A 3-Step Chemiluminescence Method for Chemical Oxygen Demand Measurement

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Direct chemiluminescence emission from the reaction of acidic permanganate and organic compounds was employed for determining the chemical oxygen demand (COD) in water (1-step CL COD). Due to the diversity of organic pollutants in water, there are no standards for COD measurements, and many compounds do not show any chemiluminescence signal in the 1-step CL COD method. As a result, this method shows a low correlation with the conventional CODMn method. In this study, a new 3-step CL COD method was developed to overcome these drawbacks. The basic principle of the 3-step CL COD method is based on the principle of “back titration” in the CODMn method: (i) the sample is treated with permanganate under heating, (ii) the excess permanganate is treated with pyrogallol, and (iii) the excess pyrogallol is measured by the chemiluminescence reaction with permanganate. The reagent concentration, sample volume, and heating temperature were optimized, and the 3-step CL COD method successfully obtained the signal from some samples that cannot be detected by 1-step CL COD method. The calibration graph is linear in the range of 0 - 12.86 mg/L with a detection limit of 0.082 mg/L. This method is continuous, sensitive and low cost compared with the conventional method, and is applicable for on-site monitoring. The effect of the chloride ion was investigated, and showed an insignificant effect after two-times dilution of high-salinity samples. The correlation with the CODMn method for various organic compounds showed a good coefficient of determination, \( R^2 = 0.9773 \) (\( n = 16 \)).

Keywords Chemiluminescence, chemical oxygen demand, 3-step method, acidic permanganate, back titration

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Introduction

Chemical oxygen demand (COD) is used to measure the oxygen equivalent of the organic matter that is susceptible to oxidation by a strong chemical oxidant. COD is widely used as a criterion for evaluating organic pollutants in water. The most common method for measuring of COD is a potassium dichromate method (CODCr), which is well-established as a standard method in many countries. The CODCr method requires 2 - 4 h of refluxing, and employs the use of highly toxic HgSO4 and Cr(VI) chemicals, which are among the most hazardous heavy metals in the environment. In the past, Japan suffered a serious breakout due to heavy metal poison: the Itai-Itai disease caused by cadmium poisoning in Toyama Prefecture, Minatama disease caused the death of more than 900 people, and 2 million suffered neurological disturbances, coma, exhibiting involuntary movements, tremors, agitation, and conclusions from eating fish contaminated with dimethyl mercury. As a result, waste that contains heavy metals needs to be strictly controlled and treated before disposal. To reduce the risk of using toxic heavy metals, a permanganate CODMn method, which uses permanganate and silver nitrate instead of dichromate and mercury sulfate, respectively, is the standard method for COD measurements in Japan. Although, the CODMn method is less time-consuming than CODCr, it still takes approximately 40 min for one measurement. CODMn also requires expensive silver nitrate for screening the effect of the chloride ion. To overcome these drawbacks of the CODCr and CODMn methods, several methods have been developed. In order to reduce the experimental time, a colorimetric determination of excess dichromate after dichromate digestion was employed in the simplified CODCr method. Potassium permanganate-glutaraldehyde chemiluminescence is applied to determine the remaining permanganate after digestion to simplify the CODCr method. An autoclaving sample at 120°C, as well as microwave digestion, was also applied to reduce the digestion time. However, these methods can still not eliminate the use of toxic chemicals. Another non-toxic oxidant (cerium(IV)) and chloride masking reagent (bismuth-based adsorbents) were used as well as employing radiation-assisted sample digestion. However, the back-titration process is still required, which is labor-intensive, and the reproducibility is dependent on a laborious technique. Other analytical methods were reported for the measurement of COD, including atomic absorption spectrometry (AAS), positive potential accompany with quartz UV lamp illumination applied on a TiO2 photocatalytic sensor, silver nitrate instead of dichromate and mercury sulfate, respectively, is the standard method for COD measurements in Japan.
Fourier-transform infrared (FTIR) spectroscopy combined with attenuated total-reflection (ATR) technology.\textsuperscript{21} UV-vis spectroscopy combined with partial-least-squares regression,\textsuperscript{22} near-infrared (NIR) transmission combined with the UV absorbance,\textsuperscript{23} inductively coupled plasma optical emission spectrometry (ICP-OES) with microwave digestion,\textsuperscript{24,25} and single-sweep polarography.\textsuperscript{26} These methods require massive and expensive instruments and, hence, it is difficult to apply for on-site monitoring.

The first chemiluminescence emission from the oxidation of domestic waste water that contains miso, soy sauce, coffee, tea, etc. was found since 1995 in our group. Then, the first application of the permanganate chemiluminescence for measuring organic pollutants and COD was reported by Fujimori.\textsuperscript{27,28} This method, which is referred to the 1-step CL COD method, is based on chemiluminescence emitted from the reaction of the permanganate reagent and organic matter in the water sample, and the use of flow-injection analysis. Due to the fast reaction, the measurement of COD could be carried out in a short time. As a result, the effect of chloride ion was also minimized, and the use of expensive, any hazardous chemical, can be omitted. Because of the simple, inexpensive equipment, the 1-step CL COD method is applicable for the on-site monitoring of organic pollutants in water. However, due to the complexity of organic pollutants present in a water body, there are no standards for the 1-step CL COD method. Many organic compounds also give no signal in the 1-step CL COD method.\textsuperscript{29}

In this study, we focus on an investigation of the reason that compounds have no signal on the 1-step CL COD method and development of a new method, called a 3-step CL COD method, to overcome this disadvantage.

**Experimental**

**Reagent**

The chemical and preparation of solutions used in this study are described in Supporting Information. Deionized (D.I.) water is generated by a Direct-QR 3 UV water system (Millipore SAS, 67120 Molsheim, France) with a resistivity $\geq 18.2$ M$\Omega$ cm was used throughout this work. First 0.96 mM permanganate (reagent R$_1$); then 200 $\mu$M pyrogallol solution; and 0.25 mM permanganate (reagent R$_2$) are used in the first, second, and the third steps, respectively. All permanganate reagent are prepared in sulfuric acid solution.

**Method and apparatus**

The experimental flow injection set. The sample was injected through a six-way valve switch (Rheodyne MXP 7900-000, IDEX Health & Science). A timer (Omron HSS timer switch) was employed to turn on the sample loading pump when an automatic six-way valve was in the loading mode, and turned off in the injection mode. By using this system, each sample was automatically injected into the reaction cell. The flow reaction cell was made of a circular Teflon disk (thickness, 10 mm; diameter, 30 mm). The channel (1.5 mm width, 2.0 mm depth) was machined from the center to the outer in a spiral configuration. The cell volume was 0.396 cm$^3$. A 6-mm diameter single inlet port (in the center of the cell) and the 6 mm diameter outlet port (at the end of the channel) were drilled through the Teflon disk (Fig. 1a). Finally, the Teflon disk was covered by transparent Pyrex glass before it was assembled into the cell holder.

**Mixing point.** To minimize the delay time, the mixing point was designed using a T-shaped connector, which was used in a two inlet ports and one outlet port configuration. The bigger tubes were used for the permanganate inlet port and the outlet port. The smaller tube was used for the sample inlet port, and it passed through the T-shaped connector and the bigger outlet tube. The outlet port of the T-shaped connector was connected to the inlet port of the reaction cell (Fig. 1b). The inlet port of the reaction cell was actually a double-layer tube, which separated the sample solution and the permanganate solution (Fig. 1b). The sample and permanganate solution were mixed just after they entered the reaction cell, and initiated in front of the detector without depending on the position of the T-shaped connector.

A photodiode detector (Kosumo Riken, Ltd.,) was used to capture the emitted light from the reaction cell. The reaction cell was fastened to a stainless-steel cell holder that contained the upper and lower part. (Fig. 1c). The photodiode detector was directed to the reaction cell. Two holes were drilled through the bottom of the lower part, which allowed the inlet and outlet lines to pass through (Fig. 1c). The cell holder was covered by aluminum foil to prevent outside light from entering the detector. **Reaction coils.** Two reaction coils (RC$_1$, RC$_2$) were used in the first and second step of the 3-step CL COD method, and the RC$_3$ was used for cooling the solution after heating. The coils were made from glass tube (i.d., 0.8 mm; o.d., 2 mm) in a helical spring shape that contained 13 turns with a diameter of 2 cm. The solution was heated or cooled by leading it to pass through the coil, which was put in a water bath.

COD$_{Mn}$. Chemical oxygen demand by potassium permanganate (COD$_{Mn}$) is the conventional method for the measurement of COD in Japan. The procedure of the COD$_{Mn}$ method was carried out as described in the standard procedure provided by Japan Industrial Standards (JIS K0102).\textsuperscript{10} A UV-VIS
spectrophotometer (Shimadzu prominence UV/Vis detector SPD-20AV) was used for a kinetics study of the reaction between permanganate ion and chlorine ion.

1-step CL COD. A tygon tube (Saint-Gobain, France) was used to continuously conduct the chemiluminescence reagent and sample into the reaction cell through a peristaltic pump (EYELA MP-2000). These solutions were mixed in the reaction cell, and the emitted light was captured by a chemiluminescence detector. The CL intensity (Volt) is proportional to the formation rate of the excited intermediate of manganese species; as a consequence, it is proportional to the concentration of organic pollutants.

3-step CL COD. The new 3-step CL COD method was developed in this study (Fig. 2). The sample was injected using the described sample injection system once per every 6 min at a flow rate of 3 mL/min. The temperature of the hot-water bath was kept constant with a heater (Magnetic stirrer REXIM RSH-1DN, As One Co.). The flow rates of reagent R1, reagent R2, and pyrogallol were 1.5, 6, and 1.5 mL/min, respectively. Reagent R1 was mixed with the sample, then passed through the reaction coil RC1 in a hot-water bath. The out-coming solution was cooled in RC2, and then was merged with a 200 μM pyrogallol solution in RC3. This solution was then mixed with reagent R2 in the reaction cell through the T-shaped connector mixing point. The bubbles that were formed under the effect of heating could cause the signal to be unstable. A degasser made of a porous Teflon tube (i.d. 1 mm × 5 cm, Sumitomo Electric Engineering) was used to separate the bubbles from the solution.

Results and Discussion

1-step CL COD method

In order to investigate the reason that many compounds do not give a signal in the 1-step CL COD method, the oxidation yield of organic compounds and permanganate was studied. The oxidation yield of organic compounds in the COD_{Mn} method was calculated based on the theoretical COD_{Mn} value and the experimental COD_{Mn} value. The detailed data are given in Table S1 (Supporting Information). For the compounds that have signals in the 1-step CL COD method, the experimental COD_{Mn} yield varies from 65 percent to approximately 90 percent of the theoretical one, depending on the compounds and concentration. While those values for the compounds that cannot be detected by the 1-step CL COD were considerably low, distributing from 1.2 percent to 14.49 percent. The lowest oxidation yield was obtained from benzoic acid at approximately 1 percent (Table S1). Oxalic acid, which can be measured by permanganate titration, could not be detected by the 1-step CL COD method. This probably due to the difference in temperature. For the titration of sodium oxalate by permanganate, it is recommended to be performed at 60°C. From the data in Table S1, the compounds that contained the aliphatic carbon-carbon double bonds (itaconic acid, fumaric acid) showed good CL signals. The highly reactivity of these compounds towards KMnO4 is due to the presence of double bonds. The less-effective π-overlap of the π-bonding causes a weakness of the π-bond compared to the sigma bond. Hence, the permanganate oxidation of carbon-carbon double bonds more easily takes place. Under an acidic condition, the cleavage products predominate over the diols and α-hydroxy ketone products.

Aromatic compounds that contain hydroxyl groups, such as pyrogallol, gallic acid, resorcinol, and sodium salicylate, showed an especially high CL intensity. Phenols and phenolate ions showed particularly strong nucleophilic activity due to the electron-rich aromatic rings and the resonance structure of phenolate ions. As a consequence of the availability of electrons in the phenolic ring, the molecule is easily attacked by an electrophilic reagent (oxidizing reagent). This susceptibility towards the oxidizing reagent increases in the polyhydroxy aromatic compounds. Oxidation of the resonance structure of phenolate ions results in the formation of quinone derivatives. The permanganate oxidizing reagent is known to readily oxidize the alkene carbon-carbon double bond. This oxidation results in diols, alpha-hydroxyl ketones or C=C bond cleaved products, depending on the conditions. Under an acidic condition, permanganate oxidation reactions are likely cleave the C=C bond, which forms a cleavage product. As a result, further oxidation of quinone derivatives probably leads to an opening of the rings at the position of the carbon double bonds, and the products of permanganate oxidation contains carbon dioxide and other organic compounds. On the other hand, the presence of substituted carbonyl groups in aromatic rings results a weak chemiluminescence emission. The oxidation yield of benzoic acid and benzaldehyde are one percent and six percent, respectively (Table S1). This can be explained by the deactivation effect of the electron-withdrawing -COOH and -CHO groups (EWG). The electron-withdrawing groups make the aromatic rings less nucleophilic; as a result, it becomes less reactive towards an electrophilic reagent (KMnO4). In short, the difference in the oxidation yield of the COD_{Mn} method for different organic compounds is because of the difference in the chemical structure.

The continuous-flow system for the 1-step CL COD method is shown in Fig S1, Supporting Information. Because the chemiluminescence signal is reported to be very dependent on the system configuration, a unit of the relative CL intensity is normalized to a signal intensity of 10 μM pyrogallol. The CL intensity of the sample is defined by subtraction of the sample signal height to the blank signal (unit: Volt). Pyrogallol was used as reference compound because the reaction of pyrogallol and permanganate precedes very efficiently, and very strong chemiluminescence was observed. The 1-step CL COD method employs the same reaction with the conventional COD_{Mn} method. Hence, it is expected that the 1-step CL COD method is correlated to the COD_{Mn} method for all organic compounds.
The 12 organic compounds of different chemical structure are: itaconic acid, gallic acid, benzoic acid, methyl propionate, sodium salicylate, fumaric acid, resorcinol, benzaldehyde, potassium hydrophthalate, pyrogallol and oxalic acid. They were used as samples for the 1-step CL COD. Humic acid and fulvic acids are some of the main substances in fresh water; however their molecular weight is unknown, so gallic acid is often used as a model compound in previous studies. In those 12 compounds, oxalic acid and potassium hydrophthalate are used as the primary standard for potassium permanganate and potassium dichromate in the determination of chemical oxygen demand by permanganate and dichromate, respectively. Among them, 6 compounds (benzoic acid, methyl propionate, ethanol, benzaldehyde, potassium hydrophthalate, pyrogallol and oxalic acid) could not be detected by the 1-step CL COD. The correlation for the other 6 compounds between the 1-step CL COD and CODMn was conducted. The result is shown in Fig. 3a with a very low correlation, the $R^2$ value is 0.052 ($n = 22$). The low correlation is due to the difference in the signals of different organic compounds; for pyrogallol the signal is much higher than the others five compounds.

Stop-pump. The main difference between the COD$_{Mn}$ method and the 1-step CL COD method is the reaction time (30 min versus approximately 4 s). This means that in the 1-step CL COD method, the emitted light is only captured in a few early seconds of the reaction. It is expected that the reaction appears to continue after the mixing solution gets out of the reaction cell. The stop-pump experiment was carried out as follows. After the signal became stable, the pump was stopped. Figure 3b shows recordings of the stop-pump signal of itaconic acid (black line) and gallic acid (red line) solutions, respectively. The time zero indicates the time when the pump was stopped. The signal of both compounds increased until it reached the maximum, and then gradually decreased to the blank values. The signal of the itaconic solution reached to the maximum after 1.5 min, while this value for gallic acid was 0.25 min. The difference in the time reaching the maximum is due to the difference in the reaction rate of the two compounds. The signal, which was obtained by stopping the pump, was a part of the kinetics curves of itaconic and gallic acid. The same experiment was carried out for pyrogallol, salicylate, fumaric acid, and resorcinol. The correlation with CODMn was plotted again using the maximum signal. A significant improvement of the correlation was obtained; the coefficient of determination $R^2$ changed from 0.052 to 0.200 (Fig. 3c).

3-step CL COD method

The COD$_{Mn}$ method involves the reaction of the sample with an excess of KMnO$_4$ at 100°C in 30 min. The remaining KMnO$_4$ is determined by a redox back titration, which uses Na$_2$C$_2$O$_4$ as a standard reagent. The 3-step CL COD method, based on a similar principle of the COD$_{Mn}$ method, was developed to remediate the drawbacks of 1-step CL COD.
The principle of 3-step CL COD method is based on flow-injection analysis and the process that resembles the backtitration process. In the first step, organic matter in the sample is oxidized by an excess amount of permanganate reagent under heating. The remaining permanganate is neutralized by pyrogallol, which is used as a standard reagent instead of $\text{Na}_2\text{C}_2\text{O}_4$ in the CODMn method (the second step). In step 3, the remaining pyrogallol was detected by the permanganate chemiluminescence reaction using a photodiode detector. The detection of pyrogallol is similar to the 1-step CL COD method. In the second step, pyrogallol was used instead of oxalic acid because pyrogallol shows strong CL emission in the third step, while oxalic acid does not.

In this study, we did not do the experiment with a 2-step method, because the signal of the remaining permanganate is inversely proportional to the amount of organic substance in the sample. Therefore, the standard deviation of the blank will be high. As a result, the detection limit of the 2-step method is significantly higher than in the 3-step CL COD method.

**The optimization conditions for 3-step CL COD method**

The optimization conditions for acidic permanganate CL were reported to be inconsistent in previous results. It appears that the optimization conditions and the CL signal are much dependent on the system configuration as well as type of acid used. Hence, the signal intensity of 20 $\mu$M pyrogallol was used for normalization of other signals. The optimization of the 3-step CL COD method system was carried out. In the first step of the 3-step CL COD method, the 0.96 mM potassium permanganate ($\text{KMnO}_4$) was used, which can be fully neutralized by the standard reagent (200 $\mu$M pyrogallol) in step 2. The potassium permanganate solution was designed to be in stoichiometric ratio in the reaction with 100 $\mu$M pyrogallol. The optimize concentration of $\text{KMnO}_4$ and $\text{H}_2\text{SO}_4$ in the permanganate reagent used in the third step was carried out with the 1-step CL COD.

**Optimization of $\text{KMnO}_4$.** Pyrogallol was used as a standard compound for optimizing the permanganate concentration. The concentration of $\text{KMnO}_4$ varied from 0.02 to 0.9 mM. Figure 4a shows the dependence of the CL intensity on the concentration of $\text{KMnO}_4$ in solution. The signal intensity increases sharply with an increase in the permanganate concentration, and reaches to the maximum at 0.25 mM. The increase in the reactant concentration (KMnO₄) accelerates the reaction; as a result, the CL intensity increases. A further increase in the concentration of permanganate beyond the maximum value results in a decrease in the signal intensity. This can be explained by the absorption of emitted light by the pink-violet color KMnO₄. Therefore, 0.25 mM was chosen as being the optimum concentration of $\text{KMnO}_4$ for all following experiments.

**Optimization of $\text{H}_2\text{SO}_4$.** The optimization of the sulfuric acid concentration was carried out in the range from 10 to 1000 mM $\text{H}_2\text{SO}_4$ acid; the concentration of $\text{KMnO}_4$ is 0.25 mM. The obtained data are shown in Fig. 4b. From these data, a significant increase in the CL intensity of the signal was observed when the $\text{H}_2\text{SO}_4$ concentration increased from 10 to 200 mM. From 200 to 1000 mM the intensity increased steadily. However, a higher concentration of acid could increase the risk for corrosion to occur on the stainless-steel 6-ways valve. In order to avoid any significant effect on the signal by changing the $\text{H}_2\text{SO}_4$ concentration, 600 mM was chose as the optimum condition.

**Sample loop volume.** The sample loop was made of a PTFE tube, which was connected to two ports of the automatic six-way valve. When the sample loop increased, the volume of sample injected to the reaction cell increased. This results in an increase in the signal intensity. Nevertheless, the signal is normalized by the signal of 20 $\mu$M pyrogallol, so the increase in the sample volume caused the enhancement in the signal of both the sample and the 20 $\mu$M pyrogallol normalized signal. As a
consequence, there was almost no effect on the relative intensity. However, the increase in the signal intensity resulted in an increase in the signal-to-noise ratio, and had a positive effect on the detection limit. Figure 4c shows the change in the absorbance at 525 nm of the sample loop volume increased from 0.5 to 3.75 mL. The S/N ratio increased by 92 (from 40 to 132) when the sample volume increased from 0.5 to 2.5 mL. A further increase in the sample volume beyond the 2.5 mL resulted in a small increase in the S/N ratio. Due to the increase in the sample volume, the measurement time also increased. The corresponding measurement times for above-examined sample volume are given in Fig. 4d. The signal peak width was reported in term of the measurement time. For the 2.5 mL sample volume, the signal peak width was 130 s (approximately 2 min). Therefore, the sample volume of 2.5 mL was chosen for the operating system.

Under the optimized condition, the signals were obtained in a sharp-shape peak. Figure 5 shows a typical signal obtained from the measurement of a 50 μM pyrogallol solution by the 3-step CL COD method under these optimum conditions. The peaks have a symmetry shape without any peak tailing.

**Effect of chloride ion**

The reaction of chloride and permanganate was slow at room temperature.41 Due to the effect of heating and longer reaction time, the interference from chloride ion in 3-step CL COD method was more than in the 1-step CL COD method. To investigate the effect of the chloride ion on the CL signal, the kinetics of the reaction between chloride ion and permanganate was studied using a UV/Vis detector. The time course of the remaining permanganate during the reaction was monitored. The details of the experiment are described in Supporting Information. From the decrease in the absorbance at 525 nm of the reaction solution at 40, 60, and 75 °C, the time dependence of the permanganate concentration was derived using the calibration curve. From the reaction rate law, it follows that:

\[
\frac{d[\text{MnO}_4^-]}{dr} = -k_1[\text{MnO}_4^-]^{a}[\text{Cl}^-]^{b}[\text{H}^+]^{c},
\]

\[
\frac{d[\text{MnO}_4^-]}{dr} = -k_1[\text{MnO}_4^-]^{a}.
\]

Here, \( k_1 = k[\text{Cl}^-][\text{H}^+] \) is the observed rate constant for the disappearance of MnO₄⁻ ion (the change in [Cl⁻] and [H⁺] throughout the experiment can be negligible). A plot ln[KMnO₄] versus time is given in Fig. 6a. We obtained three straight lines with the R² values at 40, 60 and 75 °C were 0.9949, 0.9956 and 0.9863, respectively. According to the differential rate law, the order of the reaction with respect to KMnO₄ is considered to be first order, which is in agreement with previous research.42,43

The observed rate constant \( k_1 \) values at 40, 60 and 75 °C were 0.0068, 0.021, and 0.0493 min⁻¹, respectively. From the Arrhenius equation, we have:

\[
\frac{k_1}{[\text{Cl}^-][\text{H}^+]} = Ae^{\frac{E_a}{RT}},
\]

\[
k_1 = [\text{Cl}^-][\text{H}^+]Ae^{\frac{E_a}{RT}},
\]

\[
\ln k_1 = \ln([\text{Cl}^-][\text{H}^+]A) - \frac{E_a}{RT}.
\]

Here, \( E_a \) is the activation energy; \( R \) is the universal gas constant; \( T \) is temperature in Kelvin; \( A \) is pre-exponential factor. From the graph ln \( k_1 \) versus \( 1/T \) (Fig. S4, Supporting Information), we obtained the value of \( E_a = 51 \) kcal/mol (the slope \( -E_a/R \approx -6143 \)). This value is less than the activation energy previously reported 18 – 20 kcal/mol (75 – 83 kJ/mol),42,43 because of the effect of the Mn²⁺ catalyst. The observed rate constant of permanganate \( k_1 \) at 20, 40, 60, 80, and 100 °C are 0.0018, 0.0068, 0.021, 0.0493, and 0.1575 min⁻¹ (calculated as in the Eq. (6)).

\[
k_1 = e^{\left(\ln[\text{Cl}^-][\text{H}^+]A\right)\frac{E_a}{RT}} = e^{\left(14.62-\frac{E_a}{RT}\right) \text{min}^{-1}}.
\]

The effect of Cl⁻ ions on seawater samples is reported to be less than or equal to 10% of the intensity signal, which is not so drastic; thus, the 1-step CL COD method is suitable for analyzing seawater at an estuary.24 In our research, the effect of
the chloride ion seems to be larger. Hence, optimization of the heating temperature was carried out with respect to reducing the amount of permanganate reacting with the chloride ion. From Eq. (2), it follows that:

\[
\frac{\Delta [\text{MnO}_4^-]}{\Delta t} = -k_1[\text{MnO}_4^-]_0,
\]

\[
\frac{\Delta [\text{MnO}_4^-]}{[\text{MnO}_4^-]} = -k_1\Delta t.
\]

From Eq. (7), the amount of potassium permanganate that is consumed in the reaction was calculated by time at 20, 40, 60, 80, and 100°C. The results are given in Table 1. At 80 and 100°C, the amounts of permanganate reacted after 2 min of reaction were 9.86 and 31.5%, respectively. At 40 and 20°C, the rate of permanganate consumption was slower, after 10 min 6.8 and 1.8 of permanganate were reacted. However, the temperature of 40 and 20°C was near to room temperature, so the effect of heating on the reaction rate of organic substances and KMnO₄ might be insufficient. Therefore, these temperatures cannot be employed for the 3-step CL COD method because of the huge effect of Cl⁻ on the signal. The temperature of 60°C was chosen for heating the reaction coil, because only 6.3% of KMnO₄ is consumed after 3 min of heating. However, the increase in the measurement time is not desirable, so the heating time was not extended in this research (the reaction coil was kept the same as before, which corresponds with approximately 1.5 min heating). Figure 6b shows the effect of the chloride ion on the signal intensity of sodium oxalate at 60°C. An insignificant effect of the chloride ion on the signal intensity of the sample that contains less than 2% of NaCl was observed. For a high-salinity sample, such as seawater (salinity 3.4%), two times dilution will be conducted before any analyses.

Under the optimized conditions, the calibration curve using pyrogallol as the standard was plotted to determine to detection limit of the 3-step CL COD method (Fig. S5, Supporting Information). The detection limit, estimated as three-times signal noise of the blank sample, is 0.082 mg/L (x₁₀₀). The 3-step CL COD method has a linear range up to 12.86 mg/L (using pyrogallol as standard). This linear range could be extended by extending of the first step in the 3-step CL COD permanganate concentration (noted that in this research, the first step reagent R₁ KMnO₄ is in stoichiometric ratio with 100 μM pyrogallol, theoretical CODₘₐₐ value 19.2 mg/L).

### Table 1 Percentage of permanganate consumed in the reaction with chloride ions by time (min) at 20, 40, 60, 80, 100°C

| Time/min | 100°C | 80°C | 60°C | 40°C | 20°C |
|----------|-------|------|------|------|------|
| 1        | 15.75 | 4.93 | 2.1  | 0.68 | 0.18 |
| 2        | 31.5  | 9.86 | 4.2  | 1.36 | 0.36 |
| 3        | 47.25 | 14.79| 6.3  | 2.04 | 0.54 |
| 4        | 63    | 19.72| 8.4  | 2.72 | 0.72 |
| 5        | 78.75 | 24.65| 10.5 | 3.4  | 0.9  |
| 6        | 94.5  | 29.58| 12.6 | 4.08 | 1.08 |
| 7        | 34.51 | 14.7 | 4.76 | 1.26 |
| 8        | 39.44 | 16.8 | 5.44 | 1.44 |
| 9        | 44.37 | 18.9 | 6.12 | 1.62 |
| 10       | 49.3  | 21   | 6.8  | 1.8  |

a. Calculated values.

### Table 2 Chemical oxygen demand data for real samples (obtained from CODₘₐₐ method and 3-step CL COD method)

| Sample | CODₘₐₐ | COD (arb. unit)² | Sample | CODₘₐₐ | COD (arb. unit)² |
|--------|--------|-----------------|--------|--------|-----------------|
| 1a     | 0.4    | 0.144           | 11b    | 5.2    | 0.404           |
| 2a     | 1      | 0.26            | 12b    | 5.6    | 0.315           |
| 3a     | 2      | 0.296           | 13b    | 5.9    | 0.378           |
| 4a     | 4      | 0.316           | 14b    | 7.4    | 0.405           |
| 5a     | 5.2    | 0.322           | 15b    | 14.8   | 0.382           |
| 6a     | 8      | 0.473           | 16c    | 3.1    | 0.392           |
| 7a     | 26.3   | 1.371           | 17c    | 3.5    | 0.32            |
| 8a     | 114    | 4.735           | 18c    | 3.6    | 0.302           |
| 9b     | 1.2    | 0.245           | 19c    | 4.5    | 0.354           |
| 10b    | 4.3    | 0.364           | 20c    | 7.9    | 0.422           |

a. Industrial waste water.
b. Waste water after treatment.
c. River water.
d. 3-step CL COD method.

### Fig. 7 Correlation between the CODₘₐₐ and the 3-step CL COD method for real samples. The concentrations of KMnO₄ chemiluminescence reagent, H₂SO₄, and Mn²⁺ were 0.25 mM, 600 mM, and 75 μM, respectively. The heating temperature was 60°C.

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### Correlation between CODₘₐₐ and 3-step CL COD

Using the developed 3-step CL COD method, the signal of oxalic acid, which is the standard reagent in the CODₘₐₐ method, was successfully obtained. It is noted that, although oxalic acid has a high oxidation yield in the CODₘₐₐ method, it cannot be detected in the 1-step CL COD (Table S1). The correlation between the 3-step CL COD and CODₘₐₐ method was investigated for oxalic acid and 4 other compounds. The results are given in Fig. 3d. Figure 3d shows the good correlation obtained from five organic compounds: Itaconic acid, phenol, gallic acid, pyrogallol, and oxalic acid $R^2 = 0.9773$ ($n = 16$). It is noteworthy that, this correlation is much higher than that of the 1-step CL COD method. In the 1 step CL COD method, the correlation $R^2$ was 0.052 (Fig. 3a).

### Measurement of real samples

Twenty samples of waste water, waste water after treatment and river water (provided by Hiyoshi company, Japan) have been tested using the 3-step CL COD method; the results are given in Table 2. The correlation between the CODₘₐₐ and the 3-step CL COD has been studied, and the result showed a good correlation ($R^2 = 0.9896, n = 20$) (Fig. 7). The highly significant correlation between the two methods suggested that the presented CL method is suitable for the COD determination of...
waste water. A sample of high-molecular-weight compounds, such as lactose, hydroxyl propyl cellulose, corn starch, was also tested with the 1-step and 3-step CL COD methods. The signals were obtained from the 3-step CL COD method, while no signal was observed from the 1-step COD. However, the signal from the 3-step CL COD method was small compared to the equivalent COD_{Eth} value of these samples, because it is difficult to efficiently oxidize high-molecular-weight compounds in a short time by a permanganate oxidizing reagent.

The developed 3-step CL COD method is successful in achieving the signal from compounds that have a weak CL signal, or even no CL signal, in the 1-step CL COD method, such as itaconic acid, oxalic acid. The 3-step CL COD method is applicable for the measurement of real samples, and successful in obtaining signals from some samples that cannot be detected by the 1-step CL COD method. The small signal obtained from high-molecular-weight compounds due to inefficient oxidation of permanganate as an oxidizing reagent suggest the potential of using a stronger oxidizing reagent.

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Supporting Information

Chemicals, solution preparation, theoretical COD_{Eth} calculation, and UV data are given in Supporting Information. Supplementary data related to this article can be found at: http://www.jsac.or.jp/analsci/.

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