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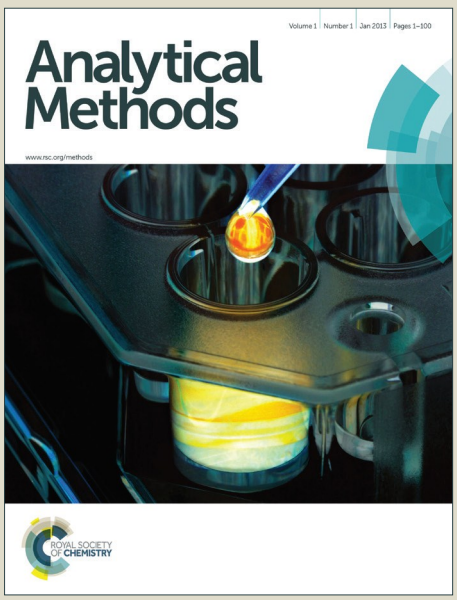

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ARTICLE

Determination of ketoacids in drinking water by DNPH derivatization and LC-ESI-MS/MS

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A quantitative method for determination of ketoacids, including glyoxylic acid, pyruvic acid and ketomalonic acid, in ozonated drinking water was developed by using 2, 4-dinitrophenylhydrazine (DNPH) derivatization combined with liquid chromatography-electrospray mass spectrometry analysis. The optimized derivatization conditions were obtained as: under a molar ratio of DNPH and the total reactive groups at 150, derivatization temperature at 40 °C and derivatization time at 1 h. The method detection limits were 0.5 μg/L for glyoxylic acid, 0.3 μg/L for pyruvic acid and 0.2 μg/L for ketomalonic acid, which were similar to those of the pentafluorobenzylhydroxylamine-gas chromatography-electron capture detection method. The mean recoveries of glyoxylic acid and pyruvic acid were in the range of 94-109%, whereas the inorganic matrix effect reduced the average recovery of ketomalonic acid to 65%. Due to this matrix effect, it is suggested the standard addition method be applied to determination of ketomalonic acid. Finally, the method was field tested by determining ketoacids in water samples collected from a drinking water treatment plant consisting of ozone and biologically active carbon (BAC) processes. The analytical results indicated that ketoacids were formed during ozonation and near completely removed through the subsequent BAC process.

1 Introduction

Ozone has been increasingly used as a strong oxidant and primary disinfectant for drinking water treatment in order to achieve the more stringent water regulations.[1-3] In 1998, only 2% of the water utilities in the United States used ozone as a primary disinfectant, while the percentage increased to 9% in 2007.[4] In China, ozonation in combination with biologically active carbon (BAC) had a total treatment capacity at approximately 20 million m^3/d by 2013.[5] While this technology has been increasing used, more recent studies have showed that ozonation would enhance the formation of halogenated disinfection byproducts (DBPs) during subsequent chlorination or chloramination.[6, 7] This phenomenon is likely caused by the reaction of ozone with natural organic matter (NOM) to form some low molecular weight ozonation byproducts, serving as DBP precursors during subsequent chlorination. Further, ozonation byproducts such as aldehydes, ketones and ketoacids have been detected at a substantial level in ozonated water. They are not only potential DBP precursors, but also are of concern as they may promote bacterial regrowth in water distribution systems.[8, 9] In addition, glyoxylic acid has been proven to be mutagenic.[10] Recently, a number of studies were conducted on the determination of aldehydes and ketones in water samples, whereas little effort has been devoted to quantitative analysis of ketoacids which constitute a major group of ozonation byproducts owing to their analytical difficulties.[11-14]

Common ketoacids in ozonated water include glyoxylic acid, pyruvic acid and ketomalonic acid. GC with electron capture detection (ECD) or mass spectrometry (MS) was commonly used to analyze ketoacids. A pentafluorobenzylhydroxylamine (PFBHA) and diazomethane double-derivatization and GC-ECD analysis procedure has been firstly reported for ketoacids analysis,[8, 11, 15] whereas a carcinogen, N-methyl N'-nitro-N-nitrosoguanidine, is used to prepare diazomethane. Because of the toxicity of N-methyl N'-nitro-N-nitrosoguanidine and the hazardous nature of diazomethane, a PFBHA-acidic methanol derivatization was developed to eliminate the use of diazomethane, while the acid methanol is not an effective methylation reagent for ketomalonic acid.[12] Owing to the double-derivatization and extraction, these GC methods are tedious, labor-intensive and require a great deal of organic solvent. Recently, several other techniques for the determination of ketoacids in various samples have been reported. Ion chromatography (IC) is

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applied to pyruvic acid analysis without derivatization,[16] however its analysis time is as long as 50 minutes. Further, it tends to suffer the interference of other anions such as fluoride, chloride and sulfate in field water samples which result in increased detection limits (DLs) within such samples.[9] Both capillary electrophoresis analysis [17] and enzymic fluorescence capillary analysis [18] need only one simple derivatization step prior to analysis, and have been commonly applied to analyze biological samples such as rat plasma and human urine. However, both methods are not suitable for determination of pyruvic acid in drinking water because of their higher DLs. In recent years, high performance liquid chromatography (HPLC)-MS or tandem MS (LC-MS or LC-MS/MS) has been of interests for its high simplicity and sensitivity. Due to the low molecular weight chemical background presented in MS analysis, a single derivatization with 2, 4-dinitrophenylhydrazine (DNPH) was developed.[14,19-21] So far, the major application of DNPH-LC/MS has been for the analysis of aldehydes and ketones in air and biological samples,[17,18,22,23] but there were only a few reports on these methods for ketoacid analysis in drinking water. Richardson et al.[14] successfully applied DNPH-LC-ESI-MS to identify new, polar DBPs in ozonated drinking water. Later, Zwiener et al.[13] modified this method for quantitative analysis of polar DBPs in water by LC-ESI-MS/MS. These studies mainly focused on aldehydes and ketones, whereas there is a lack of detailed quantitative information on ketoacids, which are formed at much greater concentrations than aldehydes in ozonated water.[8,9]

In this study, a sensitive and specific method for analyzing ketoacids, including glyoxylic acid, pyruvic acid and ketomalonic acid, in drinking water using DNPH derivatization and LC-ESI-MS/MS detection was investigated. The critical reaction conditions used for derivatization and the operating conditions for LC-ESI-MS/MS were investigated. The developed method was demonstrated by testing its application in analyzing fields samples collected from an ozonated drinking water treatment plant.

2 Experimental

2.1. Chemicals

Pyruvic acid (98%) and glyoxylic acid (98%) in liquid form were obtained as individual products from Sigma Aldrich (St. Louis, MO, USA). Sodium ketomalonate monohydrate (98%) in solid form was also purchased from Sigma Aldrich (St. Louis, MO, USA) (under the name of sodium mesoxalate monohydrate). Stock standard solutions (2 g/L in methanol) for the standard substances were prepared and stored in the dark at -20 °C. The working standard solutions were prepared by diluting the stock standard solutions with ultrapure water prior to use. Pyruvic acid-2-^{13}C sodium salt used as the internal standard was obtained from Toronto Research Chemicals Inc. (Toronto, Canada). DNPH, as a 50% water solution, was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Methanol and acetonitrile (HPLC grade) were purchased from Fisher Chemicals (Ohio, USA). Formic acid and ammonium acetate (HPLC grade) were purchased from Sigma Aldrich (St. Louis, MO, USA). Syringe driven filters (0.2 µm) were purchased from Pall Corporation (Ann Arbor, MI, USA). Ultrapure water (18 MΩ/cm) was prepared with a Milli-Q System (Millipore, Tokyo, Japan).

2.2. Sample preparation

As shown in Fig. 1, field water samples were collected at four locations, including raw water, O_3 process influent, O_3 process effluent, and O_3-BAC process effluent, in a drinking water treatment plant in Jinan, Shandong, China on November 20, 2014. Raw water contained dissolved organic carbon in the range 2-3 mg/L. Ozone dosage applied ranged from 1 to 1.5 mg/L. All samples were treated with H_2SO_4 to lower their pH to less than 2.0 for water preservation and were stored in the dark at 4 °C prior to use. All samples were filtered through 0.2 µm syringe driven filters prior to derivatization by DNPH.

2.3. Derivatization

DNPH solution at 3 g/L was prepared by dissolving 600 mg DNPH (50% water solution) in 100 mL acetonitrile. 5 mL of an aqueous sample was first adjusted to a pH 3.0 with either HCl or NaOH. Then 100 µL of a DNPH solution (3 g/L) was added to the sample. This mixture incubated in a water bath (40 ± 1 °C) for 1 h. After derivatization (Fig. 2), 1 mL of the sample was used for analysis using LC-ESI- MS/MS. The chemical structure of the derivatized glyoxylic acid, pyruvic acid and ketomalonic acid were shown in Fig. S1.

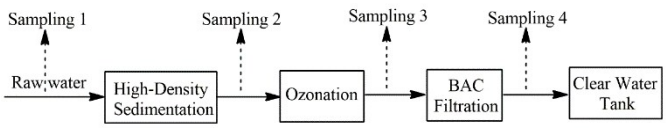
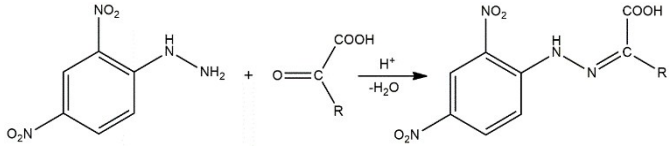


Fig. 1 Flow chart of the drinking water treatment plant.



R：-H, -CH_3, or –COOH.

Fig. 2 Derivatization of ketoacids with DNPH.

2.4. LC-ESI-MS/MS analysis

The LC apparatus was a 1290 series HPLC system (Agilent, USA). Separation of the three ketoacids was achieved using an Agilent Eclipse Plus C18 column (1.8 µm, 50 mm × 2.1 mm). The column was maintained at 35 °C with a flow rate of 0.4 mL/min and the sample injection volume was 5 µL. The mobile phases were ultrapure water containing 10 mM ammonium acetate (A) and acetonitrile (B). The gradient was increased from initial 10% to 60% of solvent B linearly within 5 minutes. Then the mobile phase B increased to 100% during the next 2 minutes and remained at 100% for the next 1 minute. Finally, the gradient returned to the initial conditions of 10% B for 1 minute prior to the next injection, for a total run time of 9 minutes.

MS/MS was performed using an Agilent 6460 triple-quadrupole mass spectrometer (Agilent, USA) equipped with an electrospray ionization (ESI) source in negative ion mode. The optimized MS parameters were as follows: gas temperature, 300 °C; gas flow, 7 L/min; nebulizer, 35 psi; sheath gas heater, 325 °C; sheath gas flow, 11 L/min; capillary, 3000 V. Finally, the data acquisition was performed in multiple reaction monitoring (MRM) mode. The operating conditions used for analysis of three ketoacids were summarized in Table 1.

3 Results and discussion

3.1. Optimizing analytical conditions

The LC conditions, especially the solvent conditions, have a great effect on the separation of target compounds and MS sensitivity.[24] The effects of mobile phase composition on sensitivity and separation for analyzing ketoacids were investigated. Acetonitrile/water, acetonitrile/water containing 0.05%, 0.1%, 0.2% and 0.3% formic acid, and acetonitrile/water with 0.5, 1, 2, 5 and 10 mM ammonium acetate were compared in this study (Fig. S2-S3). Finally, ultrapure water containing 10 mM ammonium acetate was used as the aqueous mobile phase due to the relatively good separation effect and high sensitivity for the three ketoacids.

The yields of hydrazones from the reaction between ketoacids and DNPH are strongly dependent on the molar ratio of DNPH and the total reactive groups (i.e. carbonyl and hydroxyl groups) to make the acid-catalyzed derivatization reach an equilibrium. Excess DNPH at a molar ratio of approximately 150 was sufficient for quantitative derivatization (Fig. 3). The reaction temperature and time were another two critical factors that might affect the acid-catalyzed derivatization. An increase in the yield of hydrazones was obtained by increasing the reaction temperature from 20 to 40 °C, whereas at higher temperatures, the yield of ketomalonic acid derivate decreases (Fig. 4). This decrease may be a result of the volatility, decomposition and/or complexation of the derivate at temperatures

Table 1 Operating MRM conditions for MS/MS analysis of different ketoacids

Compound	DNPH derivative $[M-H]^-$ (m/z)	Product ion for quantification (m/z)	Product ion for qualification (m/z)	Dwell time (s)	Fragmentor (V)	Collision energy (eV)	Cell Accelerate (V)
Glyoxylic acid	253	182.1	122.1	50	78	9/29[a]	3
Pyruvic acid	267	182.1	122.1	50	78	9/29[a]	3
Ketomalonic acid	297	182.1	122.1	50	90	7/35[a]	3
Pyruvic acid-2-^{13}C (IS)	268	182.1	122.1	50	78	9/29[a]	3

[a] two values for quantification and qualification, respectively.

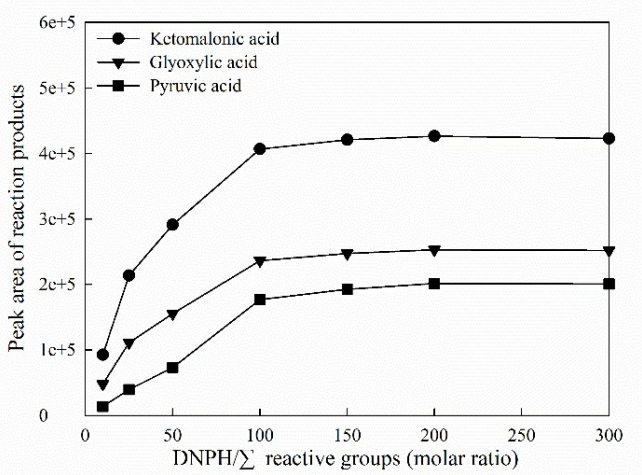


Fig. 3 Effect of the molar ratio of DNPH and the total carbonyl groups on peak areas of reaction products (5 mL of the mixed standard solution of 100 μg/L, pH 3.0, temperature 40 ± 1 °C, derivatization time 1 h).

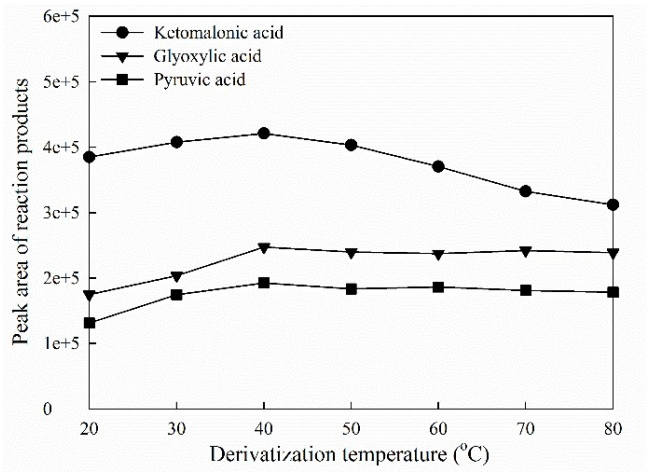


Fig. 4 Effect of derivatization temperature on peak areas of reaction products (5 mL of the mixed standard solution of 100 μg/L, pH 3.0, derivatization time 1 h, molar ratio of DNPH to the total reaction groups at 150).

greater than 40 °C. For the derivatization time experiments, trials ranged from 0.5 to 24 h. Yields of hydrazones substantially increase after1 h, indicating DNPH derivatization with ketoacids is very fast and stable (Fig. 5). Agreeing with previous studies,[13,14] a derivatization time of 1 h was selected in this study.

3.2. Quantification

The method of internal calibration was applied for ketoacid quantification. Pyruvic acid-2-^{13}C sodium salt at 100 μg/L was chosen

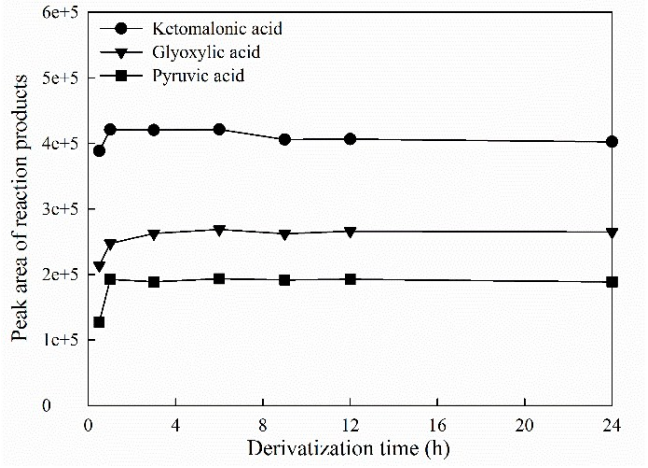


Fig. 5 Effect of derivatization time on peak areas of reaction products (5 mL of the mixed standard solution of 100 μg/L, pH 3.0, temperature 40 ± 1 °C, molar ratio of DNPH to the total reaction groups at 150).

as the internal standard. Calibration curves were constructed in the concentration range 0.5-100 μg/L (the standard concentration levels for ketomalonic acid, glyoxylic acid and pyruvic acid were 0.5, 1, 5, 10, 25, 50 and 100 μg/L). The strong linearity was observed for the three ketoacids with square of correlation coefficients (R^2) being greater than 0.99. The calibration curves were performed in three replicates during a 15-day period, and the relative standard deviations (RSDs) of the slopes for glyoxylic acid, pyruvic acid and ketomalonic acid were 2.7%, 0.4% and 1.8%, respectively. The sensitivity of this analytical procedure was evaluated in terms of the detection limits (DLs), which were calculated (i.e. 3.14 times the standard deviation) from seven replicate analysis of samples containing low levels of analytes. DLs are strongly dependent on the fortified concentration, and the US Environmental Protection Agency (EPA) guidelines require that the ratio of the fortification level to the noise level should be in the range 2:1-5:1.[25] If the ratio is greater, the fortified concentration should be reduced until the criterion is achieved. Therefore, the fortified concentration selected in this experiment was 1.0 μg/L for the three ketoacids. The DLs were estimated to be 0.5 μg/L for glyoxylic acid, 0.3 μg/L for pyruvic acid and 0.2 μg/L for ketomalonic acid, which were similar to those of the PFBHA-GC-ECD method.[11] The quantification limits were defined as 3.3 times the DLs, so the quantification limits for glyoxylic acid, pyruvic acid and ketomalonic acid were estimated to be 1.7, 1.0 and 0.5 μg/L, respectively. This indicated that without pre-enrichment

Table 2 Mean recoveries (R) and relative standard deviations (RSDs) of ketoacids and the instrument precision

Compounds	LFBs (μg/L)	R ± RSD (%)	Instrument precision (%)	
			Intra-day RSD	Inter-day RSD
Ketomalonic acid	5	94 ± 1.6	2.2	7.0
	25	104 ± 5.0	3.9	5.3
	100	103 ± 2.3	0.5	2.9
Glyoxylic acid	5	82 ± 4.1	3.4	6.3
	25	110 ± 1.2	1.5	3.6
	100	97 ± 1.0	0.2	0.9
Pyruvic acid	5	100 ± 3.2	2.8	7.3
	25	103 ± 0.5	0.5	3.1
	100	101 ± 2.2	0.5	1.2

steps, this method was sensitive enough to directly analyze glyoxylic acid, pyruvic acid and ketomalonic acid in ozonated drinking water.

3.3. Method validation

Laboratory fortified blanks (LFBs) which used ultrapure water as blank matrix were chosen to validate the method in precision and accuracy, which was expressed as RSD. Intra-day precision and accuracy was calculated in seven replicate LFBs at the concentration of 5, 25, 100 μg/L for glyoxylic acid, pyruvic acid and ketomalonic acid on the same day. The inter-day precision and accuracy was measured by the aforementioned samples on three consecutive days. As shown in Table 2, the intra- and inter-day precisions of glyoxylic acid, pyruvic acid and ketomalonic acid were below 3.4% and 7.3%, respectively. The mean recoveries of them at low, medium, and high concentrations were within 100 ± 20%, and the RSDs were below 5%. They were all within acceptance criteria set by USEPA, suggesting that the method had remarkable reproducibility and accuracy. In addition, the matrix effect was investigated with laboratory tap water and ozonated water from a pilot plant. All field water samples were filtered through 0.2 μm syringe driven filters before spiking with 100 μg/L glyoxylic acid, pyruvic acid and ketomalonic acid. Experiments were performed in five replicates. The analyte average recoveries in tap water were 99% for glyoxylic acid, 97% for pyruvic acid, 67% for ketomalonic acid, respectively. The average recoveries of glyoxylic acid, pyruvic acid and ketomalonic acid in ozonated water were 97%, 98% and 65% (Fig. S4-S5), respectively. The resulting

Table 3 Mean recoveries (R) of ketoacids from ultrapure water samples containing various ions

Compound	R(%)		
	A	B	C
Ketomalonic acid	101	77	24
Glyoxylic acid	99	100	96
Pyruvic acid	100	105	106

A: Ultrapure water; B: Ultrapure water added 1 g/L NaCl; C: Ultrapure water added 1 g/L NaCl, 0.4 g/L $CaCl_2$, and 0.4 g/L $Mg(NO3)_2$

matrix effect on ketomalonic acid analysis was likely related to the complexation between ketomalonic acid and metal ions such as calcium ion and/or magnesium ion. A series of ultrapure water containing various inorganic ions were investigated to explore this phenomenon. Each sample was spiked with 100μg/L glyoxylic acid, pyruvic acid and ketomalonic acid. As shown in Table 3, the recovery of ketomalonic acid decreased to 24% in ultrapure water containing calcium ion and magnesium ion, which supports the aforementioned speculation. In view of the matrix effect, standard addition method is suggested for the analysis of ketomalonic acid. As for glyoxylic acid and pyruvic acid, no evident matrix effect was observed (Fig. S4-S5, Table 3), indicating that the standard curve method has good reproducibility and accuracy for drinking water samples.

3.4. Determination of ketoacids in ozonated drinking water

The LC-MS/MS method was applied to the determination of ketoacids in water samples collected at four different treatment locations (raw water, O_3 process influent, O_3 process effluent, and O_3-BAC process effluent) in a drinking water treatment plant in Jinan, Shandong, China. Glyoxylic acid and pyruvic acid was determined with standard curve method, while ketomalonic acid was determined with standard addition method. Experiments were performed using three replicates. As shown in Table 4, the concentrations of ketoacids were below the detection limits in the raw water, and ketoacids were formed after ozonation. At the site of ozone application, dosages ranged from 1 mg/L to 1.5 mg/L, and glyoxylic acid was the major ketoacid detected. However, the ketoacids were near completely removed through the BAC process following ozonation, indicating biological degradation. The levels and

Table 4 Concentrations of ketoacids during drinking water treatment processes

Compound	Concentration (μg/L) [mean ± SD, n=3]			
	Raw water	O3 process influent	O3 process effluent	O3-BAC process effluent
Ketomalonic acid	ND	ND	7.3 ± 0.4	1.2 ± 0.1
Glyoxylic acid	ND	ND	23.0 ± 0.5	1.0 ± 0.2
Pyruvic acid	ND	ND	12.3 ± 0.1	1.7 ± 0.1

ND: not detected

behaviors of ketoacids in this treatment plant were similar to these reported in previous studies.[9,11]

4 Conclusions

A fast, sensitive and simple method was developed for the analysis of ketoacids (i.e. glyoxylic acid, pyruvic acid and ketomalonic acid) in ozonated drinking water using DNPH derivatization combined with LC-ESI-MS/MS analysis. This method was validated for its sensitivity, selectivity, accuracy, precision and reproducibility. The main drawback of this method was the inorganic matrix effect on ketomalonic acid, which should be determined using standard addition methods rather than the standard curve methodology. Finally, standard curve method and/or standard addition method was successfully applied in determining ketoacids in ozonated drinking water.

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