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Comparative qualitative analysis of nonylphenol isomers by gas chromatography–mass spectrometry combined with chemometric resolution

Ze-ying Wu, Zhong-da Zeng, Philip J. Marriott*

Australian Centre for Research on Separation Science, School of Applied Sciences, RMIT University, GPO Box 2476, Melbourne 3001, Australia

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ABSTRACT

The relationship between nonylphenol (NP) isomers' structures and their estrogenic potencies has been evaluated previously. However, due to their similarities in both chemical and physical properties, complete separation and identification remain strikingly difficult. In the present study, gas chromatography–mass spectrometry (GC–MS) is employed to separate commercial NP isomers. Both extracted ion chromatograms (EIC) based on selected ions known to be definitive for the suite of isomers, and the heuristic evolving latent projection (HELP) chemometric resolution method have been applied for the analysis and identification of the NP isomers. This method corrected the wrong identification of one isomer which was suspected based on the EIC data, and also was able to be applied for the determination of an additional isomer with low abundance. Overall, 15 NP isomers have been proposed by the HELP interpretation method. Pure component chromatograms and mass spectra have been extracted with the aid of chemometric resolution. The applicability of the commercial deconvolution software package automated mass spectral deconvolution and identification system (AMDIS) has also been tested against the HELP method for comparative presentation of pure component mass spectra.

1. Introduction

In the past decade, nonylphenols (NPs) have become of increasing concern in the environment due to their estrogen potency. NPs enter the aquatic environment mainly as degradation products of nonylphenol polyethoxylates (NPEOs), widely used as non-ionic surfactants worldwide following sewage treatment or wastewater disposal. However, NPs are more toxic and persistent than their parent NPEO compounds. Moreover, they possess the ability to disrupt the endocrine system in animals. They are capable of interacting with the estradiol receptor [1] and reducing the production of vitellogenin in male fishes [2]. Thus, they have been listed as priority hazardous compounds which need to be monitored in the aquatic environment by the European Water Framework Directive in 2000 [3].

NPs are a complex mixture of isomeric compounds comprising different isomeric nonyl side chain groups attached to the phenolic ring. Recently, it has been revealed that the estrogenic activity of NPs is related to the nonyl chain structures. Routledge and Sumpter reported that the estrogenicity of the alkylphenols depends on the number of the carbons in the alkyl group, as well as the position and the branching of the side chain [4]. Kim et al. employed several technologies including high performance liquid chromatography (HPLC) fractionation, gas chromatography–mass spectrometry (GC–MS) and GC-preparative fraction collection (PFC)–nuclear magnetic resonance (NMR) to separate and identify NP isomers: the relationship between the structures of the isomers and the estrogenic activities were evaluated by the recombinant yeast screen assay [5–7]. The role of preparative capillary GC–NMR and multidimensional GC–NMR for isolation of individual components of essential oils [8] and catalysis products with X-ray crystal structure analysis [9] have been recently reported. Several NP isomers have been synthesized and their estrogenic activities have been tested by Preuss et al. [10] and Uchiyama et al. [11,12]. However, due to the similarities of the chemical and physical properties of NP isomers, complete separation and identification of individual isomers remains very difficult. The separation of NP isomers has been improved by HPLC on a graphitic carbon column [13] and GC–MS using a 100 m Petrocol DH column [14]. It was further improved by the application of comprehensive two-dimensional GC combined with MS [15] although the similarity of boiling point and polarity of these compounds makes poor use of the 2D separation space. For the identification of the isomers, GC–MS in selected ion monitor-

* Corresponding author. Present address: Monash University, School of Chemistry, Clayton 3800, Australia. Tel.: +61 3 99054547; fax: +61 3 99058501.
E-mail address: Philip.Marriott@monash.edu (P.J. Marriott).

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ing mode [16] and GC–tandem MS combined with cluster analysis [17] have been used. However, overlapping of peaks results in pure mass spectra of individual isomers being difficult to obtain.

Chemometric-based resolution methods, coupled with hyphenated chromatography instrumentation, can successfully resolve overlapping chemical components from complex separation systems. This approach has been used to analyze volatile constituents in traditional Chinese medicines (TCMs) which are known to contain a large number of components [18–25]. Such methods provide "extracted" chromatograms and mass spectra for overlapping components. Commercial deconvolution software, such as automated mass spectral deconvolution and identification system (AMDIS) proposed by Stein [26], and the peak fitting module resident in Microcal Origin 7.5 software, which permits the extraction of individual GC traces arising from unresolved conjugated linoleic acid isomers in a successive manner by Blasko et al. [27], have been developed. The heuristic evolving latent projection (HELP) chemometric resolution method was proposed by Kvalheim and Liang to resolve two-way multicomponent data into mass spectra and chromatograms of pure chemical constituents by means of so-called full rank analysis after the determination of zero-component and selective regions of the target components [28,29]. This chemometric approach, hyphenated with GC–MS, has been employed for the determination and analysis of volatile components and essential oils in TCMs and Chinese herbs [19,22,24,25]. However, although the three kinds of isomers (α-, β- and γ-eudesmol) in houpo volatile oils have been simultaneously quantified with the help of HELP chemometric resolution [25], this method has not been applied for the complex mixture of structural isomers.

In this paper, an approach is proposed for the analysis of individual NP isomers. Commercial NPs were first separated by GC–MS. The HELP chemometric resolution method was then employed to resolve overlapped peaks and acquire pure chromatograms and mass spectra of individual isomers. The results from extracted ion chromatograms (EIC) and AMDIS methods were used as a comparison to the HELP approach.

2. Experimental

2.1. GC–MS separation of commercial NPs

Commercial NP samples were provided by Huntsman Chemical Company (Melbourne, Australia). 0.2 μL NP in acetonitrile (Merck KGaA, Germany) were injected into the GC–MS system, which consisted of an HP6890 gas chromatograph and an HP5973 mass detector (Agilent Technologies, USA). A 30 m × 0.25 mm I.D. × 0.25 μm film thickness BPX5 column (SGE International, Australia) was applied for the separation of NPs. Different temperature programs, carrier flow rates and narrower bore capillary columns have been used and evaluated in order to achieve best separation. However, incomplete resolution of components was still noted, and so final conditions chosen employed a temperature program from 60 °C, heated to 140 °C at 20 °C min^{-1}, then to 260 °C (5 °C min^{-1}). Helium was used as carrier gas at a flow rate of 1.5 mL min^{-1}. These conditions were proved to be acceptable for analysis of data by using the HELP resolution method.

2.2. HELP chemometric resolution method

Data obtained from GC–MS is a bilinear matrix. It can be expressed as the product of a chromatographic matrix **C** (dimension $m \times k$) and a spectral matrix $\mathbf{S}^{\mathrm{T}}$ (dimension $k \times n$):

$$\mathbf{X}_{m\times n} = \mathbf{C}\mathbf{S}^{\mathrm{T}} + \mathbf{E} = \sum_{i=1}^{k} \mathbf{c}_i \mathbf{s}_i^{\mathrm{T}} + \mathbf{E} \quad (1)$$

Table 1
Retention times of nonylphenol isomers identified by extracted ion chromatograms and HELP chemometric resolution method.

Extracted ion chromatogram		Chemometric resolution method	
Isomer	Retention time	Isomer	Retention time
NP1	10.434	NP-1	10.456
NP2	10.520	NP-2	10.556
NP3	10.610	NP-3	10.630
		NP-A	10.681
NP4	10.742	NP-4	10.762
NP5	10.765	NP-5	10.774
NP6	10.842	NP-6	10.858
NP7	10.894	NP-7	10.916
NP8	10.945	NP-8	10.961
NP9	11.109	NP-9	11.128
NP10	11.167	NP-10	11.186
NP11	11.270	NP-11	11.286
NP12	11.305	NP-12	11.331
NP13	11.440	NP-13	11.462
NP14	Wrong identification		
NP15	11.585	NP-15	11.604

The matrix **C** consists of k column vectors, each one representing the chromatographic profile of a pure chemical species. While the matrix **S** is composed of n row vectors of the corresponding spectral profiles of each of the pure chemical components. $\mathbf{S}^{\mathrm{T}}$ is the transform of matrix **S**. The matrix **E** contains instrumental and experimental noise. Thus, $\mathbf{X}_{m\times n}$ expressed k components of m chromatographic scan points measures at n atomic mass units.

The HELP chemometric resolution method used in the present study is primarily based on four elements: (1) the use of zero-component regions to establish the detection limit for the chemical species, and then simulate the data background for subtraction, (2) the use of latent-projective graphs (datascope) and rank map on the basis of the eigenstructure tracking analysis to reveal selective (one-component) chromatographic and/or mass spectral regions, (3) local rank analysis to check the selectivity of the regions found by visual detection and (4) the use of selective and zero-concentration regions for unique resolution into mass spectra and gas chromatograms of the pure chemical components. HELP works in a stepwise inductive manner to resolve GC–MS two-dimensional data into pure chromatograms and spectra.

2.3. Data analysis

Raw data were converted to a two-dimensional data matrix with GC as first dimension and mass spectra as second dimension, based on Agilent Chemstation data. Data resolution was then performed according to the HELP chemometric method. Pure chromatograms and mass spectrums were provided by the method. Programs related to the chemometric resolution were coded in MATLAB version R2008b for windows. A note on terminology: "NP*X*" is used as the label for NP isomers identified through the use of EIC, while "NP-*X*" is used to label NP isomers determined by using the chemometric resolution method. The isomers are listed in increasing retention time order (Table 1).

2.4. AMDIS analysis

GC–MS data were subjected to analysis by AMDIS software (version 2.62, NIST, USA) in simple mode. For the optimization of automated deconvolution, different specifications have been used: component width = 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15; adjacent peaks subtraction = 0, 1, 2; resolution = low, medium, high; sensitivity = very low, low, medium, high, very high; shape requirements = low, medium, high.

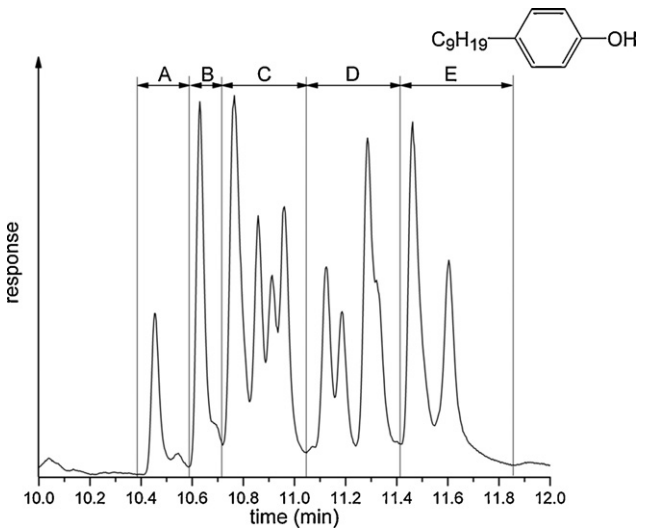

Fig. 1. Total ion chromatogram profile of commercial NP mixture by using GC–MS.

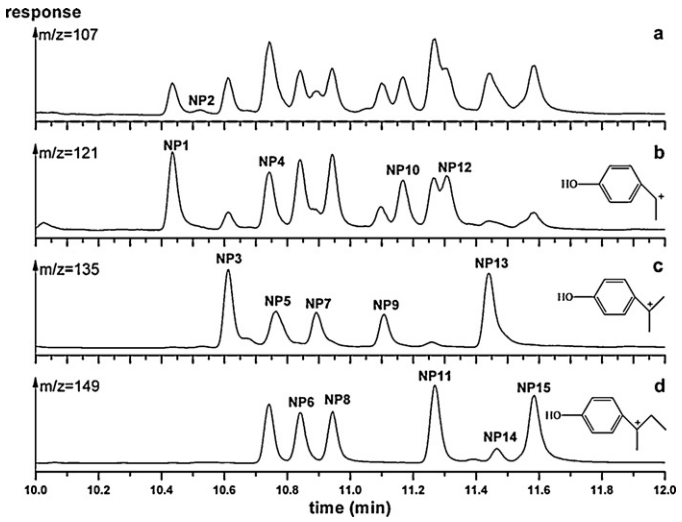

Fig. 2. Extract ion chromatograms of commercial NP mixture by using GC–MS.

3. Results and discussion

3.1. GC–MS separation of NP isomers

Fig. 1 is the total ion chromatogram (TIC) of the commercial NP sample. Since the chemical structures of the NP isomers are very similar to each other, not surprisingly many of the isomers apparently coelute (i.e. the number of isomers exceeds the peak capacity over the elution range of the components). Additionally, some peaks that seem to be of a single constituent have variable mass spectra over their elution range, which suggests that they comprise an overlapping composite peak. Such results suggest that it is problematic to precisely identify the isomers based on the mass spectra of the peaks due to poor resolution.

3.2. Extracted ion chromatograms

Ions 107 m/z, 121 m/z, 135 m/z and 149 m/z are selected to present EIC data as proposed in a prior study [16]. The fragmen-

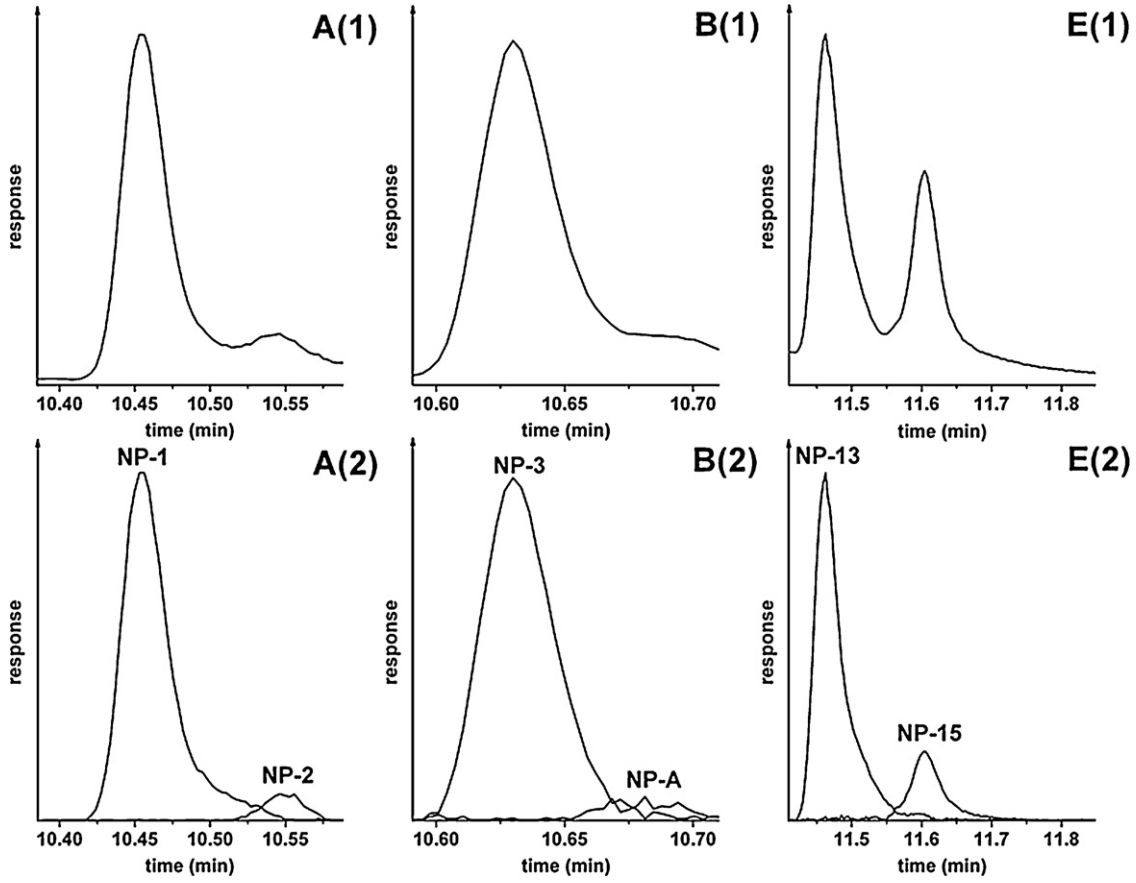

Fig. 3. The original (1) and resolved (2) peak clusters A, B and E from total ion chromatogram by using GC–MS, and the HELP chemometric resolution method.

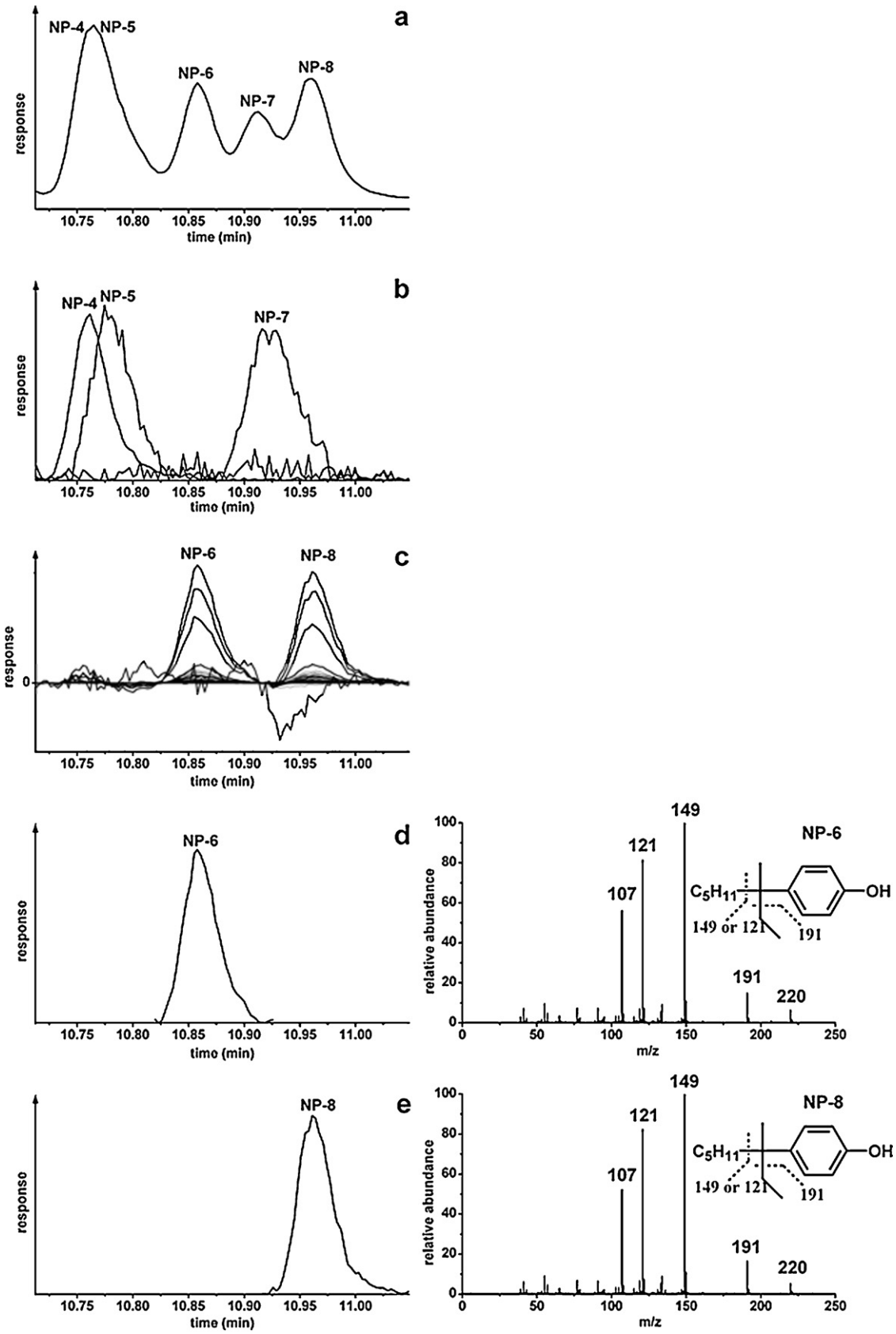

Fig. 4. The original (a), resolved (b) and residual (c) peak cluster C after one HELP chemometric analysis of the data set. Chromatograms and mass spectra of NP-6 (d) and NP-8 (e) following additional chemometric resolution.

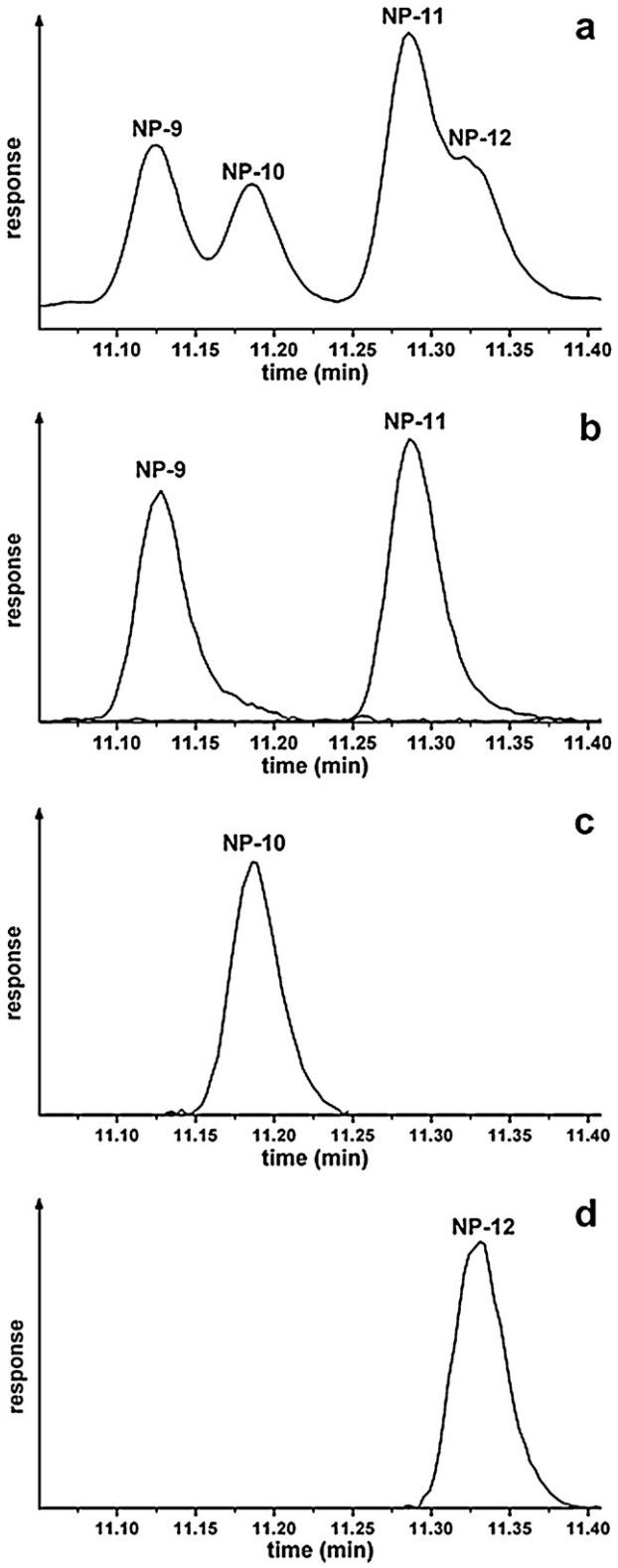

Fig. 5. The original (a) and resolved (b–d) chromatogram profiles of peak cluster D.

tation patterns of the mass spectra of NP isomers depend on the branching structure of the nonyl side chain. Benzylic cleavage provides very abundant ions at 107 m/z ($[M-C_8H_{17}]^+$) because of the charge-stabilization influence of the phenol moiety, and so this ion effectively represents a common daughter ion for all of the isomers, although it is of rather variable abundance compared with the base peak. This can be seen by the correlation of the TIC trace (Fig. 1) with the 107 m/z ion plot (Fig. 2a). However, the tropylium ion becomes more stable with alkyl groups at the α-carbon atom. Therefore, ions 121 m/z ($[M-C_7H_{15}]^+$), 135 m/z ($[M-C_6H_{13}]^+$) and 149 m/z ($[M-C_5H_{11}]^+$) resulting from single or multiple fragmentations are observed for NP isomers due to their α-methyl, α, α-dimethyl or α-ethyl-α-methyl substitution. Thus, ions 107 m/z, 121 m/z, 135 m/z and 149 m/z were selected for display of relevant EIC. The chromatogram of each selected ion is shown in Fig. 2. From the chromatograms, 15 isomers can be delineated or suspected under this mode. Each isomer is listed numerically in retention time order. The labels are applied according to the extracted ion which is most definitive for each isomer; thus as examples isomer NP1 is predominantly selected by 121 m/z, NP2 is only shown by 107 m/z, and NP3 has a dominant 135 m/z peak. The peak at about 10.75 min for 107 m/z has a broadened response indicative of an overlapping peak, and the plots of 121 m/z and 135 m/z show slightly different maxima in this region, with isomers NP4 and NP5 accorded to these respectively. While NP4 also apparently has a strong 149 m/z ion, for clarity we choose to only label the 121 m/z peak as NP4. The retention times of these isomers from EIC plots are presented in Table 1.

With the adequately optimized chromatographic separation, the pure mass spectra of NP1, NP3, NP13 and NP15 are assumed to be obtained directly from the TIC since they were apparently baseline separated without overlapping with other isomers. For NP2, which was of much lower abundance, spectral identification is difficult due to the presence of interfering background. For NP4 and NP5 isomers, these two peaks arise from at least two overlapping peaks, so obtaining their unique mass spectra using this approach may lead to erroneous results. The same applies to NP13 and NP14. For isomers NP6, NP7 and NP8, which are partially overlapping, their unique qualitative analysis and identification is difficult. Furthermore, NP11 and NP12 strongly co-eluted, and hence their pure mass spectra cannot be obtained just from spectral interpretation at a given scan number.

In conclusion, using EIC mode, it is possible to obtain a qualitative indication of the number of possible NP isomers in the commercial mixture, but their individual pure spectra are not all obtainable. In order to acquire the pure mass spectra of individual isomers, they either have to be completely separated, or an effective deconvolution method must be applied. With many overlapping isomers in this sample, complete separation was deemed to be unlikely under normal chromatographic methods.

3.3. HELP chemometric resolution method

The HELP chemometric resolution method was employed for deconvolution of all the overlapping peaks. First, since the two-dimensional data (comprising chromatographic and mass spectral information) necessarily contain response background arising from column bleed, instrumental disturbance, and other factors, signal correction was applied according to the procedure described by Liang et al. [28,29]. Second, evolving latent projective graph and rank map based on eigenstructure tracking analysis was used for the determination of number of components and acquirement of the unique information of constituents following local full rank analysis to generate the pure chromatographic profiles and mass spectra of each isomer. However, since some isomers have very similar, or even identical, mass spectra with only slight differences in ion abundance ratios, the method may still fail to extract all the respective chromatograms and mass spectra. As a strategy to simplify the data set, the total ion chromatogram was thus sectioned into five clusters based on retention time regions: A: 10.385–10.588 min, B: 10.591–10.710 min, C: 10.713–11.048 min, D: 11.051–11.408 min and E: 11.411–11.848 min. These regions are shown in Fig. 1.

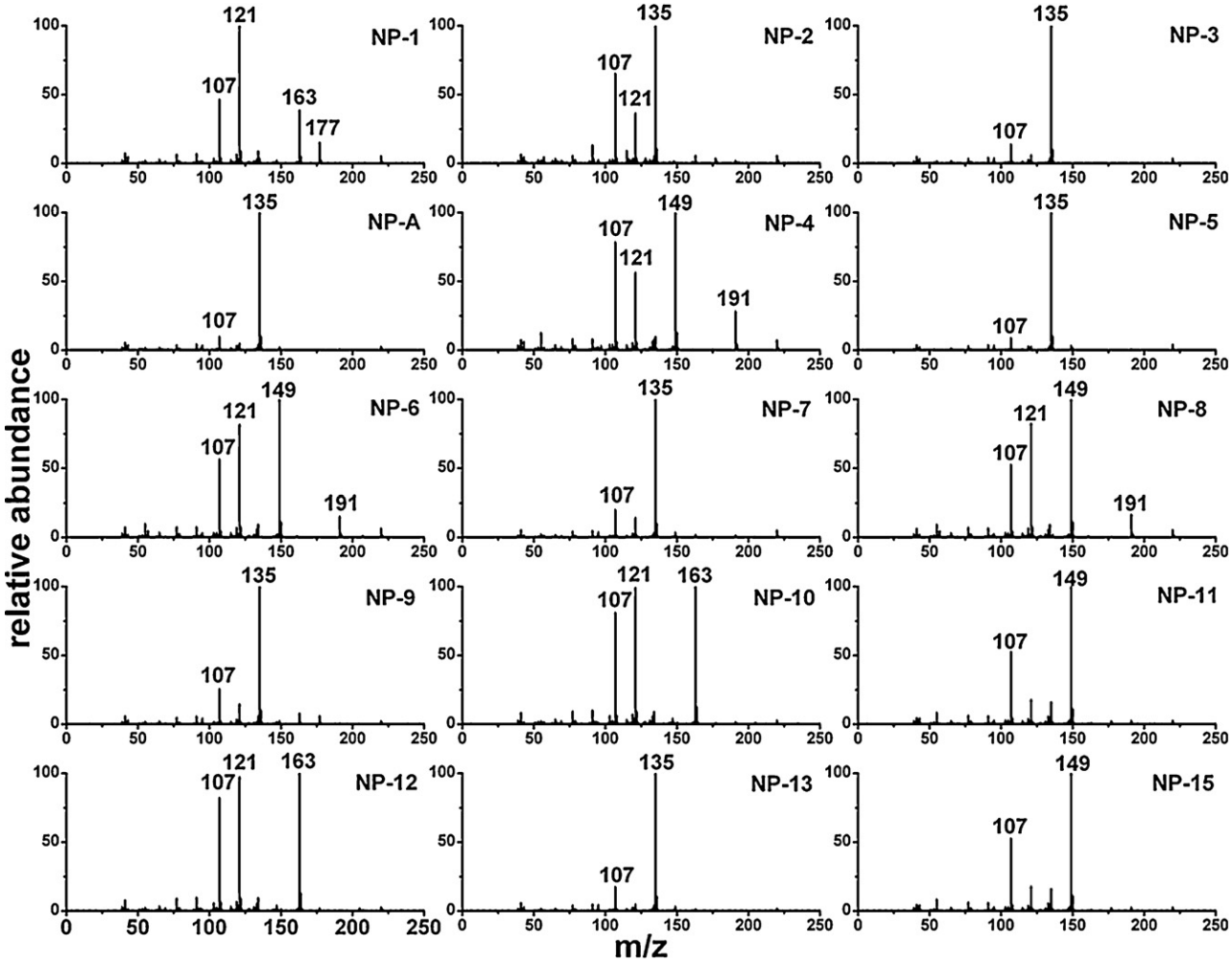

Fig. 6. Mass spectra of all the isomers determined by HELP chemometric resolution method obtained at peak maxima.

The original and resolved peak clusters A, B and E are shown in Fig. 3, where A(1), B(1) and E(1) are the original TIC traces, and A(2), B(2) and E(2) their HELP resolved analogues. The pure mass spectra arising from the HELP method could also be generated from the chemometric approach. One additional isomer is labelled NP-A because it is found by using the HELP chemometric resolution method in peak cluster B, but it was not evident in the original EIC data. As the background had been removed, the pure mass spectra could be gained for NP-2 and NP-A using the chemometric approach, even though they were both at low concentration. For NP14 in the EIC, insufficient information could be gleaned from the HELP method to identify this as a NP isomer because only one extracted ion at 149 m/z was found corresponding to this compound, and for the NP compounds, other ions are expected in their mass spectra, such as 107 m/z and the (usually very low abundance) molecular ion 220 m/z.

For peak cluster C, after HELP resolution, three isomers are observed in this chromatographic region, illustrated in Fig. 4b. After the subtraction of all the ions corresponding to these three isomers, the residual shows the presence of another two isomers (as indicated in Fig. 4c). Each line in the residual (Fig. 4c) represents one ion mass corresponding to a certain component. HELP chemometric resolution could not simultaneously provide pure chromatograms nor mass spectra of these two isomers since it was assumed that their mass spectra were nearly the same. In order to extract the pure chromatograms and mass spectra of these two isomers, ion 135 m/z which represents a strong negative signal and does not correspond to either of these two isomers was first subtracted. The residual was then isolated into two segments with only one isomer in each segment and again analyzed by HELP chemometric resolution. Pure chromatograms and mass spectra of both isomers are presented in Fig. 4d and 4e respectively, which illustrate the similarity between the structures of these two isomers. Note that the profile in Fig. 4a has now been effectively deconvoluted into its five constituent components marked as NP-4 to NP-8 respectively.

The same problem also arises for resolution of peak cluster D. Only two isomers were resolved after the initial chemometric analysis, while the other two isomers remained unresolved and are incorporated in the data residual. Further analysis was performed on the residual according to the process described before. The original and resolved chromatograms of the four isomers in peak cluster D are shown in Fig. 5. Again, the resolved components in Fig. 5b–d can be seen to derive from the unresolved GC-TIC result given in Fig. 5a.

This is the first time that the HELP chemometric resolution method has been successfully applied for the analysis of a complex mixture of isomers. Due to the high similarity of the isomers' mass spectra, the resolution of the whole GC–MS data profile cannot be achieved by a single analysis step. As a result, the original chromatogram, as well as the resolution residual of peak clusters C and D after first extraction, was further partitioned into several segments during the optimization process in order to extract pure chromatograms and mass spectra of each isomer. Overall, 15 NP isomers were determined by HELP chemometric resolution method and

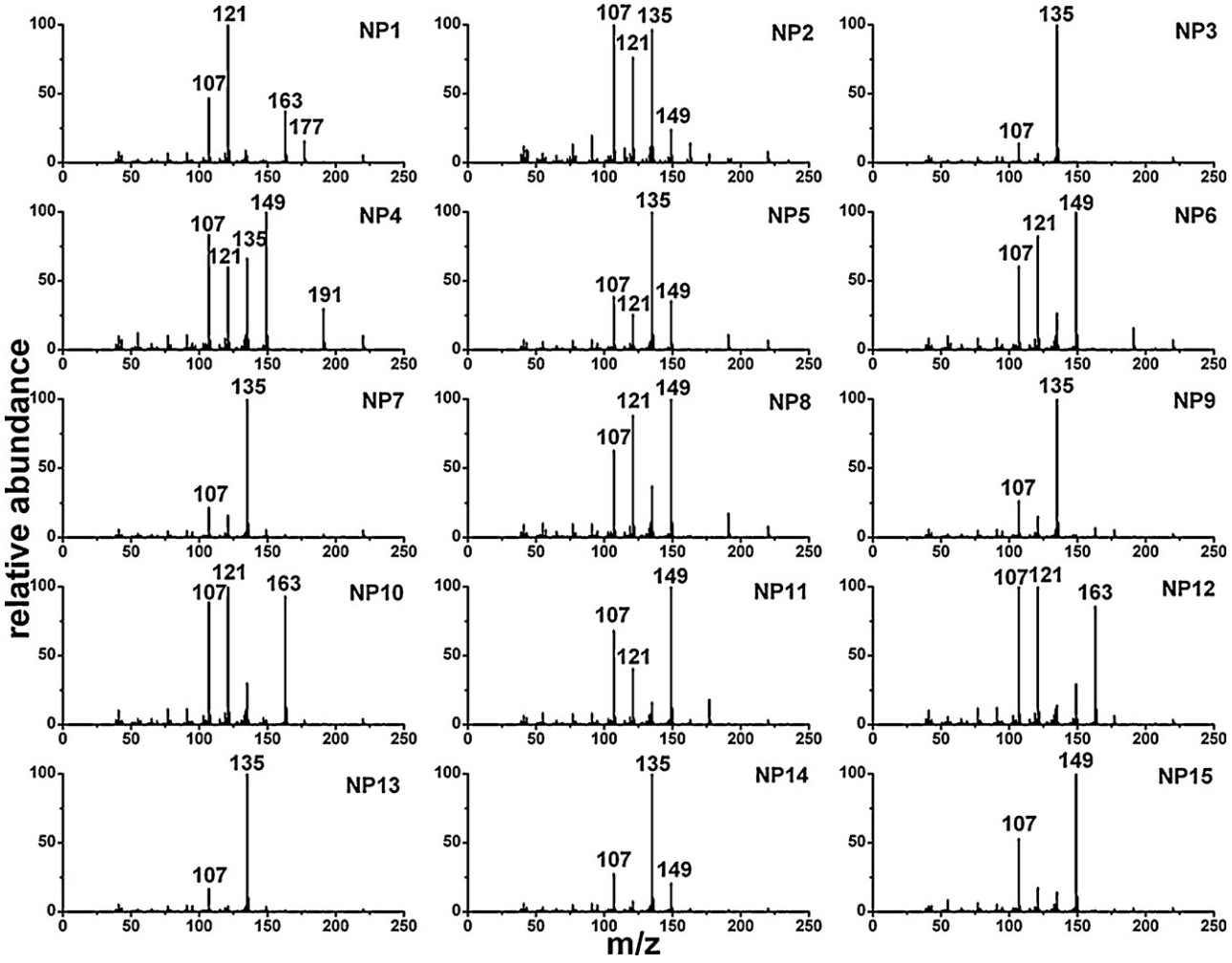

Fig. 7. Mass spectra of all the peaks in EIC obtained at peak maxima.

labelled according to their retention times (as shown in Table 1). Table 1 presents the retention times of the NP*X* components using peak maxima from the EIC data, and also NP-*X* retentions based on maxima predicted from the chemometric approach. A slight difference between the retention times obtained from EIC and HELP method is observed for each isomer. The obtainment of retention times from the EIC profile only employs the information from a single-ion and possibly incompletely resolved one-dimensional chromatogram, while the resolved chromatographic profile of pure components using HELP wholly considers two-dimensional GC–MS data, including all the chromatograms of every ion. Thus, the change of mass spectra of every single component at different elution points may have an effect to the shape of the final resolved chromatogram, which results in the change of retention times.

Unfortunately, the commercial library does not separately list individual NP isomer structures, but rather groups them generically as being a 'nonylphenol isomers'. Proposed HELP-derived mass spectra therefore cannot be matched against any library entry to provide a match statistic under a library searching routine.

3.4. Comparison of EIC and HELP chemometric resolution

Both EIC plots and HELP chemometric methods have proposed the presence of 15 NP isomers in the commercial mixture. However, the selected EIC plots identified the presence of various isomers only according to the most abundant ion in each isomer. Since many isomers overlap, the pure mass spectra cannot be reliably obtained with this method. One isomer was identified wrongly using this approach because only one single ion was represented in the pure mass spectrum of this compound, according to the results from chemometric resolution analysis. Furthermore, through chemometric resolution it was determined that one more isomer was present, but of very low abundance, whereas the EIC plot failed to identify its presence. HELP chemometric resolution provided not only the pure chromatograms but also the respective mass spectra of every isomer identified. Fig. 6 presents the mass spectra obtained by chemometric resolution for all isomers identified. The mass spectra of all the isomers determined by EIC method are also provided for comparative purposes (Fig. 7).

In cases where single isomers are known to be present in a chromatographic peak, it is possible to isolate the isomer using GC-PFC technology, as previously reported. The estrogenic activities of the individual isomers can then be tested and their structures can be confirmed with NMR analysis. With the pure mass spectrum of each isomer obtained, comparison with the mass spectra of synthesized isomers and isomers whose structures have been confirmed [14,15] is relatively straightforward. Although some of the isomers have similar or identical mass spectra, their structures have been reported to be correlated by using predicted retention indices [3]. Since the estrogenic activities of the NP isomers depend on the structure of the nonyl side chain, GC–MS interpreted through chemometric resolution could be employed to analyze the commercial NPs, in order to predict its potential estrogenic risk when discharged to waste water.

3.5. AMDIS analysis

The original GC–MS data were analyzed automatically by AMDIS software with different parameter settings. With all the specifications taken into account, deconvolution involving a total of 2025 interactions were performed. As the component width and adjacent peaks subtraction values increase, or higher resolution and sensitivity and lower peak shape requirements are used, an increasing number of hits for possible component structure could be offered by the software. The number of target components proposed by AMDIS with different parameters varies from zero to hundreds. However, due to the absence of standard mass spectra of individual nonylphenol isomers in the NIST library, no match factor could be obtained. Without any spectrum match factor, the possible hits cannot be evaluated as true positive or false negative results. Thus, none of the nonylphenol isomers could be identified. Overall, the AMDIS method for automatic resolution was unable to provide accurate and precise interpretation of the present sample as the described study outlines.

4. Conclusion

Fifteen NP isomers were identified in the commercial NP mixture by using the HELP chemometric resolution method, based upon GC–MS data. Pure chromatograms of individual isomers and their respective mass spectra for all the detected isomers, were obtained for the one analysis, which shows the advantage of HELP chemometric resolution method against the EIC and AMDIS methods. Mass spectra combined with retention indices could provide the identification of the structures of the isomers. Further confirmation of structural assignment could be achieved by the application of GC-PFC–NMR technology.

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