Isomer-specific Determination of 4-nonylphenols using Comprehensive Two-dimensional Gas Chromatography/Time-of-Flight Mass Spectrometry

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Supporting Information: 33 pages, 9 figures, 2 tables

Supporting Information

1. Acylation of tNP (Fluka)

A stock solution of tNP Fluka (~200 ng/μL in isooctane) and the acetic anhydride and pyridine reagents, all maintained in a freezer, were allowed to warm in the dark. Once they had reached room temperature, 500 μL of the tNP solution was transferred into a conical reaction vial. The solvent was evaporated under a stream of dry nitrogen gas after which 100 μL of pyridine and 100 μL of acetic anhydride were transferred into the vial. The vial was then capped under nitrogen gas flow. The contents were gently swirled to ensure that the walls of the vial were contacted by the solvent/reagent mixture; the mixture was then agitated in a sonicator for about one minute to thoroughly mix the contents. The vial was placed in a cabinet in the dark overnight. The next day, 250 μL of 3N HCL and 250 μL of hexane were added to the vial. The vial was shaken vigorously, and aqueous and solvent phases were allowed to separate in the dark. The hexane layer was then transferred to a ½-dram vial. The extraction/transfer procedure was repeated twice more after which an aliquot of the hexane extract (*e.g*. 50 μL), containing the tNP acetates, was transferred to an autosampler vial and diluted with an appropriate volume of isooctane.

Note: Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

2. Structures and names of 4-NP isomers investigated in this study

Structures and names of the 4-NP isomers investigated in this study are given in the Table S1.

Table S1. Structures[a], short name (*1*), and IUPAC (International Union of Pure and Applied Chemistry) names of 4-NP isomers.

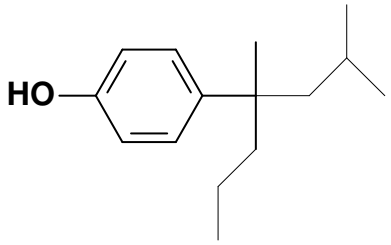
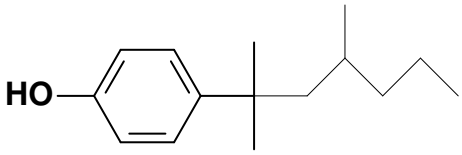
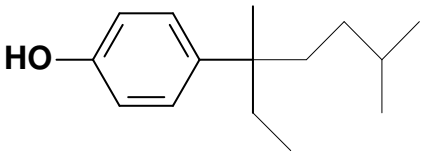
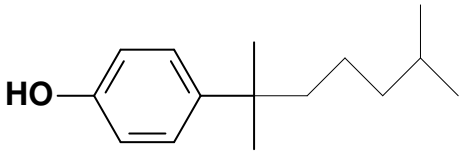
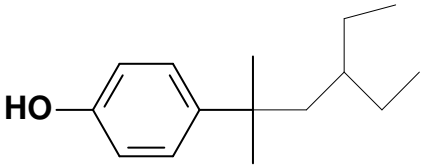
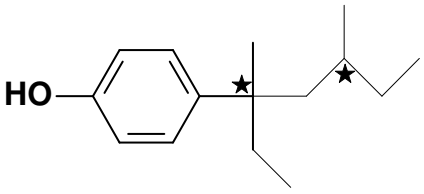
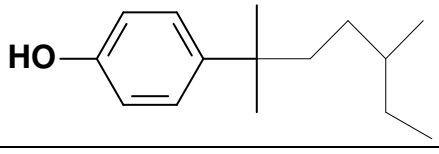
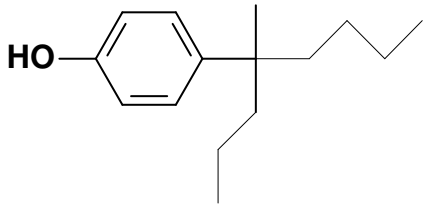
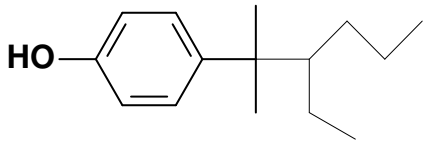
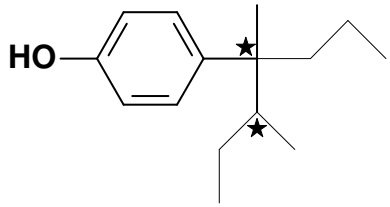
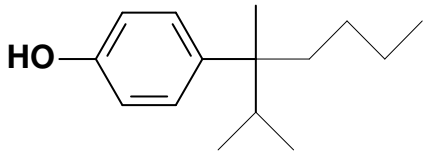
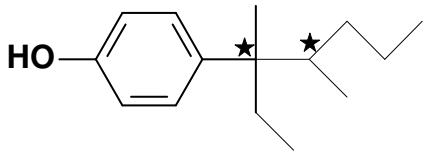
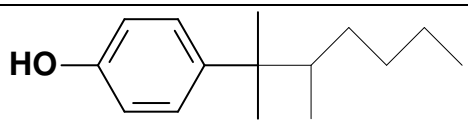
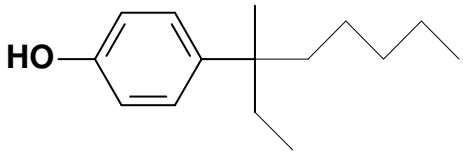
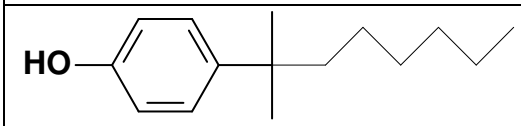
Structure	Short name	IUPAC name
	4-NP_{194}	4-[1,3-dimethyl-1-n-propylbutyl]phenol
	4-NP_{36}	4-[1,1,3-trimethylhexyl]phenol
	4-NP_{112}	4-[1-ethyl-1,4-dimethylpentyll]phenol
	4-NP_{38}	4-[1,1,5-trimethylhexyl]phenol
	4-NP_{128}	4-[3-ethyl-1,1-dimethylpentyl]phenol
	$4\text{-NP}_{111a,b}$	4-[1-ethyl-1,3-dimethylpentyl]phenol
	4-NP_{37}	4-[1,1,5-triimethylhexyl]phenol

Table S1. Continued.

	4-NP_{152}	4-[1-methyl-1-n-propylpentyl]phenol
	4-NP_{119}	4-[2-ethyl-1,1-dimethylpentyl]phenol
	4-$NP_{193a,b}$	4-[1,2-dimethyl-1-n-propylbutyl]phenol
	4-NP_{143}	4-[1-isopropyl-1-methylpentyl]phenol
	4-$NP_{110a,b}$	4-[1-ethyl-1,2-dimethylpentyl]phenol
	4-NP_{35}	4-[1,1,2-trimethylhexyl]phenol
	4-NP_{65}	4-[1-ethyl-1-methylhexyl]phenol
	4-NP_9	4-[1,1-dimethylheptyl]phenol

[a] Stars indicate location of asymmetric carbon atoms.

3. Extraction and chromatographic cleanup of environmental sample extracts

The ground-water sample collected (in 2006) from the Cape Cod, MA Toxics Substances Hydrology Program research site was acidified to pH < 2 and extracted using dichloromethane by continuous liquid-liquid extraction (*2*). The extract was subsequently purified by column chromatography using a modification of the method of Isobe *et al*. (*3*) (see below). The NP-bearing fraction was evaporated to dryness and taken up in 150 μL of the internal standard solution (4-*n*-NP in isooctane @ 20.1 ng/ μL) just prior to GC x GC/ToFMS analysis.

The biodegradation experiment extracts (day 0, day 9) described by Gabriel *et al*. (*4*) were amended with 4-*t*-OP as internal standard at the time of extraction with dichloromethane. Just prior to GC x GC/ToFMS analysis, aliquots of each extract (15μL, and 70 μL, respectively) were removed and combined with an appropriate volume of internal standard solution (4-*n*-NP in isooctane @ 20.1 ng/ μL; 310 and 240 μL, respectively).

The JWPCP unfiltered final wastewater effluent sample was collected on 8/15/79 as a flow-proportioned 24-hour composite. After adjusting its pH to 1, the effluent sample was extracted with chloroform. The extract was concentrated by rotary evaporation and treated for removal of elemental sulfur and water (*5*) after which it was transesterified using BF_3:MeOH. NPs were enriched by column chromatography using a modification of the method of Isobe *et al*. (*3*) (see below). The NP-bearing fraction was then evaporated to dryness under a stream of nitrogen gas and taken up in an internal standard solution (4-*n*-NP in isooctane @ 20.1 ng/μL) just prior to GC x GC/ToFMS analysis.

As described above, the JWPCP wastewater effluent and Cape Cod ground-water extracts were subjected to column chromatography to obtain a more purified fraction containing the 4-NPs. The procedure was a modification of that described by Isobe *et al*. (*3*). For the JWPCP

wastewater sample, gravimetric analysis was performed on the extract in order to determine the volume containing 10 mg of extractable organic matter. This volume was taken for column chromatography. In the case of the Cape Cod ground-water sample, the entire extract was used without gravimetric analysis.

Two-hundred grams of solvent-extracted silica gel (70-230 mesh, silica gel 60) was activated at 200 ℃ for 6 hours. After cooling to room temperature, the silica gel was deactivated with pre-extracted Milli-Q water (5% w/w), shaken vigorously, and allowed to equilibrate in a sealed flask in the dark overnight. The silica gel was covered with dry hexane and wet packed in a 1-cm id glass chromatography column to a height of 7.4 cm after which the sample extract, previously transferred to 250 μL of hexane, was applied to the head of the column. The fractions were eluted as follows: 1) 30 mL of 25% dichloromethane (DCM)/75% hexane (v/v), 2) 20 mL of 40% DCM/60% hexane (v/v), 3) 60 mL 65% DCM/35% hexane (v/v), and 4) 60 mL 80% DCM/20% hexane (v/v). Fractions 1, 2 and 4 were set aside. Fraction 3, containing the nonylphenols, was concentrated by rotary evaporation and transferred to a ½-dram autosampler vial using dichloromethane. The samples were then stored at -20 ℃ pending final processing just prior to GC x GC/ToFMS analysis (see above).

4. Analysis of Environmental Samples using GC x GC/ToFMS

Figure S1 shows GC x GC/ToFMS chromatograms of the Cape Cod ground-water sample and tNP (Fluka). 4-NPs are present in ground water at this site because of the infiltration of treated wastewater from the Massachusetts Military Reservation, which took place from 1936 to 1995. These wastewaters contained nonylphenol polyethoxylate (NPEO) surfactants (*6, 7*) from which the 4-NPs were formed by cleavage of the ethoxylate chain, presumably under anoxic conditions. The composition of 4-NPs in the ground-water sample appears very similar to the tNP (Fluka)

sample. Assuming that tNP (Fluka) is representative of the original NP composition of the NPEOs in the wastewater, it would appear that little, if any, isomer-specific degradation of the 4-NPs occurred prior to, during, or after discharge to the aquifer.

Despite efforts to clean up this ground-water extract using column chromatography, a significant number of co-extractants were observed in the chromatogram. As shown in the full chromatogram in Figure S1, co-extractants eluting within the same 1st dimension retention-time range as the 4-NPs in this sample would not be expected to cause significant interferences by GC/MS if fragment ions resulting from cleavage at the α-carbon were used for quantitation (*viz.* 135, 149, 163, *etc*….). Nevertheless, the GC x GC separation of the 4-NPs from other co-extractants is complete. One limitation of the GC x GC analysis for the 4-NPs with this particular column set is that the analysis time is quite long (about 3 hrs to the last eluting component). This is due to the relatively low upper temperature limit of the Supelcowax 10 stationary phase (280 °C) and the need to conduct analyses under isothermal conditions during elution of the 4-NPs to obtain optimal separation of these isomers. Given these limitations, further research could be directed at identifying suitable narrow bore secondary columns having stationary phases with higher upper temperature limits. Alternatively, methods of extraction/cleanup that would avoid or remove unwanted high molecular weight components could be used.

Figure S2 presents summed ion chromatograms of the nonylphenol-bearing fraction isolated from the JWPCP wastewater effluent sample collected on 8/15/79 along with analytical results for the eight 4-NP isomers. As evidenced by the many peak markers in the chromatogram, this fraction contains a very complex mixture of components that elute (in the 1st dimension) before, during, and after the 4-NPs. However, the chromatographic separation of the 4-NPs from other

co-extractants appears complete in the 2nd dimension. Some of these co-extractants have mass spectra containing ions that could potentially be used for quantitation in GC/MS. Consequently, the chromatographic separation of the 4-NPs by GC x GC makes their interference-free determination possible. Concentrations of the 4-NP isomers ranged from 1.0 to 4.0 μg/L and their relative abundance approximated that found in tNP ($Fluka_{WG}$; Figure S3). Based on the percentage that each of the isomers comprised in tNP ($Fluka_{WG}$), tNP concentrations in the wastewater sample were estimated at 41 to 100 μg/L (55.8 ± 19.6; mean 1 ± std dev). These concentrations are reasonable for wastewater effluent receiving primary treatment with contributions from anaerobic sludge digestion centrate during the late 1970s/early 1980s (*8-10*).

Figure S1. Summed ion chromatograms (m/z = 107+121+135+149+163+177+191) of a ground-water sample from Cape Cod, MA (a,b) and tNP (Fluka; c) showing complete 2D chromatogram (a) and 4-NP elution region (b,c).

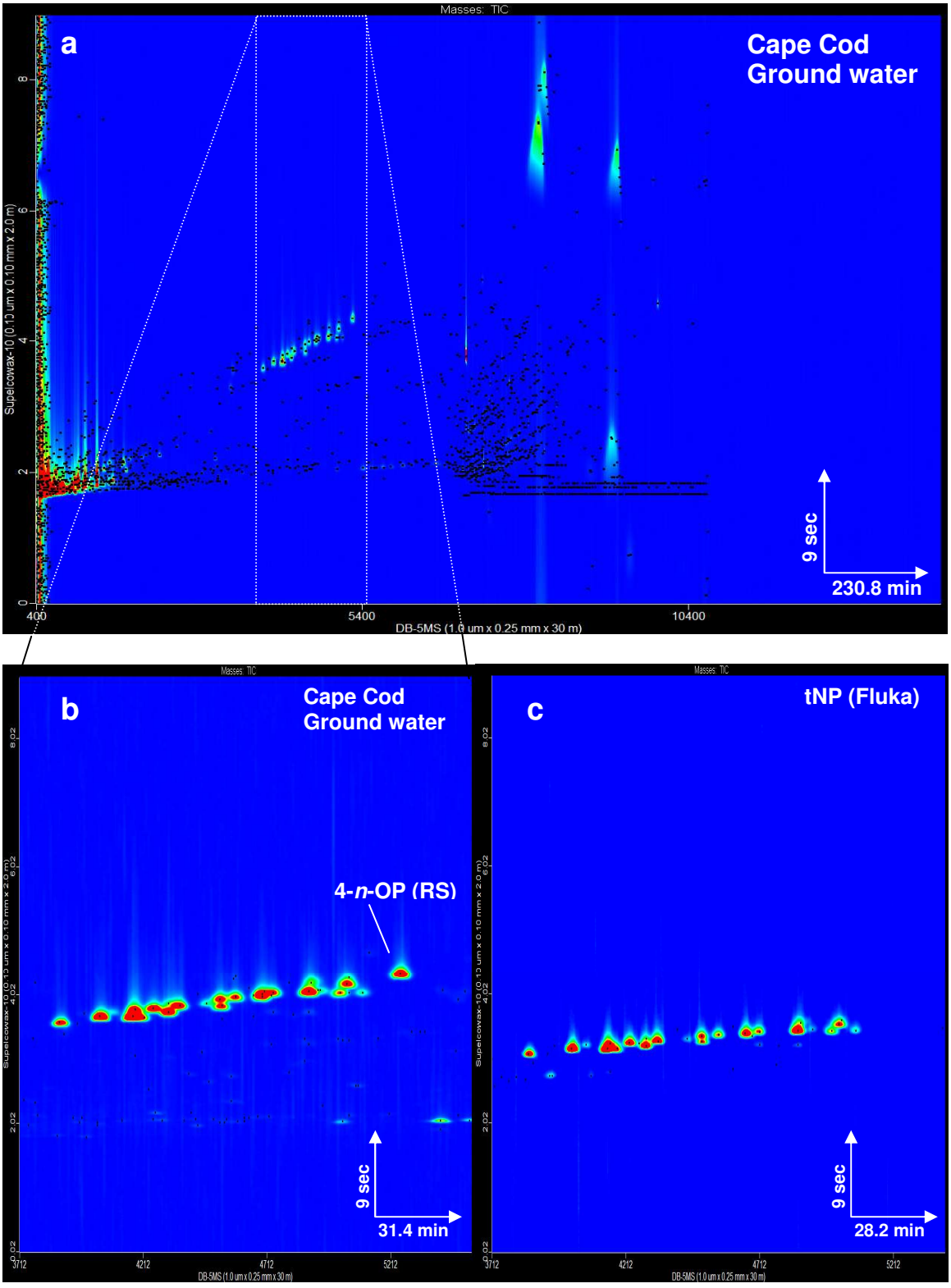
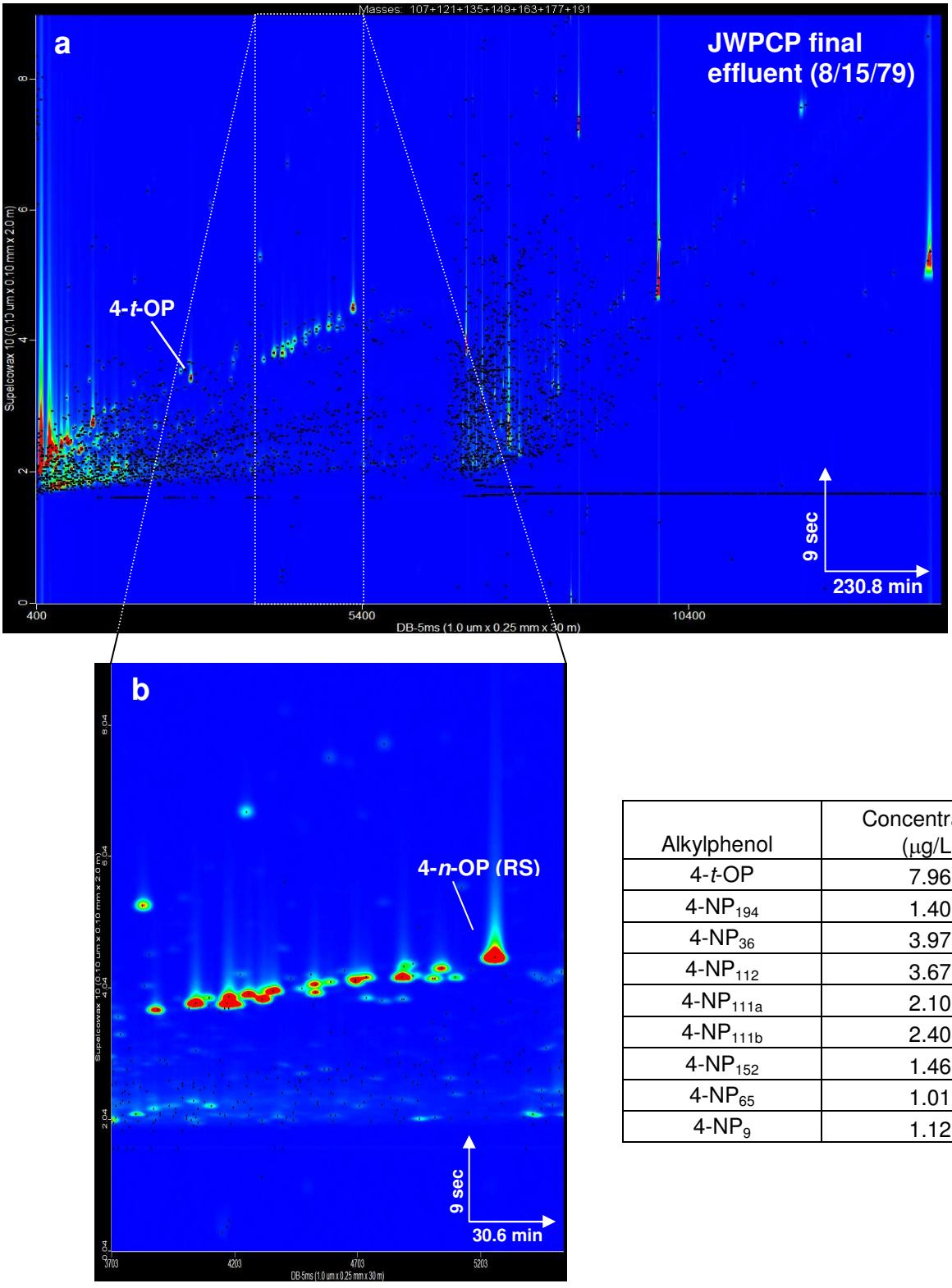
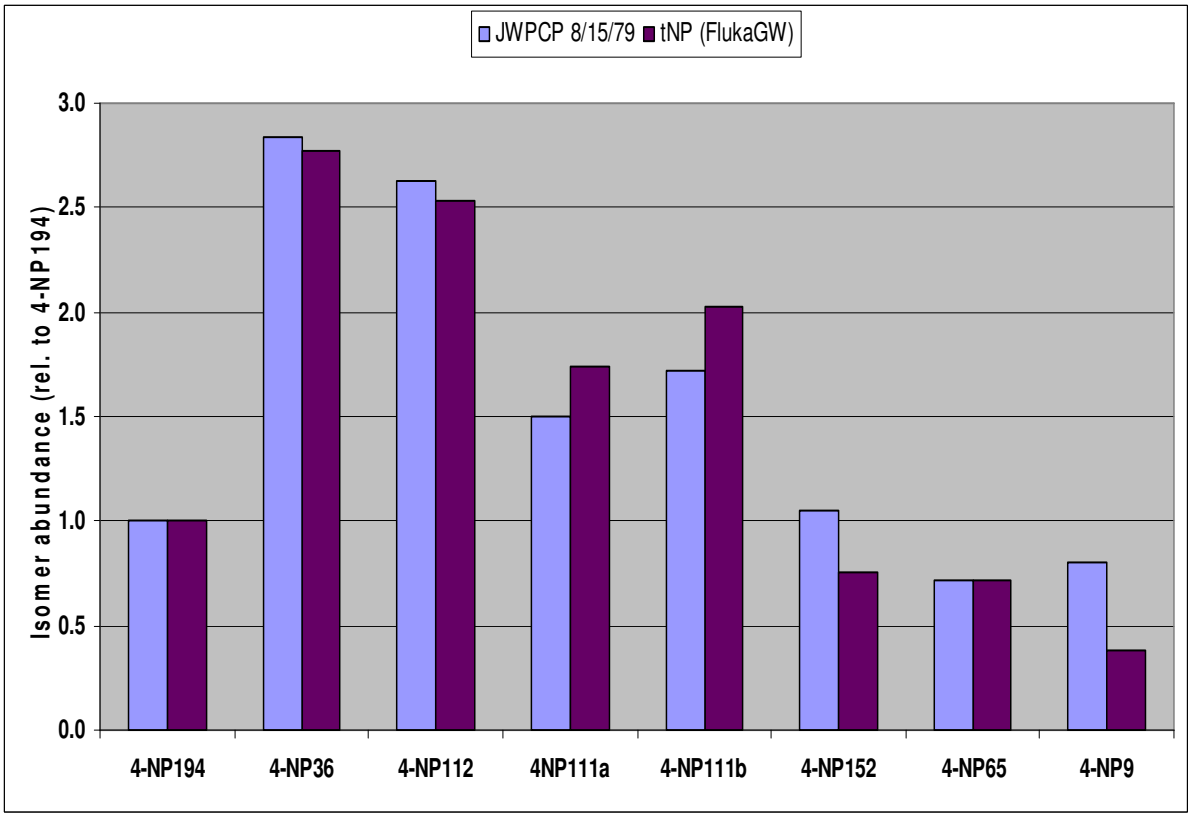


Figure S2. Summed ion chromatograms (m/z = 107+121+135+149+163+177+191) of an extract of the JWPCP municipal wastewater effluent showing a) complete chromatogram, b) 4-NP elution region, and tabulated concentrations of eight 4-NP isomers + 4-*t*-OP.



Alkylphenol	Concentration (μg/L)
4-*t*-OP	7.96
4-NP_{194}	1.40
4-NP_{36}	3.97
4-NP_{112}	3.67
4-NP_{111a}	2.10
4-NP_{111b}	2.40
4-NP_{152}	1.46
4-NP_{65}	1.01
4-NP_{9}	1.12

Figure S3. Comparison of 4-NP isomer composition of JWPCP final effluent sample (8/15/79) and tNP (Fluka$_{WG}$). Concentrations are normalized to 4-NP$_{194}$.



5. Column set testing

In an effort to establish optimal conditions of analysis for the 4-NPs, several combinations of columns were evaluated. These analyses were conducted using both unaltered and acylated tNP (Fluka) with the hope that acylation might provide some improvement in separation by reducing the influence of the hydroxyl group. Based on the reported observations of Ieda *et al.* (*11*), initial tests were conducted with a DB-5ms (0.25 mm id, 1.0 μm film, 30 m) x Supelcowax 10 (0.1 mm id, 0.1 μm film, 2.0 m) column set, whereby chromatographic conditions were systematically varied to obtain the best possible separation of the 4-NP isomers. Following this, eight other column combinations were tested, four of which were in the 'reversed configuration' (*i.e.* with a non-polar secondary column and polar primary column). Table S2 lists the columns that were used, and Figures S4 and S5 show GC x GC/FID chromatograms for the best runs with each column set for unaltered and acylated tNP, respectively.

In the case of the unaltered tNP analyses (Figure S4), best results were obtained for the **DB-5ms x Supelcowax 10** and **DB-5ms x 007-FFAP** column sets. However, the narrow bore 007-FFAP column has a significantly lower upper temperature limit (240 °C *vs*. 280 °C) and a thinner column wall thickness (0.05 mm *vs*. 0.1 mm), making it more fragile than the corresponding Supelcowax 10 narrow bore column. Among the 'normal configuration' column sets, those with BPX50 and HT8 as secondary columns proved unsatisfactory, whereas the set with BPX70 as secondary column effected a somewhat better separation. In the latter case, however, the 'maximum continuous' upper temperature limit of BPX70 (250 °C) offered no advantage over Supelcowax 10 (280 °C). Among the 'reversed configuration' column sets, no improvement in separation was obtained, and all primary columns (BPX70, α-Dex 120, β-Dex 120, γ-Dex 120) had upper temperature limits of 250 °C. Consequently, the **DB-5ms x Supelcowax 10** column set was selected for all subsequent

analyses. For the acylated tNP analyses (Figure S5), no improvement in separation of 4-NP isomers was observed for the column sets tested. For this reason, no further testing was done with nonylphenyl acetates.

Table S2. Column sets tested for separation of unaltered and acylated tNP (Fluka) by GC x GC/FID.

Primary column				**Secondary column**				
Phase	**Length (m)**	**id (mm)**	**film (μm)**	**Phase**	**Length (m)**	**id (mm)**	**film (μm)**	**Configuration**
DB-5ms[a]	30	0.25	1.00	BPX70[b]	1.5	0.10	0.20	normal
DB-5ms	30	0.25	1.00	HT8[b]	2.0	0.10	0.10	normal
DB-5ms	30	0.25	1.00	007-FFAP[c]	~2.0	0.10	0.10	normal
DB-5ms	30	0.25	1.00	BPX50[b]	2.0	0.10	0.10	normal
DB-5ms	30	0.25	1.00	Supelcowax 10[d]	~2.0	0.10	0.10	normal
α-Dex 120[d]	30	0.25	0.25	DB-1ms[a]	2.0	0.10	0.10	reversed
β-Dex 120[d]	30	0.25	0.25	DB-1ms	2.0	0.10	0.10	reversed
γ-Dex 120[d]	30	0.25	0.25	DB-1ms	2.0	0.10	0.10	reversed
BPX70	30	0.25	0.25	DB-1ms	2.0	0.10	0.10	reversed

[a] Agilent Technologies, [b] SGE Analytical Science, [c] Quadrex Corporation, [d] Supelco Analytical

Figure S4. GC x GC/FID chromatograms of tNP (Fluka) using various column configurations.

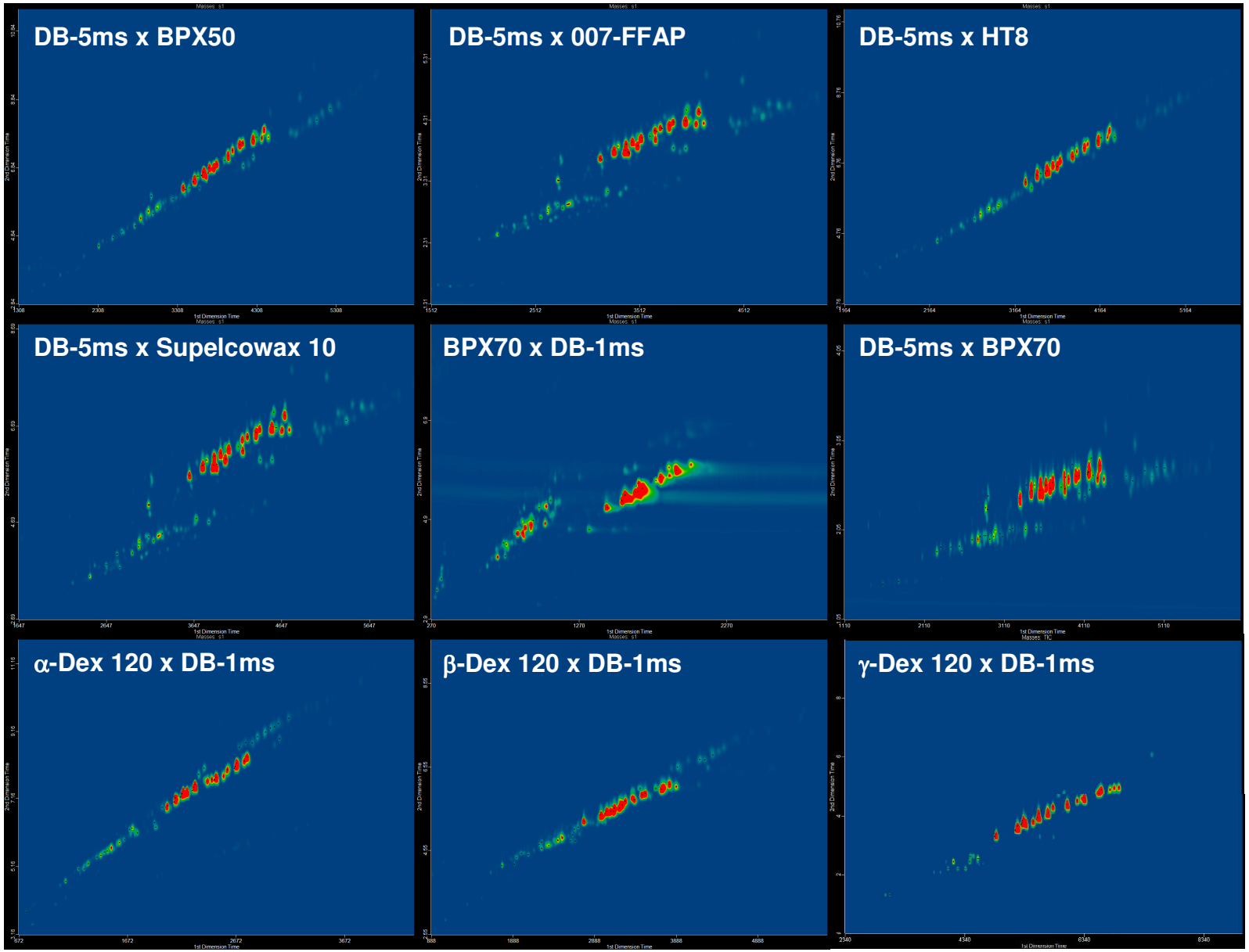
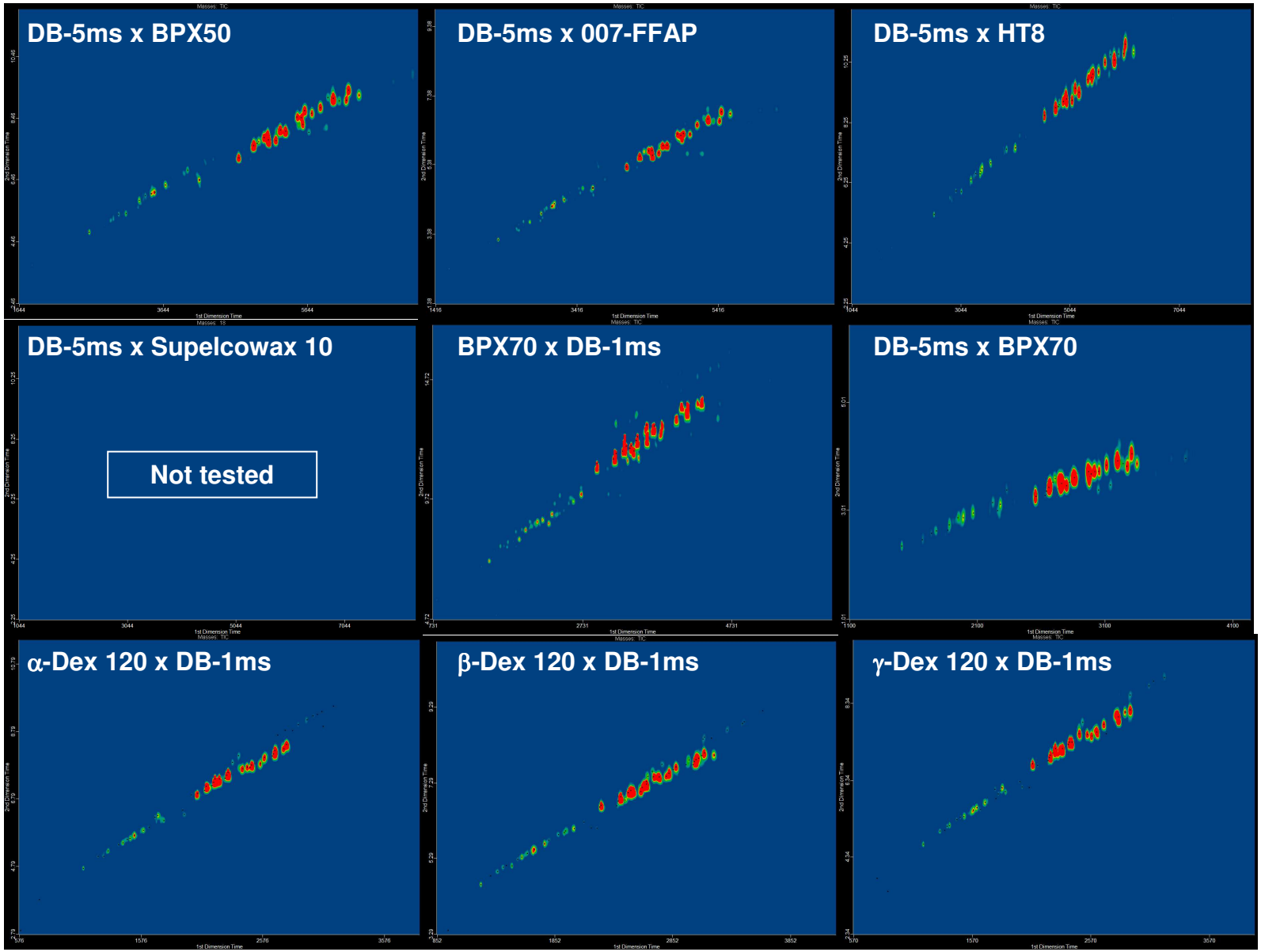


Figure S5. GC x GC/FID chromatograms of acylated tNP (Fluka) using various column configurations.



6. Quantitation ions used for GC x GC/ToFMS determination of 4-NP isomers

Quantitation requires specification of one or more ions that is/are characteristic of the analyte and which provide(s) a means of overcoming potential interference with co-eluting peaks. ChromaTOF software uses mass spectral deconvolution to identify a 'unique ion' for each analyte peak that ideally is only found in the peak of interest. In some cases, however, the 'unique ion' is not unique to the analyte but is in significantly greater relative abundance in the analyte than in co-eluting peak(s). The 'unique ion' identified by ChromaTOF for any analyte can differ from one set of analyses of the same sample to the next because of small variations in the amount of the substance entering the mass spectrometer, the data processing method parameters, and/or the chromatographic separation of sample components. As a first pass, 4-NP isomers in all samples were quantified using the 'unique ion'. However, it was observed that the 'unique ions' were sometimes not identical for the same analyte/tNP product pairs in each of the three analysis sets (*e.g.* 4-NP_{112}, 4-$NP_{111a,b}$, 4-NP_{152}, 4-NP_{65}) (see explanation below). Moreover, quantitation with the 'unique ion' did not always yield a reproducible quantitative result, particularly in the case of partially co-eluting components. In these cases, examination of the deconvoluted mass spectra of co-eluting peaks provided a means for selecting appropriate ions to minimize, if not avoid, interference during quantitation. In such instances, uncorrected (*i.e.* 'caliper') and deconvoluted (*i.e.* 'peak true') mass spectra for all co-eluting components were examined, and abundant rational fragment ions that appeared most specific to the peak in question were manually designated to be used for quantitation. Three sets of analyses (involving separate multilevel calibrations) were performed for quantitation of 4-NP isomers in the seven tNP products over a time span of 2 months. This and the fact that data on the abundance of some 4-NP isomers in tNP products have been published (*12, 13*), allowed us to evaluate the comparability of the three replicate data sets to each other and to published

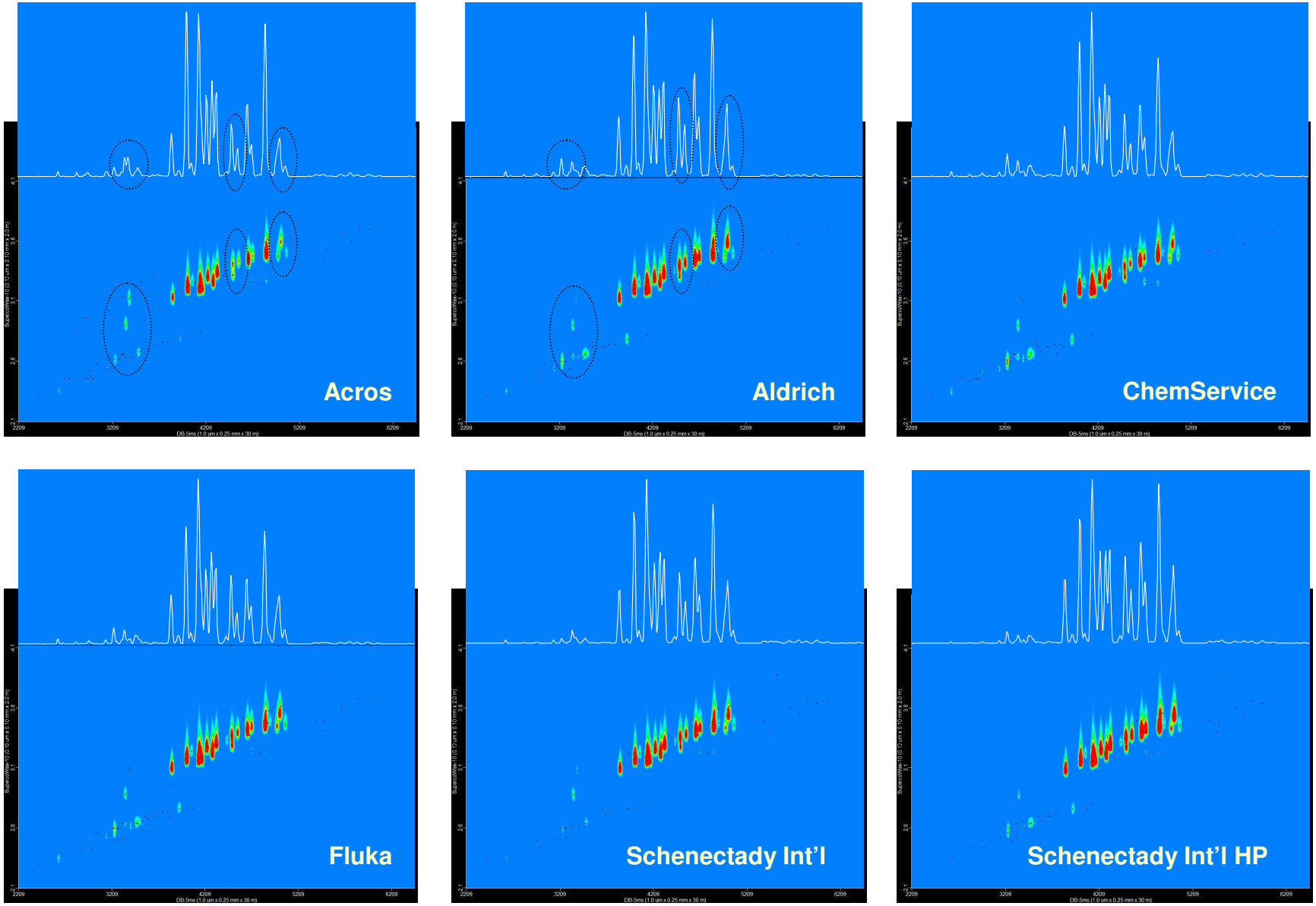
information. During the period of analysis, chromatographic separation of 4-NP isomers in the 2nd dimension declined somewhat because of deterioration of the Supelcowax 10 (secondary) column under the influence of high oven temperatures over extended periods. This provided an opportunity to evaluate the efficacy of mass spectral deconvolution and the effect of chromatography (through identification of and quantification with the 'unique ion') on determination of analyte concentrations for peaks that were only partially separated from other sample components (*e.g.* 4-NP_{112}, 4-NP_{152}, 4-NP_{65}). Among the eight available isomers, the only significant impact was noted for 4-NP_{65}, which was nearly baseline-resolved from all other 4-NPs during the first of the three analysis sets but which partially co-eluted with 4-NP_{110b} (a non-analyte) in the third analysis set. Concentrations of 4-NP_{65} in the tNP products for the first and second data sets agreed with each other and were consistent with published data, but in the third data set, concentrations were highly variable and different from those found in the first two data sets. Use of a less abundant but more specific quantitation ion for 4-NP_{65} (*e.g.* m/z = 191) in the third analysis set did not bring the abundance of this isomer (as measured during the third analysis) in all tNP products into closer agreement with data obtained in the first two analysis sets nor did it reduce the level of variability among products. (Note: The deconvoluted mass spectra of peaks identified in the third quantitation data set as 4-NP_{65} and 4-NP_{110b} were consistent with those obtained in the first two quantitation data sets and with published spectra, indicating that deconvolution was, in fact, successful.) This occurred because during mass spectral deconvolution the software was unable to find a peak corresponding to 4-NP_{65} due to inadequate chromatographic resolution. Consequently, concentrations of 4-NP_{65} determined in the third analysis set were deemed invalid and are not presented here.

For quantitation of the eight 4-NP isomers in the seven tNP products, three separate calibrations were conducted. For five of the eight isomers, the 'unique ions' identified by the ChromaTOF software were not the same for all three calibrations (*viz*. 4-NP_{112}, 4-NP_{111a}, 4-NP_{111b}, 4-NP_{152}, 4-NP_{65}). For example, in the case of 4-NP_{111a}, m/z 107 was determined to be the 'unique ion' for calibration #1, whereas m/z 121 was the 'unique ion' for calibrations #2 and #3. In such instances, testing was performed to determine if using the more frequently identified 'unique ion' (*e.g*. m/z 121 for 4-NP_{111a}) in all three quantitations resulted in significantly different results. In every case but 4-NP_{65}, the relative standard deviations for the concentrations determined in the three separate quantitations decreased when the same quantitation ion was used (see main text for discussion). In the case of 4-NP_{112}, where baseline resolution from 4-NP_{38} and 4-NP_{128} was not achieved (Figure 2, main paper), a more specific ion (m/z = 149) was used for quantitation instead of the 'unique ion' (m/z =107). Similarly, m/z = 163 was used for quantitation of 4-NP_{152} instead of the 'unique ion' (m/z =121) even though chromatographic separation was adequate under the conditions of analysis we used (Figure 2). Consequently, for quantitative data presented in this report, namely the tNP, JWPCP, and tNP biodegradation samples, the same quantitation ions were used (ions given in parentheses, <u>underlined</u> if they were the predominant or sole 'unique ion'): 4-NP_{194} (<u>121</u>), 4-NP_{36} (<u>135</u>), 4-NP_{112} (149), 4-NP_{111a} (<u>121</u>), 4-NP_{111b} (<u>121</u>), 4-NP_{152} (163), 4-NP_{65} (<u>107</u>), 4-NP_{9} (<u>135</u>).

7. GC x GC/ToFMS chromatograms of tNP products

Figure S6 presents reconstructed 1D and 2D GC x GC/ToFMS chromatograms for six of the seven tNP products (Fluka$_{WG}$ product not shown) analyzed in this study. In general, the gross compositions of the tNP products in the 4-NP region appear quite similar. However, minor, but distinct, differences can be seen in both 1D and 2D chromatograms, examples of which are indicated by circled areas on the Acros and Aldrich tNP chromatograms.

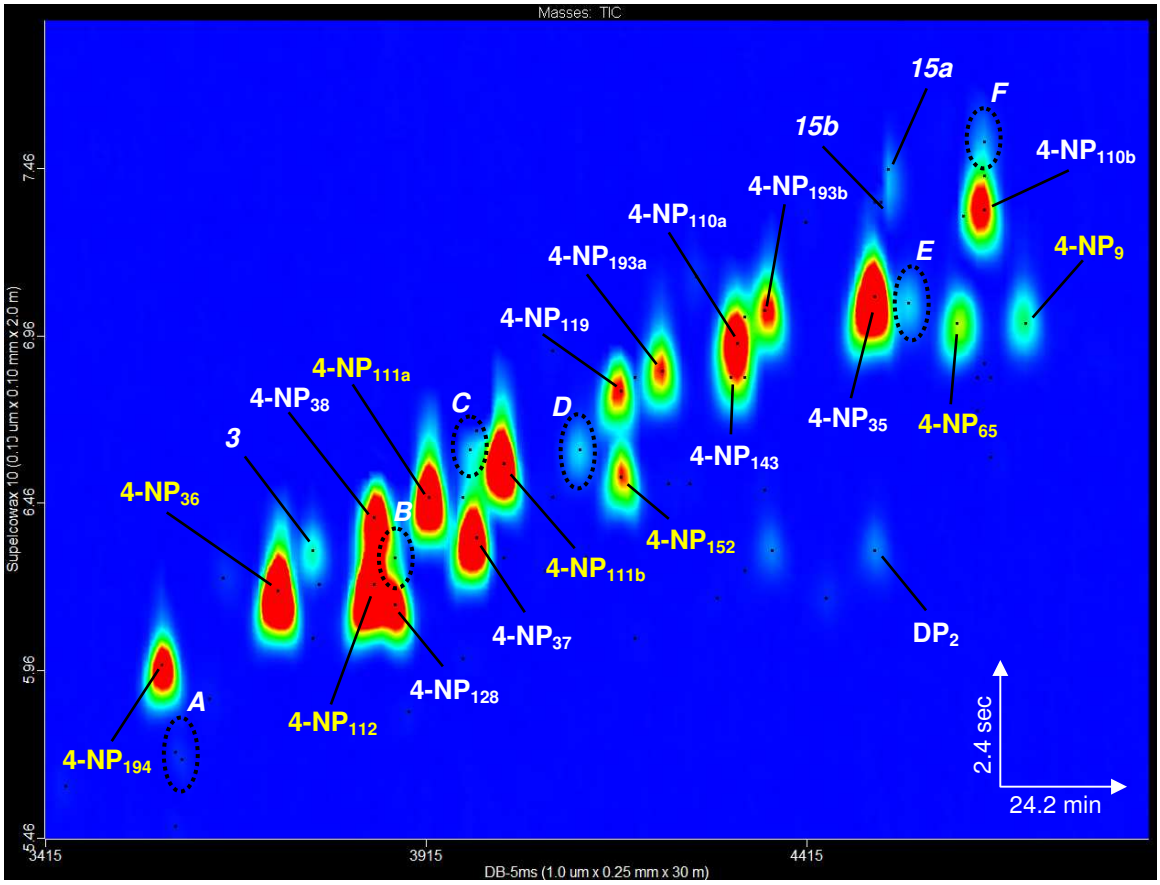
Figure S6. GC x GC/ToFMS chromatograms (reconstructed shown 1D above the 2D) of six tNP products analyzed in this study.



8. Mass spectra of NP components in tNP (Fluka)

Figure S7 shows a GC x GC/ToFMS total ion current (TIC) chromatogram of tNP Fluka with names of each peak. Figure S8 provides deconvoluted (*i.e.* 'peak true') mass spectra for the six minor 4-NP components in tNP (Fluka; peaks ***A-F*** in Figure S7), 21 identified 4-NP isomers (4-NP_x), and DP_2 shown in Figure S7.

Figure S7. GC x GC/ToFMS (TIC) chromatogram showing locations of peaks.



-Continued on next page-

Figure S8. Deconvoluted ('peak true') mass spectra of NP components in tNP (Fluka).

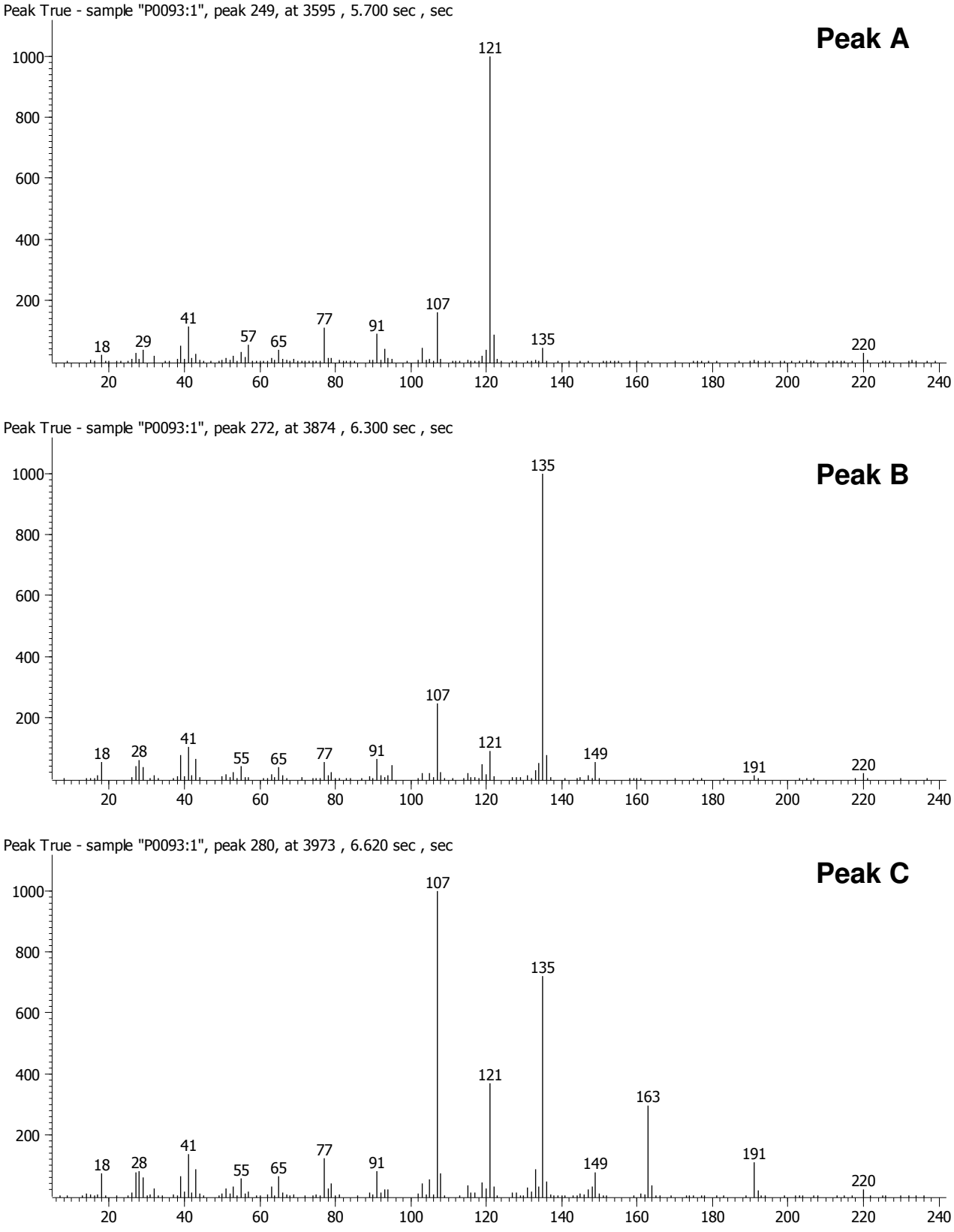


Figure S8 continued.

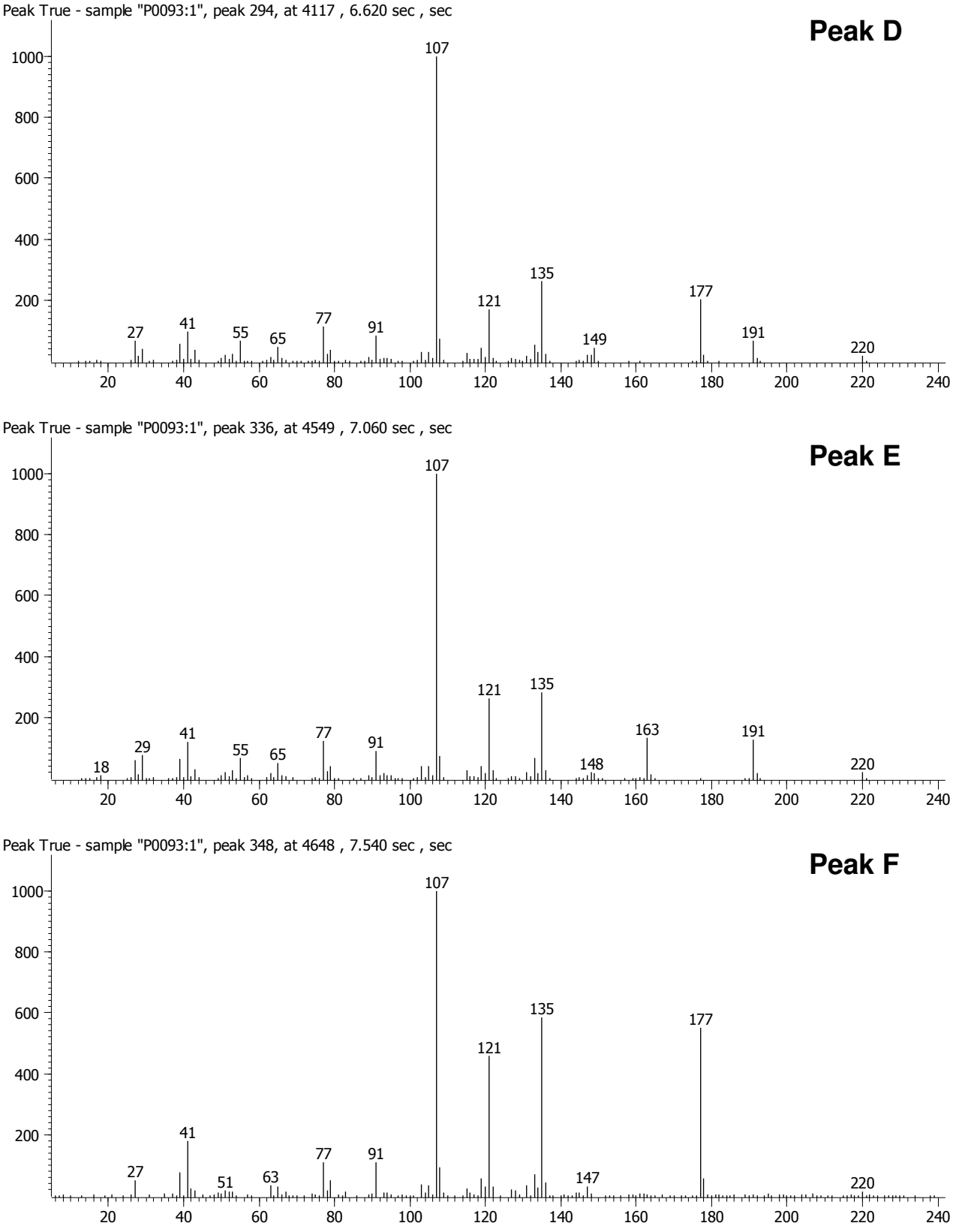


Figure S8 continued.

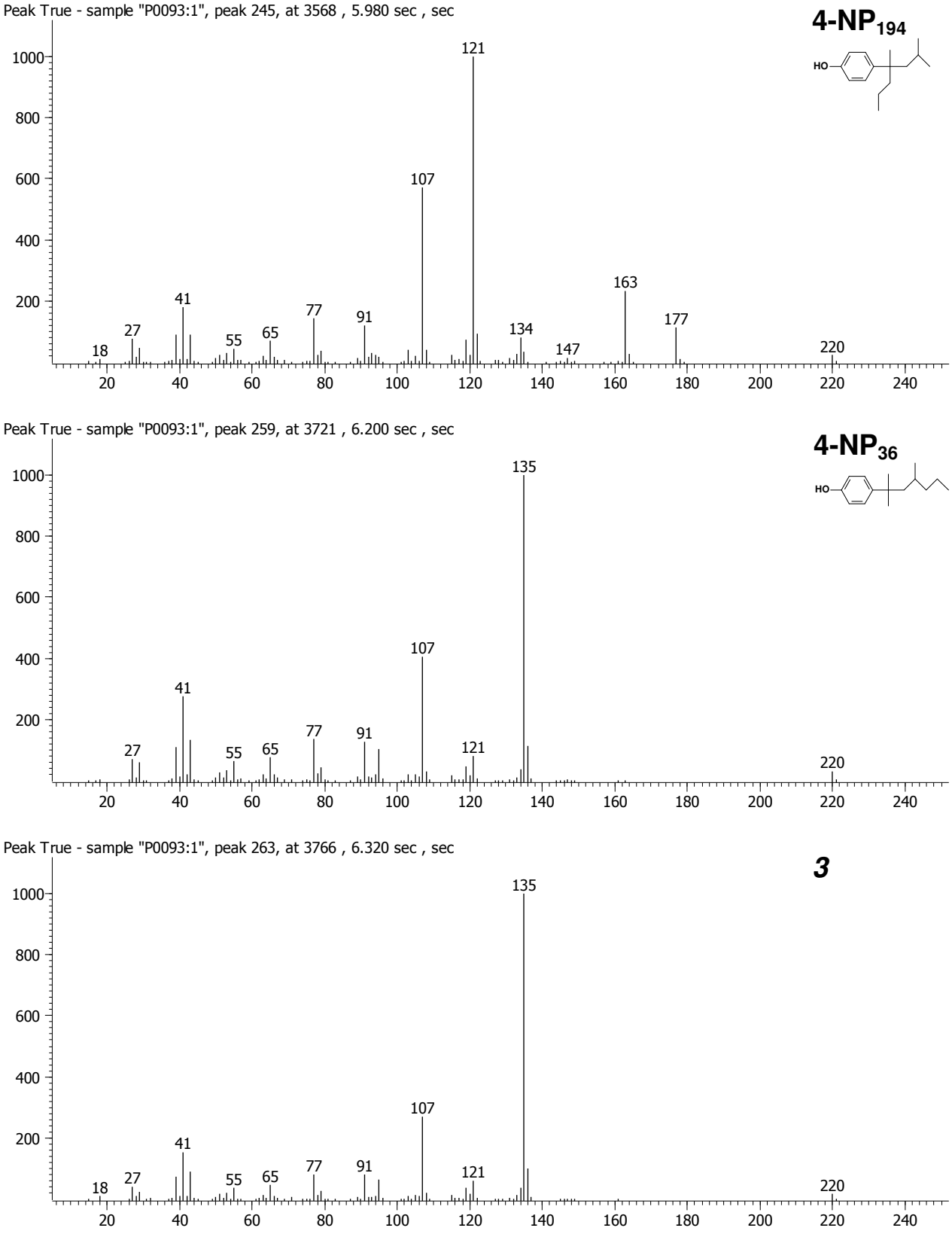


Figure S8 continued.

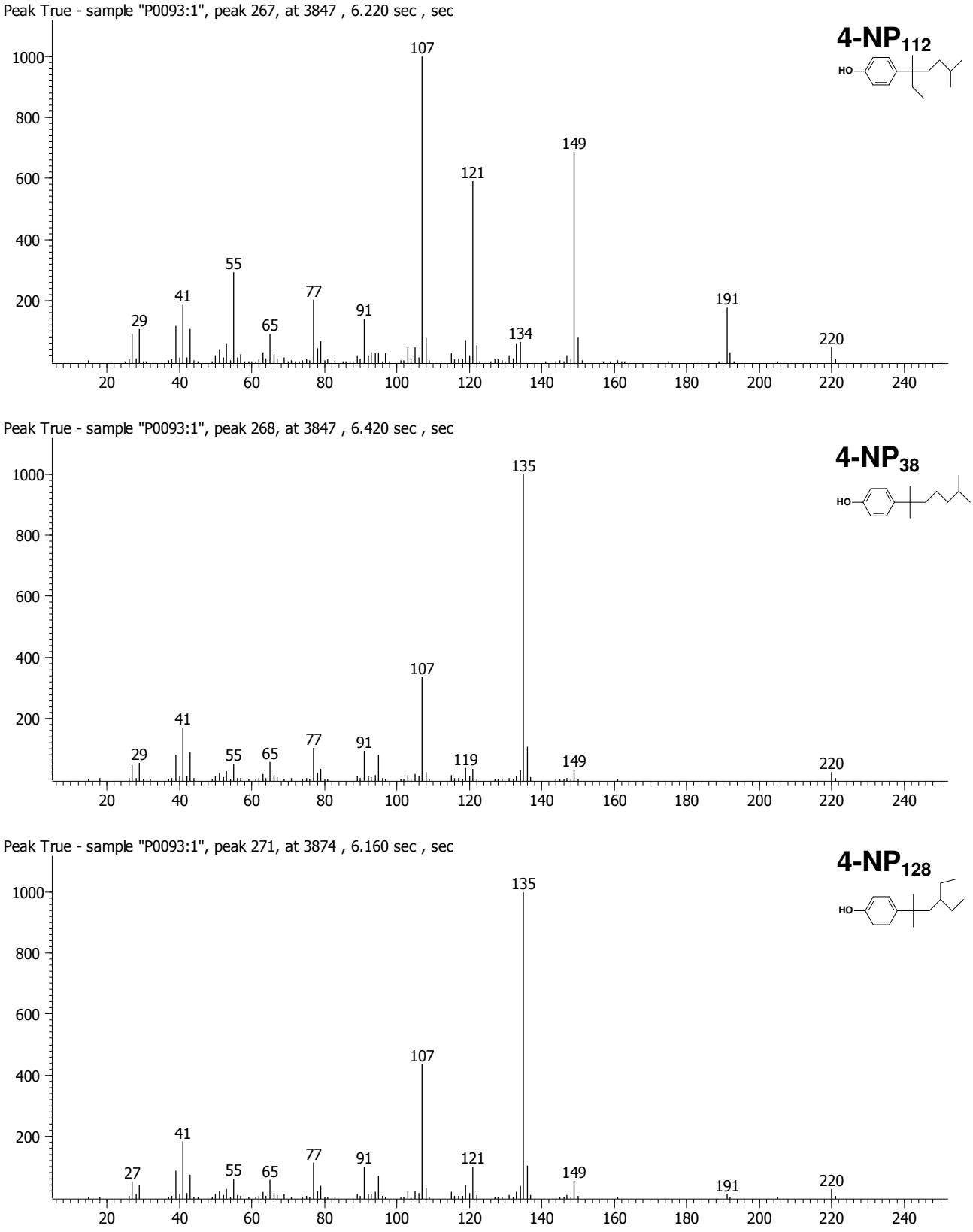


Figure S8 continued.

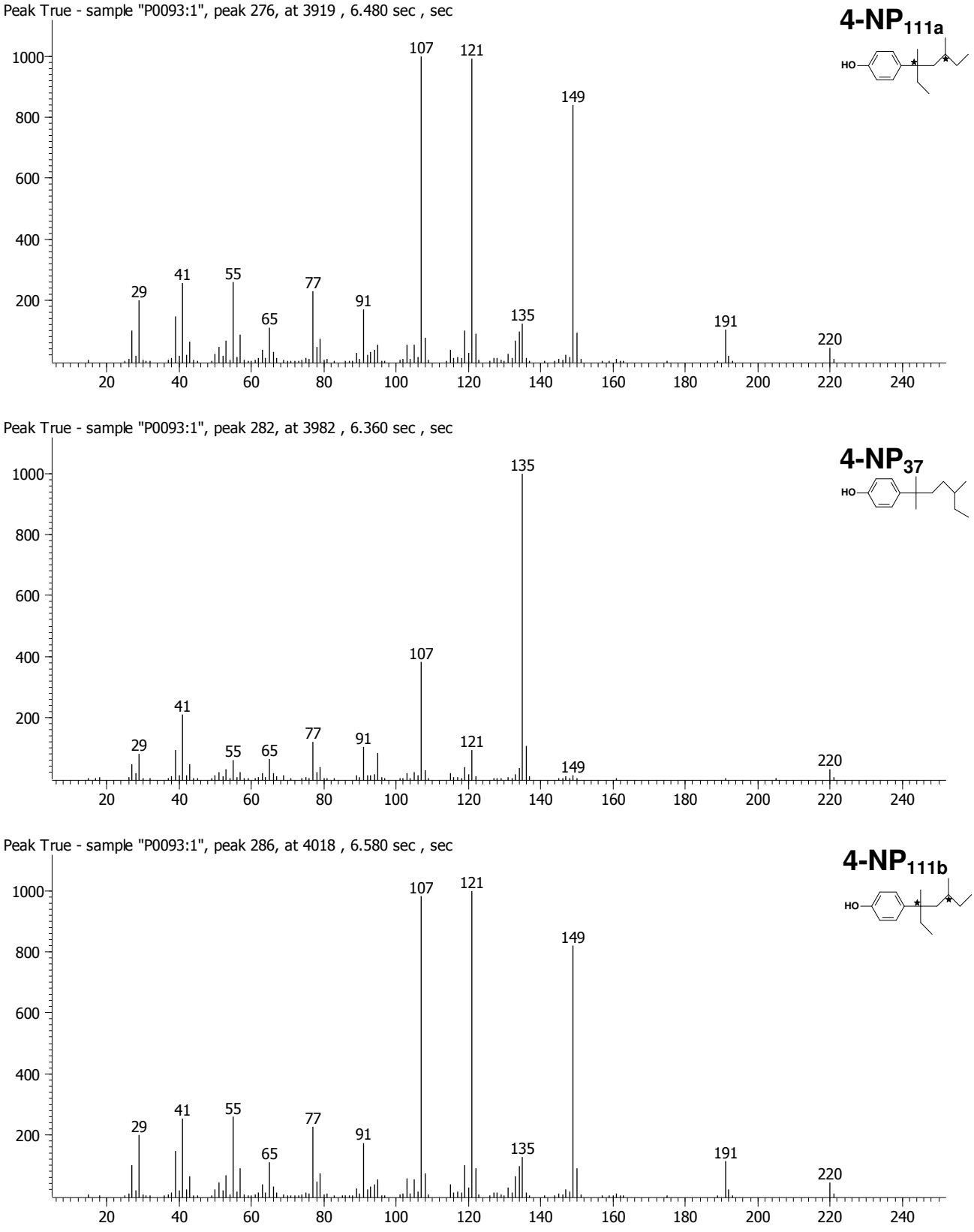


Figure S8 continued.

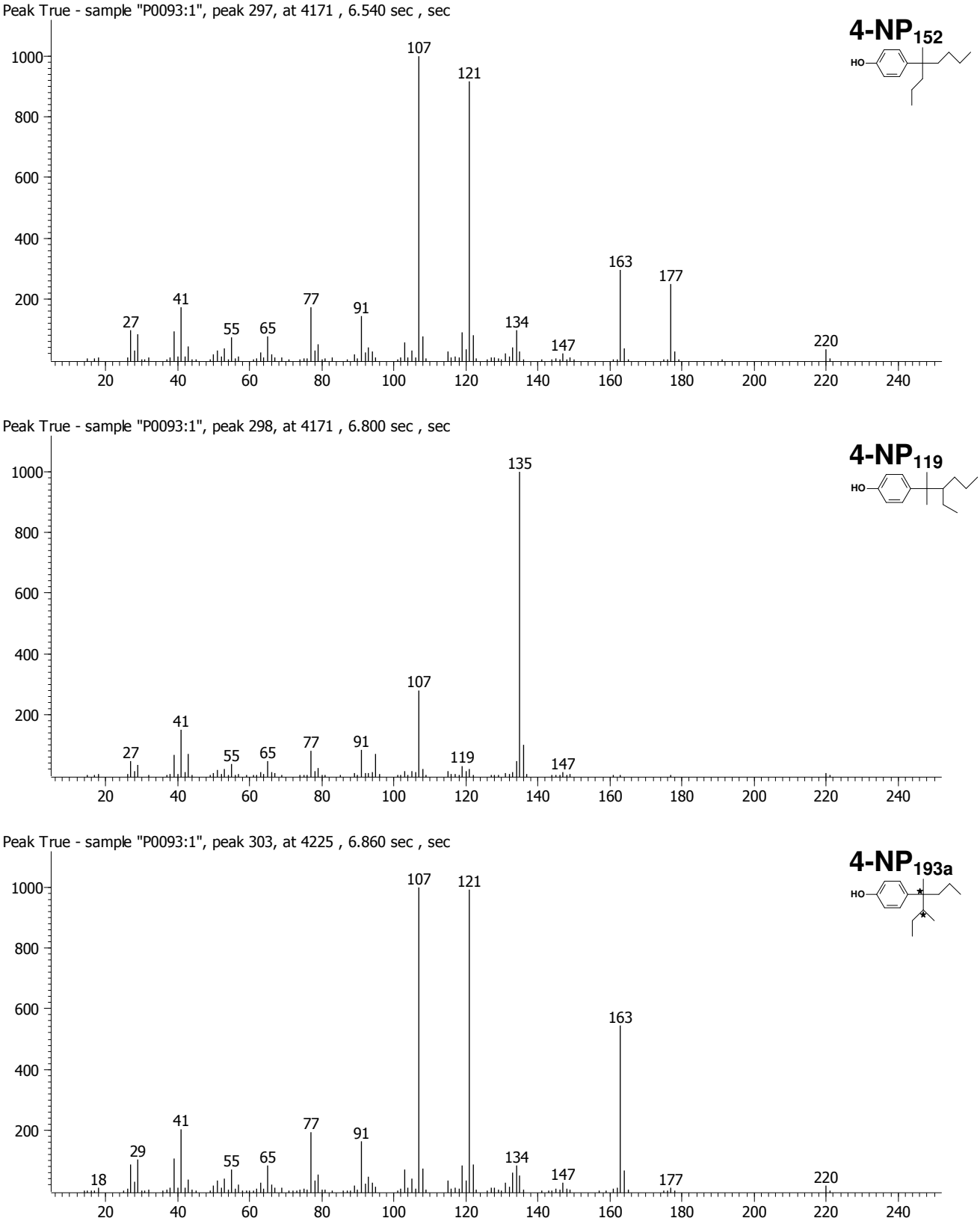


Figure S8 continued.

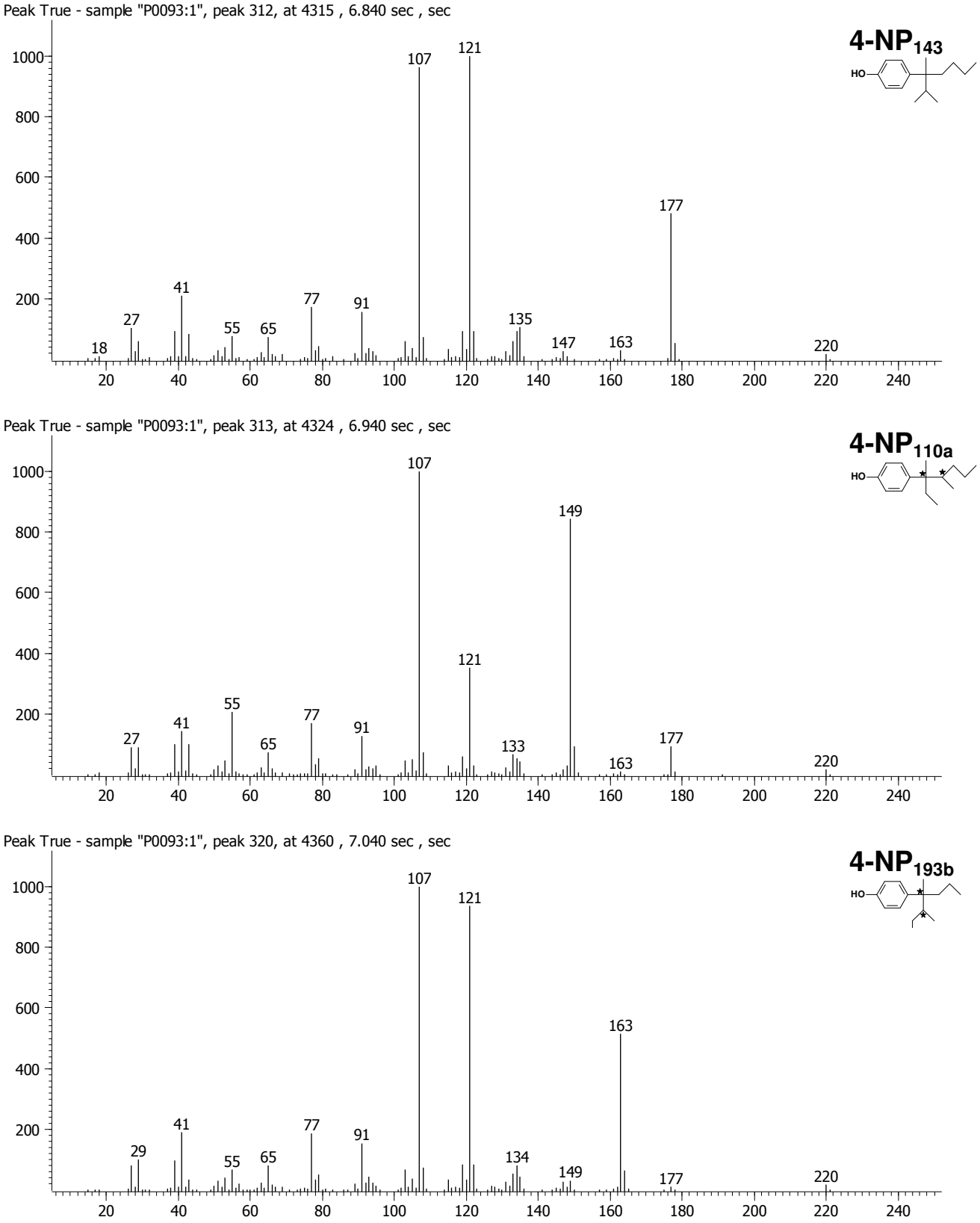


Figure S8 continued.

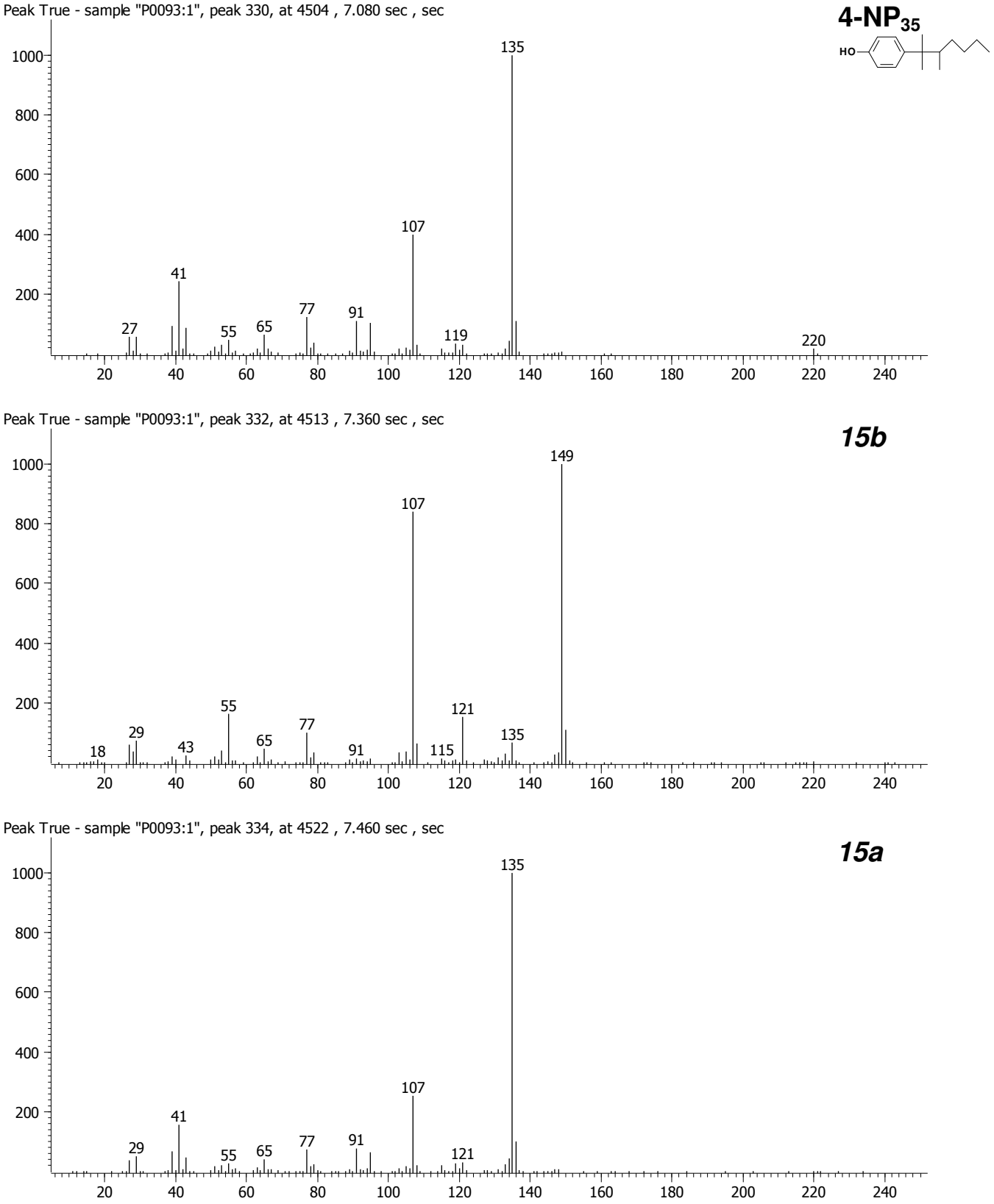


Figure S8 continued.

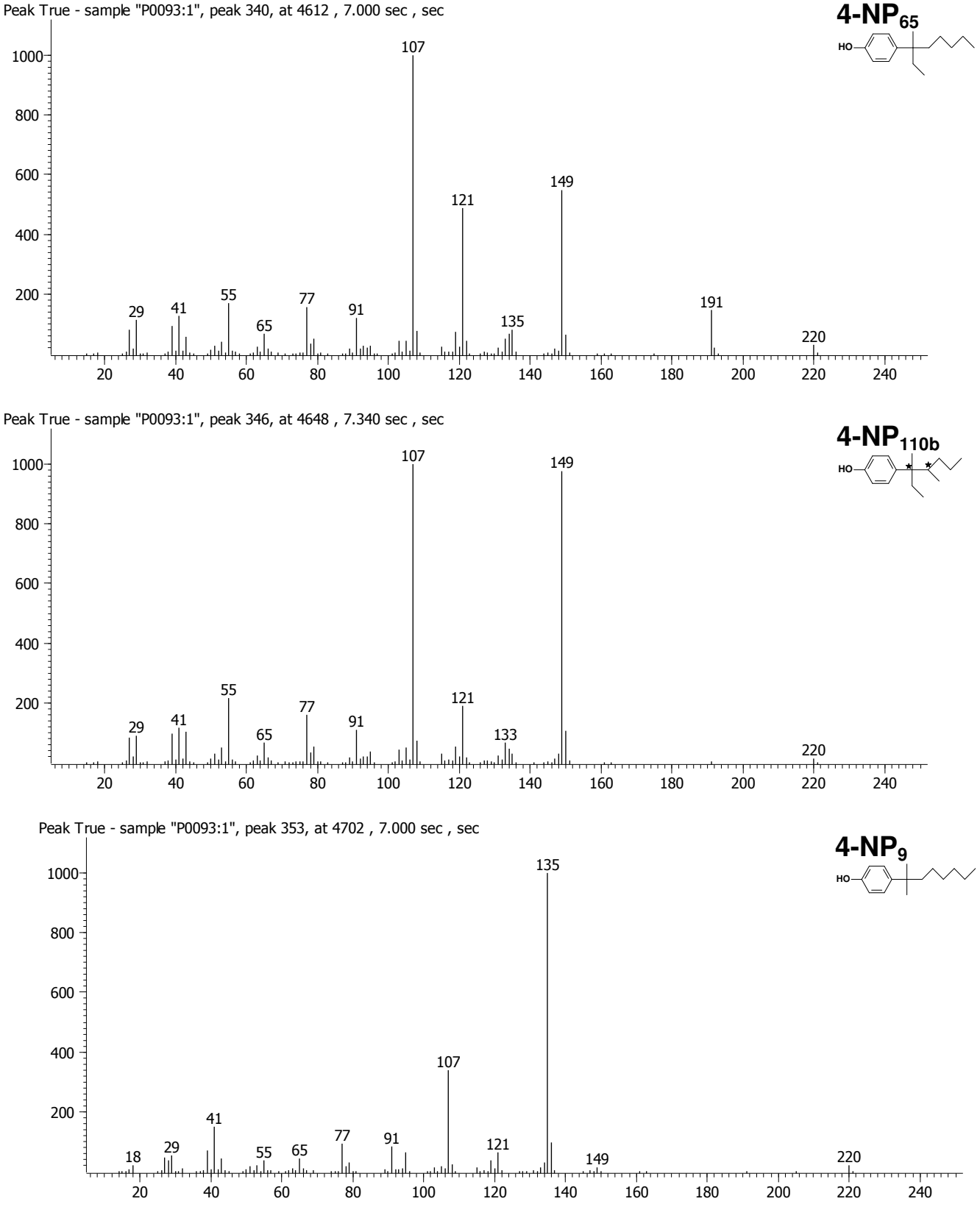
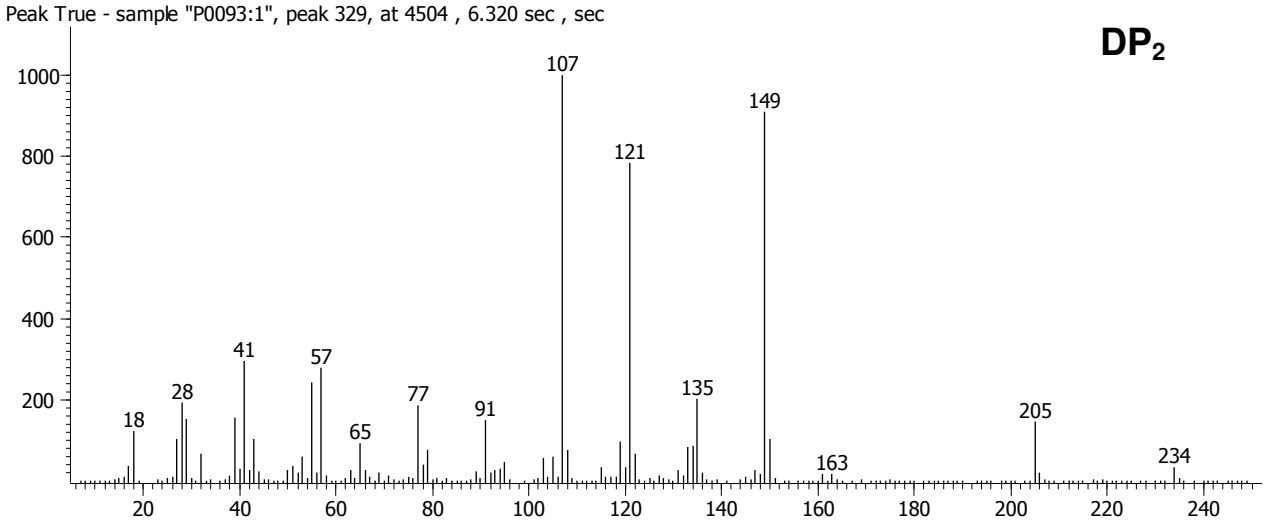


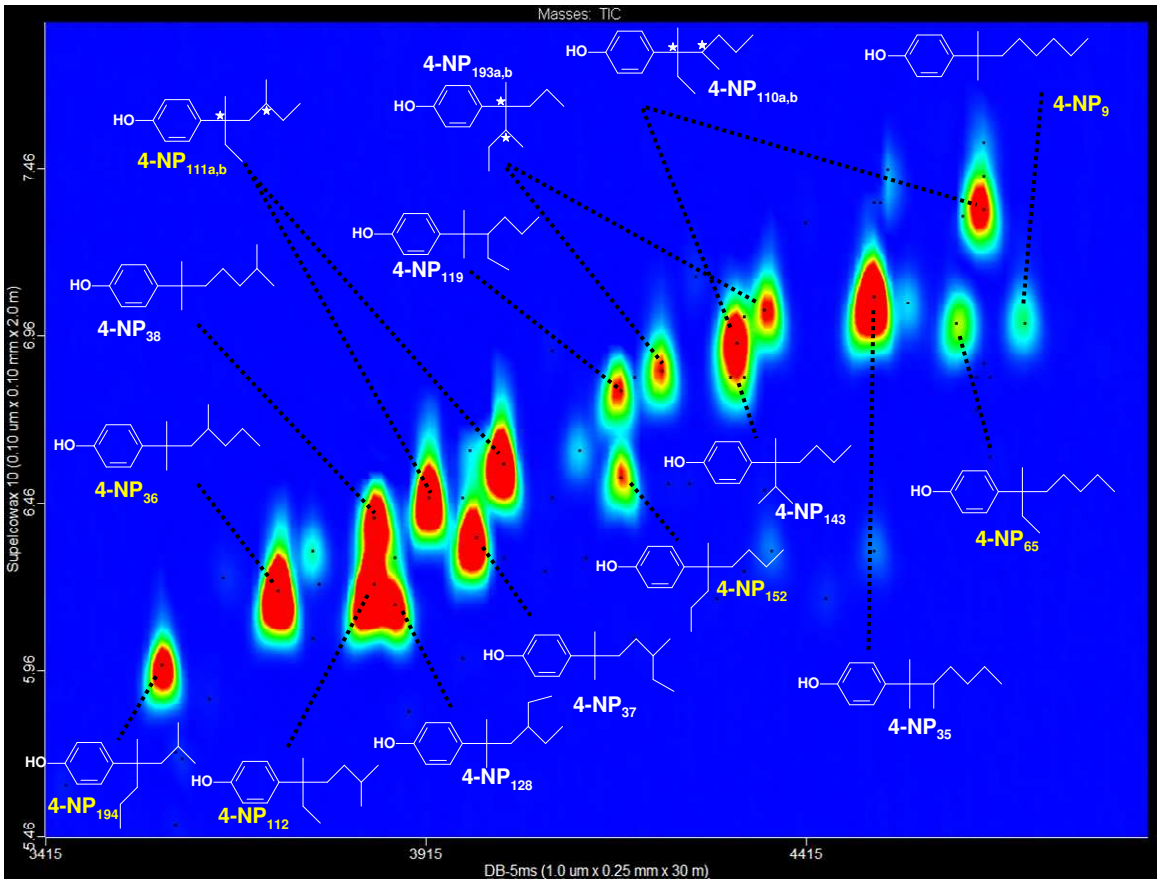
Figure S8 continued.



9. Structures of known 4-NP isomers

Figure S9 shows structures of the 4-NP isomers in tNP (Fluka) that have been elucidated to date.

Figure S9. Structures and names of 4-NP isomers in tNP (Fluka). Note: yellow text indicates isomers measured in this study.



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