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# LC-MS Analysis of Low Molecular Weight Carbonyl Compounds as 2,4-Dinitrophenylhydrazones Using Negative Ion Mode Electronspray Ionization Mass Spectrometry

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## Abstract

Organic compounds in the environment, such as pesticides, degrade into inorganics *via* transformation into carbonyl compounds, such as formaldehyde, acetaldehyde, and acetone. 2, 4-Dinitrophenylhydrazones of 37 carbonyl compounds were prepared with 2, 4-dinitrophenyl hydrazine (DNPH). Their ESI negative spectra and EI positive spectra were obtained using an HPLC-mass spectrograph. EI positive spectra showed more ions than ESI negative spectra except in the case of two hydrazones. Molecular ions ($M^+$) of carbonyl-DNPHs appeared as a base peak in the EI positive spectra in the case of all but 8 hydrazones. Many significant ions for the identification of a specific carbonyl compound are obtained by ESI negative spectra. Analysis of acetone in various waste waters was successfully conducted using a newly developed ESI negative spectroscopy method—levels found in the samples ranged from 2.1 mg/L to135.0 mg/L. Results of similar tests of the rate of acetone degradation in various water samples showed that acetone in moat water degraded completely after 18 days, whereas 70% of the acetone remained in pure water after 65 days, suggesting that microorganisms may play an important role in the degradation of carbonyl compounds in the environment.

**Keywords:** Low molecular weight carbonyl compounds; HPLC-MS; Negative ion mass spectrometry; Acetone; Wastewater; DNPH derivatives

**Abbreviations:** DNPH: 2, 4-di-Nitro Phenyl Hydrazine; LC-MS: Liquid Chromatography-Mass Spectrometry; HPLC: High Performance Liquid Chromatography; ESI: Electron Spray Ionization; EI: Electron Impact; NMR: Nuclear Magnetic Resonance; GC: Gas Chromatograph; GC-MS: Gas Chromatography-Mass Spectrometry

## Introduction

Organic compounds in the environment, such as pesticides, degrade by oxidation and are eventually changed into inorganics. Carbonyl compounds have been known as an intermediary of this process. For example, it has been proposed that chlorpyrifos and gyphosate breakdown to pyruvic acid and formaldehyde, respectively, in the environment [1]. Moreover, certain aldehydes, such as formaldehyde and acrolein, are known to be carcinogens [2]. Therefore, accurate analysis of these carbonyl compounds is important in order to investigate the fate of organic compounds in the environment. Some low molecular weight carbonyl compounds present in the environment or in biological substances such as blood, tissues, and urine, including formaldehyde, acetaldehyde, acetone, and acrolein, are very difficult to analyze because they are highly reactive and unstable.

Stable derivative methods have been widely applied to determine the presence of these carbonyl compounds [3]. 2, 4-Dinitrophenylhydrazones, which are derivatives of carbonyl compounds with 2, 4-dinitrophenylhydrazines (DNPH), have been most commonly used for the analysis of low molecular weight carbonyl compounds. Gas chromatography is not ideal for analysis of these hydrazones because they have relatively high boiling points or decompose before vaporization. Therefore, HPLC has been used to analyze these derivatives [4–8]. The sensitivity of HPLC detectors has been relatively low compared with that of GC. However, the recent development of LC-MS makes it possible to determine trace levels of reactive carbonyl compounds using these derivatives [9-15].

There are a few reports on the analysis of carbonyl compounds associated with *in vivo* metabolism of certain biological substances by LC-MS using 2, 4-dinitrophenylhydrazones [16,17]. However, there are virtually no reports on the analysis of carbonyl compounds found in the environment by LC-MS using hydrazones. Therefore, development of a selective and sensitive analytical method for these carbonyl compounds as they are found to be present in various samples collected from the environment such as air, water, and soil as well as biological substances including blood, tissue, and urine, is a pressing need. In the present study, a new methodology, LC-MS analysis of low molecular weight carbonyl compounds such as 2, 4-dinitrophenylhydrazone using negative ion mode electronspray ionization (ESI) mass spectrometry, was successfully applied to actual environmental samples.

## Experimental

### Material and reagents

Carbonyl compounds (formaldehyde, acetaldehyde, propionaldehyde acrolein, butyraldehyde, isobutyraldehyde, valeraldehyde, isovaleraldehyde, 2-oxopropanal, benzaldehyde, 2,4-dichlorobenzaldehyde, 3,4-dichlorobenzaldehyde, 5-methyl-2-furfural, 5-bromosalicylaldehyde, glyoxal, glutaraldehyde, acetone, 2-pentanone, 3-pentanone, 3-methyl-2-butanone, 3-hexanone, 2-methyl-2-hepten-6-one, cyclo-

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pentanne, 2-cyclohexen-1-one, 2-hydroxy-3-methyl-2-cyclopentenone, dicyclohexyl ketone, *L*-carvone, *D*-camphor, a-ionone, acetovanillon, 4-acetylbenzoic acid, 2-acetylfuran, acetophenone, 3-aminoacetophenone, 4-methoxyacetophenone, 2',4'-dihydroxyacetophenone, and benzophenone), 2,4-dinitrophenylhydrazine (DNPH), acetonitrile, and methanol were purchased from Waco (Osaka, Japan) or Tokyo Kasei (Tokyo, Japan). All chemicals used were analytical grade purity.

## Preparation of 2, 4-dinitrophenylhydrazones from carbonyl compounds with 2, 4-dinitrophenylhydrazine (DNPH)

A methanol or acetonitrile solution (20 mL) containing a carbonyl compound (100 mg to a few grams) was mixed with a methanol or 10 mL acetonitrile saturated solution of DNPH (3 g/L) in a 50 mL flask. The reaction solution was stirred for 5 min and allowed to stand overnight at room temperature. A precipitate of a product (corresponding hydrazone) appeared in the solution was removed by filtration under reduced pressure. The product was recrystallized from methanol or acetonitrile. Hydrazones prepared were stored at –5°C until used.

## Instruments

**LC-MS:** A Model 2695 Waters HPLC (Boston, MA, USA) equipped with a 150 mm x 2 mm i.d. Develosi PREFULLERENE column and interfaced to a Model ZQ4000 Micromass MS spectrometer (Waters/Micromass, Manchester, UK) was used. Flow rate of the HPLC solvent (water/methanol = 5/95) was 0.2 mL/min. MS conditions were as follows: ionization mode, ESI/negative; ionization voltage, 3 kV; corn voltage, 35 V; ion-source temperature, 100°C; desolvation temperature, 450°C.

**Measurement of EI spectra by direct injection:** A model MStation mass spectrometer (JEOL, Tokyo, Japan) was used. Ionization mode was EI/positive. Ionization current and energy were 300 μA and 70 eV, respectively. Accelerating voltage was 8 kV. Each sample was placed in a capillary column, which was subsequently inserted into an ion source to be vaporized at an increasing rate of 128°C/min.

**NMR:** Formaldehyde-DNPH prepared by the method described above was recrystallized from deuterated methanol ($CD_3OD$) twice. NMR spectra of this compound were obtained using a Model JNM-A500 (JEOL, Tokyo, Japan) 500 MHz NMR spectrometer.

**Method validation for acetone analysis:** The analytical method validation for acetone analysis was conducted according to the previously reported guidelines including the terms of specificity, linearity, sensitivity, and inter-day precision and accuracy [18]. Figure 1 shows a typical chromatogram of an aqueous standard acetone-DNPH solution (0.2 mg/L) obtained using the method described above. The chromatogram shows no contamination and base-line resolution. Figure 2 shows an ESI negative mass spectrum of acetone-DPNH.

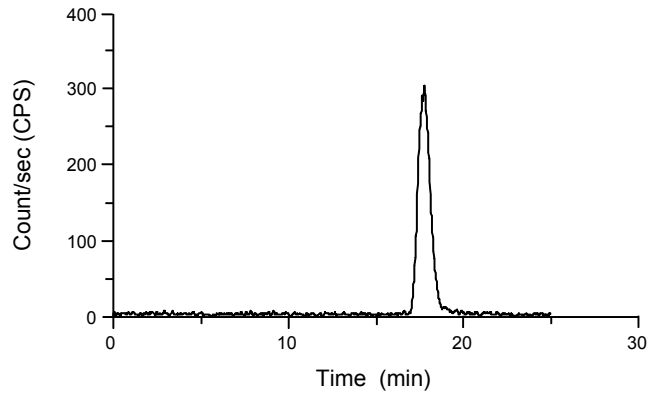


**Figure 1:** A typical chromatogram of an aqueous standard acetone-DNPH solution.

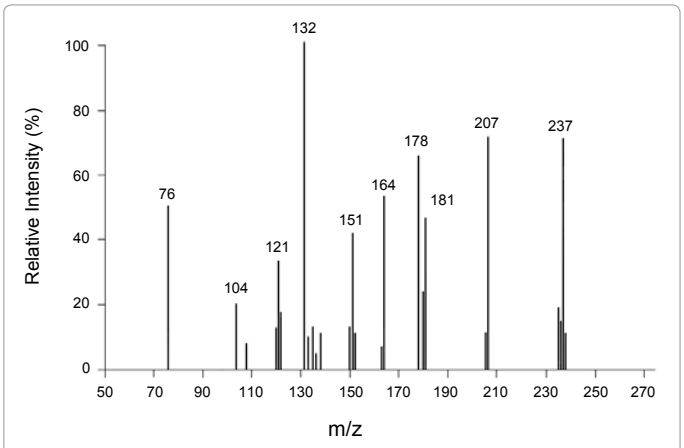


**Figure 2:** An ESI negative mass spectra of acetone-DPNH.

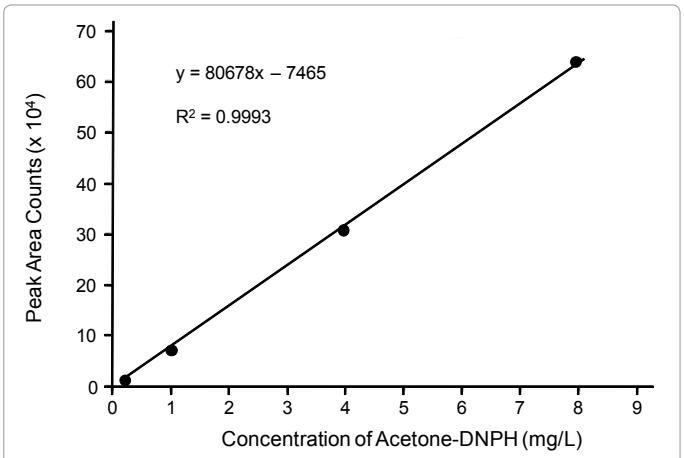


**Figure 3:** The calibration curve used for the quantitative analysis of acetone in water samples.

A four-point calibration curve (y = 80678x – 7465, $R^2$ = 0.9993) obtained from analysis of acetonitrile/water (3/2, v/v) solutions containing a standard acetone-DNPH in four different concentrations (0.2 mg/L, 4 mg/L, and 8 mg/L) is shown in Figure 3. This calibration curve was used for the quantitative analysis of acetone in water samples. The limit of detection (LOD) and the limit of quantitation (LOQ), calculated as 3- and 10-fold the signal-to noise ratio, were 0.2 mg/L and 0.7 mg/L, respectively.

In order to examine the recovery efficiency, an acetonitrile/water (3/2, v/v) solution containing acetone (4.00 mg/L) was analyzed for acetone as acetone-DPNH using the method described above. The recovery efficiency of acetone measured as acetone-DPNH was 92.8 ± 0.9%. The value was mean ± standard deviation (n = 4). The coefficient of variation was 0.01%.

When a treated-water sample spiked with a standard acetone-DNPH (4.00 mg/L as acetone) was analyzed at various time intervals (0, 2, 5, 8, 7, 13, 3, and 12 days) to investigate the inter-day precision and accuracy using the method developed in the present study, the acetone concentration was 4.12 ± 0.12 mg/L (n = 8) and the coefficient

of variation was 2.9%, suggesting that the precision and accuracy of this method were quite satisfactory. The inter-day coefficient of variation is a very important parameter when establishing routine methods for use over extended periods. The results indicate that water samples could be stored nearly three months before analysis, suggesting that analysis can be performed after all samples of interest are collected.

An LC/MS/MS multiple reaction monitoring (MRM) method was used to determine levels of acetone-DPNH in the samples. The analysis was performed using an Applied BioSystems Model 3200Q Trap LC/MS/MS equipped with a 150 mm x 2.1 mm i.d. Inters WP300 $C_{18}$ (5 m) column. The mobile phase was water/acetonitrile (6/4) at 0.2 mL/min. The oven temperature was 40°C. The injection amount was 10 μL. MS conditions were as follows: ionization mode, ESI/negative; ionization voltage, 4.5 kV; corn voltage, 30 V; collision energy, 16 eV; turboion spray temperature, 600°C; monitoring ion mass in case of acetone-DNPH, 236.6 at first stage and 207.1 at second stage.

**Analysis of acetone in wastewater samples collected from a laboratory building on different days:** Waste waters were collected from a large chemistry building (housing 40 laboratories) at the Tokyo University of Science, Tokyo, Japan. The waste waters were drained into a reservoir water tank, in where it was treated by a cleanup unit consisting of a neutralization process, an aeration process, and a coagulation-sedimentation process and then stored. When the tank was filled by the treated water, the stored water was drained through a sewage system. It took approximately 12–24 h to fill the tank. Water samples were collected once a day on different days from effluents before (untreated water) and after (treated water) the above three processes.

The typical sample preparation and the analytical method developed for carbonyl compounds including acetone in the present study are as follows: A 50 μL aqueous hydrochloric acid solution (1 mol/L) and a 30 mL DNPH acetonitrile solution (2.3 g/L) were added to a 20 mL sample solution. The solution was heated at 60°C in a water bath and then allowed to stand at room temperature overnight. The reaction solution (1 mL) was diluted to 20 mL with an acetonitrile/water (3/2) solution, which was subsequently analyzed for a corresponding hydrazone by LC/MS/MS.

**Studies of acetone degradation in various water samples:** Water samples were also collected from various sources for investigation of the acetone degradation in different water samples over prolonged times. They were collected from waste water before processing as described above (untreated water), from waste water after processing (treated water), and water from the moat of Edo Castle in Tokyo (moat water). Water samples were prepared for the acetone concentration to be 80 mg/L. The amount of acetone in the water samples was monitored as a hydrazone derivative over 65 days using a newly developed method.

## Results and Discussion

### Mass spectra of hydrazones

In the soft ionization conducted by LC/MS, generally the formation of quasi-molecular ions and cluster ions associated with a solvent is observed. However, the formation of fragments from the quasi-molecular ion hardly occurred [19-21]. It was hypothesized that two electron-attractive nitro groups reduce the electron density on a benzene ring in a hydrazone molecule (DNPH derivative), which induces electron instability in a molecule, and consequently the fragmentation occur under the soft ionization condition.

Table 1(included as supplementary data) shows ESI negative and EI positive mass spectra of carbonyl compounds- DNPHs. EI positive spectra showed more ions than ESI negative spectra except in the case of acetaldehyde-DNPH, and cyclopentanone-DNPH. All molecular ions ($M^+$) of carbonyl-DNPHs appeared as a base peak in the EI positive spectra except in the cases of valeraldehyde-DNPH, glyoxal-diDNPH, glutaraldehyde-diDNPH, 3-pentanone-DNPH, 3-methyl-2-hepten-6-one-DPNH, cyclopentanone-DNPH, L-carvone-DNPH, and α-ionone-DNPH.

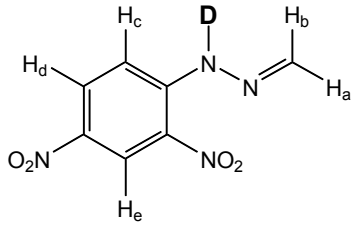


**Figure 4:** Structure of deuterated formaldehyde-DNPH.

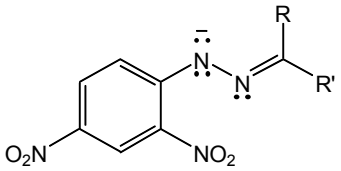


**Figure 5:** Structure of a pseudo molecular ion of carbonyl-DNPH.

Generally, the number of ions in the ESI negative spectra was fewer than in the EI positive spectra. In particular, glyoxal-diDNPH, *L*-carvone-DNPH, and α-ionone-DNPH showed only a few ions in their ESI negative spectra. However, many significant ions for the identification of a specific carbonyl compound are obtained by ESI negative spectra. Formation of a pseudo molecular ion—formed from a molecule losing one proton—was observed as one of the major ions (30–50%) in all ESI negative spectra. The intensity of pseudo molecular ions ranged from 7% (formaldehyde-DNPH) to 72% (acetone-DNPH).

As described above, formation of a pseudo molecular ion was clearly observed in the present study. An EI positive mass spectrum of deuterated formaldehyde-DNPH obtained by a direct injection method showed that one hydrogen on a molecule was replaced with a deuterium. A NMR spectrum of the deuterated formaldehyde-DNPH revealed that the location of this deuterium was bonded with a nitrogen in the hydrazine moiety as shown in Figure 4. The NMR spectral data of deuterated formaldehyde-DNPH are as follows: Ha: 6.722 ppm (d, J = 0.022 Hz); Hb: 7.326 ppm (d, j = 0.022 Hz); Hc: 7.997 ppm (d, j = 0.019 Hz); Hd: 8.355 ppm (dd, $J_1$ = 0.019 Hz, $J_2$ = 0.005 Hz), and He: 9.029 ppm (d, J = 0.005 Hz).

When the ESI negative spectrum of deuterated formaldehyde-DNPH in a deuterated acetonitrile solution was taken, the spectrum exactly matched the one from formaldehyde-DNPH. In this experiment, the samples were injected directly into the ion source using a capillary column in order to avoid the occurrence of hydrogen-deuterium exchange during HPLC. Based on these results, the structure of this pseudo molecular ion was elucidated as in Figure 5.

The ESI negative mass spectra showed several major fragments, which came from the hydrazone moiety. They are m/z = 181, 152, and 122. Figure 6 shows a typical ESI negative mass spectrum of propionaldehyde-DNPH. The proposed formation of these fragments from propionaldehyde-DNPH is shown in Figure 7. It is interesting

that the ESI negative mass spectra of many carbonyl-DNPHs as well as DNPH alone gave m/z at pseudo molecular ion minus 30 as one of the major peaks. The neutral compounds with molecular weight of 30 might be NO molecule, which proposed fragmentation from a pseudo molecular ion of DNPH is shown in Figure 8.

### Analysis of acetone in various water samples

Table 2 shows the amount of acetone analyzed in the untreated and treated waste water samples collected from the laboratory building on different days. Acetone is heavily used to wash glassware (test tubes, flasks, beakers, etc.) used for experiments in laboratories. Samples were collected before (untreated waste water) and after (treated waste water) the treatment process. Some unexpected results were obtained. The amount of acetone in the waste waters did not significantly change from before to after treatment. High concentrations of acetone were detected from waste waters after the treatment, suggesting that the cleanup units used in this laboratory building are not working for acetone.

Figure 9 shows the rate of acetone degradation in various water samples. As described above, untreated water and treated water were water samples collected before and after being placed in a cleanup unit, respectively. Pure water was distilled water. Acetone in moat water degraded completely after 18 days, whereas 70% of the acetone remained in pure water after 65 days. The results indicate that strong microbial activity was present in the moat water. On the other hand, loss of acetone from the pure water may be due to evaporation

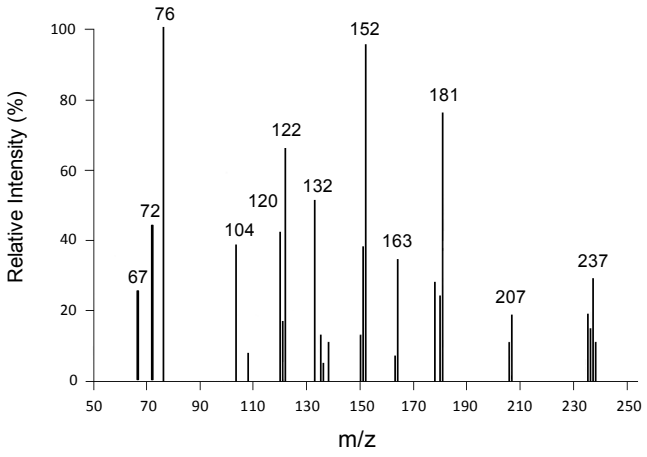


**Figure 6:** Proposed ESI negative mass spectrum fragmentation of propionaldehyde-DNPH.

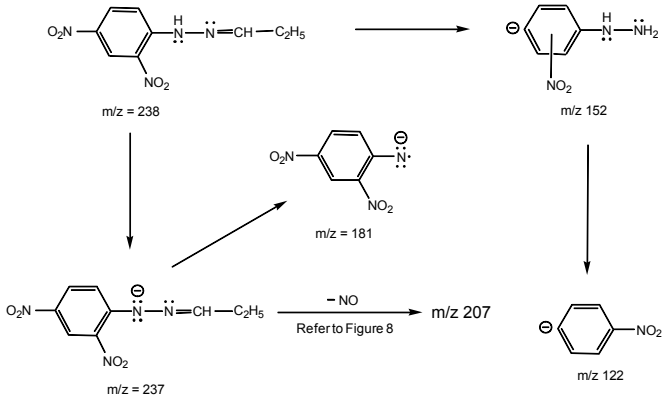


**Figure 7:** Proposed formation of the fragments from propionaldehyde-DNPH.

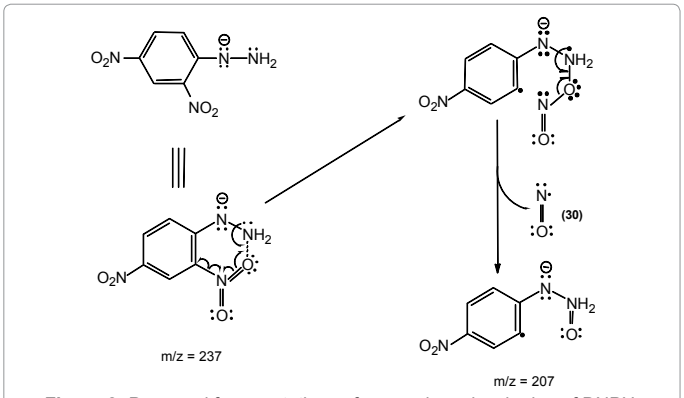


**Figure 8:** Proposed fragmentations of a pseudo molecular ion of DNPH.

| Collection date (2007) | Untreated waste water | Treated waste water |
|---|---|---|
| January/16 | 3.4 | 23.1 |
| /17 | 28.3 | 47.6 |
| /18 | 17.1 | 10.8 |
| /23 | 135.0 | 20.1 |
| /24 | 16.5 | 25.7 |
| /25 | 29.7 | 9.3 |
| /30 | 20.5 | 22.6 |
| /31 | 2.7 | 28.0 |
| February/05 | 3.4 | 2.1 |

**Table 2**: Acetone concentrations (mg/L) in waste waters from the laboratory building.

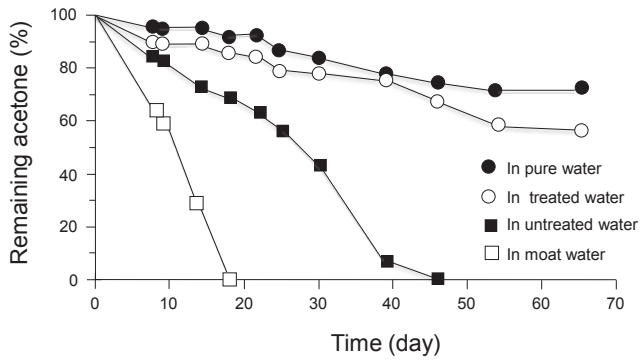


**Figure 9:** Rate of acetone degradation in various water samples.

or transformation to other chemicals because pure water did not contain any microorganisms. Additionally, acetone in the untreated water degraded 100% after 46 days, while nearly 60% of the acetone remained in the treated water after 65 days, suggesting that the level of microorganisms which degrade acetone are removed in the cleanup unit. These results may explain the presence of acetone in waste waters from the laboratory building shown in Table 2.

## Conclusions

Hydrazones, which form from a reaction between carbonyl compounds and DNPH, are stable at room temperature but unstable at higher temperatures and have relatively high boiling points. Therefore, GC or GC-MS is not easy to apply for hydrazone analysis due to these physical natures. On the other hand, with the recent development of high resolution columns and an MS detector, HPLC is seen to analyze these compounds much better than GC. Moreover, the ESI ion method developed in the present study is highly sensitive and selective for

hydrazones. This method was also validated by analyzing the level of acetone in various waste water samples. The microorganisms in the water samples may play an important role in the acetone degradation. However, investigation into the microbial degradation of acetone is outside the scope of this study.

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