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Letter to the Editor

To the Editor-in-Chief
Sir,

Site determination of phenyl glycidyl ether-DNA adducts using high-performance liquid chromatography with electrospray ionization tandem mass spectrometry

In the past decades many techniques have been used to measure DNA adducts. Among these methods the most commonly used are ^{32}P-postlabeling and immunoassays,[1–3] which are very sensitive but cannot provide any structural information. In addition, traditional DNA-sequencing methodologies are often unsuccessful in the characterization of modified oligonucleotides.[4,5] Mass spectrometry (MS), especially when coupled to separation techniques such as high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE), has the sensitivity and specificity to analyze carcinogen-oligonucleotide adducts in complex mixtures. Recently, characterization of DNA adducts has been achieved at the nucleoside level by coupling HPLC to electrospray ionization mass spectrometry (ESI-MS), and also at the nucleotide level by coupling CE to ESI-MS.[6,7] Thus, LC/MS/MS could be a useful tool for identification of the adduct structure and of the specific position of modification within a given sequence.[8–10]

Phenyl glycidyl ether (PGE, Fig. 1) is frequently used in numerous industrial chemical processes, such as in the paint and resin industries.[11–14] The US National Institute of Occupational Safety and Health surveyed the extent of occupational contact and potential damage from the glycidyl ether group of epoxides, and established threshold limits for human exposure.[15] PGE can induce mutagenicity in the Ames test, and cause a range of cytotoxic reactions including skin irritation, allergic reactions, and pulmonary edema, and can lead to modification of DNA building blocks and effects on blood composition.[16] However, compared with aromatic polycyclic hydrocarbons (PAHs),[13] there is not currently enough evidence to prove that PGEs can cause carcinogenicity and mutagenicity in humans. Only a few researchers have studied the formation of PGE adducts with individual bases or DNA itself; they found that the alkylation occurred not only on the heterocyclic moiety, but also on the 5′-phosphate group of the deoxynucleotides.[16–18]

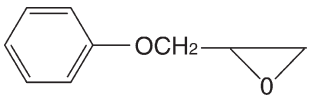

Figure 1. Structure of phenyl glycidyl ether (PGE).

However, until now, no work has been reported that aimed to determine the adduction site(s) of PGE in DNA because, in previous studies, all sequence information was lost after the enzymatic digestion of the oligomers to the individual deoxynucleosides. Nevertheless, the assignment of the sites of modification within a target sequence is very important since isomeric adducts may possess different mutagenic activities due to different biological responses to the presence of the adducts. There has been increasing interest in the development of analytical methods which can be used to measure DNA modifications at the base or nucleoside level.[19–23] Analysis of modified nucleotides and oligonucleotides is critical to development of a full understanding of the chemical behavior of a carcinogen, because it may be used to detect structurally diverse adducts.[24]

More recently, we have studied the characteristics of G- and T-rich oligonucleotides and the DNA methylation by ion-pairing reversed-phase (IP-RP) HPLC coupled with ESI-MS/MS.[25–27] In the present study, three synthetic single-stranded oligonucleotides were used to investigate the sites of modification by PGE within the known sequences.

Phenyl glycidyl ether (PGE) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Oligonucleotides (5′-TATCTGTC-3′, 5′-ACCCGCGTCC-3′ and 5′-ACCCGCGTCCGCGCC-3′) were obtained from Asia Biochemistry (Shanghai, China) and were used as received. Triethylamine (TEA, 99.5%), glacial acetic acid (99.99%), and HPLC-grade acetonitrile were purchased from Merck (Darmstadt, Germany). Water was purified with an Elix-Milli-Q system (Millipore Corp., Bedford, MA, USA).

The 400 mM hexafluoroisopropanol/16 mM triethylammonium buffer, pH 7.7, was prepared by dissolving 22 mL HFIP in 400 mL water and then slowly titrating with 1.2 mL TEA. The mobile phase was filtered through a 0.22 μm film before use in the HPLC analysis.

Aqueous solutions (0.2 nmol μL^{-1}) of each oligonucleotide were prepared. To 10 μL of this solution were added 20 μL methanol and 2–10 μL of a 1 M methanolic solution of PGE. Then the mixtures were incubated at 37°C for either 12 or 48 h. All reactions were terminated by evaporating the methanol using a stream of nitrogen gas. Then the white residue was removed by syringe, and the mixture was extracted three times with 60 μL of chloroform in order to remove the residual PGE.

All samples were analyzed using an Agilent 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled with an API 4000 triple-quadrupole mass spectrometer (Applied Biosystems/MDS-Sciex, Foster City, CA, USA). The liquid chromatograph was equipped with an online vacuum degassing system, a quaternary pumping system, an autosampler and a variable wavelength detector. The chromatographic separation was performed on a C18 column (150 × 3.0 mm i.d., 3.0 μm particle size, ZORBAX 300SB, Agilent Technologies) at room temperature. A mobile phase flow rate of 200 μL min^{-1} was used in the analysis, and the conditions are detailed in the figure captions. The injection volume was 5 μL, and UV detection used a wavelength of 260 nm. The LC effluent was introduced into a TurboIonSpray interface without splitting. ESI mass spectra were acquired in the negative ion mode with a spray

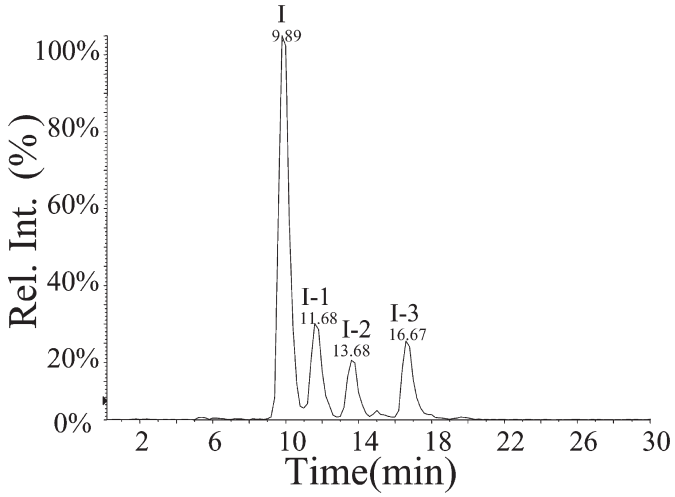

Figure 2. LC/negative ion ESI-MS chromatograms obtained from the injection of a reaction mixture of PGE with 5′-TATCTGTC-3′. Peak I is the unmodified oligonucleotide, while peaks I-1 to I-3 are PGE-modified versions.

voltage of −4 kV and declustering potential of −100 V. Nitrogen was used as the curtain gas (setting 16), nebulizer gas (setting 20), and turbo-gas (setting 20). The turbo-gas temperature was 400°C. MS/MS was performed using nitrogen as collision gas (CAD gas setting 8). The collision energy was set to 35 eV in order to obtain as complete sequence information as possible. The mass spectrometer was operated at unit mass resolution for both Q1 and Q3 (peak widths 0.6–0.8 Th at FWHM). The data were acquired using the Sciex Analyst software, version 1.3.1.

Some carcinogens will react with oligonucleotides to form adducts not only in different numbers, but also at different sites of modification within a given sequence. The determination of the adduction sites, especially in cases where isomeric adducts are possible, is very important for determination of which site of modification within a target sequence should be ascribed to the mutagenic activities. In this paper, three synthesized oligonucleotide sequences were used to react with PGE in order to investigate the adduction sites.

Figure 2 displays the total ion current (TIC) chromatogram of the PGE-oligonucleotide (5′-TATCTGTC-3′) reaction mixture. It is apparent that three adducts (I-1, I-2, I-3) are observed in addition to the original oligonucleotide. The unmodified oligonucleotide was characterized by its $[M-2H]^{2-}$ ion at *m*/*z* 1186.6, while the three adducts were positional isomeric modified oligonucleotides characterized by the same *m*/*z* value (1261.1) of their $[M-2H]^{2-}$ ions, which corresponds to the addition of one molecule of PGE to the original oligonucleotide. MS/MS experiments were performed to identify the sites of adduct formation via the characteristic series of fragment ions a-B (derived from the 5′-end of the oligonucleotides) and w (derived from the 3′-end). The nomenclature of the oligonucleotide fragments is that introduced by McLuckey *et al.*;[28] Fig. 3 is shown as an example (5′-TATCTGTC-3′ and 5′-TATCTGTC*-3′).

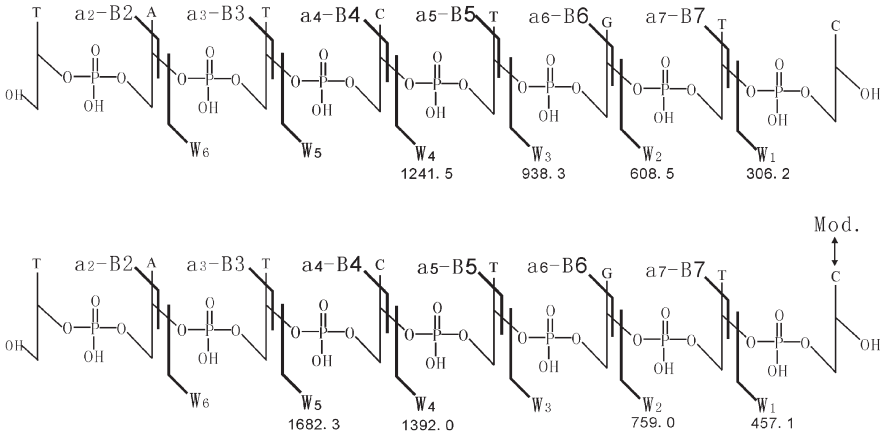

Figure 3. Definition and nomenclature[28] for the MS/MS fragmentations of the unmodified oligonucleotide I (5′-TATCTGTC-3′) and of the modified version I-1 (5′-TATCTGTC*-3′). The mass shift of modified ions is 150.2 Da (PGE molecular mass).

Figure 4 shows the MS/MS spectra of the $[M-2H]^{2-}$ ions for modified and unmodified oligonucleotides, in which the modified oligonucleotides are indicated with an asterisk on some bases and fragments. On comparison of the product ion spectra for modified and unmodified oligonucleotides, w1, w2, w4 and w5 ions are observed indicating PGE modification at C while the a-B series ions are not observed. The key fragment ion (w1+mod) at *m*/*z* 457.1 in the MS/MS spectrum of 5′-TATCTGTC*-3′ indicates the PGE-modification position. In the MS/MS spectrum of I-2 (5′-TA*TCTGTC-3′), a2-B, a3-B and a6-B ions, especially the abundant fragment at *m*/*z* 862.5 (a3-B +mod), provides the evidence for modification at A. Diagnostic fragments a4-B, a5-B and w_5 are observed in the MS/MS spectrum of I-3 (5′-TATC*TGTC-3′), which can be used to determine the adduction site in this sequence.

Since some additional fragment ions (such as *m*/*z* 990.9 ion for I-1, and *m*/*z* 757.8, 1393, and 992.8 ions for I-2), observed in Fig. 4, are not interpretable in this fashion, it may be that the spectra for I-1 and I-2 in Fig. 4 were the result of the fragmentation of two co-eluting isomeric oligonucleotide adducts. In fact, formation of isomeric adducts can occur even if the modification takes place on the same base in the sequence; this feature has already been described by several authors working on the modification of DNA bases by PGE or other electrophilic species.[13,29]

The other two oligonucleotides used in this study were derived from the p53 gene sequence. The reason for selecting exon 5 of the p53 tumor-suppressor gene was based on the finding that this region of p53 contains the multiple hot-spot characteristics for lung cancer

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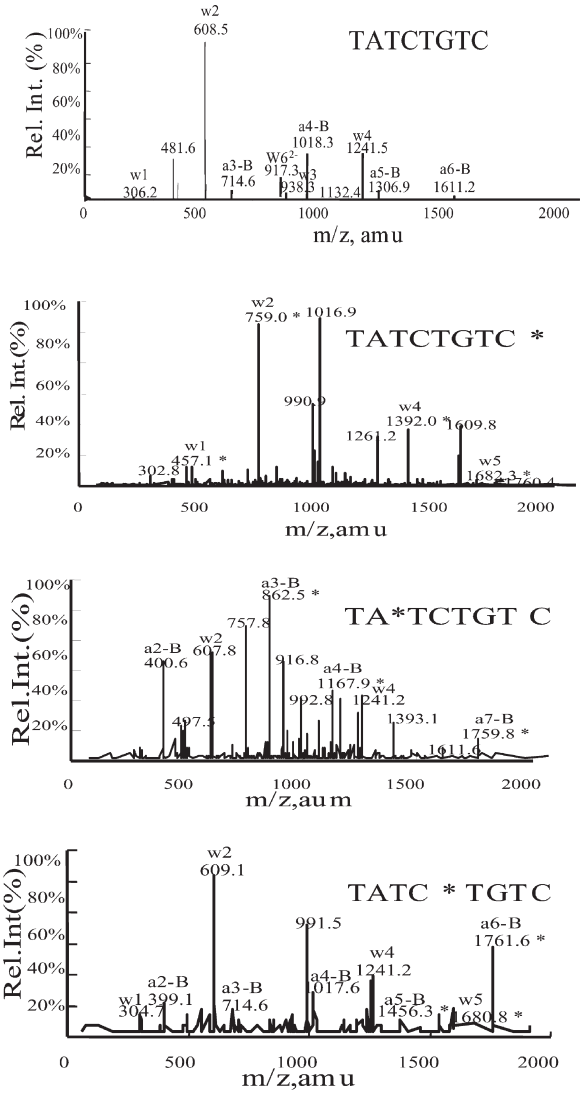

Figure 4. Fragment ion spectra for $[M-2H]^{2-}$ ions of unmodified I and modified oligonucleotides I-1 to I-3, obtained by on-line HPLC/MS/MS. Asterisk * indicates ions carrying the modification.

(codon 157 and 158). Hecht has reported that other highly reactive pre-mutagenic components may also react preferentially at such hot-spots.[30] For example, mutations attributed to BaP and aminobiphenyl exposure have been observed in the same codons in the p53 gene.[31] Hence, it is important to investigate the susceptibility of this sequence to the adduction of PGE. One of the oligonucleotides is 5′-ACCCGC**G**$_7$TCC-3′, which corresponds to a portion of exon 5 of the p53 tumor-suppressor gene that contains the codon 157 mutation hot-spots with two Gs.[32,33] The results of LC/MS analysis of the reaction mixture of PGE with 5′-ACCCGC**G**TCC-3′ are shown in Fig. 5; two major peaks (II, II-1) eluted at 15.24 and 19.5 min, and the corresponding $[M-3H]^{3-}$ ions were observed at *m/z* 981.7 and 1031.8, respectively. The mass shift from *m/z* 981.7 to 1031.8 should correspond to the adduction of one molecule of PGE to the oligonucleotide. A comparison of MS/MS spectra of the $[M-3H]^{3-}$ ions of the modified and unmodified oligonucleotides is shown in Fig. 6; the fragments w1, w2 and w3 are the same while the fragments w4 to w8 are different, and the fragments a2-B to a7-B are the same whereas the a8-B ions are different. The absence of modification in the w1 to w3 and a2-B to a7-B series, as well as observation of the modified fragment ions w4 to w8 and a8-B, indicate that the covalently modified site was located at G_7, which is a known hot-spot in codon 157. This result is in contrast to the case of the first sequence investigated (see above), in which A and C were more readily modified than T and G. The reason may be that the higher-order structures of oligonucleotides can influence the chemical selectivity of PGE towards potential modification sites.

Another sequence derived from the p53 gene is 5′-ACCCGCGTCCGCGCC-3′ which includes the codon 157 and 158 mutation hot-spots, with four Gs. The TIC chromatogram of the reaction mixture of III (5′-ACCCGCGTCCGCGCC- 3′) with PGE is presented in Fig. 7. In addition to the unmodified oligonucleotide eluted at 6.2 min, three PGE adducts were formed and eluted at 12.0, 15.2 and 17.6 min. Three isomeric

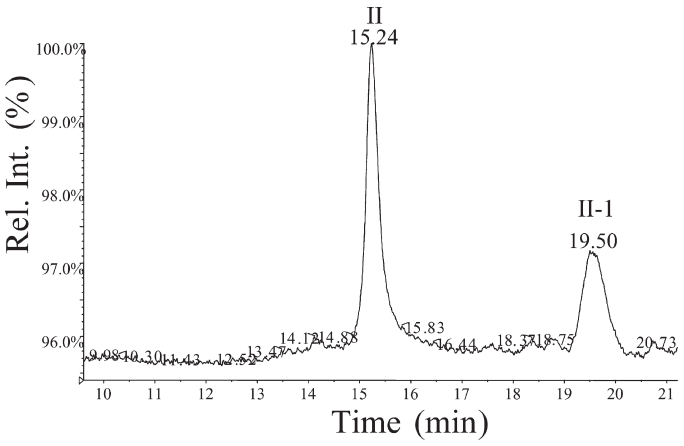

Figure 5. LC/negative ion ESI-MS chromatograms obtained from the injection of a reaction mixture of PGE with oligonucleotide II (5′-ACCCGCGTCC-3′). Peak II is the unmodified oligonucleotide and II-1 is the modified version.

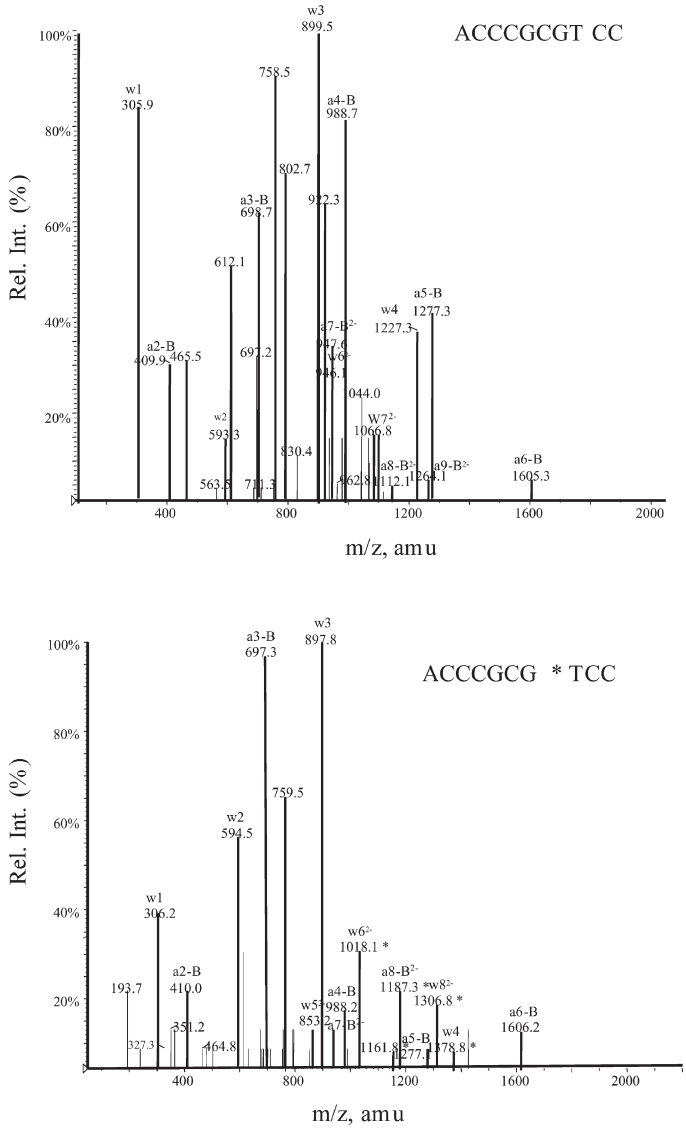

Figure 6. Fragment ion spectra for $[M-3H]^{3-}$ ions of unmodified II and modified oligonucleotide II-1, obtained by on-line HPLC/MS/MS. Asterisk * indicates ions carrying the modification.

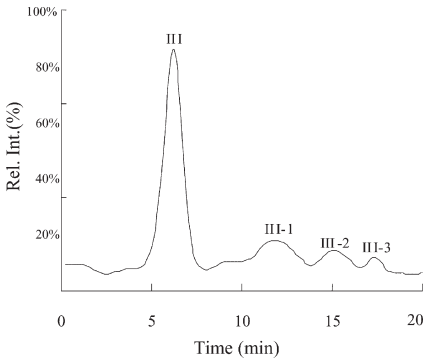

Figure 7. LC/negative ion ESI-MS chromatograms obtained from the injection of a reaction mixture of PGE with oligonucleotide III (5′-ACCCGCG-TCCGCGCC-3′). Peak III is the unmodified oligonucleotide, and III-1 to III-3 are modified versions.

adducts (III-1, III-2 and III-3) were identified by their $[M-3H]^{3-}$ ions, all at *m/z* 1539.4, while that of the unmodified oligonucleotide was observed at *m/z* 1489.5. The mass shifts from *m/z* 1489.5 to 1539.4 again indicated the adduction of one molecule of PGE to the oligonucleotide. In addition, the TIC intensity of this oligonucleotide was much weaker than those of the other two sequences discussed above. This may be due to the fact that this sequence is more hydrophilic because of the high content of G and C; it is known that, in general, the more hydrophilic an analyte is, the weaker its ESI signal intensity.[32] The MS/MS fragmentation data for the$[M-3H]^{3-}$ ions of the modified and unmodified oligonucleotides are listed in Table 1. The fragment ons that identify the modification positions are shown in bold font in this table. The indicated fragment ions of III-1 (5′-ACCCG$_5$C**G$_7$*** TCC**G$_{11}$**CG$_{13}$CC-3′) locate the adduction site at G7, since fragments a7-B to a10-B and w9 to w12 were different from those of the unmodified oligonucleotide whereas a2-B to a6-B and w1 to w8 were the same as for the unmodified sequence. The other two PGE-modified oligonucleotides were also identified by analysis of the MS/MS data as described above. Table 1 indicates that the other two modified sites were at G_{11} (5′-ACCCG$_5$C**G$_7$** TCC **G$_{11}$***CG$_{13}$CC-3′) and at C_4(5′-ACC**C$_4$*** GCGTCCGCGCC-3′). These results show that two of the covalently modified sites were located at the hotspots G_7 and G_{11} in codons 157 and 158 in the p53 gene, and thus may explain the occurrence of some relevant diseases in environments involving exposure to PGE. In contrast with the other p53 sequence discussed above, PGE can be adducted at C (C_4) in this sequence. This may be related to the higher GC content, which will tend to more readily form secondary structure.[34] It is known that secondary structure and sequence can affect adduct formation.[7,35]

In conclusion, we have shown that PGE can be covalently bound to oligonucleotides, and have identified the sites of PGE adduction with three oligonucleotides, for the first time to our knowledge. The adduction positions were related to the sequences and secondary structures of the target oligonucleotides.

However, it must be noted that the experiments performed in the present work involved only single-stranded oligonucleotides. Although in a biological system the carcinogen is presented to the double-stranded DNA, the present work can be still used as reference information for studies of the PGE-modification of DNA.

Acknowledgements

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Table 1. MS/MS data for the $[M-3H]^{3-}$ ions of the unmodified (III) and modified (III-1 to III-3) oligonucleotides

Fragments	III	III-1	III-2	III-3
a2-B	410.1	410.1	409.6	410.5
a3-B	698.6	698.7	697.3	698.6
a4-B	988.3	987.5	987.2	**1136.7**
a5-B	1278.1	1277.6	1276.8	**1428.7**
a6-B	1606.0	1605.6	1605.1	**1756.2**
a7-B	1895.0	**2046.8**	1865.3	nd
$(a8-B)^{2-}$	1112.0	**1187.2**	nd	nd
$(a9-B)^{2-}$	nd	**1339.1**	1264.8	nd
$(a10-B)^{2-}$	nd	**1483.6**	1408.7	nd
$(a11-B)^{2-}$	nd	nd	**1648.1**	nd
$(a12-B)^{2-}$	nd	nd	nd	nd
W1	305.3	306.1	304.4	304.9
W2	595.2	596	593.6	593.8
W3	924.2	923.1	923.4	924.4
W4	1212.5	1210.9	1213.6	1213.8
W5	1542.3	1542.6	**1692.8**	1541.2
$W6^{2-}$	914.8	914.9	**990.2**	nd
$W7^{2-}$	1059.1	1059.5	**1135.6**	1059.4
$W8^{2-}$	1212.5	1212.3	**1287.9**	nd
$W9^{2-}$	1375.3	**1451.2**	nd	1375.5
$W10^{2-}$	nd	**1596.1**	nd	1521.6
$W11^{2-}$	1685.9	**1760.2**	nd	nd
$W12^{2-}$	nd	**1904.7**	nd	nd

Three peaks are from the chromatogram depicted in Fig. 7. Boldface means presence of the modification in the indicated fragment.

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