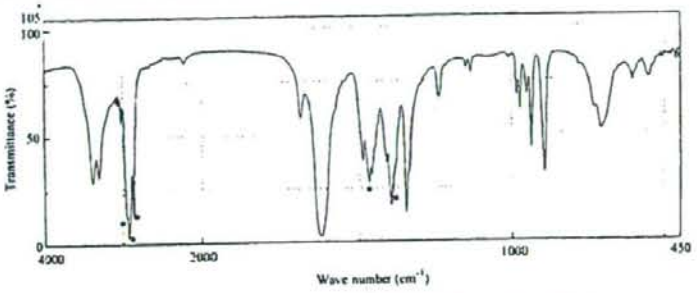


Fig. 6. IR spectrum of commercial betaine (the paste method)
*Bands due to liquid paraffin.

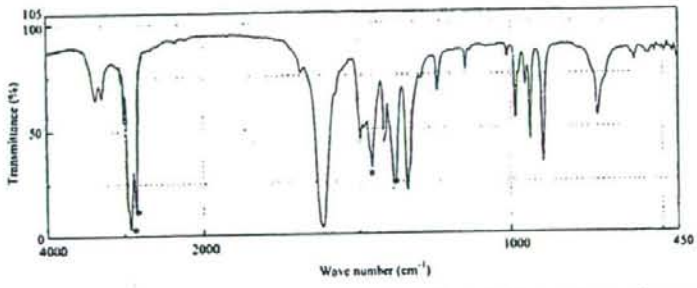


Fig. 7. IR spectrum of betaine dried over phosphorus pentoxide at 105℃ for 3 hours (the paste method)
*Bands due to liquid paraffin.

2. Examination by the paste method

In the paste method, a target substance is coated with liquid paraffin when preparing a paste for measurement. Therefore, the IR spectrum should be littel affected by humidity, even if the substance is hygroscopic. Figure 6 shows the IR spectrum of a betaine product (commercially available bulk powder) measured by the paste method without drying treatment. The high similarity of the IR spectrum in Fig. 6 to that of betaine hydrate in The Sigma Library[6] indicates that the bulk powder contained predominantly hydrate. Because the water content generally varies from one betaine product to another, the IR spectrum of a single product cannot serve as a standard IR spectrum. Another bulk powder also gave a similar IR spectrum to that shown in Fig. 6.

We then examined suitable drying conditions for obtaining the anhydride in a reproducible manner. Drying commercial betaine at 105℃ for 3 hours did not affect the IR spectrum according to the General Method of "Loss on Drying Test" in the Voluntary Specifications of Existing Food Additives: 3rd Ed.[1] Thus, drying was conducted over phosphorus pentoxide at 105℃ for 3 hours. The IR spectrum of this dried betaine is shown in Fig. 7. The IR spectrum in Fig. 7 agreed with that of betaine anhydride provided by The Sigma Library[6]. Further, the spectrum in Fig. 7 was in agreement with that of betaine anhydride measured by the KBr method[2], except that the former also contains absorption bands due to liquid paraffin.

When drying was conducted at different temperatures, 120, 140, 170 or 200℃, over phosphorus pentoxide for three hours, the IR spectra of the dried betaine samples were identical. We, therefore, concluded that the IR spectrum shown in Fig. 7 could be available as a standard IR spectrum. Needless to say, when this spectrum is used as a standard in the identification of a betaine product, the spectra must be compared in the region other than that containing the absorption bands marked by "*", which are due to liquid paraffin.

As mentioned above, the IR spectra of betaine hydrate measured by the KBr and paste methods were different. On the other hand, the IR spectrum of betaine anhydride measured by the KBr disk method at low humidity (relative humidity; less than 12%)[2] was in agreement with that of betaine anhydride measured by the paste method. Accordingly, when using the IR spectrum measured by the KBr method in the identification test of betaine, it is necessary to confirm that the spectrum is identical to the spectrum measured by the paste method in order to ensure the reliability of the test results. The identification method based on the IR spectrum has various advantages, including energy saving, and wide applicability, and is a convenient alternative to chemical identification methods. It is therefore important to ensure good reproducibility of the spectrum.

Conclusion

We examined suitable conditions and methods to obtain a reliable and reproducible IR spectrum applica-

ble to identification of betaine as a standard spectrum. It was found that a reproducible spectrum can be obtained by drying betaine at 105℃ for 3 hours over phosphorus pentoxide and then measuring the IR spectrum by the paste method. The IR spectrum thus obtained is suitable to be used as a standard reference IR spectrum.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Health, Labour and Welfare of the Japanese Government. We thank Associate General Manager M. Karasawa (Ajinomoto Co., Inc.) for his advice. We also thank Japan Food Additives Association, Nippon Beet Sugar Manufacturing Co., Ltd. and Danisco Japan Co., Ltd. for providing betaines.

References

1) Japan Food Additives Association Ed. "Voluntary Specifications of Existing Food Additives: 3rd Ed.", Japan Food Additives Association, Tokyo, Japan (2002).
2) Viertorinne, M., Valkkonen, J., Pitkanen, I., Mäthlouthi, M., Nurmi, J., Crystal and molecular structure of anhydrous betaine, $(CH_3)_3NCH_2CO_2$. J. Mol. Struct., 477, 23–29 (1999).
3) Toccalino, P. L., Harmon, K. M., Harmon, J., Hydrogen bonding: Part 26. Thermodynamics of dissociation and infrared spectra-crystal structure correlations for betaine monohydrate and trimethylamine oxide dehydrate. J. Mol. Struct., 189, 373–382 (1988).
4) Dega-Szafran, Z., Jakólski, M., Szafran, M., OHO hydrogen bond and electrostatic interactions in a complex of pyridine betaine with phenylacetic acid studied by X-ray diffraction, FTIR spectroscopy and PM3, DFT calculations. J. Mol. Struct., 555, 191–201 (2000).
5) Baran, J., Barnes, A.J., Engelen, B., Panthöfer, M., Pietraszko, A., Ratajczak, H., Sledz, M., Structure and polarised IR and Raman spectra of the solid complex betaine–trichloroacetic acid. J. Mol. Struct., 550–551, 21–41 (2000).
6) Keller, R. J., "The Sigma Library of FT-IR Spectra 1st Ed.", Sigma Chemical Co., Inc. (1986).
7) Ministry of Health, Labour and Welfare Ed. of "7th Ed. Japan's Specifications and Standards for Food Additives" published by The Ministry of Health and Welfare of Japanese Government (1999). This book can also be viewed at http://www.ffcr.or.jp/zaidan/FFCRHOME.nsf/pages/spec.stand.fa
8) Ketelaar, J. A. A., Hass, C., van der Elsken, J., Infrared absorption spectra of bifluorides in alkali halide disks. The formation of mixed crystals. J. Chem. Phys., 24, 624–627 (1956).
9) Pliskin, W.A., Eischens, R.P., Effect of using the pressed salt technique to obtain the spectrum of chemisorbed ammonia. J. Phys. Chem., 59, 1,156–1,159 (1955).
10) Famer, V. C., Effects of grinding during the preparation of alkali-halide disks on the infra-red spectra of hydroxylic compounds. Spectrochim. Acta, 8, 374–389 (1957), see also Text Book for the IR spectroscopy, for example Jikken-Kagaku-Kouza Zoku Vol.10 "Infrared Spectra" in Japanese, Maruzen, Tokyo (1964).

Food Additives and Contaminants, February 2006; 23(2): 212–218

Taylor & Francis
Taylor & Francis Group

Migration of formaldehyde and acetaldehyde into mineral water in polyethylene terephthalate (PET) bottles

M. MUTSUGA, Y. KAWAMURA, Y. SUGITA-KONISHI, Y. HARA-KUDO, K. TAKATORI, & K. TANAMOTO

National Institute of Health Sciences, Tokyo, Japan

(Received 10 May 2005; revised 14 September 2005; accepted 6 October 2005)

Abstract
The levels of formaldehyde (FA) and acetaldehyde (AA) in polyethylene terephthalate (PET) bottles and in commercial mineral water are reported. All the water samples bottled in Japan contained detectable levels of FA (10.1–27.9 $\mu g l^{-1}$) and AA (44.3–107.8 $\mu g l^{-1}$). Of 11 European bottled water samples, eight did not contain either FA or AA, while the remaining three had detectable levels of FA (7.4–13.7 $\mu g l^{-1}$) and AA (35.9–46.9 $\mu g l^{-1}$). In three North American bottled water samples, two contained FA (13.6 and 19.5 $\mu g l^{-1}$) and AA (41.4 and 44.8 $\mu g l^{-1}$), and one did not. Regardless of the region of origin, all the sterilized water samples contained FA and AA, whilst in contrast, none of the unsterilized water without carbonate contained FA or AA. Of the carbonated water samples, three contained FA and AA, and one did not. When fortified with FA and AA, the commercial water sample without otherwise detectable FA and AA was able to reduce levels, although the commercial water sample containing FA and AA could not. The presence of bacteria in the commercial water samples was investigated using an ATP-based bioluminescent assay and heterotrophic plate count method. The commercial water without FA and AA contained heterotrophic bacteria, whilst the commercial water with FA and AA did not contain detectable bacteria. It is suggested that in this case both FA and AA migrated from PET materials, but were subsequently decomposed by the heterotrophic bacteria in the unsterilized water.

Keywords: *Polyethylene terephthalate (PET), commercial mineral water, formaldehyde, acetaldehyde, heterotrophic bacteria.*

Introduction

Acetaldehyde (AA) has been reported as being present in polyethylene terephthalate (PET) bottles (Dong et al. 1980; Wyatt 1983; Duflos et al. 1993; Linssen et al. 1995) and bottled mineral water (Nijssen et al. 1996; Sugaya et al. 2001; Dabrowska et al. 2002; Nawrocki et al. 2002; Ewender et al. 2003; Hirayama et al. 2003). AA was reported to migrate from the PET plastics, resulting in an undesirable slightly sweet and fruity taste in the mineral water, particularly in the case of carbonated mineral water (Nijssen et al. 1996; Dabrowska et al. 2002; Nawrocki et al. 2002). On the other hand, there are only a few reports of formaldehyde (FA) in PET bottles and bottled water (Villain et al. 1994; Sugaya et al. 2001; Ewender et al. 2003; Hirayama et al. 2003).

The determination of the AA content of PET is generally carried out using headspace gas chromatography (HS/GC). In contrast, the determination of FA in PET samples using HS/GC is difficult because FA is generated by the heating of PET in the headspace sample. Previous papers reported an analytical method for FA and AA in PET products (Mutsuga et al. 2003). In this method, the PET samples are not heated, allowing the accurate measurement of free FA without decomposition of the PET samples. The levels of FA and AA were measured in PET products including bottles for mineral water (Mutsuga et al. 2005). The findings were that most of the PET products contain FA to the same extent as AA.

In the present study, the content of FA and AA in PET bottled commercial water and the bottle material were determined, and subsequently the origin

Correspondence: M. Mutsuga, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo, Japan. E-mail: mutsuga@nihs.go.jp

ISSN 0265-203X print/ISSN 1464-5122 online © 2006 Taylor & Francis
DOI: 10.1080/02652030500398361

and disappearance of FA and AA in commercial water was studied.

Materials and methods

Sample

Twenty PET-bottled commercial mineral water samples were purchased in Japan between April 2003 and March 2004; six were bottled in Japan, 11 were bottled in Europe and three were bottled in North America.

Reagents

Formaldehyde solution (37%), hydrochloric acid for precision analysis grade (36%) and sodium sulfate were purchased from Sigma Aldrich Japan (Tokyo, Japan). Acetaldehyde was purchased from Aldrich Chemical Co., Inc. (Milwawkee, WI, USA). 2,4-Dinitrophenylhydrazine (DNPH) hydrochloride for HPLC labelling grade, formaldehyde 2,4-dinitrophenylhydrazone (FA-DNPH) and acetaldehyde 2,4-dinitrophenylhydrazone (AA-DNPH) were purchased from Tokyo Kasei Kogyo Co, Ltd (Tokyo, Japan). Trifluoroacetic acid, potassium carbonate and dichloromethane for dioxin analysis grade were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Acetonitrile for high-performance liquid chromatography (HPLC) grade were purchased from Merck Co., Inc. (Darmstadt, Germany). Sterilized water was prepared by autoclaving the commercial mineral water in glass bottles.

The CheckLite-HS kit (ATP-based bioluminescent assay kit, containing luciferin-luciferase reagent, ATP-eliminating reagent (ATPase) and ATP-releasing reagent), and CheckLite ATP standard (ATP standard solutions kit) were purchased from Kikkoman International (Chiba, Japan). The LIVE/DEAD BacLight bacterial viability kit (stains mixture of SYTO 9 and propidium iodide) was purchased from Molecular Probes (Eugene, OR, USA), and R2A agar was purchased from Difco Laboratories (Detroit, MI, USA). The membrane filter (0.45 μm, i.d. 13 mm and 0.22 μm, i.d. 47 mm) used was Millex-LH and contains nitrocellulose (Millipore). The carbon membrane filter (0.2 μm, i.d. 19 mm) used was from Track-Etch Membrane (Whatman, ME, USA).

Apparatus

FA and AA were quantified using an HPLC system consisting of the Shimadzu LC-10A (Shimadzu Co., Kyoto, Japan).

Bioluminescence was measured using a Lumitester C-100N (Kikkoman International). Direct counting of bacteria was performed with an epifluorescence microscopy OLYMPUS BX60 (Olympus Co., Tokyo, Japan).

Preparation of standard solution

Stock solution (100 $\mu g\,ml^{-1}$ each FA and AA) was prepared by dissolving 70.0 mg FA-DNPH and 50.9 mg AA-DNPH in 100 ml acetonitrile. Standard solutions with which to calculate curves were prepared by stepwise dilution with the acetonitrile/water (1:1) ranging in concentration from 0.05 to 5 $\mu g\,ml^{-1}$ for FA and AA.

Analytical procedure for mineral water

Commercial water (100 ml) was collected in 200 ml glass flasks, 5 ml DNPH/hydrochloric acid solution (1 $mg\,ml^{-1}$) added and then derivatized for 2 h at room temperature. Approximately 3.7 g potassium carbonate were added slowly with agitating to adjust the pH to approximately 3. The solution was transferred into a separatory funnel and derivatives extracted with dichloromethane (25 ml × 2). The dichloromethane layers were collected and added about 2 g sodium sulfate and filtrated. The filtrate was evaporated completely under reduced pressure. The residue was dissolved in 2 ml acetonitrile.

Analytical procedure for PET bottle material

PET bottle material (0.5 g) was cut into small pieces and placed in a 5 ml centrifuge glass tube equipped with a glass plug, after which 2.5 ml DNPH/trifluoroacetic acid solution (1 $mg\,ml^{-1}$) was added and the mixture left overnight for dissolution and derivatization. Dichloromethane (10 ml) was then added and approximately 12 ml potassium carbonate solution (0.2 $g\,ml^{-1}$ dissolved in sterilized water) was added to adjust the pH to 7, when the solution changed to a thick yellow liquid. The precipitates were removed by filtration under reduced pressure and washed with dichloromethane (2 × 10 ml). The filtrate and washes were combined and transferred to a separatory funnel. The dichloromethane layers were separated from the aqueous layer, which was subsequently extracted using dichloromethane (10 ml). The dichloromethane layers were collected and dried via the addition of sodium sulfate and evaporation. The residue was dissolved in 2.5 ml acetonitrile and diluted to 5 ml with water, then filtered using a membrane filter (0.45 μm).

HPLC condition

Column: TSKgel ODS-80Ts (4.6 mm i.d. × 250 mm) (Tosoh Co., Tokyo, Japan), guard column: stainless column (1.0 mm i.d. × 45 mm)