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QUANTITATIVE BENEDICT TEST FOR REDUCING SUGAR AND MILK ANALYSES

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Abstract: *Benedict method for reducing sugers offers rapid semi-quantitative analysis without dangerous materials. The method is mainly used for determining glucose in urine in cases of suspected diabetes or in other samples. It provides information about the concentration of reducing sugars presented in possible ranges of values. This study optimized the method to become quantitative with numerical values for the color of each test sample. The detection was made without any expensive equipment. A free available mobile phone app was used for color parameter determination. The lactose concentrations from 0 to 2.5% were determined in standard solutions and also in milk. Linear trendlines and R^2 were presentend. The Benedict test was used with other sugars – galactose, glucose amd fructose, that had positive reaction with Benedict reagent, and sucrose and starch that had negative reaction with the reagent. The obtained results are discussed. The proposed method for conversion of semi-quantitative method to quantitative method could be applied for lactose determination on field and also could be adapted for other analytes.*

Keywords: *quantitative method, reducing sugars, saccharide test, milk lactose determination, Benedict* ***test.***

INTRODUCTION

Carbohydrates (sugars) are vital organic components and the organism's main energy source. Due to their structure, carbohydrates could be classified as mono-, di-, oligo- or polysaccharides. The monosaccharides (such as glucose, galactose and fructose) are directly used as energy supply, while disaccharides (such as lactose and sucrose) and polysaccharides (such as starch) serve as energy reserves (Febriyossa et al., 2024).

Lactose (also called milk sugar) is the main carbohydrate in milk and dairy products. Its molecule is composed of one molecule D-galactose and one molecule D-glucose, which are linked with a β-(1,4') glycosidic bond. Milk and dairy products represent huge nutritional value for the human organism, yet some individuals are lactose intolerant and thus cannot consume most of the dairy products. Lactose intolerance represents the condition that individuals are unable to break down lactose to its monomers (Chengolova et al., 2024). Therefore, easy tests for determination and quantification of lactose in different products are needed.

Carbohydrates could be grouped as reducing and non-reducing sugars. Sugars with free carbonyl (aldehyde or ketone) groups are referred to reducing sugars (RS). In alkaline solution the carbonyl group is able to act as an electron acceptor or reductant (Yahia et al., 2019). The chemistry behind RS' reactivity lay on the presence of a hemiacetal functional group in their formulas. As "hemiacetal" is considered a group where an OH group and O-R group are attached to the same carbon atom, while two O-R groups attached to the same atom are called acetal functional group (Figure 1). The hemiacetal sugars open to acyclic aldehydes under basic conditions, while acetals are stable in basic conditions. Carbohydrates which exist as acetals do not revert to aldehydes in the presence of basic solutions, thus they do not reduce the metal ions and are called non reducing sugars (Ouellette & Rawn, 2015). The ketones usually do not oxidize in alkaline conditions; however, fructose makes an exception as it contains an α-carbon with a hydroxyl group (fructose is an α-hydroxy ketone). Thus, the ketone is in equilibrium with an aldehyde through Lobry de Bruyn-Alberda van Ekenstein rearrangement (Angyal, 2001; Ekeberg et al., 2007).

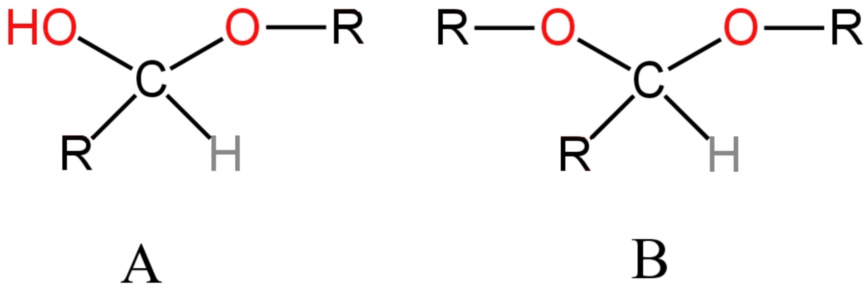

Fig. 1. Chemical structures of hemiacetal (A) and acetal (B) functional groups

Due to the reducing properties of the carbohydrates' carbonyl groups in alkaline medium, RS could be easily detected with a Benedict's test (Figueira et al., 2014). The Benedict's test for reducing sugars was invented by Stanley R. Benedict in 1907 as a method of qualitative detection of reducing sugars (Benedict, 1909, Ratanawimarnwong et al., 2022). Furthermore, the method is the most commonly performed test for diabetes in clinical laboratories because it detects the presence of glucose in urine (Hernández-López et al., 2020; Ratanawimarnwong et al., 2022).

There are other tests for detection of RS. Comparison of Benedict's test and other methods is presented in Table 1.

Table 1. Comparison of different methods for reducing sugar detection

Method	Reagents	Mechanism	Advantages (+) Limitations (-)	Ref.
Folin-Wu method	1. Alkaline copper reagent (Na_2CO_3, tartaric acid and $CuSO_4$) 2. Phosphomolybdic acid reagent (sodium tungstate,phosphomolybdic acid, NaOH and orthophosphoric acid)	- RS reduce cupric ions to cuprous in alkaline medium - obtained Cu_2O reduces phosphomolybdic acid to phosphomolybdous acid (blue) - measurement of optical density at 420 nm	+ partly easy method - use of many acids - inability to differentiate the carbohydrates	Mahesha, 2012
Picric acid test	1. 10% sodium carbonate 2. saturated picric acid solution	RS reduce picric acid (yellow) to picramic acid (red) in alkaline medium	+ easy method - highly acidic solutions - inability to differentiate the carbohydrates	Jain et al., 2024
Fehling's test	1. Fehling's solution I 2. Fehling's solution II	- the Cu^{2+} ions from copper tartrate complex are reduced to copper(I) hydroxide in alkaline conditions	+ easy method - inability to differentiate the carbohydrates	Jain et al., 2024

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		- through dehydration of copper(I) hydroxide is formed copper(I) oxide precipitate - the carbonyl group of RS is oxidized to carboxyl acid, which is then deprotonated	- Fehling's solution II could cause eye damage and skin irritation	
Benedict's test	1. Benedict's solution (sodium citrate, sodium carbonate and copper sulfate)	- RS convert into enediols in hot alkaline medium - the enediols reduce Cu^{2+} (cupric) ions from Benedict's solution to Cu^{+} (cuprous) ions the result is a brick-red precipitate of Cu_2O	+ easy and quick method + optimized Fehling's test with only one solution, which is more stable and non-corrosive - inability to differentiate the carbohydrates	Jain et al., 2024
Tommer's test	1. Tommer's solution (sodium hydroxide and copper sulfate)	- in alkaline medium Cu^{2+} (cupric) ions from Tommer's solution are reduced to Cu^{+} (cuprous) by RS - as result of the reaction red-colored precipitate forms	+ easy and quick method - inability to differentiate the carbohydrates - sodium hydroxide could cause skin irritation	Jain et al., 2024
Nylander's test	1. Nylander's solution (potassium hydroxide, bismuth subnitrate and potassium sodium tartrate, also called Rochelle salt)	- in alkaline medium bismuth subnitrate transforms into bismuth hydroxide - the bismuth hydroxide is then reduced by RS to metallic bismuth, resul-ting in black precipitate	+ easy and quick method - inability to differentiate the carbohydrates - potassium hydroxide is corrosive	Jain et al., 2024
Tollens's test (silver mirror)	1. Tollens's solution (silver nitrate, sodium hydroxide and ammonia)	- the RS reduce Ag^{+} ions from Tollens's solution to Ag^{0} - as result the silver precipitates on the walls of the test tube	+ easy method - inability to differentiate the carbohydrates	Benet et al., 2011
Dinitrosalicylic acid (DNS) method	1. Dinitrosalicylic acid	- under hot alkaline conditions the DNS reacts with the free carbinyl group of RS - the result is formation of 3-amino-5-nitrosali-cylate - the optical absor-bance is recorded at 540nm	+ quantitative test for RS + easy and quick method - use of acidic reagent	Du et al., 2023

High Performance Liquid Chromatography (HPLC)	1. specific thermostat column	- based on the difference in the adsorption characteristics of different RS	+ diferentiates all types of RS + very high specificity- expensive apparatus and consumatives - need for addition-nnal tool for peaks indentification	Tiwari et al., 2023

The methods for RS detection are overall quick and easy. The best choise for detection, identification and quantification of the different carbohydrates is HPLC, however the analysis with this system is expensive and includes specific apparatus. Most of the listed methods are old, however, they are easily performed and use available materials and reagents. The Folin-Wu test and the DNS method are quantitavive methods that use standard spectrophotometers to detect RS quantities in solutions, but they cannot differentiate them. The rest of the tests (Picric acid test, Fehling’s test, Benedict’s test, Tommer’s test, Nylander’s test and Tollens’s test) are all qualitative methods for detection of RS (although Benedict proposes a modification of his method to make it semi-quantitative (Hernández-López et al., 2020)). Aldough easy to perform a lot of the tests include highly acidic, skin irritating or corrosive reagents, which makes them more dangerous to perform, hence the ones that include less dangerous and corrosive reactives are Benedict’s test, Tollen’s test and HPLC.

Benedict’s test is an alternative to Fehling’s test; however, Benedict’s reagent contains stable alkaline agents which are not very corrosive. The method is based on the reducing capacity of the free carbonyl groups in sugars, which in alkaline medium reduce the Cu^{2+} ions to Cu^{+} ions, which then precipitate as Cu_2O (Hernández-López et al., 2020). In detail the reducing sugars in alkaline medium convert into enediol, or alkenes with a hydroxyl (-OH) group on each of the C=C carbons. The enediols are very strong reducing agents, hence the cupric ions (Cu^{2+}) from Benedict’s reagent are reduced to their cuprous form (Cu^{+}) as cuprous oxide (Cu_2O) which in turn forms a brick-red precipitate (Jain et al., 2024). Benedict’s reagent contains copper sulfate ($CuSO_4$), sodium citrate ($Na_3C_6H_5O_7$) and sodium carbonate (Na_2CO_3) (Febriyossa et al., 2024). The copper sulfate is the source of cuprous ions, the sodium carbonate is used to create the alkaline medium and the sodium citrate is used to stabilize the cupric ions (making complexes) and prevents their deterioration to cuprous ions upon storage (Ratanawimarnwong et al., 2022; Jain et al., 2024).

In the recent years many mobile apps have increasing application in science. For example, instead of using expensive spectrophotometers, there are apps which recognize and analyze colors using the mobile phone’s camera. These apps are often used when it comes to food analysis as they are an easier and cheaper alternative to the expensive apparatus (Tjandra et al., 2025). For example, Park et al., 2024 describe a mobile app which recognizes a food product’s information through the phone’s camera. Other apps are used to recognize food products and calculate their nutritional value to create a healthier and more balanced diet (Meyers et al., 2015; Akter et al., 2021). There are some new mobile apps designed to recognize food allergens (such as peanuts) or toxins in food products (Rateni et al., 2017), as well as apps developed to recognize dual quality of the food products in EU contries’ market (Di Marcantonio et al., 2024). There are some authors who propose the use of color recognition apps for chemical quantification of colorimetric test strips (Solmaz et al., 2018).

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EXPOSITION

Materials

Copper sulfate pentahydrate, sodium citrate dihydrate, anhydrous sodium carbonate, galactose, glucose, fructose, sucrose and starch pucrchased from Sigma–Aldrich, Germany. Lactose purchased from SUNLIFE, Germany. Lactose-free (LF) milk from the local market.

Methods

Benedict reagent preparation and test steps

For the preparation of 1000 mL of Benedict's reagent, 27.06 g copper sulfate pentahydrate ($CuSO_4.5H_2O$), 197.16 g sodium citrate dihydrate ($Na_3C_6H_5O_7.2H_2O$) and 100 g anhydrous sodium carbonate (Na_2CO_3) were used. The reagents were dissolved in distilled water in a water bath and filtered through filter paper (Benedict, 1909; Figueira et al., 2014; Jain et al., 2024).

The procedure of Benedict's test includes the following steps:

1) 1 mL of sugar sample was pipetted into a clean test tube;

2) 2 mL of Benedict's reagent were added to the sample tube;

3) the mixture was heated into a boiling water bath for 5 min;

4) observation of the test samples for color change, color intensity and presence of precipitate was made

5) determination of the RGB (red, green, blue) parameters of a 500 µL drop of the sample was made.

The color solutions obtained by Benedict's test were analyzed using the free available mobile app "Color Identification" (released by Google Commerce Ltd. on 22.04.2018; required OS: Android 8.1 and up), which can determine the RGB parameters through a mobile phone's camera. The target for analysis was the whole drop of the sample placed on a white plate.

Optimization of the method

Different quantities of the 1% lactose dissolved in water and Benedict's reagent were tested, to observe any changes in the test's parameters. The tested quantities for the 1% lactose solution were 1 mL, 2 drops (using standard Pasteur pipette), 50 µL, 20 µL and 10 µL. The quantities of Benedict's reagenet used for these lactose probes are 2 mL, 1 mL, 500 µL, 500 µL and 500 µL respectively.

Analyzis of the influence of reaction time was made using the 1% lactose solution. In three test tubes were pipeted 1 mL of 1% lactose and 2 mL of Benedict's reagent. The test tubes were then incubated on boiling water bath. The first test tube was removed from the water bath on the 3rd minute, the second on the 4th and the last one on the 5th minute of incubation. At the end of each incubation period the RGB results were collected.

Tested sugar preparation

Standard lactose solutions were prepared with concentrations from 0% to 2.5% in water. Furthermore, 1% sugar solutions were prepared with different types of saccharides: galactose, glucose, fructose, lactose, sucrose and starch.

Milk analysis optimization

Using a commercially available brand LF milk (1.5% fat, 4.9 g total sugar in 1000 mL) were prepared different concentrations. The test was performed with ratios milk/water: pure milk, 1:1, 1:2, 1:5 and 1:10. Standard lactose solutions (from 0% to 2.5%) were added to LF milk diluted 1:10.

RESULTS AND DISCUSSION

Solution of 1% lactose was tested the productivity of Benedict's test using different quantities of the lactose and Benedict's reagent (Table 2). The R (red), G (green) and B (blue) values from the app were measured and recorded for furder analyses.

Table 2. Optimization of Benedict's test with different quantities of lactose solution and Benedict's reagent

№ of probe	1% lactose	Benedict's reagent	R	G	B	Satisfaction
1	1ml	2ml	44	88	88	very good
2	2 drops	1ml	5	115	138	insuficient
3	50µl	500µl	3	119	142	insuficient
4	20µl	500µl	2	137	159	insuficient
5	10µl	500µl	1	139	167	none

Only the probe in test tube 1 gave a positive reaction to the test, the precipitation of test tubes 2 and 3 was very weak, while test tubes 4 and 5 gave no precipitation at all. Nevertheless, the R values vary in all the test tubes, which is an indication that a reaction with the Cu^{2+} ions has occurred even though it is not visible to the naked eye (Figure 2).

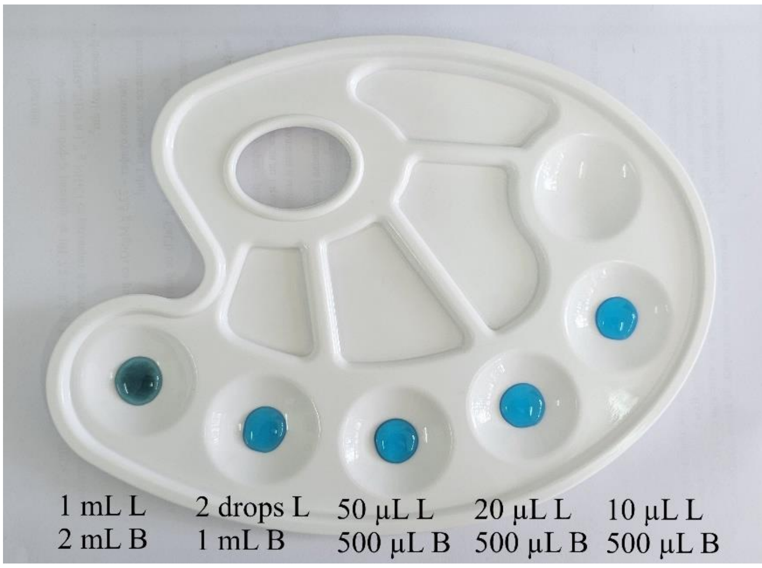

Fig. 2. Oprimization of the reagents in the Benedict test (L – lactose, B – Benedict's reagent)

In the subsequent analyses the ratio of 1 mL of lactose to 2 mL of Benedict's reagent were preferred as a standard, giving satisfactory results to the examined lactose concentrations.

The incubation time for reaction proceeding was analyzed. Test tube incubated for 5 minutes gave the strongest positive reaction with Benedict's reagent. The other two probes (for 4 and 3 minutes) also were positive to Benedict's test. However, the color and precipitation values were lower compared to the 5-min probe. Therefore, for subsequent analyses an incubation time of 5 minutes was preferred as optimal.

The Benedict's reagent was used for standard lactose solutions, and the color of the probes was deterined. Due to RGB data for each probe a trendline for R (the red color) was obtained, with R^2 of 0.9908 and linear equation: y=36.589x-4.3818 (**Figure 3**). The value of R^2 gives very good and reliable results (Gupta et al., 2024).

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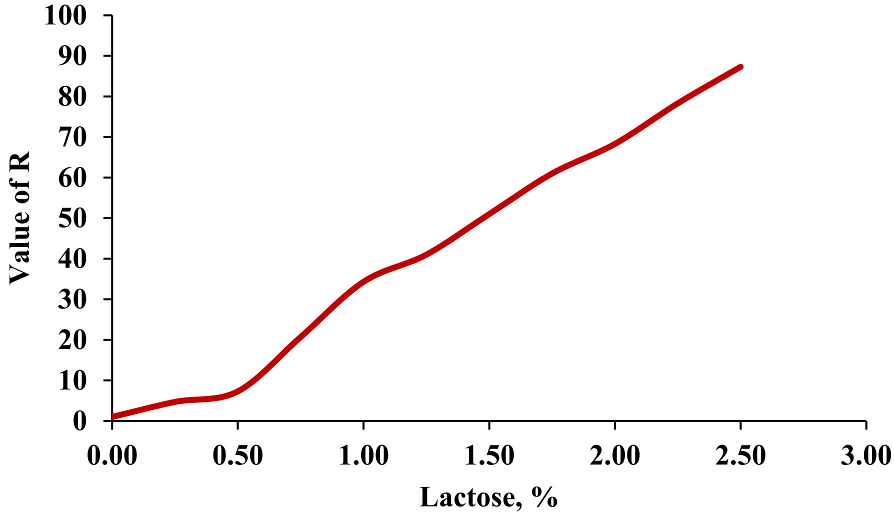

Fig. 3. Lactose standard solutions analyzed with the Benedict method.

On Figure 4 are presented the results for each standard solution and its RGB values. The R color in the tested drops increased with increasing lactose concentration. On the other hand, the B and G colors had higher values with lower lactose concentrations and with increasing the lactose, B and G decreased. The end point of the decreased color values was 1.75% lactose then the line was flat.

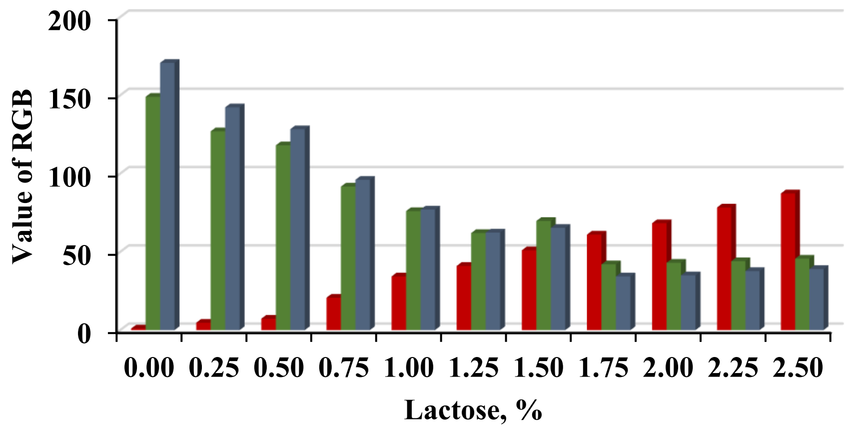

Fig. 4. Lactose standard solutions and their RGB values with Benedict method.

The Benedict's test with 1% solutions of different sugars was made (Figure 5). It was observed positive colorful reaction with the tested monosaccharides. The reaction with the fructose had the strongest red color intensity. From the disaccharide probes only the lactose solution gave positive reaction, while sucrose solution remained unchanged. Sucrose has only acetal groups and cannot acquire an open aldehyde group (Akyüz et al., 2021). The reaction of the polysaccharide starch was negative. The starch only has one terminal hemiacetal group, which is not nearly enough to reduce Cu^{2+}. Starch is composed of two types of glucose polymers called amylose and amylopectin. The molecule of amylose has approximately 200 to 1000 glucose units and amylopectin have 2000 to 2000000 glucose units, yet in starch only the terminal unit has a free hemiacetal group, which is not nearly enough to test positive as RS (Yahia et al., 2019). Therefore, starch is not considered as RS.

Fig. 5. Results from Benedict's test with different carbohydrates – from left to right: galactose, glucose, fructose, lactose, sucrose and starch

On Table 3 are shown the RGB results obtained from Benedict's test with the different sugars.

Table 3. Benedict's test with different sugars

Sugar	**R**	**G**	**B**
Galactose	65	71	69
Glucose	71	61	69
Fructose	96	29	21
Lactose	50	95	98
Sucrose	3	145	167
Starch	9	144	166

It was examined how commercially available LF milk reacts through Benedict's test. The LF milk was diluted in a few concentrations (pure, 1:1, 1:2, 1:5 and 1:10). As a result, all of the probes gave positive reaction with Benedict's reagent, therefore LF milk did not contain lactose but had other RS in its composition. It is also observed a precipitation of the milk proteins, due to the Benedict reagent's alkaline properties (Table 4).

Table 4. Benedict's test with different dilutions of LF milk

LF milk dilution	**R**	**G**	**B**
pure	160	74	23
1:1	154	56	21
1:2	113	60	18
1:5	90	73	17
1:10	67	93	46

To decrease effect of the milk proteins' precipitation, the milk was diluted 1:10 and different lactose concentrations were added. Those samples simulate the real milk samples and

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thus the milk's matrix effect is minimized. Figure 6 presents a graph of the results from lactose determination in milk using Benedict's reagent, where the obtained RGB data from the Benedict test for lactose in milk, was used to construct a trendline for R (the red color) with R^2 of 0.9759 and linear equation: y=26.358x+8.2298.

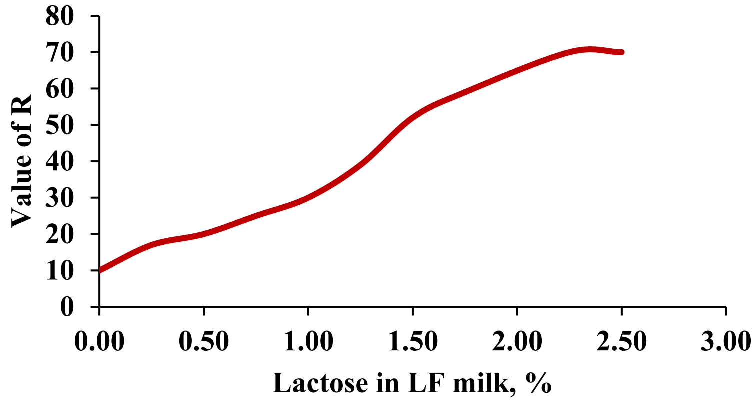

Fig. 6. Qantitative Benedict test for lactose in milk.

CONCLUSION

Benedict's test is a quick method for qualitative detection of RS, which does not require dangerous and corrosive reactives. The test is reliable and easy to perform, giving brick-red precipitate which can be detected without the need for expensive machinery and equipment. Benedict's test could also become a quantitative test for RS.

The presented study shows how to made semi-qantitative method to quantitative method with free available app for mobile phone. This opens up horizons to apply the rapid quantitative lactose milk method on field. Also, an interesting and modern exercise for students can be developed, and the idea can be extended to any analysis with obtaining color in the visible region. After all, mobile phones are part of our everyday life, and due to the development of technology, we can now use them for scientific purposes.

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