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Liquid chromatography fractionation with gas chromatography/mass spectrometry and preparative gas chromatography–nuclear magnetic resonance analysis of selected nonylphenol polyethoxylates

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ABSTRACT

Commercial nonylphenol polyethoxylates, designated as NP*n*EOs, where *n* is the number of ethoxy groups, comprise a range of ethoxylate groups. According to the starting material nonylphenol, they may also be composed of a complex mix of isomeric nonyl substituents. In order to study more fully the heterogeneity arising from both the ethoxylate and nonyl groups, a mixture of NP*n*EOs is first fractionated by normal phase liquid chromatography (NPLC) into separate fractions comprising individual ethoxymers, *n*. Preparative collection of each early elution ethoxymer fraction allows further separation of different isomeric nonyl group components by using analytical gas chromatography/mass spectrometry (GC/MS). The nonyl isomers are not resolved in the NPLC method. The distribution of the isomeric nonyl side chain of different ethoxymers bears close resemblance with each other, and also with the original nonylphenol starting material, although separation efficiency of the nonyl isomers for each ethoxymer decreases with increasing ethoxymer number. Mass spectrometry of the separated isomers display close similarity for presumed equivalent isomers in each fraction, based on elution order of the nonyl isomers. This suggests that each corresponding peak has the same isomer structure. Mass spectra are interpreted based on branching within the nonyl side chain. Preparative GC coupled with MS and nuclear magnetic resonance spectroscopy elucidated the molecular structure of one of the resolved isomers as 4-(1,3-dimethyl-1-propyl-butyl)-phenol diethoxylate.

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1. Introduction

Nonylphenol polyethoxylates (NP*n*EOs, where *n* designates the number of ethoxy units) have been extensively used for more than 50 years as non-ionic surfactants in both industrial and domestic detergents because of their effectiveness, economy and ease of handling and formulation. Commercial NP*n*EOs are manufactured by the addition of ethylene oxide to nonylphenol (NP) and the number of condensed ethoxy (EO) groups, is usually between 3 and 20. Since the starting NP material is manufactured by the alkylation of phenol with isomeric C9 species, NP*n*EOs are often a mixture of oligomers with various isomeric and branched nonyl groups.

NP*n*EOs are primarily used in aqueous solutions and are later introduced into the environment via wastewater discharge. Environmental and biological degradation of NP*n*EOs starts at the hydrophilic part of their molecules, leaving the hydrophobic part untouched, and this creates more persistent metabolites: NP, nonylphenol monoethoxylate (NP1EO), nonylphenol diethoxylate (NP2EO), nonylphenoxy acetic acid (NP1EC) and nonylphenoxy ethoxy acetic acid (NP2EC) [1]. NP*n*EOs and all these breakdown products bioaccumulate [2,3] and their toxicity has been reported [4]. The aquatic toxicity of NP*n*EOs varies with ethoxymer chain length as well as with branching of the nonyl chain [5]. However, the main environmental concern is not their toxicity but rather the estrogenic potential of the metabolites, especially NP, even at low concentrations [6–9]. It has been reported that branching (tertiary > secondary = normal) of the nonyl group affects estrogenic activity of NP [10]. Several NP isomers have been successfully synthesized or separated and their different estrogenic potencies have been evaluated [11–16]. As a result, separation and analysis of individual NP*n*EO isomers are required to assess the risk and determine the fate of these compounds in the environment.

Due to limited volatility and high polarity of higher NP*n*EO ($n > 6$) oligomers, gas chromatographic methods are less suited for the quantitative determination of commercial NP*n*EOs over their full ethoxylate range. Therefore, high performance liquid chromatography (HPLC) and supercritical fluid chromatography (SFC) have been recognised as particularly valuable methods. HPLC using a graphitic carbon column enabled the separation of NP and select NP*n*EOs into component isomers although considerable overlap still occurred

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[17]. In normal phase HPLC (NPLC) and SFC systems, individual ethoxylated oligomers are eluted in the order of increasing number of oligomeric ethoxy units, and the isomeric heterogeneity within the hydrophobic alkyl part has virtually no influence on the chromatographic process (i.e. they remain unresolved) [18–21]. Although NP*n*EOs with *n* ranging from zero (NP) to tens have been successfully separated using these technologies, they have several drawbacks, such as lower separation efficiencies compared to gas chromatography (GC) techniques and the inability to speciate the isomeric nonyl group.

Mass spectrometry (MS), hyphenated with LC or GC, has been widely employed for the identification of components' molecular structures. However, it is unable to provide sufficient information for the absolute identification of structural isomers which exhibit very similar or even identical mass spectra. In order to confirm the structures of these isomers, other spectroscopic techniques need to be applied, such as nuclear magnetic resonance (NMR) spectroscopy which requires compounds of high purity. Thus, isolation of a single isomer has to be achieved prior to NMR analysis. While on-line GC–NMR remains difficult to implement [22,23], preparative GC (prep-GC) and multidimensional GC (prep-MDGC) systems have been developed and employed to provide ^{1}H NMR samples [24–28] from mixtures of varying complexity.

Prior study has reported technical NP mixture characterisation through fraction collection using LC, synthesis of individual branched nonyl isomers, and GC/MS analysis [11–16]. Through prep-GC and fraction collection, the isomers from the technical mixture were isolated and then analyzed by using MS [29] and NMR [14]. In a later study, environmental NP was isolated from river water and analyzed by comprehensive two-dimensional GC (GC × GC) methodology [30]. Subsequent work also investigated various structural relationships of NP, and correlated the results with retention in the GC × GC space [30].

In this study, prior research on technical and environmental NP samples has been extended to their polyethoxylate analogues. Since NP*n*EOs isomers are degraded to NP isomers, and the latter have different estrogenic potencies, structural similarities of NP and their NP*n*EO progenitor are of interest. Commercial NP*n*EOs were firstly separated into individual oligomers by NPLC. Collected individual fractions of lower NP*n*EOs ($n < 7$) oligomers were further resolved by GC into their alkylated isomers, which allowed comparison of the gas chromatograms and mass spectra of different nonyl isomers within each oligomer, and with similar data for the NP isomers. One of the separated isomers was subsequently compared with MS and NMR data from the literature for NP, and its structure could be inferred. A sample of the original NP, which was used to synthesize NP*n*EOs was analyzed as a comparison, and data from prior literature [11,13–16,30–32] were used to assign individual alkyl isomer structures in various NP*n*EOs.

2. Experimental

2.1. Materials

All solvents used were of HPLC grade. Acetonitrile was purchased from Merck Pty Ltd. (Kilsyth, Australia). 1,2-Dichloroethane and 2-propanol were obtained from BDH Laboratory Supplies (Kilsyth, Australia). Commercial Teric N8, Teric N2 (NP*n*EOs with 8 and 2 ethoxy units on average respectively) and NP used to produce NP*n*EOs were supplied as gifts by Huntsman Chemical Company (Deer Park, Australia). Stock solutions of Teric N8 and NP were prepared in *n*-hexane (Ajax Finechem, Taren Point, Australia) and 1,2-dichloroethane (7:3). $(CD_3)_2CO$ (Riedel – de Haën, Seelze, Germany, degree of deuteration min. 99.5 at%) was used for NMR analysis.

2.2. Fractionation by NPLC

The NPLC fractionation system consisted of a four-channel solvent delivery system, an auto-sampler with a 10 μL sample loop, a UV–VIS detector (at 227 nm) and an auto fraction collector (all components comprised an LC-10A system, Shimadzu, Rydalmere, Australia). A Hypersil 3 μm APS-2 (NH_2) column (100 mm × 4.6 mm internal diameter (I.D.); Alltech, Grace-Davidson, Rowville, Australia) was applied for the analytical scale separation of Teric N8. Eluent composition included *n*-hexane (A), acetonitrile (B), 1,2-dichloroethane (C) and 2-propanol (D) solvents and were degassed prior to use. A gradient program was used at a flow rate of 1.0 mL/min as follows: initial condition of 63.0% A, 7.4% B and 27.0% C (held for 2 min) with 3 min gradient to 52.5% A, 18.5% B and 22.5% C, then 20 min gradient to 46.9% A, 24.4% B and 20.1% C (all eluents made to 100% with D), resulting in a run time of 25 min. The system was re-equilibrated at the commencing conditions for 15 min between runs. The first eluted four fractions were separately collected for a total of about 100 injections. Each fraction was then concentrated by evaporation under a gentle stream of nitrogen to about 5 mL.

2.3. Separation and identification by GC/MS

GC/MS experiments were performed on an HP6890 gas chromatograph with a model 7683 auto-injector connected to an HP5973 mass selective detector (all from Agilent Technologies, Nunawading, Australia). 0.2 μL of each of the concentrated fractions were injected in splitless mode at an injector temperature of 300 °C. A 30 m × 0.25 mm I.D. × 0.25 μm film thickness BPX5 column (SGE International, Ringwood, Australia) was used. For the separation of NP, the GC oven temperature program was 60 °C initial (held for 1 min), increased to 150 °C at 30 °C/min, and then increased to 260 °C at 5 °C/min. The mass spectrometer was operated in the electron ionization (EI) mode with an electron energy of 70 eV. For the fractions of Teric N8 from NPLC, the GC separation started at an initial oven temperature of 90 °C. The temperature was then increased at 30 °C/min to 180 °C, and then at 5 °C/min to 330 °C. Helium was used as carrier gas with a flow rate of 1.5 mL/min for all oven temperatures. The data were acquired in full scan mode from m/z 35 to 300 for NP and m/z 35 to 600 for NP*n*EOs respectively.

2.4. Heart-cut prep-GC

The heart-cut prep-GC separations were conducted on a 15 m × 0.32 mm I.D. × 1 μm film thickness DB5 column (Agilent). The model 6890A GC system (Agilent) was equipped with a split/splitless inlet and a flame ionisation detector (FID). A three-channel electronic pressure control module (EPC) was used to provide pressure to the Deans switch (DS). This setup provided sufficient peak separation even though relatively high amounts of sample were injected.

The Agilent G2855B micro-fluidic DS was used to isolate the peak of interest eluting from the outlet of the separation column, directing the flow to either the FID (to record the chromatogram) or the trapping capillary, via parallel 2 m × 0.18 mm I.D. deactivated fused silica (DFS) transfer lines. A length of megabore DFS tubing (trapping capillary) was connected with a press-fit (Agilent) inside the oven to collect the trapped heart-cut peak as it issues from the oven.

An Agilent model 7683 auto-injector was used for splitless sample injection of 1 μL (1 min, at 300 °C). A flow rate of 2.7 mL/min was provided using hydrogen carrier gas at a constant pressure of 80.6 kPa. At the DS, a constant pressure of 71.7 kPa was supplied by the EPC to divert the peaks of interest to the external trapping assembly (xTA). The FID detector was set to 340 °C with an acquisi-

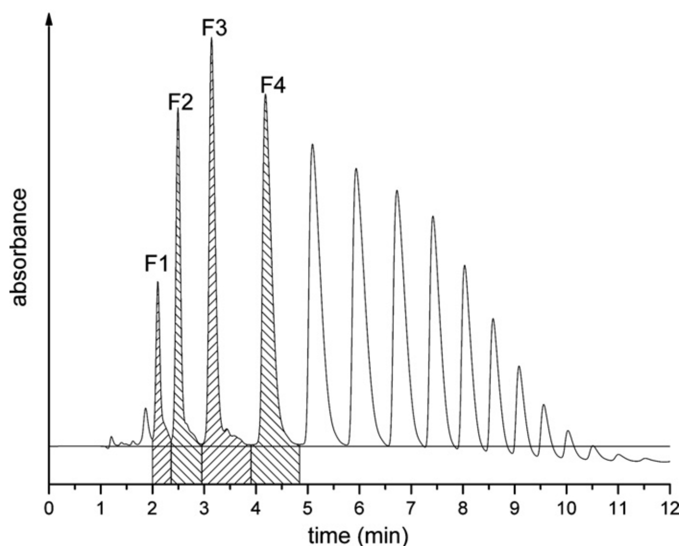

Fig. 1. Normal phase high performance liquid chromatogram of Teric N8 (NP*n*EOs with eight ethoxy units on average) with zones corresponding to fractions F1, F2, F3 and F4 selected for collection.

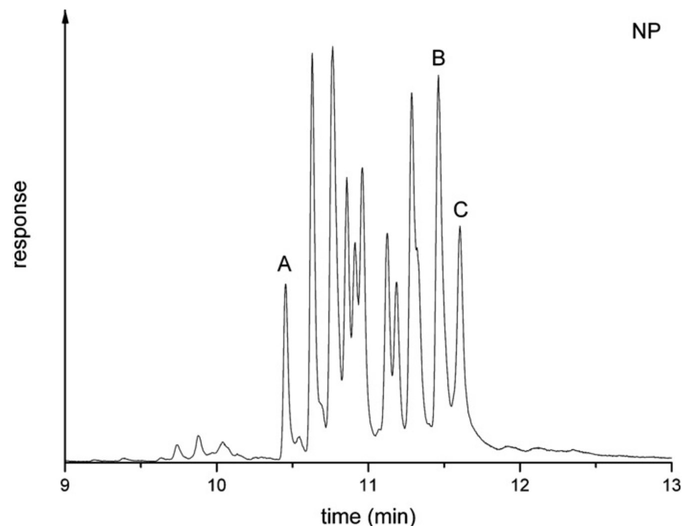

Fig. 2. GC/MS total ion chromatogram of NP. Mass spectra of marked peaks A, B and C are presented in Figs. 4–6.

tion rate of 20 Hz. Back-flushing, where carrier flow is reversed to expel late eluting components from the column back out through the injector, is achieved by reducing the inlet pressure to 1.3 kPa while the DS pressure was maintained at 121.3 kPa for 3 min. This operation can be implemented as soon as the target compound has been cut to the xTA.

The initial oven temperature was set to 40 °C (held for 1 min), ramped to 220 °C (40 °C/min), ramped to 250 °C (3 °C/min), ramped to 320 °C (40 °C/min) and held for 3 min during the back-flushing step. Forward carrier gas flow was re-established prior to the next injection.

The selected isomer was isolated from 325 injections of the Teric N2 (10 mg/mL) sample, and was eluted from the trapping capillary using $(CD_3)_2CO$ into a 3 mm thin wall NMR tube (Wilmad LabGlass, Buena, NJ) for further analysis. A 1 μL volume of this solution was injected into the GC/MS system to confirm purity.

2.5. NMR spectroscopy

NMR spectroscopy of the isolated compound was performed on a Varian Inova 500 MHz NMR spectrometer (Varian Inc., Mulgrave, Australia) using a 3 mm indirect triple resonance probe. The spectrum was referenced to the residual solvent resonances (at 2.05 ppm). The collected component was analyzed by ^{1}H NMR.

3. Results and discussion

3.1. Analysis of NP and collected fractions by GC/MS

Under NPLC conditions, all the NP*n*EO oligomers were baseline separated according to the length of their ethoxy chain within 12 min, while all nonyl isomers co-eluted (Fig. 1). Since NP*n*EO oligomers with long ethoxy chains have relatively low volatility in GC, only the selected four fractions: F1, F2, F3, and F4, as shown in Fig. 1, were collected. From these, the underlying nonyl group isomeric pattern will be identified.

In order to achieve the separation of individual isomers, NP and concentrated fractions were injected onto the GC/MS system. The GC/MS total ion chromatogram of NP and extracted ion chromatograms using the common ion *m*/*z* 45 of F1, F2, F3 and F4 are shown in Figs. 2 and 3 respectively. A group of 8–12 peaks were observed for NP and each fraction, although in reality, it is appreciated that many more isomers will or could be present. Although as the number of ethoxy units increases, the separation becomes worse due to the larger molecular size, the similarity of the chromatograms suggested that each fraction comprised a similar structural isomeric distribution. Mass spectra of all the peaks in NP and F1 obtained at the peak maxima are provided in supporting information (Figs. S1 and S2). For the NP sample, although the isomers were best separated of all the samples analyzed by GC/MS, the mass spectra differ at different elution points over some of the peaks, which indicates the presence of unresolved peaks and the complicated composition of this sample and the resultant peaks. After the application of chemometric resolution, fifteen isomers were identified in our previous study [33]. Furthermore, similar mass spectra were observed for some different peaks within the suite of peaks for a single collected ethoxylate fraction, which suggests difficulty in identification of structures of these isomers purely based on their mass spectra. For F1, coelution of peaks becomes more extensive, and the mass spectrum at any point over a peak becomes more complex due to component overlap. For F2, F3 and F4, almost all of the peaks suffer multiple component overlap. Thus, the mass spectra recorded at each peak maximum are not presented. However, as a model for discussion of the similarities of isomers across the different fractions, three peaks, marked as A, B and C in Figs. 2 and 3, remained apparently best separated of all the isomers (i.e. suffered least overlap with other peaks in NP and each of the fractions) and so they were selected to display their mass spectra for further comparison and discussion (Figs. 4–6).

The fractions F1, F2, F3 and F4 were assigned as NP3EO, NP4EO, NP5EO and NP6EO according to their molecular masses, (i.e. all compounds in fraction F1 had a molecular ion of *m*/*z* 352 and so are assigned as NP3EO) (Table 1). For each peak A, B and C for each fraction, and in NP, the mass spectrum is strikingly similar

Table 1
Mass spectrometry molecular ion and fragment ion masses for NP and NP*n*EOs.

Compound	NP	NP2EO	NP3EO	NP4EO	NP5EO	NP6EO
Molecular ion mass; $M^{+\cdot}$	220	308	352	396	440	484
$[M-15]^+$	205	293	337	381	425	469
$[M-29]^+$	191	279	323	367	411	455
$[M-43]^+$	177	265	309	353	397	441
$[M-57]^+$	163	251	295	339	383	427
$[M-71]^+$	149	237	281	325	369	413
$[M-85]^+$	135	223	267	311	355	399
$[M-99]^+$		209	253	297	341	385

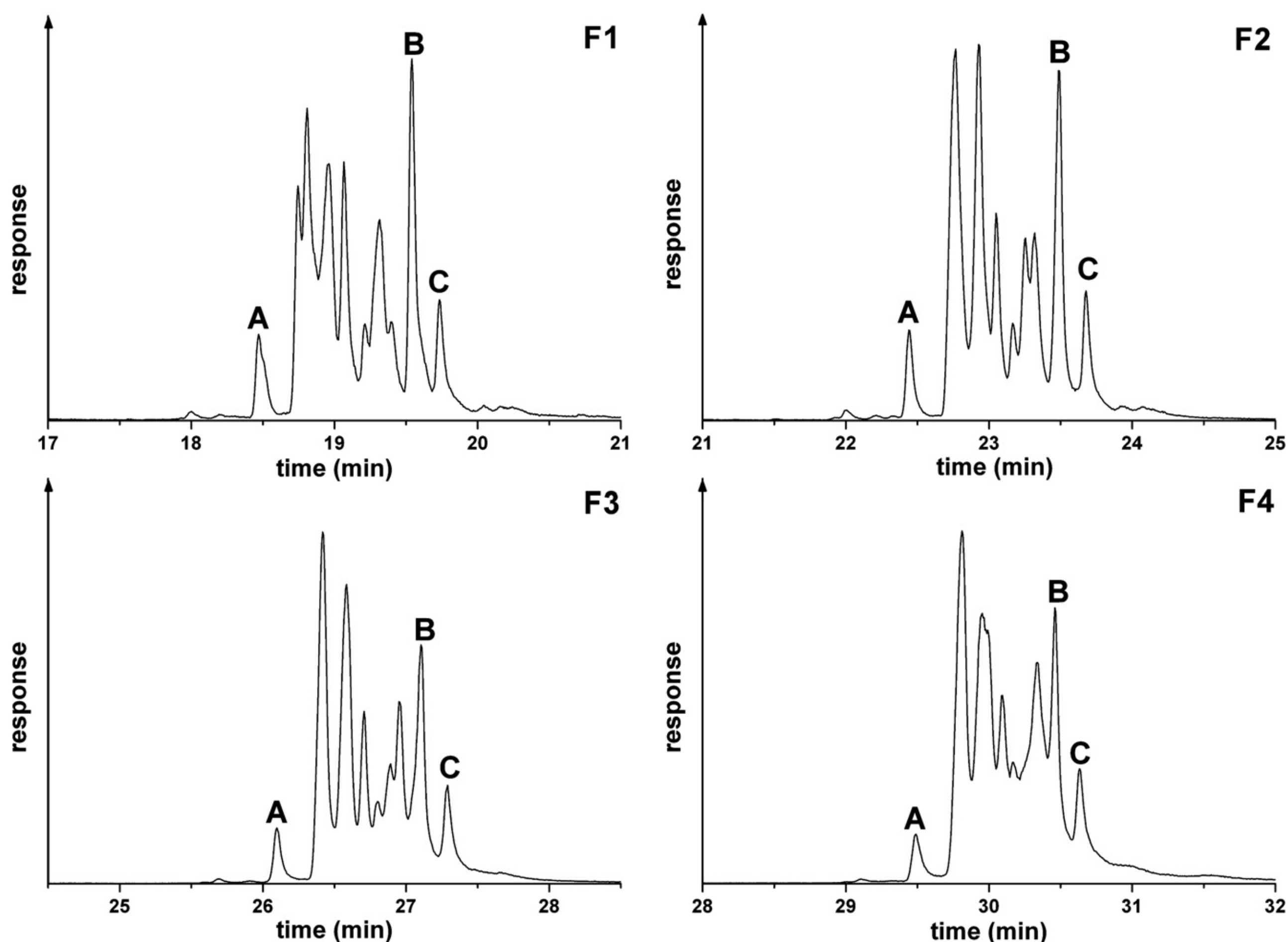

Fig. 3. Extracted ion chromatograms (*m*/*z* 45) of F1, F2, F3 and F4. Mass spectra of marked peaks A, B and C are presented in Figs. 4–6.

in respect of the fragmentation pattern – apart from the general trend in reduced abundance of the higher mass ions as the molar mass increases. Thus, this suggests that the spectrum is specific to a particular nonyl-isomer structure in the molecule, and that the relative elution order of a particular alkyl moiety in each of the fractions and in NP is relatively consistent. Each ethoxymer increases by 44 mass units, which also demonstrated the successful separation of NP*n*EO ethoxymer homologues by NPLC.

For peak A, the mass spectrum of the NP isomer shows the fragment ion of mass *m*/*z* 121 as the most abundant ion, suggesting the presence of a methyl group at the α-carbon to the phenol ring. A common abundant fragment formed by loss of a butyl radical $[M-57]^+$ is observed with NP and all NP*n*EO isomers for peak A. Another distinctive common ion which is 14 mass units higher than the major high-mass ion is also observed. It is formed by loss of a propyl radical $[M-43]^+$. All these suggest the nonyl chain to have an α-methyl and a butyl and a propyl side chains on the α-carbon, i.e. $H(OCH_2CH_2)_nO–(C_6H_4)–C(CH_3)(C_4H_9)(C_3H_7)$. At this time, the structure of the butyl and propyl side chains is not determined from the mass spectral result.

For peak B, only base peaks of *m*/*z* 135 for NP and *m*/*z* (135 + 44*n*) for NP*n*EOs are observed, without any other abundant ion peaks between the base peak and the molecular ion. Isomers with such a mass spectrum may be considered to contain an α,α-dimethyl structure [31]; again the structure of the side chain C_6H_{13} group is not evident from the MS result, but the structure is proposed as $H(OCH_2CH_2)_nO–(C_6H_4)–C(CH_3)(CH_3)(C_6H_{13})$, where $n = 0, 3, 4, 5$ or 6 for the spectra given in Fig. 5. As above the hexyl group structure is not derived from the MS, and it could be *n*-hexyl.

For peak C, all the mass spectra have fragment ions with loss of 71 u $[M-C_5H_{11}]^+$ as the most prominent peak in the high mass region of the spectrum. Loss of 85 u $[M-C_6H_{13}]^+$ led to another distinguishable ion in each spectrum. While it is not possible to propose two different C_5 and C_6 moieties on the α-carbon, there must be a distinctive fragmentation process that yields both possible losses from the molecular ion. Ions *m*/*z* 107 ($[M-C_8H_{18}]^+$) and *m*/*z* 121 ($[M-C_7H_{16}]^+$) show relative high abundance in the mass spectrum of NP for all the peaks A, B and C. However, their corresponding ions: *m*/*z* (107 + 44*n*) and *m*/*z* (121 + 44*n*) are absent in the mass spectra of peaks in F1, F2, F3 and F4. This results from the tendency towards preferential fragmentation of the ethoxy chain as the number of ethoxymer groups increases, rather than facile loss of fragments from the nonyl side chain.

Collectively these results indicate that NPLC has successfully separated the NP*n*EO oligomers and each fraction contains similar isomers to the NP starting material. However, due to the lack of structural information, identification of the single isomer structure is impossible. Therefore, prep-GC with NMR was employed as a model study of one isomer for this application.

3.2. Structural elucidation of trapped isomer by heart-cut prep-GC, GC/MS and NMR spectroscopy

Since absolute structural identification of these isomers cannot be achieved by GC/MS, heart-cut prep-GC followed by GC/MS and NMR spectroscopy was further applied. The gas chromatograms of NP2EO from Teric N2 sample before and after heart-cut by the prep-GC system are presented in Fig. 7. Teric N2 was used since it

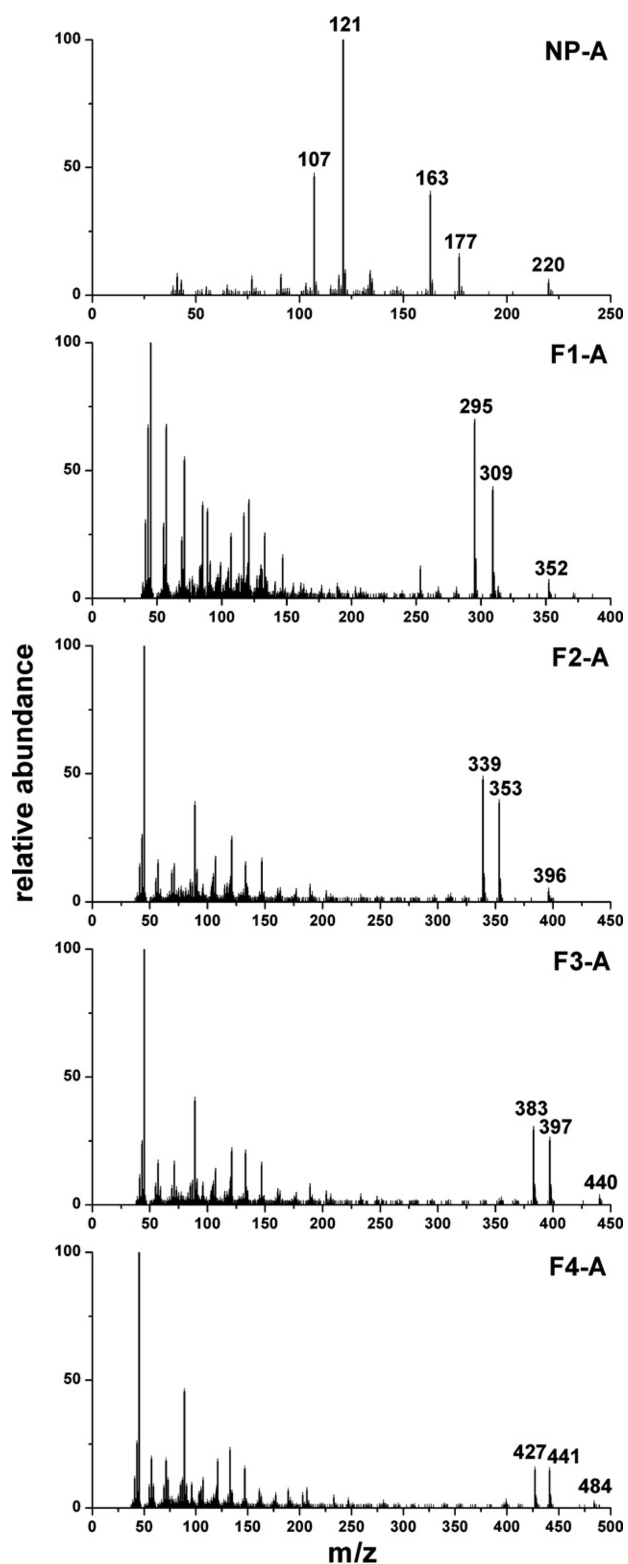

Fig. 4. Mass spectra of peak A (shown in Figs. 2 and 3) for NP, F1, F2, F3 and F4.

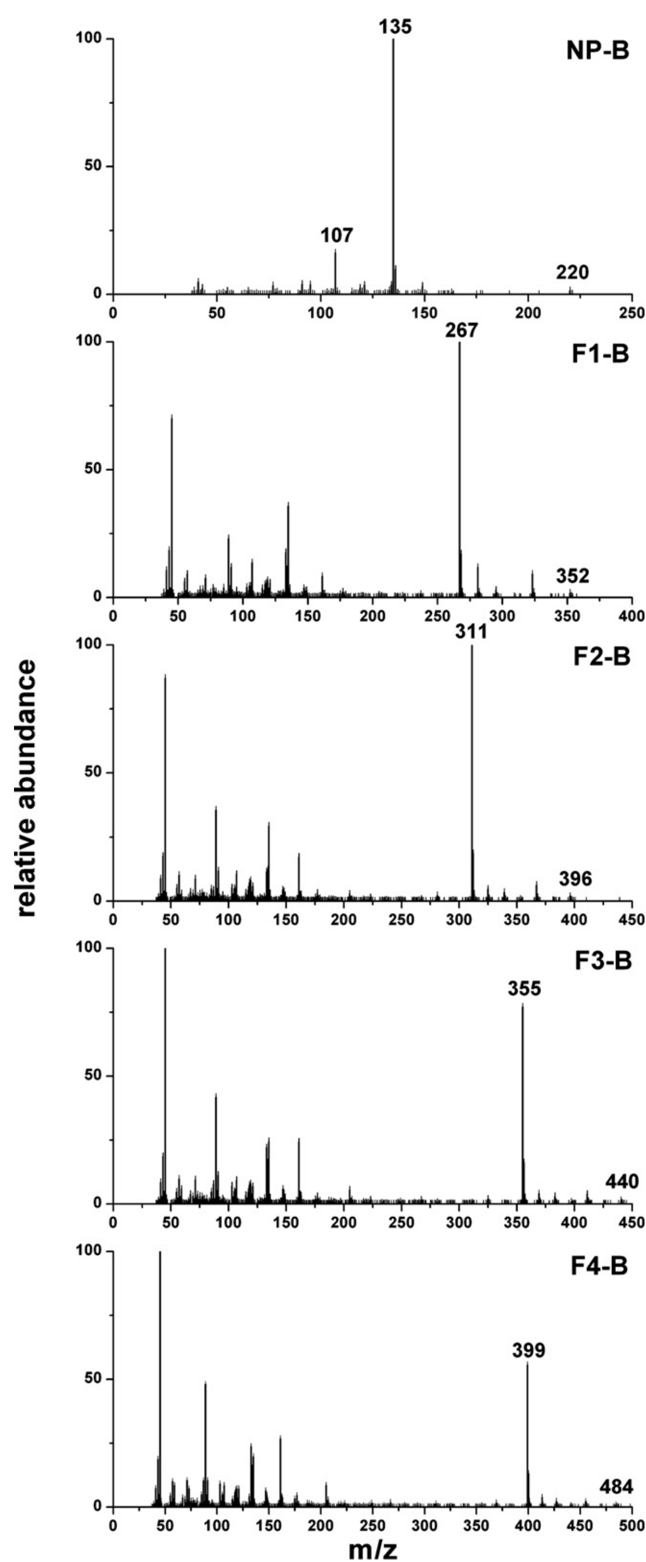

Fig. 5. Mass spectra of peak B (shown in Figs. 2 and 3) for NP, F1, F2, F3 and F4.

allows a faster turn-around of injected sample for multiple component collection, and this component (peak A) was believed to be the same as that for each collected ethoxymer fraction, so serves as a useful model. The first eluted peak was heart-cut and then trapped by the trapping capillary (the region to be heart-cut is shown in Fig. 7(a)). The chromatogram in Fig. 7(b) shows that the peak has been successfully and precisely heart-cut and sent onto the trapping capillary. GC/MS was used to confirm its purity. The chromatogram and mass spectrum of the trapped peak are shown in Fig. S3 (Supporting Information). A minor impurity has been observed (as shown in Fig. S3(a)) though it could be inconsequential for NMR of a single target compound as it is less than 5% of the main peak. For the further optimization of this method, this impurity should be ideally removed by using a narrower heart-cut

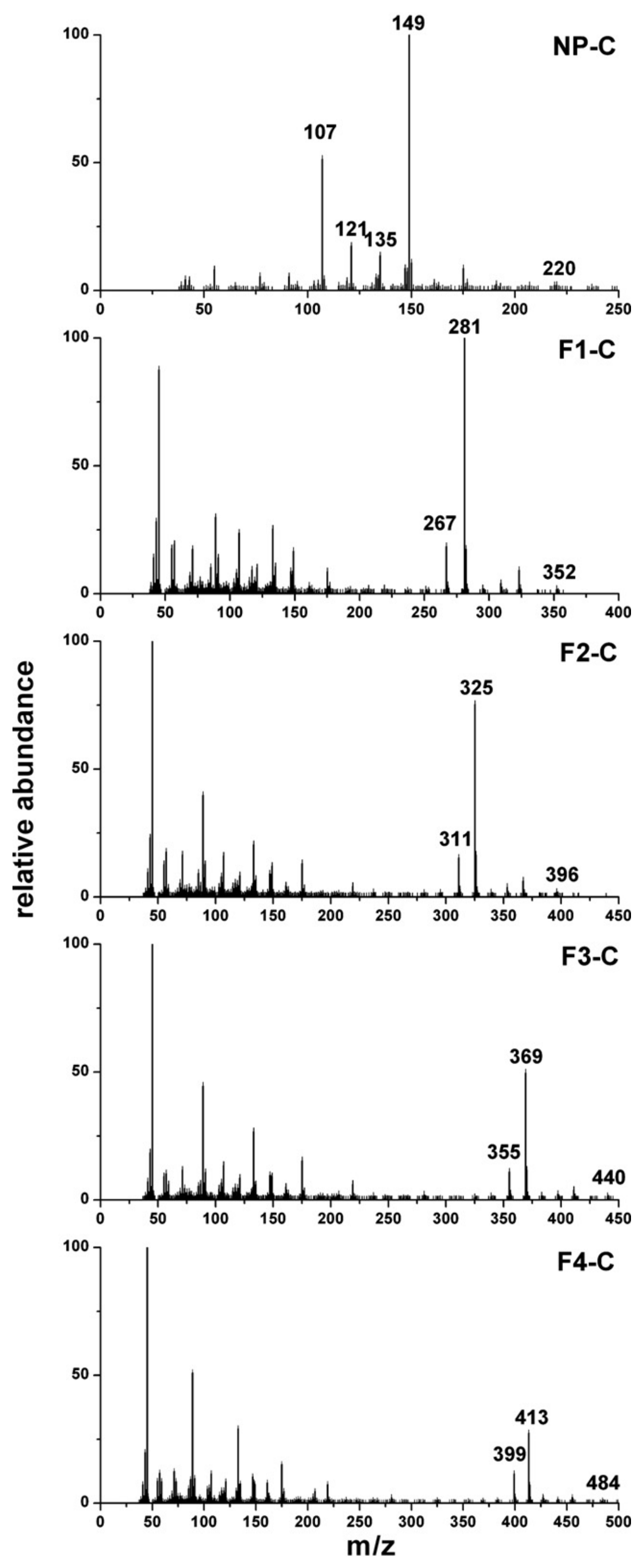

Fig. 6. Mass spectra of peak C (shown in Figs. 2 and 3) for NP, F1, F2, F3 and F4.

window. The mass spectrum of the main component indicates the presence of an NP2EO isomer according to its molecular ion *m*/*z* 308 (Fig. S3(b) and Table 1). The fragment ion *m*/*z* 251 as base peak was formed by eliminating a butyl radical while loss of a propyl radical formed ion *m*/*z* 265. Cleavage of both butyl and propyl groups lead to the occurrence of another abundant ion *m*/*z* 209. The formation of ions *m*/*z* 107 and *m*/*z* 121 also suggests an α-methyl structure.

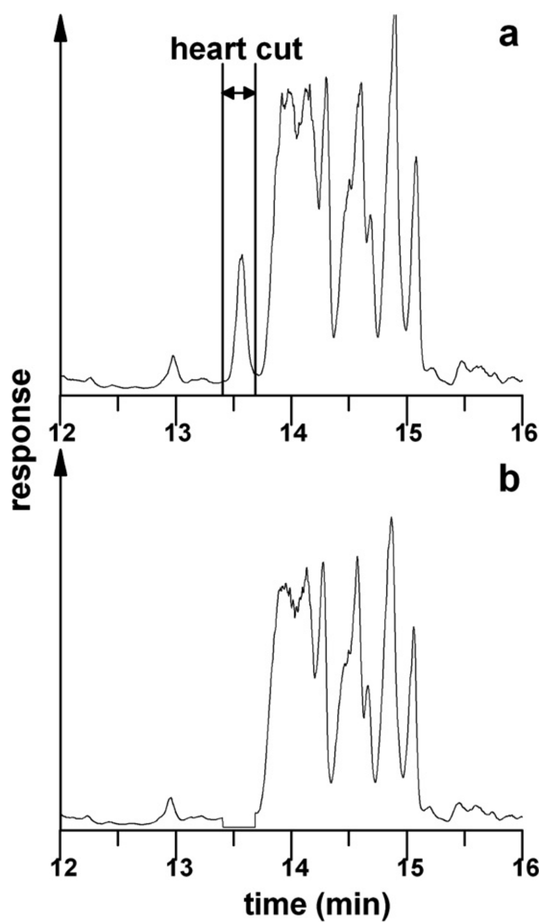

Fig. 7. Gas chromatograms of NP2EO before (a) and after (b) heart-cut of the first peak by using single-dimensional prep-GC system. Note the clean zone absent in (b) where the band has been transferred to the collection device.

All these indications confirm the same structure of the isomeric nonyl group of the trapped component (the first eluted isomer for NP2EO) with the first eluted isomer in NP and each fraction F1–F4.

The branched nonyl side chain led to a complex NMR spectrum, which makes the assignment of the signals quite difficult. Thus, the ^{1}H NMR results obtained from NP isomers with confirmed structure were used as comparison [14,15,31]. The NMR spectrum yielded two doublet peaks at 6.88 ppm and 7.25 ppm, indicating the presence of a para-substituted phenol ring. The methyl group at α-carbon gave a singlet peak at 1.30 ppm, suggesting the tertiary substitution at α-carbon. The spectrum also shows the signals due to one primary methyl group (δ = 0.81, triplet) and two secondary methyl groups (δ = 0.79, doublet; δ = 0.56, doublet). Some multiplet peaks were observed for CH_2 and CH protons in the nonyl group in the range of 0.84–1.71 ppm. The triplet peak at 4.11 ppm corresponds to the OH proton while the other four triplets in the region of 3.26–3.84 ppm correspond to the CH_2 protons in the ethoxy chain.

Correlating the present MS and NMR spectra with mass spectra and the NMR data in previous references for the same presumed NP isomer, the NP2EO isomer trapped by prep-GC was identified as 4-(1,3-dimethyl-1-propyl-butyl)-phenol diethoxylate (shown in Table 2). The estrogenic activities of the final degradation product of this isomer, has been evaluated in previous papers [14–16]. Although it did not exhibit the highest estrogenic activity among the NP isomers tested, it does have estrogenic potency. It is reasonable to analyze and elucidate the molecular structures of NP*n*EO isomers before disposing them into the environment.

Table 2
Structure and ^{1}H NMR spectra of NP2EO isomer.

NP2EO isomer	^{1}H NMR spectra
	δ: 7.25 (2H, d, H-3 and H-5), 6.88 (2H, d, H-2 and H-6), 4.11 (1H, s, OH), 3.84–3.26 (2H, t, H-16; 2H, t, H-17; 2H, t, H-18; 2H, t, H-19), 1.71-0.84 (5H, m, H-8, H-9 and H-13; 2H, m, H-14), 1.30 (3H, s, H-11), 0.81 (3H, t, H-15), 0.79 (3H, d, H-10), 0.56 (3H, d, H-12)

4. Conclusion

Commercial NP*n*EO ethoxymers have been successfully separated by NPLC. Four early-eluting oligomers (those with fewer ethoxy units) were fractionated and identified as NP3EO, NP4EO, NP5EO and NP6EO respectively by GC/MS. The GC data of these four fractions and original NP indicate a similar compositional distribution of the various isomers in each ethoxymer based on relative retention order of the isomers. Since limited information about the chemical structures of the well resolved NP*n*EO isomers have been obtained from mass spectra, absolute structural identification failed to be achieved. Thus, the first eluting NP2EO isomer was heart-cut and then trapped by using a prep-GC system. It was shown to be relatively pure by GC/MS, and its structure was then elucidated by NMR spectroscopy. This method can be applied to the problem of identification of other isolated NP and NP*n*EO isomers, although multidimensional GC methods should be employed to further enhance resolution of the nonyl isomer peaks. If sufficient pure single isomers of the ethoxylates can be isolated, the estrogenic activity of each isomer can also then be individually tested on the recombinant yeast screen system.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.04.079.

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