The effect of methylene blue on water hardness and antibacterial activity and the environment

O efeito do azul de metileno na dureza da água e na atividade antibacteriana e no meio ambiente

Efecto del azul de metileno en la dureza del agua, la actividad antibacteriana y el medio ambiente

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Abderezak Guemache
Professor of sciences and environment at the University of M'Sila-Algeria/Faculty of Technology/Laboratory of Water, Environment and Renewable Energies
M'Sila. Algeria
E-mail: abderezak.guemache@univ-msila.dz

Drif Mahmoud
Professor of Renewable Energies at the University of M'Sila-Algeria/Faculty of Technology
M'Sila. Algeria
E-mail: mahmoud.drif@univ-msila.dz

Kahoul Fares
Professor of sciences and environment at the University of M'Sila-Algeria/Faculty of Technology
M'Sila. Algeria
E-mail: fares.kahoul@univ-msila.dz

Louanes Hazioui
Professor of sciences and environment at the University of M'Sila-Algeria/Faculty of Technology
M'Sila. Algeria
E-mail: louanes.hamzioui@univ-msila.dz

Temim Djamel
Professor of sciences and environment at the University of M'Sila-Algeria/Faculty of Technology/Laboratory of Water, Environment and Renewable Energies
M'Sila. Algeria
E-mail: djamel.temim@univ-msila.dz

ABSTRACT
This research was carried out to compare complexometric titration methods for the determination of water hardness in the presence of two colored indicators. The complexometric titration with EDTA method has evolved from the typical method of determining water hardness rates used in various references in several countries. Our objective is to make a comparison between Methylene Blue (MB) and Eriochrome black T (EBT) used as color indicators to determine the hardness in drinking water as a function of the flow. Several chemical – physic were used to determine the maximum absorbance of the two dyes UV-visible, identification of functional groups by infrared spectroscopy (FT-IR), supported by the effect of concentration conductivity, pH and turbidity and
density. Test results to determine water hydrometric title (TH) in the presence of two colored indicators indicated that the equivalent volume values ($V_{eq}$) are doubled for the BM indicator compared to the EBT indicator however with impressive TH values in two different intervals between $10 < TH < 20$ and $30 < TH < 50$. This shows that the colored indicator Methylene Blue has important differences. An antibacterial activity is observed on the dye by the disk method on four bacteria in the agar culture medium, from which it can be said that there is sensitivity to antimicrobial agents against methylene blue.

**Keywords:** complexometric, titration, methylene blue, eriochrome black T, FT-IR, UV-visible, hydrometric title, antibacterial activity.

**RESUMO**
Esta pesquisa foi realizada para comparar os métodos de titulação Complexométrica para a determinação da dureza da água na presença de dois indicadores coloridos. A titulação Complexométrica com o método EDTA evoluiu a partir do método típico de determinação das taxas de dureza da água usado em várias referências em vários países. Nosso objetivo é fazer uma comparação entre Azul de Metileno (MB) e Eriocromo preto T (EBT) utilizados como indicadores de cor para determinar a dureza em água potável em função do fluxo. Vários químicos - físicos foram utilizados para determinar a máxima absorbância dos dois corantes UV-visíveis, identificação de grupos funcionais por espectroscopia de infravermelho (FT-IR), apoiada pelo efeito da condutividade de concentração, pH e turbidez e densidade. Os resultados dos testes para determinar o título hidrométrico da água (TH) na presença de dois indicadores coloridos indicaram que os valores de volume equivalente ($V_{eq}$) são duplicados para o indicador BM em comparação com o indicador EBT, no entanto, com valores de TH impressionantes em dois intervalos diferentes entre $10 < TH < 20$ e $30 < TH < 50$. Isso mostra que o indicador colorido Azul de Metileno tem diferenças importantes. É observada atividade antibacteriana no corante pelo método do disco em quatro bactérias no meio de cultura agar, do qual se pode afirmar que há sensibilidade aos agentes antimicrobianos contra o azul de metileno.

**Palavras-chave:** complexométrico, titulação, azul de metileno, eriocromo preto T, FT-IR, UV-visível, hidrométrico title, atividade antibacteriana

**RESUMEN**
Esta investigación se llevó a cabo para comparar los métodos de valoración complexométrica para determinar la dureza del agua en presencia de dos indicadores coloreados. La valoración complexométrica con el método EDTA ha evolucionado a partir del método típico para determinar los índices de dureza del agua utilizado en varias referencias de diversos países. Nuestro objetivo es realizar una comparación entre el azul de metileno (MB) y el negro eriocromo T (EBT) utilizados como indicadores de color para determinar la dureza del agua potable en función del caudal. Se utilizaron varias pruebas químico-físicas para determinar la absorbancia máxima de los dos colorantes UV-visible, la identificación de los grupos funcionales por espectroscopia infrarroja (FT-IR), apoyándose en el efecto de la concentración conductividad, pH y turbidez y densidad. Los resultados de las pruebas para determinar el título hidrométrico del agua (TH) en presencia de los dos indicadores coloreados indicaron que los valores de volumen equivalente ($V_{eq}$) se duplican para el indicador BM en comparación con el indicador EBT, sin embargo, con valores TH impresionantes en dos rangos diferentes entre $10 < TH < 20$ y $30 < TH < 50$. Esto demuestra que el indicador coloreado Azul de Metileno presenta diferencias importantes. Se observa actividad antibacteriana en el colorante por el método del disco sobre cuatro bacterias en medio de cultivo agar, de lo que se puede afirmar que existe sensibilidad a los agentes antimicrobianos frente al azul de metileno.

**Palabras clave:** complexométrico, valoración, azul de metileno, eriocromo negro T, FT-IR, UV-visible, título hidrométrico, actividad antibacteriana
1 INTRODUCTION

Drinking water is an indispensable resource for human life. However, water quality can vary depending on many factors, including the presence of minerals such as limestone. Limestone, also known as calcium carbonate (CaCO$_3$), is a substance commonly found in drinking water (Gurgatz, 2017). In order to measure the level of limestone in drinking water, specific tests are necessary. These tests are performed in the laboratory or with commercially available test kits. They quantify the calcium concentration in the water by measuring the amount of calcium or calcium carbonate present (Balch, 2005). The use of colored indicators in the study of water hardness dates back centuries (Pahlevan, 2022). Scientists and researchers have long sought ways to determine the presence of minerals and ions in water, particularly calcium and magnesium ions that contribute to water hardness (Aram, 2022). Colored indicators are chemical substances capable of reacting with these ions and producing a distinctive staining, allowing their detection and measurement. Colored indicators are chemicals that change color depending on pH or other specific parameters in a solution (Orouji, 2022). They are widely used to determine the hardness of water, which refers to the concentration of minerals, including calcium and magnesium, in water [(Otto, 2021)]. The use of colored indicators in water hardness analysis offers several advantages. First, they allow quick and visual determination of water hardness without the need for sophisticated laboratory equipment. Second, they offer an economical and practical method for monitoring water quality in domestic, industrial and environmental systems (Kozisek, 2020). Among the color indicators commonly used to assess water hardness are complex indicators such as black erichrome T (EBT), calmagite (Nielsen, 2017). These indicators form, color complexes with calcium and magnesium ions present in hard water, allowing visual assessment of mineral concentrations (Karmanovskaya, 2021). Note that the colored indicators provide only a rough estimate of water hardness. For more accurate measurement, more complex titration methods or laboratory analysis may be required. Colored indicators play an important role in the study of water hardness and have wide application in various fields (Bozorg, 2021). Here are some key areas where colored indicators are used: Drinking water testing: Colored indicators are used in water testing laboratories to assess drinking water quality. By measuring the concentration of calcium and magnesium ions, the colored indicators determine whether the water meets the standards of portability in terms of hardness (Dohare, 2014). Water treatment, colored indicators is also used in the field of water treatment to evaluate the effectiveness of the demineralization and water softening processes (Stanciulescu, 2020). By monitoring, color changes due to reduced hardness, operators can adjust the treatment parameters to achieve optimal water quality (Aliasrafi, 2021). In this note, we will study a comparison of an Eriochrome Black T dye (EBT) which is a colored indicator almost exclusively used during the determination of metal...
ions in solution by complexometric in the presence of EDTA in analytical laboratories since the 60s by methylene blue (MB), where we will see the possibility of replacing it with MB dye. Physicochemical analyzes were used to compare the effectiveness of the two indicators with respect to the effect of concentration, the effect of pH, conductivity, turbidity and finally density.

2 MATERIALS AND METHODS

In this work, the complexometric titration of calcium carbonate by EDTA was investigated in the presence of two colored indicators Eriochrome Black T (EBT) and Methylene Blue (MB) for the purpose of determining the hydrometric title (TH). A solution of calcium carbonate at the concentration of 0.1 g/L was prepared in the laboratory. In each experiment, 50ml of CaCO₃ is taken at different concentrations ranging from 10 mg, 20 mg, 30 mg, 40 mg, 50 mg and 5mL of buffer solution at pH =10 is added, followed by a colored indicator (EBT, MB) and the assay is started.

Figure 1. Determination of Hardness by the EDTA method using MB and EBT

Drawing the absorbance λmax curve (UV-Vis spectroscopy)

Using a spectrophotometer, the absorbance of the two colored indicators (EBT and MB) was measured and the maximum wavelengths were obtained, λmax = 550 nm for EBT and λmax = 650 nm for MB, which is the absorbance in terms of change in concentration. The absorption wavelengths at values of λmax >500 nm and therefore there is a bathochromic effect would confirm that the indicators are colored. The wavelength that corresponds to the high absorption is usually called lambda-max (λmax).
Spectral and structural properties

The spectral analysis methods used are UV-Vis spectroscopy and infrared spectroscopy (FTIR). The first step is the determination of the maximum wavelength (λ max) belongs to the 200-700 nm range of the two dyes (EBT, MB). The second step is to determine the crystalline structures and the vibrational energy state of the molecules CaCO₃, EDTA, EBT and MB, will be detected by the IR devices. We studied the effect of the main parameters influencing hardness, the effect of dye discoloration will be noticed by UV analysis, and acidity will be examined by pH, turbidity by turbidity measurement, and conductance by conductimeter and density by a densimeter.

Infrared spectroscopy and UV-Vis spectroscopy for Eriochrome Black T (EBT)

Eriochrome Black T (EBT) is a "black powder, very dark blue, water-soluble. Figure 2 show the UV-visible spectrum and Infrared spectroscopy of the EBT. This spectrum has a maximum absorbance peak λ max = 550 nm, which corresponds to the complementary color of the EBT color. In the infrared spectrum of the EBT dye, there is a low absorption range between 500 and 1700 cm⁻¹ (Pourshirband, 2020). This indicates that the molecular vibrations associated with the functional groups present in this specific range have minimal absorption of infrared energy. However, from the range of 700 cm⁻¹ to 4200 cm⁻¹ the absorption rate increases (BANSAL, 2020). This increased absorption indicates that specific molecular vibrations in this infrared energy range are more active.

Infrared spectroscopy and UV-Vis spectroscopy for Methylene Blue (MB)

Figure 3 shows the UV-Visible absorption spectrum and Infrared spectroscopy of the BM. This spectrum shows the λ max adsorption wavelength at 660 nm (Karuppasamy, 2021). Signature of methylene blue, indicating its absorption (Giovanazzi, 2024). In the infrared spectrum, the bands appeared at 1394 and 1339 cm⁻¹ belong to the aromatic cycles of the BM. The characteristic C=C
band of the aromatic rings observed a 1179 cm\(^{-1}\) in the spectrum of methylene blue appeared at 1175 cm\(^{-1}\) (Verma, 2022).

Figure 3. FT-IR and UV-Vis spectroscopy for Methylene Blue (MB)

![FT-IR and UV-Vis spectroscopy for Methylene Blue (MB)](image)

Table 1. Physicochemical characteristics of used dyes

<table>
<thead>
<tr>
<th>Name</th>
<th>Molecular structure</th>
<th>MW (g/mol)</th>
<th>pH</th>
<th>Color change</th>
<th>(\lambda_{\text{max}}) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eriochrome BlackT(EBT)</td>
<td></td>
<td>461.38</td>
<td>8</td>
<td>Red-Bleu</td>
<td>550</td>
</tr>
<tr>
<td>Methylene Blue(MB)</td>
<td></td>
<td>319.85</td>
<td>10</td>
<td>Blue-Colourless</td>
<td>660</td>
</tr>
</tbody>
</table>

Infrared spectroscopy CaCO\(_3\) and EDTA

Figure 4 shows the interpretation of these spectra to suggest the presence of specific absorptions in this frequency range, which are attributed to the chemical bonds present in the molecule EDTA and CaCO\(_3\). These absorptions are influenced by the characteristic functional groups of EDTA, such as carboxylate and amino bonds. Peak absorption for carbonyl group is 1690 cm\(^{-1}\) for EDTA: Zone 1 (between 400 - 300 cm\(^{-1}\)): most of the elongation vibration bands are present, Zone 2 (between 1300-600 cm\(^{-1}\)). It contains most of the bands of deformation vibration (zone often difficult to analyze, called fingerprint zone) (Dampang, 2021). CaCO\(_3\) is composed of calcium (Ca), carbon (C) and oxygen (O). The peaks in the IR spectrum are due to the absorption of light energy by the chemical bonds present in the CaCO\(_3\) molecules. Calcium calcite or carbonate (CaCO\(_3\)) is characterized by the three C-O elongation moods of the carbonate group, which appear
as a triplet consisting of: - A wide and intense absorption band at 1459 cm\(^{-1}\); - A thin, intense band at 874 cm\(^{-1}\); - A thin and weak band at 712 cm\(^{-1}\) (Chaurasia, 2018).

3 RESULTS AND DISCUSSION

In this work, the complexometric titration of calcium carbonate by EDTA was investigated in the presence of two colored indicators (EBT, MB) for the purpose of determining the hydrometric title (TH). A solution of calcium carbonate at the concentration of 0.1 g/l was prepared in the laboratory. In each experiment, 50ml of CaCO\(_3\) is taken at different concentrations ranging from 10 mg, 15 mg, 20 mg, 25 mg, 30 mg, 35 mg and 5ml of buffer solution is added at pH =10 and then some drops of colored indicator (EBT, MB) and starts the assay until the color change from red to blue for the EBT indicator and from blue to almost colorless for the MB indicator.

**Effect of concentration**

The titrating solution is an ion solution of EDTA (anion ethylenediaminetetraacetate tetravalent and symbolized by Y\(^{-4}\)), that is a sodium salt solution of EDTA, Na\(_4\)Y totally dissociated in 4 Na\(^+\) and Y\(^{-4}\)

\[
\begin{align*}
\text{Ca}^{2+} (aq) + Y^{4-} (aq) & \rightarrow [\text{CaY}]^{2-} (aq) \quad (1) \\
\text{Mg}^{2+} (aq) + Y^{4-} (aq) & \rightarrow [\text{MgY}]^{2-} (aq) \quad (2)
\end{align*}
\]

The hardness of the water is dependent on the amount of calcium salts and magnesium in the water. EDTA is a strong chelator, it «captures» metal ions and forms complexes Mg-EDTA and Ca-EDTA.
\[ TH = [Ca^{2+}] + [Mg^{2+}] \quad (3) \]

\[ 1^\circ TH = 1 \times 10^{-4} \text{ mol.L}^{-1} ([Ca^{2+}] + [Mg^{2+}]) \quad (4) \]

In a very simplified approach, and to know how many drops you need to add, just know that 1 ml is about 30 drops. The number of drops is calculated from the equivalent volume needed for the dosage. The hydrometric title of the water is determined. When water hardness increases, this is usually associated with an increase in calcium ion concentration (Ca\(^{2+}\)). Water hardness is primarily caused by the presence of dissolved calcium and magnesium salts in the water (Guemache, 2023). These salts dissolve in water and release calcium and magnesium ions. However, calcium salts are generally more abundant than magnesium salts, resulting in a higher concentration of calcium ions in hard water. In Figure 5 it is important to note that water hardness in the presence of the colored indicator the MB is majoring. In the values of TH < 15 and in the values of TH > 35 but is linear vis-à-vis the colored indicator EBT in a domain 15 < TH < 25, thus pH values decrease as the concentration of limestone decreases in the water. This is due to the fact that limestone, or calcium carbonate, tends to react with the hydrogen ions present in the water, which reduces their concentration and lowers the pH according to the Figure 6.

Figure 5. Hardness of water for indicators EBT and MB at pH =10

Effect of pH

pH can be used as an indicator of the concentration of hydrogen ions in water at different concentrations. Generally, the pH value decreases as the concentration of limestone decreases in the water (Guemache, 2024) this is due to the fact that limestone, or calcium carbonate, tends to react with the hydrogen ions present in the water, which reduces their concentration and lowers the pH (Figure 6). Therefore, when the limestone concentration decreases, fewer hydrogen ions react with
the limestone, resulting in a decrease in the water pH (SUTOMO, 2019). When EDTA is added in the presence of the EBT and MB indicators in the water, it reacts with the calcium and magnesium ions present in the water. This reaction forms stable complexes between EDTA and metal ions, thus forming insoluble compounds at different turning zones from dark blue to almost colorless blue for MB and from red to blue for EBT. When these EDTA-metal complexes form, they consume the hydrogen ions present in the solution.

![Figure 6. The effect of changing the pH](image)

**Effect of conductivity**

The addition of EDTA in the presence of EBT and MB indicators of water at different concentrations results in a decrease in electrical conductivity (Figure 7). EDTA reacts with the calcium and magnesium ions present in the solution, forming insoluble and stable complexes. These insoluble complexes reduce the concentration of conductive ions in the solution, which leads to a decrease in electrical conductivity (Verma, 2018).

![Figure 7. The impact on conductivity](image)
Effect of turbidity

Water turbidity may be affected by the addition of MB and EBT in the presence of limestone particles. When MB and EBT are added, it can form complexes with the limestone particles present in the solution. The formation of these complexes can cause a decrease in turbidity of the water after reaction, especially when the limestone concentration is high (Figure 8). However, the reaction between MB and EBT and limestone particles can also lead to an increase in water turbidity, especially when the limestone concentration is low. This may be due to the fact that there are fewer particles available to form complexes with MB and EBT, which can lead to increased turbidity after reaction (Guemache et al., 2024). Adding EDTA to water to complex calcium and magnesium ions can help remove CaCO$_3$ particles from water, which can reduce turbidity in water. However, it is important to note that the reduction in turbidity may vary with the CaCO$_3$. At higher concentrations, turbidity reduction may be more significant, as more CaCO$_3$ particles will be suspended in water. At very low concentrations, turbidity reduction may be less pronounced, as there will be fewer CaCO$_3$ particles suspended in water.

![Figure 8. The impact on turbidity](image)

Effect of density

Figure 9 shows it important to note that the increased presence of minerals in water can influence its density. Indeed, minerals add mass to water, which can increase its absolute density. It should be noted, however, that the relationship between water hardness and density is not linear and may be influenced by other factors, such as temperature and the presence of other dissolved substances. Therefore, it is important to consider several parameters for an accurate assessment of water density in relation to its hardness (Chermette, 1999).
Antibacterial activity

Microbiological analysis of Methylene Blue (MB) is to provide information on water quality. Under certain conditions, the quantity of micro-organisms may exceed the tolerance threshold of the organism and be responsible for infections that cause disease [29]. It is therefore essential to take certain precautions to reduce the rate of proliferation of its microorganisms and thus avoid the deterioration of food. These pathogenic microorganisms include viruses, bacteria, protozoons and helminths. In our case, common sense is to express or reduce contamination after treatment. Four strains of bacteria (Pseudomonas Aeruginosa, Klebsiella pneumonia, Escherichia coli, and Staphylococcus aureus [30]. Were used to see the impact of these bacteria. The reading of four boxes of betri incubated at 37°C for 48 hours informs us that water contaminated with insecticide has a domestic use, has a significant antibacterial activity revealed by the rings that locks out bacterial strains of a visible diameter.
4 CONCLUSIONS

In this research, we studied the comparison of two colored indicators, methylene blue and erichrome black T, used in the complexometric dosage in presence of EDTA to determine the hydrometric title of drinking water, and we determined the efficiency of each of them under different initial conditions, where we studied the ability to give the best results at different initial concentrations, at different pH values at different conductivity values, turbidity and also at different density. The results showed with regard to the effect of change of the initial concentration of the colored indicators, that the effect is more important, it is noted that the hardness of the water in the presence of the colored indicator MB is a majority. In addition, the study of the effect of pH on the titration process showed results. When, EDTA is added in the presence of the EBT and MB indicators of water, it reacts with the calcium and magnesium ions present in the water. This reaction forms stable complexes between EDTA and metal ions, thus forming insoluble compounds at different zones of almost identical pH, followed by a change of color at the turning point from dark blue to almost colorless blue for MB and from red to blue for EBT.

When these EDTA-metal complexes are formed, they consume the hydrogen ions present in the solution. With regard to the conductivity effect, the study showed that the results are almost identical in an interval of CaCO₃ concentration >20 mg/L, in the presence of the indicator colored the MB. On the other hand, they are lower for the dye EBT because EDTA reacts with the calcium and magnesium ions present in the solution, forming insoluble and stable complexes.

These insoluble complexes reduce the concentration of conductive ions in the solution, resulting in a decrease in electrical conductivity compared to previous studies, because the process of complexometric titration is proportional to pH. Adding EDTA to water with complex calcium and magnesium ions can help remove CaCO₃ particles from the water, which can reduce water turbidity. However, it is important to note that turbidity reduction can vary with CaCO₃ concentration. However, the reaction between MB and EBT and limestone particles can also lead to an increase in water turbidity, especially when limestone concentration is low. This may be due to the fact that there are fewer particles available to form complexes with MB and EBT, which can lead to increased turbidity after the reaction. It should be noted, however, that the relationship between water hardness and density is not linear and may be influenced by other factors, such as temperature and the presence of other dissolved substances.

It is interesting to note that antibacterial activity is observed on water polluted by the method of discs on four bacteria in the gelosed culture medium from where it can be said that there is a sensitivity to antimicrobial agent’s against the insecticide. In addition, the results obtained during
this study confirm the practical interest of the use of clays in the field of pollution of water contaminated by the pollutants.

Finally, it can be said that methylene blue is a much less toxic product, and also an antidote against nitrite and ammonia poisoning; it has favorable capacities for complexometric titrations for the determination of a mixture of different metal ions in solution. It is interesting to note that the antibacterial activity on methylene blue does not pose sensitivity to antimicrobial agents against the dye. In addition, the results obtained during this study confirm the practical interest of using methylene blue in water analysis laboratories to determine water hardness in water contaminated by pollutants.

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