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Estrogen equivalent concentration of 13 branched *para*-nonylphenols in three technical mixtures by isomer-specific determination using their synthetic standards in SIM mode with GC–MS and two new diasteromeric isomers

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Abstract

Thirteen isomers of branched *para*-nonylphenols (*para*-NP) in three technical mixtures were isomer-specifically determined using their synthesized standards by SIM of structurally specific ions, *m*/*z* 135, 149 or 163 with GC–MS. Of the 13 isomers, four isomers, 4-(2,4-dimethylheptan-4-yl)phenol, 4-(4-methyloctan-4-yl)phenol, 4-(3-ethyl-2-methylhexan-2-yl)phenol (3E22NP) and 4-(2,3-dimethylheptan-2-yl)phenol synthesized for their determinations were first used as standard substances. The 13 isomers in the technical mixtures individually occurred at mass percent portion of more than 2%. The total mass percent portions in the mixtures from Tokyo Chemical Industry (TCI), Aldrich, and Fluka covered with 89 ± 2%, 75 ± 4% and 77 ± 2%, respectively. The abundance of 4-(3,6-dimethylheptan-3-yl)phenol in the three mixtures was the largest with 11.1 ± 2% to 9.9 ± 0.3%, while that of 4-(2-methyloctan-2-yl)phenol was the smallest with 2.9 ± 0.3% to 3.0 ± 0.2%. Additionally, structures of four new isomers of more than 1% portion present in a technical mixture were elucidated as two pairs of diastereomeric isomers: two types of 4-(3,4-dimethylheptan-4-yl)phenol (344NP) and those of 4-(3,4-dimethylheptan-3-yl)phenol (343NP). By estrogenic assay of 13 isomers with yeast estrogen screen system, the activity of 3E22NP was the highest, while that of 4-(3-methyloctan-3-yl)phenol was the least. Their relative activities to that of 3E22NP were individually calculated. Estrogenic equivalent concentrations of the three technical mixtures were predictively evaluated. The ratio of the EEC to the conventional concentration, total mass percent portions of the 13 isomers in technical mixtures were 0.208 for TCI, 0.206 for Aldrich and 0.205 for Fluka. The predicted estrogenic activity of measured concentration of *para*-NP in technical mixtures was approximately 5-fold greater than the measured estrogen agonist activity.

Keywords: Single nonylphenol isomers; Technical nonylphenol; Estrogen equivalent concentration; GC–MS

1. Introduction

Nonylphenol (NP) is widely used as plastic additive and antioxidant; a derivative of NP, nonylphenol ethoxylate (NPE) is commonly used as a nonionic surfactant in detergents, paints, emulsifying agents, pesticides herbicides as

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well as a dispersing agent for industrial applications such as production of paper, fiber, metal and agriculture chemicals (White et al., 1994; Nimrod and Benson, 1996; Khim et al., 1999). NPEs are produced by the addition of ethylene oxide to NP and may have 1–20 ethoxy units per molecule. Although the estrogenic activity in vitro of NP was reported to be 10^{-6} times less than 17β-estradiol (E2) at the minimum (Jobling and Sumpter, 1993) to 2×10^{-3} times less at the maximum (Flouriot et al., 1995). Müller (2004) recently pointed out that xenoestrogens may interfere with the endocrine systems of living organisms. Dodds and Lawson (1937) first reported that propyl and propenyl phenols are weakly estrogenic substances. Routledge and Sumpter (1997) reported that the estrogenic activity of alkylphenols for human estrogen receptor (hER) on the structure–activity relationships study was found to be dependent on the number of carbon atoms in the alkyl chain, and branching (tertiary > secondary = normal) of the alkyl group affects estrogenicity. Nonylphenol isomers differing in estrogenic activity were recently discussed by Preuss et al. (2006).

NP is manufactured by the alkylation of phenol with nonene isomers. Therefore, it is expected that commercial NP would be a mixture of *para*-substituted monoalkylphenols with various isomeric and branched nonyl groups. The mixture of nonene isomers was industrially synthesized by trimerization of propylene ($CH_3CH{=}CH_2$) (Lee and Peart, 1995). Wheeler et al. (1997) first elucidated on the NP structure and idea of the alkyl group at the α-position in the side chain. There are theoretically 211 kinds of isomers of NP (Guenther et al., 2006), caused from the structure of the nonyl side chain in NP. Ieda et al. (2005) reported that comprehensive two-dimensional gas chromatography combined with mass spectrometry enabled tentative identification of 102 components of NP from a technical mixture although they did not clear if there are 102 or less different isomers. Nevertheless, commercially available technical NPs were used as standard samples for biological activity test and analyses of environmental samples.

Total NPs were determined mass-spectrometrically in environmental samples by using NP-specific ions (*m*/*z* 107, 121, 135, 149, 177 and 220) (Isobe et al., 2001; Isobe and Takada, 2004; Nakada et al., 2004), Nakada et al., 2004), and an isomer-specific quantification method for a few individual NP isomers using selected ions, *m*/*z* 121 for peak 1, *m*/*z* 135 for peaks 2, 3, 5, 7 and 11, *m*/*z* 149 for peaks 4, 6, 9 and 13, *m*/*z* 163 for peaks 8 and 10, *m*/*z* 191 for peak 12 by gas chromatography–mass spectrometry (GC–MS), was developed (Horii et al., 2004). Both of the above-mentioned methods by Isobe and Horii were, however, assumed that each single peak contains only one isomer, and that all isomers have the same response to mass spectrometric detector and also flame ionization detector (FID). Both the methods were, therefore, not for isomer-specific determination regarding NP isomers but for individual peaks on chromatogram by GC–MS. Enantioselective separation and determination of two NP isomers were recently reported by Zhang et al. (2007). Although Zhang et al. used an internal standard for determination, the single NP was not used as standard substance. We preliminary studied on variation in estrogenic activity of the isomers isolated from fractions of a commercial NP by high-performance liquid chromatography (HPLC) (Kim et al., 2004) and an estrogen-equivalent concentration of NP isomers in Ariake Sea water by the determination of individual GC-peaks based on the Horii's method (Kim et al., 2005a). The predicted estrogenic activity for measured concentrations of NP in Ariake Sea was calculated to be 2.7–3.0-folds greater than the measured estrogen agonist activity (Kim et al., 2005a). Therefore, a reliable analytical method for each isomer using synthesized standards of individual NP isomers is helpful for a risk assessment. Fourteen single isomers of branched *para*-NPs were confirmed to be present in technical NP mixtures previously by Thiele et al. (2004), Ruß et al. (2005) and Shioji et al. (2006). The 10 isomers were quantified in two technical mixtures using the standards synthesized by Ruß et al. (2005) although their detail quantification was not described there. In our previous works, 11 isomers were fractionated from the technical mixtures by HPLC and a gas-chromatograph equipped with a preparative fraction collector (GC-PFC) and their structures were elucidated by NMR and MS (Kim et al., 2004, 2005b). Furthermore, Uchiyama et al. (in press) and Saito et al. (2007) in our research group synthesized 13 isomers to be used as standard substance for bioassay and environmental analyses.

In the present study, the 13 isomers of branched *para*-NPs in three technical mixtures were isomer-specifically determined using their synthesized standard by selected ion monitor (SIM) for GC–MS of *m*/*z* 135, and *m*/*z* 149 and *m*/*z* 163 of all the mass-fragments regarding their isomers, which were contained more than 1% in the technical mixtures and systematically determined by only the three selected fragment ions. Moreover, estrogenic activities of individual single isomers by recombinant yeast estrogen screen assay (YES) were determined, then finally estrogen equivalent concentration (EEC) of three technical commercial NP was calculated for evaluation of risk assessments of NP isomers. Additionally, structures of two new diastereomeric isomers, 4-(3,4-dimethylheptan-4-yl)phenol (344NP) and 4-(3,4-dimethylheptan-3-yl)phenol (343NP) of more than 1% portion present in a technical mixture were elucidated by MS and NMR. Details for the identification of diastereomers from commercial NP mixture and for their synthesis by Friedel-Crafts reaction will be published elsewhere in the near future.

2. Experimental

2.1. Materials

2.1.1. Standards of synthetic para-NP isomers

For preparing calibration curves, following 13 *para*-NP isomers synthesized in our laboratory were used: 4-(2,4-

dimethylheptan-4-yl)phenol (244NP), 4-(2,4-dimethylheptan-2-yl)phenol (242NP), 4-(2,6-dimethylheptan-2-yl)phenol (262NP), 4-(3,6-dimethylheptan-3-yl)phenol (363NP), 4-(4-ethyl-2-methylhexan-2-yl)phenol (4E22NP), 4-(3,5-dimethylheptan-3-yl)phenol [353NP (NP-E, locally named)], 4-(2,5-dimethylheptan-2-yl)phenol (252NP), 4-(3,5-dimethylheptan-3-yl)phenol [353NP (NP-G, locally named, diastereomer of NP-E)], 4-(4-methyloctan-4-yl)phenol (44NP), 4-(3-ethyl-2-methylhexan-2-yl)phenol (3E22NP), 4-(2,3-dimethylheptan-2-yl)phenol (232NP), 4-(3-methyloctan-3-yl)phenol (33NP) and 4-(2-methyloctan-2-yl)phenol (22NP). The 13 NP isomers were synthesized by two different synthetic methods, A and B (Uchiyama et al., in press; Saito et al., 2007). The method A using 4-benzyloxyacetophenone as a starting material was used for NP isomers having two methyl groups at α-carbon. The benzyl group, a protective group of phenolic hydroxyl was interestingly deprotected on conversion of the tertiary hydroxyl group to the methyl by the action of $(CH_3)_3Al/TiCl_4$. 4E22NP and 252NP could be synthesized by this method A. The method B, which is convenient for the synthesis of NP isomer if requisite nonylalcohol is commercially available or readily accessible synthetically, was achieved by Friedel–Crafts alkylation of phenol with the corresponding nonylalchohols (Vinken et al. 2002; Ruß et al., 2005). Ten branched NP isomers, 244NP, 242NP, 262NP, 363NP, 353NP (diastereomer synthesized as mixture), 44NP, 3E22NP, 232NP, 33NP and 22NP were synthesized by this method B. The 3E22NP, 232NP and 22NP were synthesized by both methods A and B. Their purities were more than 98% by GC (Table 1). The ratio of diastereomers synthesized as mixture used in the present study was 353NP (NP-E): 353NP (NP-G) = 0.918:1 by GC determination.

Two new diastereomeric isomers, 344NP and 343NP, which could be chromatographically separated each other as four isomers, were used for structural elucidation by NMR and MS. The four isomers, 244NP, 44NP, 232NP and 3E22NP, which have not been synthesized by Lalah et al. (2001) who synthesized 262NP and Vinken et al. (2002) who synthesized 262NP, 363NP and 353NP, and Thiele et al. (2004), Ruß et al. (2005) and Shioji et al. (2006) as shown in Table 1, were first used as the standard substance in the present work.

NP isomers were recently named by nomenclature of IUPAC. However, considering that there are 211 theoretically possible constitutional isomers of *para*-nonylphenols (Robinson et al., 1976), these abbreviations are unsuitable for describing the complete system in order to get designations for all these isomers by Guenther et al. (2006) developed a practicable numbering system. It is important that the branching is indicated in the name as the estrogenic effect of *para*-nonylphenols is dependent on the structure of the side chain, that is, the branched and bulky side chains have more effect. Appendix A in the present study, therefore, was written down both names by IUPAC and Guenther et al. (2006).

2.1.2. Analytical sample of technical para-NPs

Three kinds of technical *para*-NP (CAS. No. 84852-15-3), Tokyo Chemical Industry Co. Ltd. (TCI) (Product No. N0300, Lot. No. FGE01), Fluka (Product No. 74430, Lot. No. 1092230) and Aldrich (Product No. 29085-8, Lot. No. LU00504CU) used in the present study

Table 1
Thirteen synthetic *para*-nonylphenol isomers present in technical mixtures in this study

Synthetic isomers (Saito et al., 2007 and Uchiyama et al., in press)					Previous synthetic isomers		
IUPAC name	Abbreviation	Local ID	GC retention time (min)	Purities by GC	Shioji et al. (2006)	Ruß et al. (2005)	Thiele et al. (2004)
4-(2,4-Dimethylheptan-4-yl)phenol	244 NP	NP-A	8.369	>98	n.s.	n.s.	+
4-(2,4-Dimethylheptan-2-yl)phenol	242 NP	NP-B	8.459	>98	+	+	n.s.
4-(2,6-Dimethylheptan-2-yl)phenol	262 NP	NP-C′	8.533	>98	+	+	n.s.
4-(3,6-Dimethylheptan-3-yl)phenol	363 NP	NP-C	8.543	>98	n.s.	+	+
4-(4-Ethyl-2-methylhexan-2-yl)phenol	4E22 NP	NP-D	8.560	>98	+	+	+
4-(3,5-Dimethylheptan-3-yl)phenol (NP-E)	353 NP[a]	NP-E	8.610	>98	n.s.	+	+
4-(2,5-Dimethylheptan-2-yl)phenol	252 NP	NP-F	8.622	>98	+	+	n.s.
4-(3,5-Dimethylheptan-3-yl)phenol (NP-G)	353 NP[a]	NP-G	8.673	>98	n.s.	+	+
4-(4-Methyloctan-4-yl)phenol	44 NP	NP-H	8.776	>98	+	n.s.	+
4-(3-Ethyl-2-methylhexan-2-yl)phenol	3E22 NP	NP-I	8.781	>98	n.s.	n.s.	n.s.
4-(2,3-Dimethylheptan-2-yl)phenol	232 NP	NP-M	8.979	>98	+	n.s.	+
4-(3-Methyloctan-3-yl)phenol	33 NP	NP-N	9.045	>98	+	+	+
4-(2-Methyloctan-2-yl)phenol	22 NP	NP-O	9.068	>98	+	+	+
4-Nonylphenol[b]	4- NP	–	10.454	>99.5	–	–	–

n.s.: Not synthesized.
[a] Diastereomeric pair synthesized as mixture.
[b] Purchased from Dr. Ehrenstorfer GmbH, Germany.

were purchased from each company. Anthracene-d_{10} purchased from Supelco (Product No. 44-2456) was used as injection internal standard (IIS). 4-Nonylphenol, the so-called *normal* nonylphenol was purchased from Dr. Ehrenstorfer GmbH, Germany.

2.1.3. Other reagents

2,2,4-Trimethylpentane (isooctane) (Kanto Chemical Co. Inc.) in special grade was used after distillation as solvent for chemicals.

2.2. Analytical procedures

2.2.1. Selected ions by structural types for determination of NP isomers

Three kinds of fragment ions, m/z 135 for seven isomers, 242NP, 262NP, 4E22NP, 252NP, 3E22NP, 232NP and 22NP, m/z 149 for four isomers, 363NP, 353NP (NP-E), 353NP (NP-G) and 33NP, and m/z 163 for two isomers, 244NP and 44NP were selected for isomer-specific determination of structurally different types of 13 NP isomers by GC–MS.

2.2.2. Calibration curves of single NP isomers by GC–MS

Isooctane solutions of 13 synthetic isomers for making calibration curves were individually prepared at 0.05, 0.2, 0.6, 1.2 and 2.4 mg l^{-1}. Anthracene-d_{10} isooctane solution of 1 mg l^{-1} as IIS was added into each screw vial for making calibration–curve-solutions.

The GC–MS analysis was carried out on an Agilent 5973Network quadrupole mass spectrometer fitted with an Agilent 6890N gas chromatograph (Agilent Technology). The fused silica capillary column with 30 m, 0.25 mm i.d., and 0.25 μm film thickness (HP-5 MS, J&W Scientific) was used with helium as the carrier gas at 100 kPa. For GC–MS, ionization potential was at 70 eV. The temperatures of the ion source and the transfer-line were 240 °C and 310 °C, respectively. Target compounds were measured based on SIM mode on the following quantitation ions; $m/z = 107$, 121, 135, 149, 163, 177, 191 and 220 for NP, and $m/z = 188$ for anthracene-d_{10}. The data handling were carried out using an Agilent Chemstation software G1701DA (Agilent Technology). The injection port was maintained at 300 °C, and the sample of 1 μl was injected with splitless mode followed by purge 2 min after the injection. The column oven temperature was held at 70 °C for the initial 2 min, then programmed at 30 °C min^{-1} to 180 °C, 2 °C min^{-1} to 200 °C, 30 °C min^{-1} to 310 °C, and held for 10 min.

2.2.3. Sample preparation of technical NPs

As analytical samples, adequate amount of isooctane solution of three technical NPs were prepared and finally anthracene-d_{10} isooctane solution as IIS was added for determining by GC–MS.

2.3. Estrogenic activity

Twelve single NP isomers were tested for estrogenic activity by the YES (Kim et al., 2004) based on the method by Routledge and Sumpter (1997). The yeast was kindly supplied by Dr. Sumpter, Brunel University, UK. In this system, the hER is expressed in a form capable of binding to estrogen-responsive sequence (ERE). The yeast cells also contain expression plasmids carrying the reporter gene, *lac*Z, which is regulated by the ERE. Activation of the receptor by binding of ligand causes expression of the reporter gene *lac*Z which produces the enzyme β-galactosidase. The activity of the estrogen-inducible β-galactosidase was measured by the coloration of chlorophenyl-red-β-galactopyranoside (CPRG). Each of single NPs were diluted with dimethyl sulfoxide and added to the yeast culture media in wells of microtiter plates. These plates were incubated for two days at 28 °C. Then CPRG were added to wells and incubated for 90 min. Color development caused by absorption was measured at 540 and 620 nm and the difference in the measurements was assumed to represent the activity of β-galactosidase which correlated well with the estrogenicity of 17β-estradiol (E2), which was used as a standard.

The amounts of color development by absorbance were plotted against the molar concentrations of sample to give a dose–response curve. From this curve, the minimal effective concentration was calculated as that gives the half of the maximum effect. Under our conditions, the minimal effective concentrations varied 20–50% depending on samples. We therefore carried out 4–6 independent experiments and calculated the mean value. For comparison of the activities between the single NP samples, the minimal effective concentration of each sample was compared with that of E2, which was included in all the assay plates as the standard.

Estrogen-equivalent concentration (EEC) is defined as follows:

$$\mathrm{EEC} = A \times B$$

where A the mass percent portion in technical NP mixture, B the estrogenic activity of single NP isomers relative to E2.

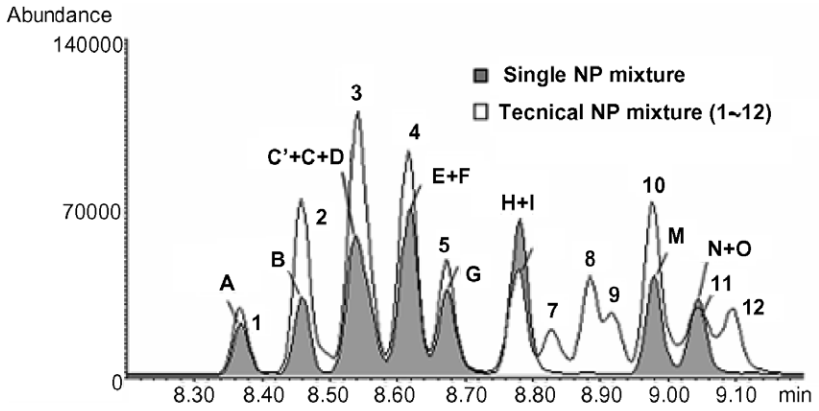
2.4. NMR and EI-MS analyses

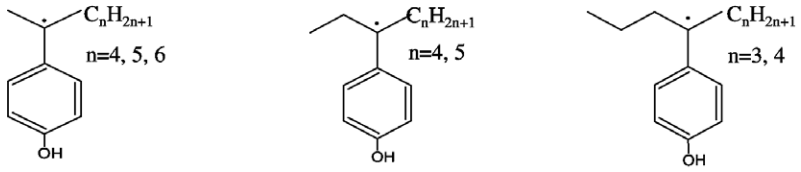
^{1}H and ^{13}C NMR spectra were recorded on a JEOL (Tokyo, Japan) JNM-LA600 FT-NMR in $CDCl_3$ containing tetramethylsilane as an internal standard. EI-MS analyses were carried out with a JEOL JMS-GCMATE.

3. Results and discussion

3.1. Chromatograms and mass spectra of single NP isomers and technical NP

Typical gas-chromatograms of 13 single NP isomers and the technical NP are shown in Fig. 1. Approximately 5.8 mg l^{-1} of a mixture of the single isomers and 10 mg l^{-1}





m/z 135
2-(4-hydroxyphenyl)alkane type

m/z 149
3-(4-hydroxyphenyl)alkane type

m/z 163
4-(4-hydroxyphenyl)alkane type

Selected ions	Structural alkane types	NP isomers quantified: A	B	C'	C	D	E	F	G	H	I	M	N	O	Peak Nos.
m/z 135	2-heptane type		+	+				+				+			7
	2-hexane type					+					+				
	2-octane type													+	
m/z 149	3-heptane type				+		+		+						4
	3-octane type												+		
m/z 163	4-heptane type	+													2
	4-octane type									+					

Fig. 1. Chromatograms of synthetic NP isomers and technical NP mixture from Aldrich For operational conditions, see text 2-2. Peaks A–N indicate A = 244NP, B = 242NP, C′ = 262NP, C = 363NP, D = 4E22NP, E = 353NP, F = 252NP, G = 353NP (diastereomer of E), H = 44NP, I = 3E22NP, M = 232NP, N = 33NP and O = 22NP, and for peaks 7 [344NP (J)], 8 [343NP (K)], 9 [344NP (L)] and 12 [343NP (P)], see text 3-3 and Appendix B.

of a technical mixture from Aldrich were used for injection to GC–MS. The chromatograms were drawn by total ion monitor summarized with *m/z* 107, 121, 135, 149, 163, 177, 191 and 220. As shown in Table 1, the retention times of each peak of the isomers on chromatogram were indicated differently. The peaks due to 13 synthetic isomers were, however, overlapped partly as shown in Fig. 1 (closed square), and chromatographically behaved similarly to those of technical NP mixture (open square). The peak No. 3 overlapped with three isomers, and the peaks No. 4, 6 and 11 individually overlapped with two isomers. As shown in Fig. 2, the peaks, No. 6 and 11 are new. Relationship between mass spectra and structure of NP were already shown in earlier study (Ieda et al., 2005). However, their spectra were not corresponding to anthropogenic single isomers. There are not significant meanings of mass spectra for mixture of single isomers. For example, the biggest problem is that the spectrum of 4-(1,4-dimethy-1-ethylpentyl)phenol, the so-called NP 3 shown in Fig. 6 in a reference from Ieda et al., 2005 but IUPAC-named 4-(3,6-dimethylheptan-3-yl)phenol (363NP) should not have such a strong fragment ion *m/z* 135 as shown in mass spectrum of 363NP(c) in Appendix A. In the present study, more comprehensive mass spectra of the 13 single NP isomers and corresponding chromatographic peaks No. 1–12 of technical NP mixture (Fig. 1) are shown in Appendix A. The eight above-mentioned strong fragment ions in mass spectrum of each peak on the chromatogram were selected for total ion monitoring. The 13 NP isomers were classified into three branched structural types: One type having intensive fragment of *m/z* 135 was for seven isomers, 242NP, 262NP, 4E22NP, 252NP, 3E22NP, 232NP and 22NP, another one having that of *m/z* 149 was for four isomers, 363NP, 353NP (NP-E), 353NP (NP-G) and 33NP, and the other one having that of *m/z* 163 was for two isomers, 244NP and 44NP. As shown in the following section, new 344NP and 343NP had the intensive fragment of *m/z* 149 and that of *m/z* 163, respectively. Single isomers

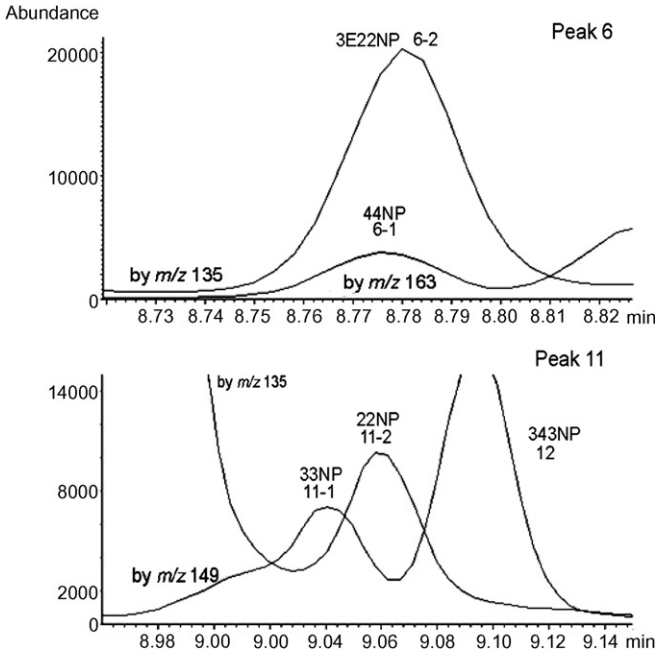


Fig. 2. Multiplicity of chromatographic peaks 6 and 11 of technical NP mixture. For numbers on peaks, see Fig. 1.

corresponding to peaks No. 7, 8, 9 and 12 on the chromatogram in Fig. 1 were not yet synthesized but already elucidated as 344NP (NP-J), 343NP (NP-K), 344NP (NP-L, diastereomer of NP-J) and 343NP (NP-P, diastereomer of NP-K), respectively.

3.2. Selected ion chromatograms for the determination

Three kinds of fragment ions, *m*/*z* 135, 149 and 163 were selected for the determination of structurally different types of 13 NP isomers by GC–MS (Fig. 3). Three ion chromatograms by *m*/*z* 135, 149 and 163 are shown in Fig. 3. These possible fragment structures are shown in Fig. 1 (middle), and relationship of quantitations between individual isomers and selected ions is summarized in Fig. 1 (bottom). Four peaks 3, 4, 6 and 11 were found to overlap with more than two isomers. The peak 3 that overlapped with 262NP, 363NP and 4E22NP was determined by using the selected ions at *m*/*z* 149 for 363NP, and at *m*/*z* 135 for 262NP and 4E22NP. In order to quantify 262NP and 4E22NP, the inseparable peak 3 was split into peak 3-1 and peak 3-3 by vertical dotted line as shown in Fig. 3 (top). When a 100 m-column (Petrocol DH, Supelco) was used, both peaks of 3-1 and 3-3 could be experimentally separated completely but also time-consuming. It took 90 min that only one sample could be determined. The peak 4 that overlapped with 353NP (NP-E) and 252NP could be determined by the ions at *m*/*z* 149 for 353NP (NP-E) and *m*/*z* 135 for 252NP, respectively. The peak 6 which consists of 44NP and 3E22NP could be determined at *m*/*z* 163 for 44NP and at *m*/*z* 135 for 3E22NP, while the peak 11 which consists of 33NP and 22NP could be determined at *m*/*z* 149 for 33NP and at *m*/*z* 135 for 22NP. The four other single peaks were determined at *m*/*z* 163 for 244NP, at *m*/*z* 135 for 242NP (corresponding to peak 2), at *m*/*z* 149 for 353NP (NP-G, corresponding to peak 5) and at *m*/*z* 135 for 232NP (corresponding to peak 10), respectively.

The isomer, 3E22NP was the most estrogenic-active isomer in our previous study (Uchiyama et al., in press). Therefore, the important peak corresponding to 3E22NP was peak 6 which consists of 44NP and 3E22NP. The most important NP-isomer, 3E22NP could be determined at *m*/*z* 135, while 44NP could be determined at *m*/*z* 163.

3.3. Amounts of NP isomers in three technical NP mixtures by using calibration curves of synthetic NP-isomer standards by GC–MS

Typical calibration formulas of single NP-isomer standards by GC–MS are shown in Table 2. Quadratic regression curves were applied for calibration curves

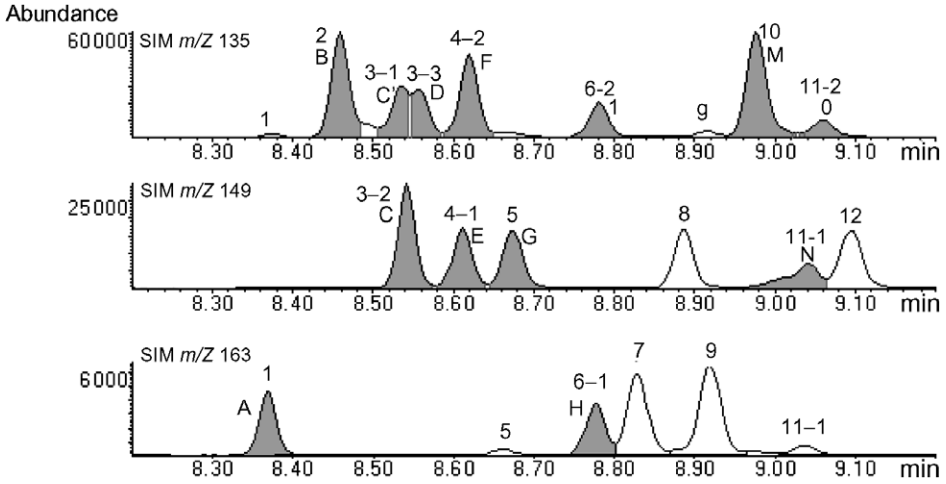


Fig. 3. Ion chromatograms by structurally selected ions for quantitation of NP isomers. For alphabets and numbers on peaks, see Fig. 1. E and G, 8 and 12, and 7 and 9 are individually diastereomeric pairs. For 8, 12, 7 and 9, see Fig. 1.

Table 2
Calibration formula, coefficient of determination, and limits of quantitation and detection of *para*-nonylphenol isomers

NP isomers	Quadratic regression equation				Linear regression equation
	Calibration formula[b]	Coefficient of determination of calibration curves (R^2)	Quantitation limit (mg l^{-1})	Detection limit (mg l^{-1})	Coefficient of determination of calibration curves (R^2)
244 NP	$0.01045x^2 + 0.02517X + 0.00005$	0.99998	0.064	0.019	0.98263
242 NP	$0.02313x^2 + 0.24463X - 0.01225$	0.99943	0.012	0.004	0.99696
262 NP	$0.02998x^2 + 0.16158X - 0.00518$	0.99988	0.012	0.004	0.99312
363 NP	$0.01429x^2 + 0.06397X - 0.00190$	0.99986	0.032	0.010	0.99122
4E22 NP	$0.02293x^2 + 0.09679X + 0.00108$	0.99993	0.012	0.004	0.99063
353 NP (NP-E)[a]	$0.01890x^2 + 0.06884X - 0.00179$	0.99960	0.016	0.005	0.98904
252 NP	$0.01173x^2 + 0.23607X - 0.01476$	0.99657	0.008	0.002	0.99575
353 NP (NP-G)[a]	$0.01650x^2 + 0.09286X - 0.00364$	0.99963	0.016	0.005	0.99288
44 NP	$0.00641x^2 + 0.04274X - 0.00226$	0.99938	0.064	0.019	0.99438
3E22 NP	$0.00421x^2 + 0.20962X - 0.01383$	0.99690	0.012	0.004	0.99675
232 NP	$0.04651x^2 + 0.25754X - 0.00668$	0.99996	0.012	0.004	0.99345
33 NP	$0.01881x^2 + 0.07085X - 0.00328$	0.99955	0.008	0.002	0.98887
22 NP	$0.01186x^2 + 0.03309X + 0.00027$	0.99993	0.012	0.004	0.98599
4-NP	$0.04740x^2 - 0.02231X + 0.00722$	0.99627	0.011	0.003	0.90518

[a] Diastereomeric pair synthesized as mixture.
[b] $Y = aX^2 + bX + C$, Y = (peak area of NP-isomer)/[(peak area of IIS (mg l^{-1}))], X = concentration of individual solution of NP isomers (mg l^{-1}).

because coefficients of determination (R^2) of quadratic regression curves were better than those of linear regression curves. The R^2 of quadratic regression curves were between 0.99627 and 0.99998, while those between of linear curves between 0.90518 and 0.99696 (Table 2). The calibration curves for determination of the concentration between 0.05 and 2.4 mg l^{-1} of the standards were applied to their determination of technical NP mixtures. Coefficients of variation of determination in triplicate were between 2% at 2.4 mg l^{-1} for 363NP and 28% at 0.05 mg l^{-1} for 232NP. For environmental samples of water and sediments, however, concentration of standard solution between 0.0024 and 0.05 mg l^{-1} for all isomers was found to be able to be determined in our experiment. Quantitation limit with noise ratio of more than 10 was between 0.008 mg l^{-1} for 252NP and 33NP, and 0.064 mg l^{-1} for 244NP and 44NP, and detection limit with noise of more than 3 was between 0.0024 mg l^{-1} for 252NP and 33NP, and 0.019 mg l^{-1} for 244NP and 44NP (Table 2). All the coefficients of variation were less than 10% in triple determinations of the NP isomers in three technical NPs.

As shown in Table 3, the four isomers, 244NP, 44NP, 3E22NP and 232NP were first determined in technical

Table 3
Portions of *para*-nonylphenol isomers in three technical mixtures

IUPAC name	Abbreviation	Mass % in technical mixture				
		TCI	Aldrich	Fluka	Fluka[b]	Acros[b]
4-(2,4-Dimethylheptan-4-yl)phenol	244 NP	6.1 ± 0.1	3.8 ± 0.1	3.8 ± 0.2	n.d.	n.d.
4-(2,4-Dimethylheptan-2-yl)phenol	242 NP	9.4 ± 0.2	7.0 ± 0.2	7.9 ± 0.2	14	12
4-(2,6-Dimethylheptan-2-yl)phenol	262 NP	3.9 ± 0.3	4.4 ± 0.1	4.4 ± 0.3	5	2
4-(3,6-Dimethylheptan-3-yl)phenol	363 NP	11.1 ± 0.3	9.9 ± 0.3	10.1 ± 0.3	13	9
4-(4-Ethyl-2-methylhexan-2-yl)phenol	4E22 NP	8.2 ± 0.4	5.9 ± 0.3	7.3 ± 0.2	6	3
4-(3,5-Dimethylheptan-3-yl)phenol (NP-E)	353 NP[a]	8.8 ± 0.4	7.4 ± 0.8	7.4 ± 0.5	10	10
4-(2,5-Dimethylheptan-2-yl)phenol	252 NP	7.7 ± 0.3	7.2 ± 0.7	7.1 ± 0.5	7	3
4-(3,5-Dimethylheptan-3-yl)phenol (NP-G)	353 NP[a]	7.3 ± 0.3	6.0 ± 0.7	6.1 ± 0.4	10	10
4-(4-Methyloctan-4-yl)phenol	44 NP	3.2 ± 0.1	3.6 ± 0.3	3.3 ± 0.1	n.d.	n.d.
4-(3-Ethyl-2-methylhexan-2-yl)phenol	3E22 NP	5.4 ± 0.1	4.4 ± 0.4	4.3 ± 0.3	n.d.	n.d.
4-(2,3-Dimethylheptan-2-yl)phenol	232 NP	9.2 ± 0.4	6.4 ± 0.3	7.4 ± 0.1	n.d.	n.d.
4-(3-Methyloctan-3-yl)phenol	33 NP	5.9 ± 0.2	5.2 ± 0.5	5.0 ± 0.3	4	2
4-(2-Methyloctan-2-yl)phenol	22 NP	3.0 ± 0.2	3.4 ± 0.3	2.9 ± 0.3	2	1
Total		89 ± 2	75 ± 4	77 ± 2	71	52

n.d., not determined.
[a] Diastereomeric pair.
[b] Quantified by Ruß et al. (2005).

mixtures. The portions of the *para*-NP isomers determined in TCI sample were between $11.1 \pm 0.3\%$ for 363NP and $3.0 \pm 0.2\%$ for 22NP. The tendency of those in the two other Fluka and Ardrich was almost similar to that of TCI. Total 13 mass percent portion in the NP mixture from TCI, Aldrich and Fulka covered with $89 \pm 2\%$, $75 \pm 4\%$ and $77 \pm 2\%$, respectively. The residual percent portions will cover with several not-yet-determined isomers. In our study, peaks 7, 8, 9 and 12 shown in Fig. 1 will correspond to those isomers. They were two pairs of diastereomeric isomers and their structures of these corresponding isomers by MS and NMR were elucidated as two types of 344NP (NP-J) for peak 7 and 344NP (NP-L) for peak 9, and those of 343NP (NP-K) for peak 8 and 343NP (NP-P) for peak 12. Their isomers, however, were not synthesized yet but chromatographically separated. More than 1 mass percent of 344NP and 343NP in the technical NP mixture were estimated by semiquantification comparing with responses of neighbor peaks of single NP isomers. The spectral data of MS and NMR and purities of these fractions are listed in Appendix B. Information on detail fractionation will be described elsewhere by Makino et al.

More than 1% portions of *para*-NPs in technical mixtures have been studied in our work. However, a less than 1% mass portion of the *para*-NP, 4-(2,5,5-trimethylhexan-2-yl)phenol (2552NP) was quantified in both mixtures from Fluka and Acros (Ruß et al., 2005). In our study, the 2552NP having retention time of 8.010 min under the conditions as described in text, 2-2 was also found at less than 1% portion in a technical mixture. It depends on their estrogenicities in the further studies, whether or not the small portions of the isomers present in technical mixtures will be determined.

3.4. Estrogenic activity

There was some deference in estrogenic relative activity to E2 between fractions by HPLC and GC-PFC, and the estrogenic activity of *n*-NP was considerably lower when compared with that of a technical mixture from a commercial source (Kim et al., 2004, 2005b). The difference in estrogenic activity for hER between *n*-NP and technical NP was found before (Routledge and Sumpter, 1997). The relative estrogenicity of twelve isomers of *para*-NP by YES is given (Table 4). The 3E22NP exhibited the greatest estrogenic activity, which was 25×10^{-4} as potent as E2. The relative potency of 33NP was the least. In our previous work (Kim et al., 2005b), the greatest estrogenic activity of NP-7 exhibited 19×10^{-4} as potent as E2. The fraction NP-7, which corresponded to GC-peak 6 in the present work, was found to contain both 44NP and 3E22NP as shown in Figs. 1 and 2. As shown in Table 4, the estrogenic activity of 44NP in the present work was fairly lower when compared with that of 3E22NP. Therefore, the present method, in which 44NP and 3E22NP could be separately determined, is important especially from a viewpoint of estrogenic study.

3.5. Estrogenic equivalent concentration

The pattern of relative portions of *para*-NP isomers varied among three technical mixtures (Table 3). The EEC of *para*-NP isomers in the technical mixture was calculated because individual isomers exhibited different activities (Table 4). The EEC of 13 *para*-NP in technical mixtures was predicted, base on the use of portion of the *para*-NP and their respective potencies, to be 0.205–0.208 times less

Table 4
Estrogen-equivalent concentration (EEC) of branched *para*-nonylphenol isomers in technical NP

NP isomers	Relative activity of NP-isomers:		EEC %		
	to 17β-estradiol(E2), $\times 10^{-4}$	to 3E22 NP (NP-I)	TCI	Aldrich	Fluka
244 NP	3.62	0.143	0.87	0.54	0.54
242 NP	3.89	0.154	1.45	1.08	1.22
262 NP	6.35	0.251	0.98	1.11	1.10
363 NP	2.42	0.096	1.06	0.95	0.96
4E22 NP	7.71	0.305	2.51	1.81	2.22
353 NP (NP-E)[a]	3.58	0.142	1.24	1.05	1.04
252 NP	3.23	0.128	0.98	0.92	0.91
353 NP (NP-G)[a]	3.58	0.142	1.03	0.85	0.87
44 NP	2.75	0.109	0.35	0.39	0.35
3E22 NP	25.3	1.000	5.36	4.43	4.26
232 NP	4.39	0.173	1.59	1.11	1.29
33 NP	1.37	0.0543	0.32	0.28	0.27
22 NP	6.6	0.261	0.79	0.88	0.75
Total	–	–	18.5	15.4	15.8
EEC %	–	–	0.208	0.206	0.205
Mass percent portion[b]					

[a] Diastereomeric pair synthesized as mixture,and for their activity, it is assumed for diastereomer of 353NP to be same. For information on assay, see Section 2.3 (estrogenic activity).

[b] For mass percent portion, see Table 3.

than the activity actually measured in technical mixtures. Some more effective analytical data for fish and water, marine sediment should be collected by the present method in the near future.

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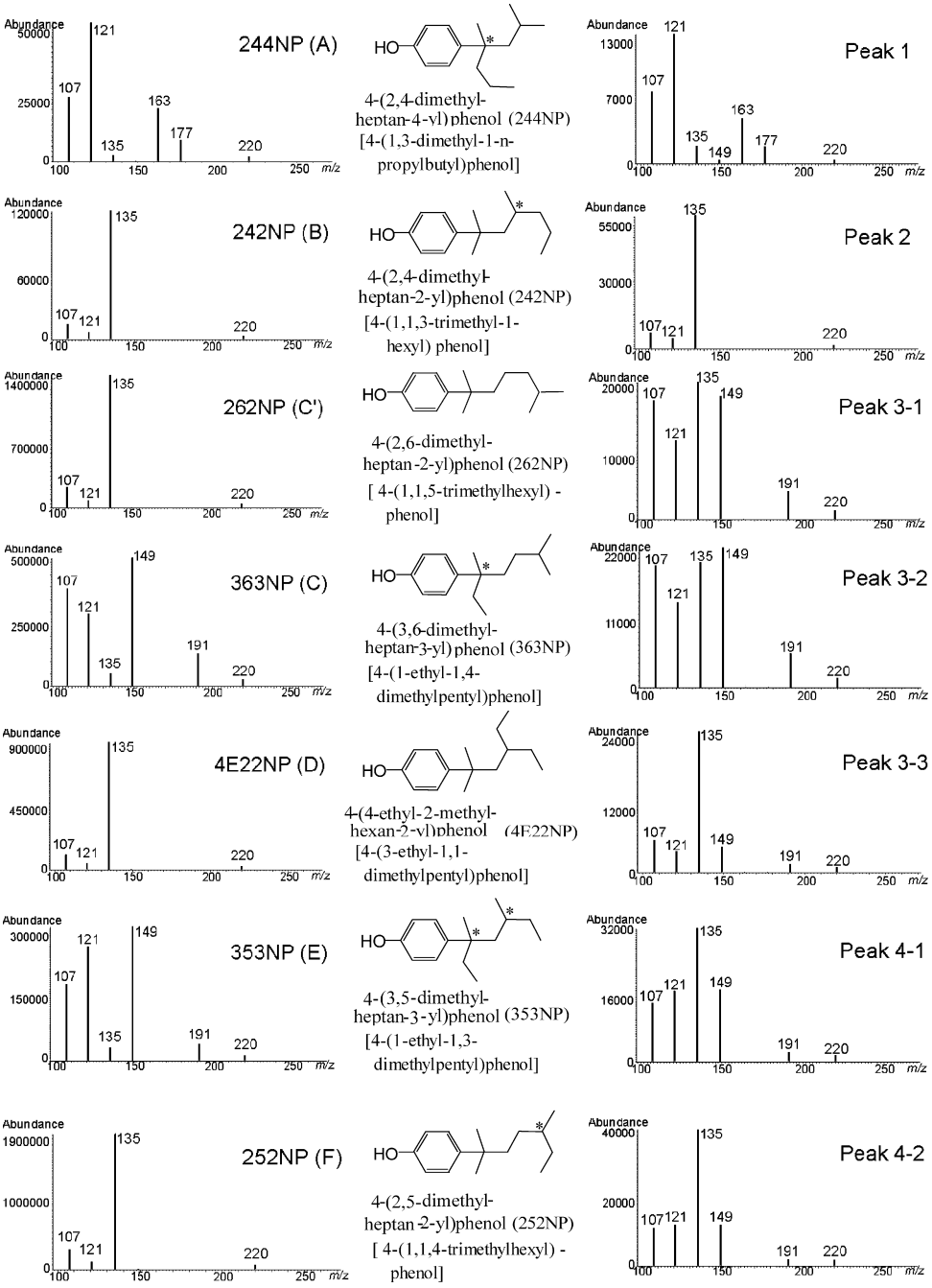
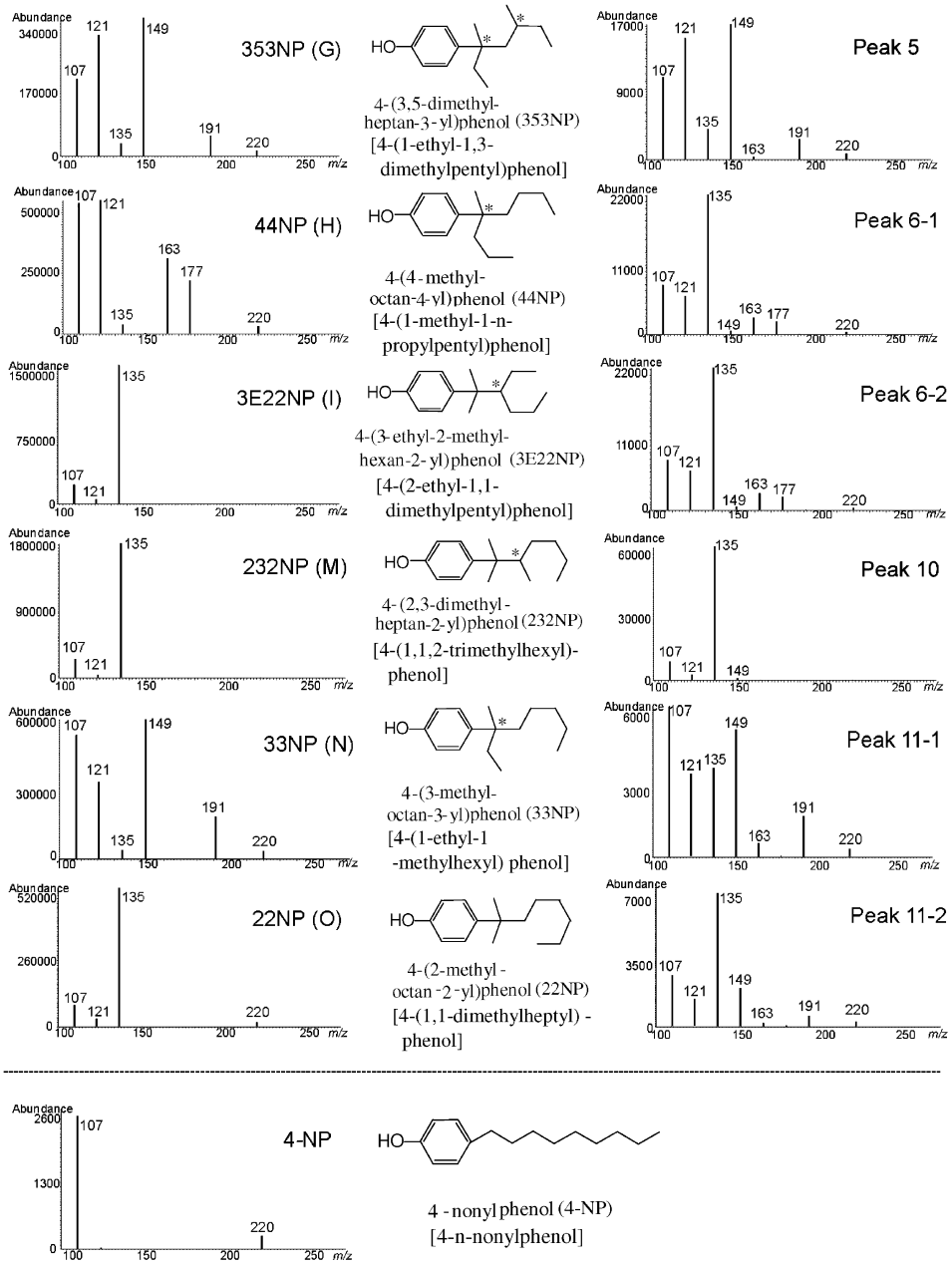
Appendix A. Mass spectra of synthetic NPs and corresponding GC-peaks

Mass spectra of single isomers, left and corresponding peaks on chromatogram, right.



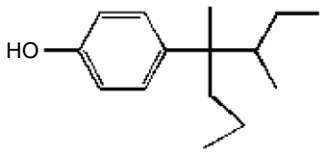


[ ]: named by Guenther et al., 2006. *: Asymmetric carbon

Major mass fragment ions of not-yet-synthesized but elucidated single isomers corresponding to peaks 7, 8, 9 and 12 were determined as follows: m/z107 (78%), m/z121(91%), m/z163(base) and m/z220(M^+, 5.5%) for peak 7, 4-(3,4-dimethylheptan-4-yl)phenol (344NP) (J) [4-(1,2-dimethyl-1-n-propylbutyl)phenol], m/z107 (61%), m/z12 (40%), m/z149 (base) and m/z220 (M^+, 3.7%) for peak 8, 4-(3,4-dimethylheptan-3-yl)phenol (343NP) (K) [4-(1-ethyl-1,2-dimethylpentyl)phenol], m/z107 (89%), m/z121(91%), m/z163 (base) and m/z220(M^+, 5.5%) for peak 9, 4-(3,4-dimethylheptan-4-yl)phenol (344NP) (L) [4-(1,2-dimethyl-1-n-propylbutyl)phenol], and m/z107 (41%), m/z121(14%), m/z149(base) and m/z220(M^+, 3.3%) for peak 12, 4-(3,4-dimethylheptan-3-yl)phenol (343NP) (P) [4-(1-ethyl-1,2-dimethylpentyl)phenol].

Appendix B. Spectral data of EI-MS and NMR, and purities for four fractions of a technical mixture from TCI

B.1. 4-(3,4-Dimethylheptan-4-yl)phenol [344NP (NP-J)], corresponding to peak 7

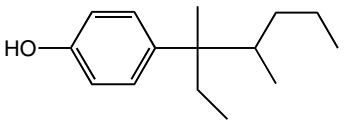


EI-MS *m/z* (relative intensity): 220 (M^+), 163 (base). ^{1}H NMR (500 MHz, $CDCl_3$, δ): 0.73 (3H, t, $J = 7.0$ Hz, CH_3), 0.67–0.81 [3H, m, $CH_3CH_2CH_2$ and $CH_3CH(H)$], 0.78 [3H, t, $J = 6.5$ Hz, CH_3], 0.88 [3H, d, $J = 6.7$ Hz, CH_3], 1.02–1.08 [1H, m, $CH_3CH(H)$], 1.13 (3H, s, CH_3), 1.49–1.62 [3H, m, $CH_3CH_2CH_2$ and $CH_3CH_2CH(CH_3)$], 4.54 [1H, brs, OH], 6.75 [2H, d, $J = 8.6$ Hz, CH = ×2], 7.11 [2H, d, $J = 8.6$ Hz, CH = ×2]. ^{13}C NMR (125 MHz, $CDCl_3$, δ): 13.1 (CH_3), 13.4 (CH_3), 14.9 (CH_3), 17.6 (CH_2), 18.3 (CH_3), 24.6 (CH_2), 43.3 (CH_2), 43.8 (C),

45.5 (CH), 114.5 (CH × 2), 127.9 (CH × 2), 140.7 (C), 152.8 (C).

Composition based on GC analysis (OV-1701, 0.25 mm × 50 m): *J* (71%), and four NPs (each one is less than 8%).

B.2. 4-(3,4-Dimethylheptan-3-yl)phenol [343NP (NP-K)], corresponding to peak 8



EI-MS *m*/*z* (relative intensity): 220 (M^+), 149 (base). ^{1}H NMR (500 MHz, $CDCl_3$, δ): 0.55 [3H, t, $J = 7.0$ Hz, CH_3], 0.72 [3H, t, $J = 7.2$ Hz, CH_3], 0.78–0.83 [1H, m, $CH_3CH_2CH(H)$], 0.87 [3H, d, $J = 7.0$ Hz, CH_3], 0.96–1.05 [2H, m, $CH_3CH(H)CH_2$ and $CH_3CH_2CH(H)$], 1.12 [3H, s, CH_3], 1.27–1.34 [1H, m, $CH_3CH(H)CH_2$], 1.56–1.69 [3H, m, CH_3CH_2 and $CH_3CH_2CH_2CH$], 4.61 [1H, brs, OH], 6.75 [2H, d, $J = 8.8$ Hz, CH = ×2], 7.11 [2H, d, $J = 8.8$ Hz, CH = ×2]. ^{13}C NMR (125 MHz, $CDCl_3$, δ): 8.8 (CH_3), 14.0 (CH_3), 14.2 (CH_3), 17.8 (CH_3), 21.4 (CH_2), 32.9 (CH_2), 34.3 (CH_2), 42.8 (CH), 43.9 (C), 114.5 (CH × 2), 128.2 (CH × 2), 140.1 (C), 152.8 (C). Composition based on GC analysis (OV-1701, 0.25 mm × 50 m): *K* (95%).

B.3. 4-(3,4-Dimethylheptan-4-yl)phenol [344NP (NP-L), diastereomer of NP-J] corresponding to peak 9

EI-MS *m*/*z* (relative intensity): 220 (M^+), 163 (base). ^{1}H NMR (500 MHz, $CDCl_3$, δ): 0.57 [3H, d, $J = 6.7$ Hz, CH_3], 0.73–0.83 [1H, m, $CH_3CH(H)CH_2$], 0.80 [3H, t, $J = 7.5$ Hz, CH_3], 0.86–0.90 [1H, m, $CH_3CH(H)$], 0.88 [3H, t, $J = 7.2$ Hz, CH_3], 1.05–1.10 [1H, m, $CH_3CH(H)CH_2$], 1.14 [3H, s, CH_3], 1.46–1.52 [1H, m, CH_3CH_2–$CH(CH_3)$], 1.58–1.68 [3H, m, $CH_3CH_2CH_2$ and $CH_3CH(H)$], 4.65 [1H, brs, OH], 6.75 [2H, d, $J = 8.6$ Hz, CH = ×2], 7.11 [2H, d, $J = 8.6$ Hz, CH = ×2]. ^{13}C NMR (125 MHz, $CDCl_3$, δ): 13.3 (CH_3), 14.0 (CH_3), 14.9 (CH_3), 17.7 (CH_2), 18.8 (CH_3), 24.0 (CH_2), 42.5 (CH_2), 43.7 (C), 45.9 (CH), 114.6 (CH × 2), 127.9 (CH × 2), 140.6 (C), 152.8 (C).

Composition based on GC analysis (OV-1701, 0.25 mm × 50 m): L2 (82%) and five NPs (one is 9% and others are less than 2%).

B.4. 4-(3,4-Dimethylheptan-3-yl)phenol [343NP (NP-P), diastereomer of NP-K] corresponding to peak 12

EI-MS *m*/*z* (relative intensity): 220 (M^+), 149 (base). ^{1}H NMR (500 MHz, $CDCl_3$, δ): 0.58 [3H, d, $J = 6.6$ Hz, CH_3], 0.59 [3H, t, $J = 7.2$ Hz, CH_3], 0.79–0.80 [1H, m, $CH_3CH(H)CH_2$], 0.88 [3H, t, $J = 7.4$ Hz, CH_3], 0.87–0.95 [1H, m, $CH_3CH_2CH(H)$], 1.12 [3H, s, CH_3], 1.41–1.45 [1H, m, $CH_3CH(H)CH_2$], 1.49–1.52 [1H, m, CH_3CH_2-$CH(H)$], 1.57–1.70 [3H, m, CH_3CH_2 and CH_3CH_2 CH_2CH], 4.58 [1H, brs, OH], 6.75 [2H, d, $J = 8.6$ Hz, CH = ×2], 7.10 [2H, d, $J = 8.6$ Hz, CH = ×2]. ^{13}C NMR (125 MHz, $CDCl_3$, δ): 8.9 (CH_3), 14.4 (CH_3), 14.8 (CH_3), 18.1 (CH_3), 21.7 (CH_2), 32.2 (CH_2), 33.8 (CH_2), 43.4 (CH), 43.8 (C), 114.5 (CH × 2), 128.2 (CH × 2), 140.1 (C), 152.8 (C). Composition based on GC analysis (OV-1701, 0.25 mm × 50 m): P (75%) and seven NPs are less than 10%.

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