Identification of Components in Waste Streams by Electrospray and Tandem Mass Spectrometry

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Highly polar, non-gas-chromatographable compounds have few unambiguous analysis protocols for environmental applications. A recent environmental investigation, concerning the identification of a non-gas-chromatographable yellow component in chemical waste water and in effluents from a biological wastewater treatment plant required the use of a number of analytical approaches. Electrospray mass spectrometry, tandem mass spectrometry, high-performance liquid chromatography, nuclear magnetic resonance, and molecular spectroscopy of commercial and synthesized chlorodinitrophenol isomers were required in order to identify the specific isomer causing the color. The present report summarizes the electrospray ionization and tandem mass spectrometric studies that were used. The mass spectrometric study shows that two different isomers of chlorodinitrophenol exhibit very different collision-induced dissociation (CID) spectra. Differences in the tandem mass spectra can be attributed to the different structures of the anions formed from these two different isomers. Instrumentation that uses electrospray ionization and produces CID mass spectra and optical absorption spectra in a single analysis may be required in order to produce highly specific information on non-gas-chromatographable compounds found in the environment. *(J Am Soc Mass Spectrom 1993, 4, 604–610)*

Highly polar, non-gas-chromatographable compounds are important in environmental studies. With the exception of draft Environmental Protection Agency (EPA) Method 553 for benzidine and substituted benzidines and three SW-846 methods,* no other standard methods are available for the large number of small (i.e., molecular weights < 400 u) non-gas-chromatographable molecules that may be present in environmental matrices.

Because the need exists for the accurate identification of environmental contamination, it is important that instrumental methods which are developed for this class of compounds must produce unambiguous information. The need for unambiguous data was recognized in the late 1970s when the EPA published acceptable methods for the analysis of gas-chromatographable compounds in water [1]. The replacement of packed-column chromatography with capillary chromatography improved the specificity of those originally published gas chromatography mass spectrometric (GC/MS) methods because complete mass spectra and characteristic ion profiles of pollutants could be measured at more specific retention times. The increased specificity has increased the reliability of information on chromatographable compounds found in environmental samples [2]. These highly specific analysis methods are probably the most heavily used in analytical and environmental laboratories.

The lack of standard protocols and the need for extending these protocols to non-gas-chromatographable compounds are demonstrated in the present study, which describes the identification of an unknown compound in process waste streams and in effluents from a waste treatment plant. The compound has a pK_a of less than 3.0, suggesting high polarity and difficulty with GC/MS analysis. This report describes the use of various types of information that may produce definitive information on the presence of other polar compounds in the environment.

A method has recently been drafted by the EPA for drinking-water analysis of compounds with intermediate volatilities. EPA Method 553 [3–6] uses a particle-beam high-performance liquid chromatography (HPLC) interface [7]. Three additional methods have also been proposed for the analysis of non-gas-chromatographable compounds in hazardous wastes [8–10].

A number of studies reported the use of the particle

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* Includes Method 8325 for substituted benzidine, Method 8350 for aromatic sulfonic acids, and Method 8321A for phenoxyacetic acids.

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beam interface in environmental analyses. The use of the particle beam interface for the analysis of selected Appendix IX compounds in environmental matrices was also reported by Cornell et al. [11]. A recent report by Miles et al. [12] describes how the particle beam interface can also be used for a large number of polar, water-soluble compounds in the National Pesticide Survey. A study describing the use of this interface for the analysis of Publicly Owned Treatment Works effluents was also reported by Clark et al. [13]. All of these particle beam interface studies, however, require the target compounds to be volatilized in the ion source and ionized by using electron ionization of the gas-phase target molecule. Thermal instability of the analytes also contributes to variable mass spectra [14].

One important feature of polar solvents in very large field strengths was first described by Iribarne and Thomson [15, 16]. Henion and co-workers [17] used this process for the analysis of small molecules, and Fenn et al. [18] applied it to much larger molecules of biological interest. They showed that ionic species that are present in polar solvents need not be volatilized in order to produce gas-phase ions. Ion evaporation of acidic or basic organic compounds via the electrospray process occurs at room temperature when high electrical fields produce submicron-sized droplets of water containing ion clusters. Solvent evaporation occurs from these droplets, and the large clusters of polar solvents that originally surround the anions and cations are eventually removed. The same process was also described for nonpolar compounds dissolved in nonpolar solvents [19]. This process is thought to occur in thermospray and electrospray processes and can produce desolvated anions, cations, and/or even-electron molecular ions of these molecules, which can then be manipulated in a mass spectrometer.

One limitation of electrospray ionization is that these anions or cations alone do not produce the degree of unambiguous information that is required to identify the exact compound or isomer of the compound. At best, the information gives a formula weight of the suspected contaminant or of an ion cluster of an acid or base form of the contaminant [20]. Further structural information, however, can be obtained through collision-induced dissociation (CID) in the ion source [21–23] or by using tandem mass spectrometry (MS/MS) [16, 24, 25].

Just as with conventional mass spectrometric analyses, to produce more specific information, elution information from HPLC, CID spectra, and/or absorption spectra of the compounds of interest can also be used. Examples of the use of similar types of information in a study of gas-chromatographable compounds in the environment have been reported by Alber et al. [26]. In that study, GC/MS retention times, mass spectra, and absorption spectra were used to identify dinitrophenol isomers in rainwater. In the present study, yellow chlorodinitrophenol isomers were detected in a wastewater treatment plant (WWTP) discharge and from a chemical process. Although the intensity of the color of the WWTP effluent has decreased significantly over the past 5 years, the continued faint yellow appearance of the effluent led to the sampling and analysis studies described in this report. The compounds detected in this study, however, are very polar and acidic and thus could not be analyzed by using normal GC/MS environmental analysis methods.

The electrospray mass spectrometry (ES/MS) and CID information were obtained in the present study in an "off-line" manner. The low concentrations in the WWTP discharge (< 0.5 ppm) precluded obtaining this information in an HPLC/ES/MS and/or HPLC/ES/MS/MS experiment without prior concentration. Therefore, ES mass spectra and CID spectra were produced by infusion of a solution that contained either a standard of a pure compound or a concentrated portion of a liquid chromatographic fraction that contained the compound of interest. On the other hand, adequate sensitivity was available to obtain absorption spectra of various isomers in an HPLC experiment. This latter information was produced by using HPLC with diode array detection. The combined information from these different identification approaches was used to identify the principal compound causing the color in the WWTP effluent. The major isomer present in the wastewater from the chemical process was shown to be the same isomer that was observed in the WWTP effluent. The following sections describe how these techniques were used to draw unambiguous conclusions concerning the identity of the major yellow component of the WWTP effluent.

Experimental

Electrospray Mass Spectrometry

A Sciex API III triple quadrupole mass spectrometer equipped with an atmospheric pressure ion source was used to sample negative ions produced from an ion-spray (pneumatically assisted electrospray) interface. Samples and standards of the chlorodinitrophenols were dissolved in a 1% ammonium hydroxide solution and introduced continuously through the ion-spray interface at the rate of 4 μL/min. Negative gas-phase ions, which were created during nebulization and desolvation of analyte solutions, were sampled through a 100-μm inner diameter (i.d.) conical orifice into the vacuum chamber for mass analysis. The atmospheric side of the conical orifice was bathed with a curtain of high-purity, dry nitrogen gas. This gas prevented contamination of the vacuum system with solvent vapors and atmospheric gases and helped desolvate ions formed during nebulization of sample solutions. Ions entering the mass spectrometer were focused into the mass analyzer and separated from neutral nitrogen molecules that were frozen onto cryogenically cooled surfaces (15–20 K) that surrounded

the first and second quadrupoles. A working pressure of 2×10^{-5} torr was maintained in the analyzer chamber during routine operation of the instrument.

Mass analysis of sample ions was accomplished by scanning the first quadrupole in 0.1-u increments from 50 to 600 u in approximately 5 s. The mass-selected ions were passed through the second and third quadrupoles, which were operated in the radiofrequency-only mode. The multiplier was operated in the pulse-counting mode. Mass spectra were averaged over at least five scans.

Collision-Induced Dissociation

Tandem Mass Spectrometry. Product-ion tandem mass spectra of all anions were acquired by passing the $[M - H]^-$ of individual chlorodinitrophenol compounds, which had been mass selected with the first quadrupole, into the second quadrupole, where they were dissociated by collision with ultrapure argon gas. The third quadrupole was scanned in 0.1-u increments from 10 u up to and including the mass of the parent ion in approximately 5 s, and at least 20 scans were averaged for each tandem mass spectrum. The argon target gas thickness was maintained at 4.9×10^{14} atoms/cm^2 and laboratory collision energies of 50 eV were chosen to obtain the CID spectra presented in this report. The first and third quadrupoles were adjusted to obtain unit mass resolution for all mass and tandem spectra.

Ion Source. CID spectra of sample compounds were also obtained in this study to help identify fragment ions. In one experiment, the ion extraction energy in the ion source of the Sciex API III was increased, and a secondary ion (m/z 144.8) produced from the dissociation of the 2-^{37}Cl-4,6-dinitrophenol anion (m/z 218.7) was selected for MS/MS. These mass spectra were obtained by first adjusting the potential difference in the entrance region of the mass spectrometer to −90 V (−35 V was the normal setting for obtaining mass spectra devoid of fragmentation) to generate fragment ions of the sample compounds. The fragment ions were then subjected to MS/MS analysis, as described above.

Concentration of Waste Treatment Plant Effluent

Eight liters of the lightly colored WWTP effluent were reduced in volume by evaporation from boiling and gentle heat. Preliminary experiments showed that this procedure concentrated the yellow color. The WWTP effluent volume was reduced to approximately 200 mL. Further increase in the intensity of the colored compound was accomplished by using C-18 bonded silica gel.

A column (3-cm i.d. × 13 cm long) with a bottom frit was packed with Worldwide Monitoring C-18 column packing material. Another frit was placed on top of the packing. The C-18 column was packed approximately one-third full and rinsed in the following order: (1) 200 mL methylene chloride (Burdick and Jackson) (distilled in glass), (2) 200 mL methanol, (3) 100 mL deionized water, and (4) 100 mL NaCl saturated water. The 200-mL concentrate of the WWTP effluent was saturated with NaCl using approximately 0.1 g NaCl/mL concentrate. This solution was then eluted through the C-18 column. After elution, the top one-third of the column appeared yellow. The C-18 column was allowed to air dry for approximately 5 min. After drying, the column was then eluted, and the fractions were collected in the following order: (1) 200 mL methylene chloride, (2) 120 mL methanol, and (3) 60 mL acidified water (with HCl) and 60 mL basified water (with KOH).

The methylene chloride was very light yellow; the methanol fraction was a much deeper yellow; and the water fraction was very light brownish yellow. No significant yellow color remained on the C-18 column. The methanol fraction was purified further by using HPLC with fraction collection, as described next.

Liquid Chromatography with Absorption Spectroscopy

The methanol fraction described above was then subjected to HPLC analyses. All HPLC analyses and fraction collections were conducted by using a 0.46-cm i.d. × 15-cm C-18 bonded phase column (Burdick and Jackson, product no. 9511*DK). All analyses were performed on a Hewlett-Packard 1090A liquid chromatograph (Avondale, PA) equipped with a Hewlett-Packard 1050A diode array detector. Although the samples were analyzed by using several slightly different conditions throughout the study, typical liquid chromatographic conditions were the following:

1. Reservoir A contained 0.01 M ammonium acetate buffer adjusted to pH 8.2 with ammonium hydroxide. Reservoir B contained 100% acetonitrile. Reservoir C contained 50% acetonitrile and 50% methanol. The flow rate was 1 mL/min.
2. The initial HPLC solvent composition was 90% A, 5% B, 5% C. Injection volume was 50 μL. This solvent composition was maintained for 4 min and linearly programmed to 0% A, 50% B, and 50% C at 10 min. This composition was maintained until 15 min. The solvents were then linearly programmed to the initial conditions of 90% A, 5% B, and 5% C at 17 min. This composition was maintained for 20 min, and a 2-min hold was added before the next 50-μL injection was made. The monitoring wavelength for data shown in Figure 1 was 230 nm.

Fraction collection was performed using an Isco (Foxy) fraction collector located after the diode array

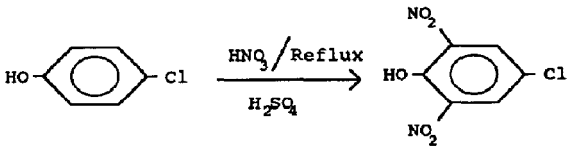
detector. The high-performance liquid chromatogram and component absorption spectra were examined for the presence of yellow compounds. Yellow-colored compounds were defined as compounds with absorption in the 300–500-nm portion of the visible spectrum and no absorption in the 500–600-nm region. Only one yellow peak, with a retention time of approximately 9.5 min, was detected in the methanol concentrate of the WWTP effluent sample. Approximately 250 injections were collected by using this system. The combined fractions were dried under a nitrogen stream in a warm sand bath. This material was subsequently analyzed by ES/MS, MS/MS, nuclear magnetic resonance (NMR), and HPLC.

Synthesis of Compounds

Only one isomer of chlorodinitrophenol (2-chloro-4,6-dinitrophenol, purchased from Aldrich Chemical Co., Milwaukee, WI, with a purity > 99%) was commercially available. The following describes how the 4-chloro-2,6-dinitrophenol and 3-chloro-2,6-dinitrophenol were synthesized to compare their ES mass spectra and CID spectra, HPLC retention times, and optical absorption spectra with those measured in various chemical plant wastewater samples and WWTP effluent samples.

4-Chloro-2,6-dinitrophenol. 4-Chloro-2,6-dinitrophenol was synthesized by nitration of 4-chlorophenol (Aldrich Chemical Co., 99 + % purity) [27]. Approximately 2 g of 4-chlorophenol was dissolved in approximately 75 mL methylene chloride (Burdick and Jackson). Cold concentrated nitric acid was cautiously added through the condenser with a 5-mL pipette bulb. This procedure was repeated until 30 mL nitric acid was added. The solution was refluxed for four different 8-h periods. On the second day of refluxing, approximately 3 mL concentrated sulfuric acid was added to the flask through the condenser, and the solution was refluxed for two more 8-h periods. Methylene chloride was added periodically during the refluxing period to keep the solution in a less flammable environment. The methylene chloride was then removed, and the material was reduced to dryness under a nitrogen stream. Scheme I describes this reaction.

The pK_a of the synthetic 4-chloro-2,6-dinitrophenol was determined by titration to be 2.79 [28], indicating that the compound is very acidic. This pK_a is in very good agreement with literature values measured for this compound (pK_a values of 2.6 and 2.9 have been reported by Dobson [29a] and Bates and Schwarzenbach [29b]). This high acidity is consistent with observations that the compound was (1) inefficiently absorbed by C-18 bonded silica gel, (2) inefficiently extracted by methylene chloride, and (3) non-gas-chromatographable.



Scheme I

Results

Isolation and Analysis of the Yellow Color

Eight liters of the water effluent from the WWTP sample were concentrated by using the techniques described above. It was observed that the yellow color could easily be concentrated by boiling water and passing an NaCl-saturated solution of the concentrate through a C-18 bonded silica gel column. The compounds of color were then eluted from the silica gel column by using methanol and were further purified using HPLC with fraction collection. Figure 1 shows the high-performance liquid chromatogram and the absorption spectrum of the yellow component detected at 9.54 min.

An ES mass spectrum of the highly concentrated effluent sample showed two major ions at m/z 216.7 and 218.7, with relative intensities of 3:1. (The m/z 112.9 ion, which is the major ion in the spectrum, was probably due to the anion of trifluoroacetic acid.) The CID spectrum (Figure 2a) of the major ion (m/z 216.7), which shows losses of NO, NO_2, and (NO, NO_2), and the m/z 217 and 219 ions in a 3:1 ratio in the ES mass spectrum were used to speculate that a chlorodinitrophenol isomer was the most likely source of these mass spectra.

Comparison with Standards

2-Chloro-4,6-dinitrophenol. Although the ES mass spectrum for 2-chloro-4,6-dinitrophenol is very similar to

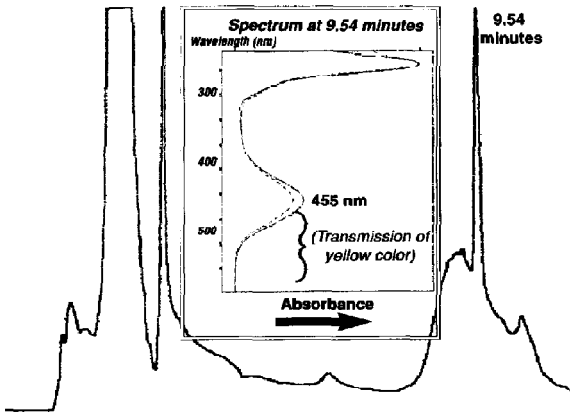


Figure 1. High-performance liquid chromatogram and absorption spectrum of a yellow component in a concentrated WWTP effluent.

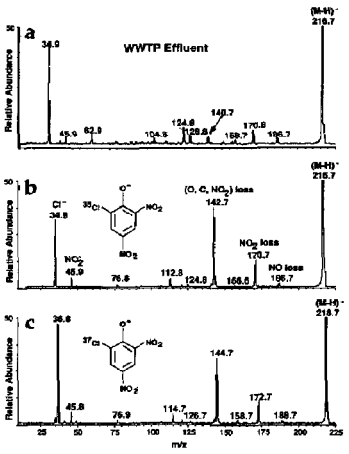


Figure 2. (a) CID spectrum of the m/z 216.7 ion in a fractionated WWTP effluent concentrate and CID spectra of $[M - H]^-$ ions from a 2-chloro-4,6-dinitrophenol standard; (b) m/z 216.7 CID spectrum; (c) m/z 218.7 CID spectrum.

that of the unknown component, the CID spectrum of the m/z 216.7 anion (Figure 2b) appeared to be very different from that shown in Figure 2a. Figure 2c shows the CID spectra for the m/z 218.7 anions from this purchased standard. The probable identities of the major fragments in the CID spectrum of these anions are also shown in Figure 2b. The absorption spectrum (Figure 3) of 2-chloro-4,6-dinitrophenol was also measured by using a diode array detector. Comparison with the absorption spectrum in Figure 1 confirms that the yellow component in the WWTP effluent is not the 2-chloro-4,6-dinitrophenol isomer.

4-Chloro-2,6-dinitrophenol. Figure 3 also shows the absorption spectrum of 4-chloro-2,6-dinitrophenol, which was synthesized from the nitration of 4-chlorophenol. The absorption spectrum and the HPLC retention time

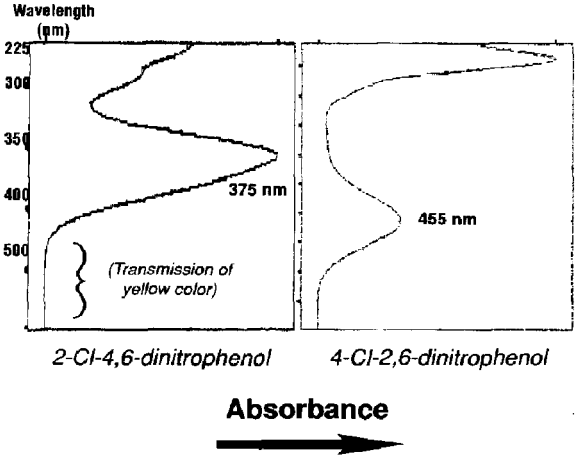


Figure 3. Absorption spectra of 4-chloro-2,6-dinitrophenol and 2-chloro-4,6-dinitrophenol.

agree with that of the compound with yellow color shown in Figure 1. Furthermore, the ES mass spectrum of 4-chloro-2,6-dinitrophenol agrees with the ES mass spectrum of the WWTP effluent concentrate. Finally, the CID spectra of the m/z 216.6 anions from 4-chloro-2,6-dinitrophenol and the WWTP effluent concentrate are consistent (Figure 5). Figure 4 shows the CID spectra of the m/z 216.7 and 218.7 anions from 4-chloro-2,6-dinitrophenol, and summarizes the probable identities of the major fragments.

The major difference in the CID spectra of the two chlorodinitrophenols is the (O, C, NO_2) loss, which results in the m/z 143 fragment in 2-chloro-4,6-dinitrophenol and is completely missing in 4-chloro-2,6-dinitrophenol. To determine conclusively the composition of this fragment, a CID/MS/MS experiment was performed. The extraction voltage in the ion-spray source was increased during the analysis of 2-chloro-4,6-dinitrophenol so that the tandem mass spectrum of the m/z 144.8 fragment could be measured. This latter fragment corresponds to the ^{37}Cl-containing fragment from the CID of the m/z 218.7 anion. The CID spectrum of the m/z 144.8 fragment shows that the m/z 144.8 ion contains at least one chlorine atom and one NO_2 group. This fact leads to the conclusion that the m/z 143/145 fragments shown in Figure 2 are produced by loss of (O, C, NO_2) from 2-chloro-4,6-dinitrophenate.

2-Chloro-4,6-dinitrophenol and 3-chloro-2,6-dinitrophenol. A mixture of 2-chloro-4,6-dinitrophenol and 3-chloro-2,6-dinitrophenol was synthesized by using the method described above. Two major HPLC peaks were observed when 1 g of 2-chlorophenol (Aldrich, 98% purity) and 1 g of 3-chlorophenol (Aldrich, 99% purity) were substituted for the 2 g of 4-chlorophenol in the 4-chloro-2,6-dinitrophenol synthesis. The first component was identified as 2-chloro-4,6-dinitrophenol by

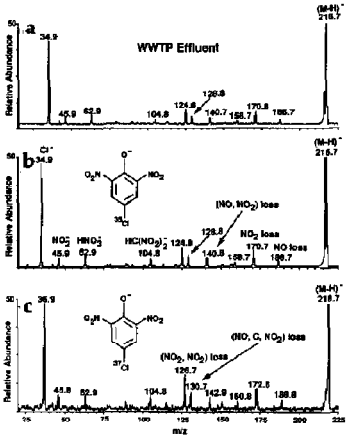


Figure 4. Comparison of CID spectra of m/z 216.7 ions in (a) fractionated WWTP effluent concentrate and (b) 4-chloro-2,6-dinitrophenol standard; and (c) CID spectrum of the m/z 218.7 ion from a 4-chloro-2,6-dinitrophenol standard.

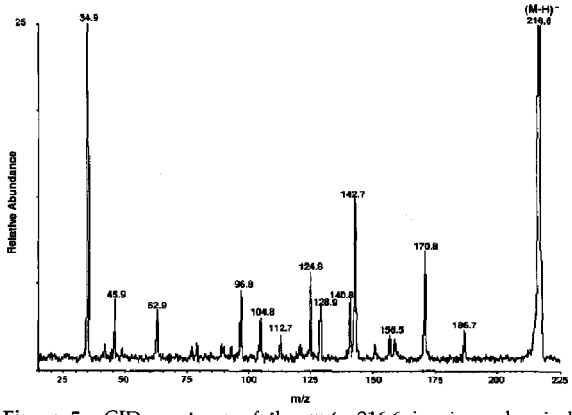


Figure 5. CID spectrum of the m/z 216.6 ion in a chemical waste stream.

comparing retention times and optical absorption spectra with those of an authentic standard. Therefore, the remaining major peak was assumed to be that of 3-chloro-2,6-dinitrophenol, whose retention time and absorption spectrum are very different from that of the synthetic 4-chloro-2,6-dinitrophenol. This mixture was used as a calibration standard to identify the HPLC retention times and absorption spectra of these two isomers.

Nuclear Magnetic Resonance Analyses. The proton NMR spectrum of the WWTP fractionated concentrate indicated that the compound of interest contained at least one strong electron-withdrawing group on an aromatic molecule; there is with one peak downfield at 8.3 ppm. The proton NMR spectrum of the concentrated sample, however, contained a number of extraneous peaks not present in that of the synthesized 4-chloro-2,6-dinitrophenol, and, therefore, these results were inconclusive but consistent with the synthesized 4-chloro-2,6-dinitrophenol. The commercial 2-chloro-4,6-dinitrophenol had an NMR spectrum containing two doublets, indicating equal amounts of two nonequivalent hydrogens in the molecule. These two doublets were not present in the fractionated WWTP effluent, indicating that the hydrogens in the yellow compound were equivalent. Reference NMR spectra for 2-chloro-4,6-dinitrophenol and 4-chloro-2, 6-dinitrophenol published in the Sadtler NMR Library [30] were in good agreement with results obtained from NMR analyses of the commercial and synthetic chlorodinitrophenol compounds [31].

Comparison with a Process Waste

A final set of experiments were conducted to confirm the identity of the chemical process that generated the 4-chloro-2,6-dinitrophenol in the WWTP effluent. The whole effluent from the suspected chemical process was analyzed using HPLC absorption spectra and MS/MS methods. As expected, the HPLC analysis showed a major yellow component whose absorption spectrum matched that of the WWTP effluent concentrate and the 4-chloro-2,6-dinitrophenol standard. A second yellow component, which was much less abundant than 4-chloro-2,6-dinitrophenol, was also detected in the HPLC analysis. The retention time and absorption spectrum of this minor component agree very well with those of the 2-chloro-4,6-dinitrophenol standard. A final confirmation experiment was conducted by using MS/MS. The CID spectrum of the anion (Figure 5) is almost identical to the sum of the two spectra shown in Figures 2b and 4b and is consistent with the presence of two different chlorodinitrophenol isomers in the chemical process wastewater.

Discussion

The CID spectra of the two chlorodinitrophenol isomers shown in Figures 2b and 4b are consistent with those of similar gas-chromatographable nitro aromatics. Electron ionization (EI) and positive and negative ion chemical ionization (CI) spectra of more than 40 nitro aromatics were reported by Feltes et al. [32]. Mononitro aromatics showed significant NO and NO_2 losses in EI mass spectra but lacked these two losses in positive and negative CI mass spectra. EI spectra of dinitro and trinitro aromatics showed less facile abundant NO and NO_2 losses, whereas the positive and negative CI mass spectra contained an order of magnitude increase in these two losses.

CID mass spectra were also reported for a number of positive ion molecular ions of nitro aromatics by Yinon [33, 34], and mechanistic studies of this class of compounds were reported by Meyerson et al. [35]. The latter study (and others referenced therein) report significant differences in fragmentation for ortho-substituted nitro arenes when the substituent contains α-hydrogens. Further differences in NO losses for between meta- and para-substituted compounds were also observed by Bursey and McLafferty [36]. Many of the differences in reactivity of positive ions, which were discussed by Yinon [33, 34], Meyerson et al. [35], Bursey and McLafferty [36], and Beynon et al. [37] involve increased stability of the positive molecular ion owing to resonance stabilization for electron-donating groups in the para position and decreased stability in the meta position.

In the present case, the striking difference between the CID spectra of the anions of the two chlorodinitrophenol isomers studied can also be explained by differences in stability of the two different phenate structures. For the 4-chloro-2,6-dinitrophenol isomer, the two strongly electron-withdrawing nitro groups adjacent to the phenate structure stabilize the negative charge. The 2-chloro-4,6-dinitrophenol isomer, however, has only one nitro group and a more weakly

withdrawing Cl atom ortho to the O^-. The difference in anion stability probably results in the complete lack of (O, C, NO_2) loss from the more stable 4-chloro-2, 6-dinitrophenate ion. More detailed comparisons of CID spectra of other chlorodinitrophenol isomers with isotopically labeled compounds and other mechanistic studies, however, are required to completely understand the role of stabilization of these anions by different substituents.

Conclusions

ES/MS and MS/MS are invaluable tools for understanding complex mixtures containing small non-gas-chromatographable polar molecules. This study has demonstrated how these techniques can be used in conjunction with HPLC and molecular spectroscopy to determine specific substituted phenol isomers that may be present in process wastes. This information can be useful in modifying the process or in developing waste treatment approaches that will improve the environmental quality of chemical processes. The importance of producing unambiguous information on which to base these conclusions cannot be overstated. Without this information, even such a simple system as the one shown in this study would be difficult to completely understand. This points out the need for commercial instrumentation that can provide this type of information in a single experiment so that non-gas-chromatographable analyte identification and quantitation may become as routine as for gas-chromatographable analytes.

Acknowledgments

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