VARIATION OF ESTROGENIC ACTIVITY IN A COMMERCIAL NONYLPHENOL PREPARATION FRACTIONATED BY HPLC

Yun-seok Kim[1], Takao Katase[1], Tadashi Inoue[1], Sayaka Sekine[1], Yasuo Fujimoto[2] and Nobuyoshi Yamashita[3]

[1] College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa, Kanagawa, 252-8510, Japan; [2] College of Pharmacy, Nihon University, 7-7-1 Narashinodai, Funabashi, Chiba, 274-8555, Japan; and [3] National Institute of Advanced Industrial Science and Technology, 16-1 Onogawa, Tsukuba, Ibaraki 305-8569, Japan

Introduction

Nonylphenol (NP) is widely used surfactants in detergents, paints, emulsifying agents, pesticides as well as in the manufacture of paper, fiber, metal and agriculture chemicals[1,2]. In Japan, NP is produced at a rate of 50,000 tons per year. In the 1930's, Dodds[3] demonstrated estrogenic activity of propylphenols. Subsequently, Routledge and Sumpter[4] found that the estrogenic activity of alkylphenols was dependent on the structure of the alkyl chain. In our recent study on environmental endocrine disrupting chemicals, we identified a NP mixture by GC-MS in extracts from a number of wrapping films obtained from commercial sources. Fractions of the NP mixture that were separated using two-dimensional capillary gas chromatograph equipped with a preparative fraction collector (2DGC-PFC) differentially showed estrogenic activity in a mammalian cell line, MVLN (MCF-7)[5]. In view of the potential human exposure to NP, which consists of more than thirty individual chemicals, it is worthwhile to study structure-activity relationships of the NP mixture components. In this study, we report optimal conditions for the fractionation of a commercial NP mixture using high performance liquid chromatography (HPLC). Although the fractions are not homogenous, their activities are quite different from each other. This suggests that it may be possible to develop a technical NP mixture with relatively low estrogenic activity. Our on-going study includes developing a method for quantitative structure-activity relationship (QSAR) of individual component in the technical NP mixtures for its estrogenic activity.

Materials and Methods

Preliminary studies on the separation of commercially available 4-*n*-nonylphenol (4-*n*-NP, Dr. Ehrenstorfer GmbH, Co., 80407) and nonylphenol mixture (NP-mix) by normal phase thin layer chromatography (TLC; MERCK, silica gel 60 F254) using hexane and ethyl acetate (10:1) as the mobile phase provided good results. Therefore, we tested the preparative HPLC (Shenshu Science, Co., SSC-3160 with PEGASIL silica column (I.D. 200 mm, length 250 mm), 10 ml/min flow rate and refractive index detector) for same mixture. NP-mix (500 mg, Tokyokasei Co., N0300) was dissolved in *n*-hexane and fractionated by HPLC for six times. Some of the fractions were further fractionated by the HPLC under the same conditions. Structures of the major components in each fraction were determined by analysis of their ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra.

Each fraction was tested for estrogenic activity by the recombinant yeast screen assay[6]. The yeast

was kindly supplied by Dr. Sumpter, Brunel University, UK. In this system, human estrogen receptor (hER) is expressed in a form capable of binding to estrogen-responsive sequence (ERE). Yeast cells also contain expression plasmids carrying the reporter gene, *lacZ*, which is regulated by the ERE. Activation of the receptor by binding of ligand causes expression of the reporter gene *lacZ* which produces the enzyme β-galactosidase. The activity of the estrogen-inducible beta-galactosidase was measured by the coloration of chlorophenyl-red-β-galactopyranoside (CPRG). Fractionated NP-mix were diluted with dimethyl sulfoxide (DMSO) and added to the yeast culture media which contained CPRG in wells of micro titer plates. Plates were incubated for four days at 28°C. Color development was measured at 540nm and 620nm 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) that was used as a positive control.

Results and Discussion

Figure 1A shows an elution profile of the first HPLC showing a major peak and some minor peaks. The major peak was further separated by a second HPLC (Figure 1B and 1C) and 10 fractions were finally obtained. A schematic diagram of the fractionation and yield of each of the fractions is shown in Figure 2. Estrogenic activity of the straight chain NP (4-*n*-NP) was considerably lower when compared with that of NP mixture from a commercial source (Figure 3a). Fractions separated from the NP mixture exhibited estrogenic activity to various extents (Figure 3b). Relative activities of the 10 fractions are summarized in Figure 4. F5-3 showed the highest estrogenic activity corresponding to 1/10,000 that of E2. NMR studies revealed that this fraction contained 4-(1,1,2-trimethyl-hexyl)-phenol and 4-(1,1,4-trimethyl-hexyl)-phenol. Since fractions generally contained more than one component, additional high resolution separation techniques are required for further purification the fractions and for elucidating the structure-activity relationship of the alkylphenols.

Acknowledgments

The authors are thankful to Mrs. Makino Mitsuko (Nihon University, College of Pharmacy) for her help in analytical experiments. We are also thankful to Dr. B. G. Loganathan (Murray State University, Murray, KY, USA) and colleague for their helpful and critical review of this report.

References

1. Nimrod, A.C. and Benson, W.H. (1998) in: *Chemically Induced Alterations in the Functional Development and Reproduction of Fishes (Roland, R., Ed)*, SETAC, Pensacola, FL.
2. White, R., Jobling, S., Hoare, S.A., Sumpter, J.P. and Parker, M.G. (1994) *Endocrinol.* 135, 175-182.
3. Dodds, E.C. (1936) *Helv. Chem. Acta* 19, E49-57.
4. Routledge, E.J. and Sumpter, J. P. (1997) *J. Biol. Chem.* 272, 3280-3288.
5. Yamashita, N., K. Kannan, K., Hashimoto, S., Miyazaki, A. and Giesy, J.P. (1999) *Organohalogen Compounds* 42, 121-124.
6. Routledge, E.J. and Sumpter, J.P. (1996) *Environ. Toxicol. Chem.* 15, 241-248.

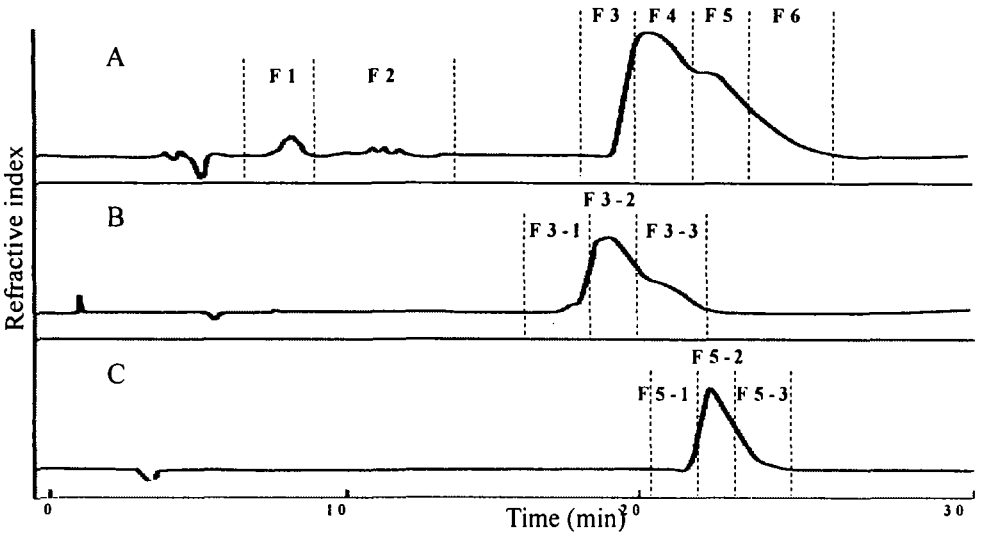

Figure 1. Elution profiles of NP-mix by HPLC.

A: Elution profiles of the first HPLC. B and C: Elution profiles of the second HPLC. The fraction F3 and F5 from the first HPLC were individually applied to the second HPLC and eluted under the same conditions. The fractions separated by vertical dotted lines were pooled.

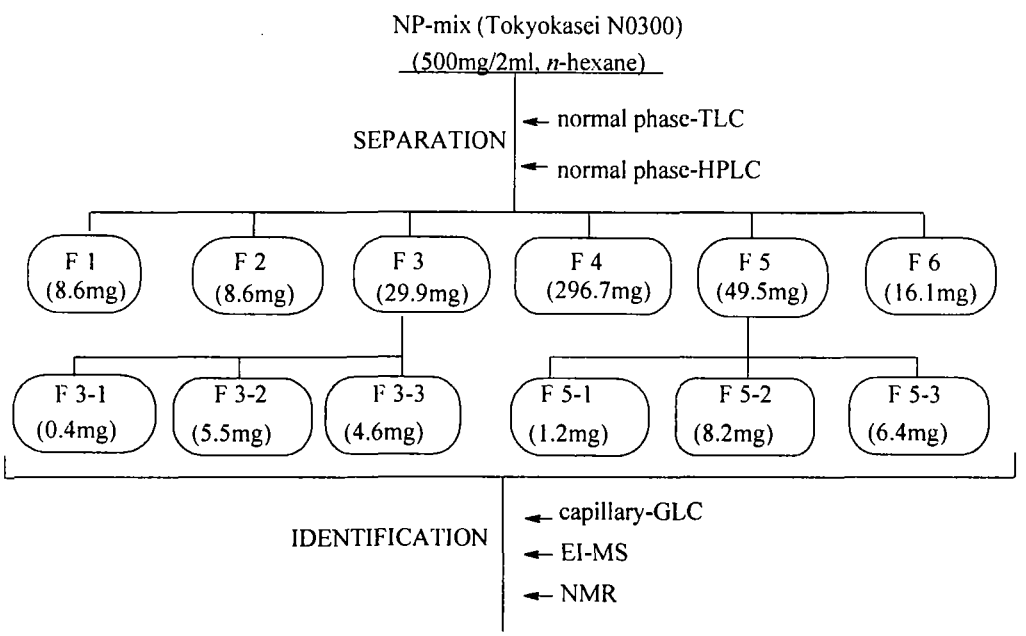

Figure 2. Schematic representation of the separation and yield of the each fraction

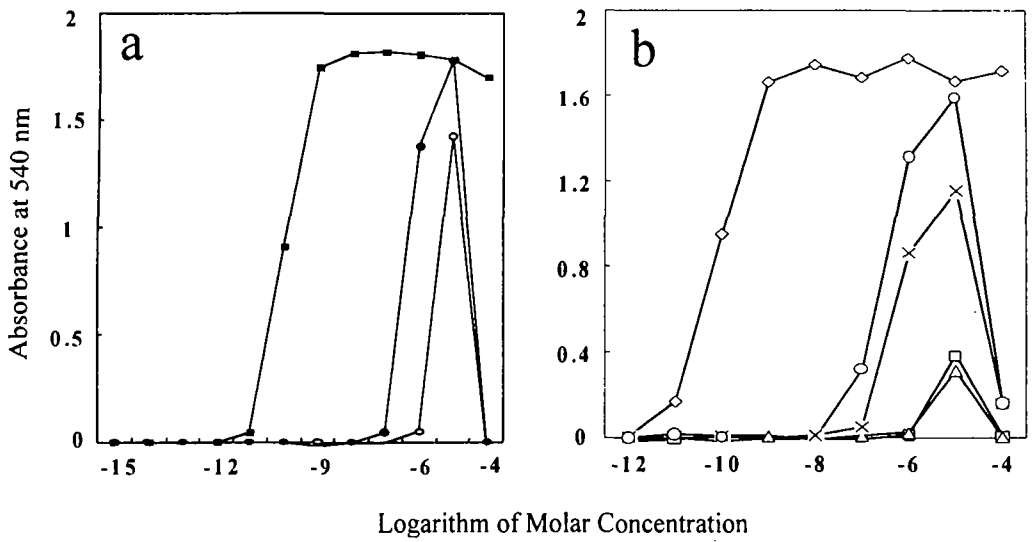

Figure 3. Estrogenic activity of 17beta-E2 , NP-mix and 4-n-NP.

(a) applied chemicals ; 17β-E2 (■), NP-mix (●), 4-*n*-NP (○). (b) fractions (F) ; 17β-E2 (◇), F1 (□), F2 (△), F5-2 (×), F5-3 (○).

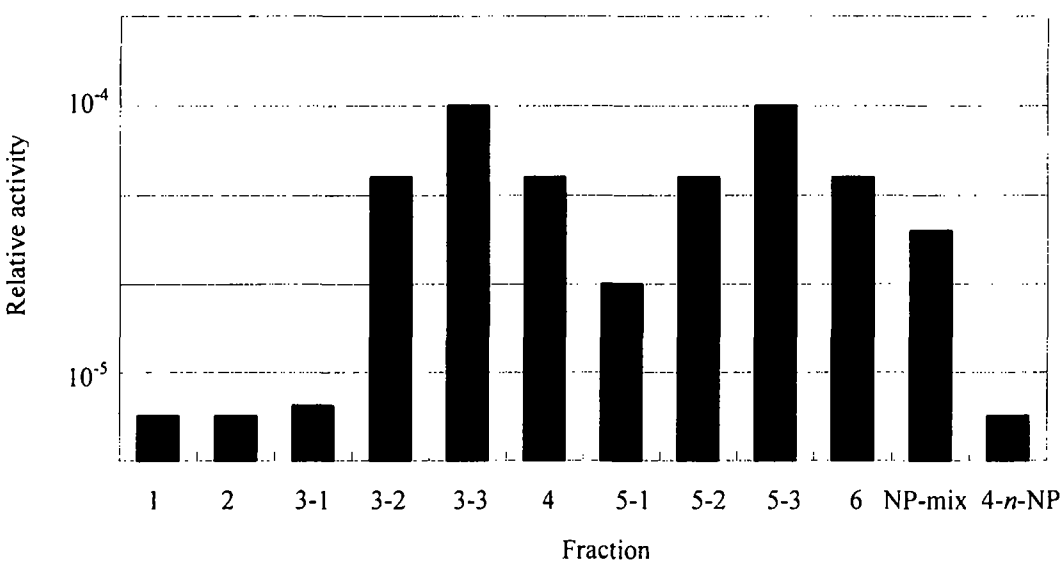

Figure 4. Relative estrogenic activities of each fractions.

Minimal concentration at which estrogenicity was detectable by the yeast screen was experimentally determined for each sample and the extent of estrogenicity of the fraction was calculated as a relative value to that of E2.