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COMPARATIVE STUDY OF LABORATORY BASED CHLOROPHYLL - A DETERMINATION METHODS

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Abstract

Protecting the quality and quantity of the available surface water resources is one of the most important points. Water quality is a complex concept that includes the physical, chemical, biological and hydrological parameters of water. One of the most significant biological water quality parameters is the chlorophyll-a content. Several different laboratory and field measurement methods are used to determine the chlorophyll-a content of water samples. The most frequently used laboratory methods are the Felföldy 1987 and the ISO 10260:1992 methods. Among field determination methods, submersible probes are frequently used to measure the chlorophyll-a concentration. Previously mentioned methods are based on fluorescence techniques. The main aim of this recent study is to compare different laboratory chlorophyll-a determination methods. Among the applied approaches, the Felföldy 1987 method and the ISO 10260:1992 method were compared. Furthermore, we measured the effect of the different solvent on the determination methods. Based on this, we investigated the Felföldy determination method using different solvents (methanol substituted with ethanol). The measurements were carried out under the same conditions from samples of Chlorella vulgaris culture in three repetitions by preparing a dilution series.
Keywords: *chlorophyll-a concentration, chlorophyll-a determination methods, comperative study*

LABORATÓRIUMI KLOROFILL - A TARTALOM MEGHATÁROZÁSI MÓDSZEREK ÖSSZEHASONLÍTÓ VIZSGÁLATA

Összefoglalás

Rendelkezésre álló felszíni vízkészleteink minőségi és mennyiségi védelme a legfontosabb feledatok közé tartozik. A vízminőség egy összetett fogalom, amely magába foglalja a vizekre vonatkozó fizikai, kémiai, biológiai és hidrológiai paraméterek körét. Biológiai vízminőségi paraméterek közül az egyik legfontosabb az klorofill-a tartalom. Vízminták klorofill-a tartalmának meghatározására több különböző laboratóriumi és terepi módszert alkalmaznak. A leggyakrabban alkalmazott laboratóriumi módszerek közé tartozik Felföldy Lajos 1987-ben publikát mérési módszere és és a Nemzetközi Szabványügyi Testület által elfogadott ISO 10260:1992 módszer. Terepi meghatározási módszerek közül legelterjedtebbek a fluoreszcens meghatározáson alapuló vízbe meríthető szondák. A jelen kutatás fő célja, különböző laboratóriumi a-klorofill tartalom meghatározási módszerek összevetése. A vizsgálati módszerek közül Felföldy 1987-es módszerét és az ISO 10260:1992 módszert hasonlítottuk össze. A kutatás céljai között szerepelt a mérési módszereknél alkalmazott különböző oldószerek vizsgálata, hogy milyen hatással van a meghatározásra, ha más oldószert használunk a vizsgálat során. Ebből kiindulva Felföldy meghatározási módszerét vizsgáltuk különböző oldoszerek (metanolt etenollal helyttesítve) alkalmazásával. A méréseket azonos körülmények

között Chlorella vulgaris tenyészetből származó vízmintából végeztük el három ismétlésben hígítási sor készítésével.
Kulcsszavak: *a-klorofill, meghatározási módszer, összehasonlító vizsgálatok*
JEL kód: *Q25*

Introduction

One of the most significant natural resources is water. Information of the quality and quantity of it play an important role in the ecosystem. Water quality measurements provide us with many different data from different aspects (integrated water management, agricultural water management, environmental monitoring etc.). Quality of a water body is a complex expression because it covers many parameters such as physical, chemical and biological factors. As for the biological water quality, it contains numerous difficult to evaluate quality index and parameters. In point of proceedings, one of the easiest to determine biological parameters is the chlorophyll-a content of the water (PADISÁK, 2005).

Chlorophyll-a is the most common type of chlorophyll. It is generally agreed today that chlorophyll-a is essential for most photosynthetic organisms to release chemical energy or in other words to capture sunlight for photosynthesis (FELFÖLDY, 1987).

Measuring the chlorophyll-a content, we can gather many useful information about the intensity of the productivity and draw conclusions about the water body's health.

It is a well-known fact that the chlorophyll-a content is closely related to the amount of the algae in the water (REYNOLDS, 2006). Actually, this means that more chlorophyll-a indicates that there are more phytoplankton present in the water. Phytoplankton can be used as an indicator organism for the status of the water quality. Monitoring the chlorophyll-a concentration is the most common way to track phytoplankton's population dynamics (WETZEL, 2001). Keeping tabs on the algae's population growth is a very significant effort because it can predict many harmful processes in the water ecosystem (KALF, 2002).

For instance, the nutrients excess can cause sudden phytoplankton bloom which later indicates dissolved oxygen level decrease. The final consequences of the whole process might be the biodiversity decline or other devastating environmental problems.

As for the chlorophyll-a measurement, it can be measured in several different ways. Generally in research, the most common ways of the determination are spectrophotometry, the high performance liquid chromatography (HPLC), and the fluorometry (DERENBACH et al., 1979; DOERFFER – SCHILLER, 2007). Anyway, alternative subdivision can be used for the classification of chlorophyll-a measurement by the researchers. In point of fact, this division can be the in-situ measurements (GREGOR – MARŠÁLEK, 2004). Spectrometric way is the classical method of determining the quantity of chlorophyll-a in the water. To measure the concentration by spectrometry, there are many sample preparation methods in practice. One of the most common methods are the ISO 10260:1992 (Water quality - Measurement of biochemical parameters -Spectrometric determination of the chlorophyll-a concentration) and Felföldy 1987 (Chlorophyll-a concentration measurement) (FELFÖLDY, 1987; ISO 10260:1992). The ISO 10260:1992 standard is using ethanol while the Felföldy 1987 method is preferring methanol during the extraction process.

These measuring methods are very similar to each other but the main difference is in the process, what kind of solvents have been used during the preparation.

Among the measuring techniques, the most commonly used solvent are the methanol, ethanol and acetone.

Nowadays, a more preferable solvent is ethanol because the methanol has a higher health risk factor. Authors of other studies have proposed that using methanol for measurement is a more

efficient way of the determination than the ethanolic method. According to many studies, ethanolic determination might have many possible-error and accuracy problems during the process. Methodical problem of chlorophyll content determination was examined by (BÖBBI – PÁPISTA, 1998). Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophyll was investigated by (PORRA et al., 1989).

Regarding of these studies, the most usable and accurate methods of chlorophyll content determination are the methanolic and acetonic way. In hydrology and algology, one of the most preferable ways of chlorophyll content determination is the Felföldy - methanolic- method (ÁCS et al.,2004; KISS, 1998). In some other recent studies, the ethanolic measuring method is being used by the researcher (BORICS, 2015). Actually, the spectrometric determination methods can be accurate, very different and would be extremely time-consuming. With reference to the other part of the division, that is in situ measurements, the most preferable determination is the fluorometry. In general, to measure the water chlorophyll-a content with this method, the researchers use a submersible probe. These kinds of instruments are very user-friendly and time-saving applications. Many studies in the world are dealing with proper use of the submersible probes.

The main objective of this recent study is to make a comparison between the different chlorophyll-a measurement methods, especially the accuracy of the different ways and the used different solvents. To achieve the listed main objective, we took the following steps:

1. Creation of a complex measuring techniques focusing on the same laboratory conditions.
2. Establishing correlations between the different methods and different applied solvents during the measurements.

Material and methods

A few chlorophyll-a measuring methods were applied in this study to compare the different ways. Two main measuring methods were the ISO 10260:1992 and the FELFÖLDY (1987). In addition to the main objectives, we were focusing on used methods with some modifications. During the comparative measurements, the effects of different solvents, ethanol and methanol, were investigated beside the basic methods.

Measured water samples derive from a controlled *Chlorella Vulgaris* culture. Dilution series (1, 2, 4, 5, 10 times) was made from the stock solution in five repetitions during the measurements. Detailed description about the applied methods can be seen on Table 1.

First measuring method was the FELFÖLDY, (1987). As for the applied solvent, methanol was used to extract chlorophyll-a pigments from the cells. During the measurement, Jenway 6400 spectrometer and 0,45 μm filter paper were used to measure the chlorophyll-a concentration of the samples.

Second measuring method was the ISO 10260:1992. As described in the standard, ethanol was applied during the extraction process. In the case of the second method, the same spectrometer and filter paper was used.

Third measuring approach was again the Felföldy method with some modification. Originally it uses methanol as we described previously but we exchanged the solvent for ethanol. The other measurement parameters and conditions were unchanged.

Fourth and the last measuring method was the previously modified Felföldy methods with an addition, an extra treatment of the samples and that was the ultrasonic destruction (600 sec long ultrasonic bath). The other measurement parameters and conditions were unchanged.

Table 1. Detailed information about the applied methods

Applied methods	Method 1	Method 2	Method 3	Method 4
Name	Felföldy 1987. (Determination of chlorophyll-a concentration)	ISO 10260:1992 (Water quality - Measurement of biochemical parameters - Spectrometric determination of the chlorophyll-a concentration)	Modified Felföldy 1987.	Modified Felföldy 1987.
Modification	No modification	No modification	Solvent modification: methanol replaced by ethanol	Method 3 completed with ultrasonic destruction

We made another measurement to compare the Method 1 with pure ethanol, with ultrawave bath, without any boiling. The processes were the following:

The original method (Method 1) suggest to filter 250 ml liquid on a 0,45 μm filter paper. To extract the chlorophylle-a from these algae, 10 ml methanol was put in a tube with the paper, closed, and boiled in water bath. When the methanol reached the boiling point, another 10 ml of methanol added into the tube, and centrifuged for 10 minutes on 5000 rpm. The supernatant measured on 3 wavelengths (653, 666 and 780 nm) and the chlorophylle-a content was calculated by the equation:

$$Chlorophyll - a\ \left(\frac{mg}{l}\right) = \frac{(17.12 \times \boldsymbol{x_1} - 8.68 \times \boldsymbol{x_2}) \times 15 \times 100}{50}$$

Where x1 = 666 nm – 750 nm, and x2: 653 nm – 750 nm absorbance values.

Ultrawave method: 250 ml liquid filtered on a 0.45 μm filter paper. 20 ml of methanol was added with paer in a centrifuge tube. This sample goes through a 600 sec long ultrawave bath. After that, the solution was centrifuged and the supernatant measure on a photometer as the previous method described.

Results

The Table 2. contains the measured absorbance values with the average and deviation numbers.

Table 2. Original and ultrasonic methods absorbance results

Boiling methode															
	1a	**1b**	**1c**	**2a**	**2b**	**2c**	**4a**	**4b**	**4c**	**5a**	**5b**	**5c**	**10a**	**10b**	**10c**
	653 nm	**666 nm**	**750 nm**	**653 nm**	**666 nm**	**750 nm**	**653 nm**	**666 nm**	**750 nm**	**653 nm**	**666 nm**	**750 nm**	**653 nm**	**666 nm**	**750 nm**
V1	0.056	0.101	0.004	0.038	0.059	0.005	0.021	0.033	0.003	0.012	0.020	0.006	0.005	0.010	0.005
V2	0.055	0.102	0.005	0.039	0.058	0.003	0.020	0.032	0.004	0.015	0.021	0.005	0.003	0.010	0.006
V3	0.054	0.100	0.003	0.037	0.057	0.004	0.021	0.031	0.001	0.013	0.020	0.004	0.006	0.011	0.005
V4	0.056	0.101	0.001	0.037	0.059	0.007	0.020	0.032	0.003	0.014	0.021	0.006	0.006	0.010	0.004
V5	0.055	0.101	0.004	0.038	0.058	0.004	0.021	0.032	0.001	0.015	0.020	0.004	0.005	0.009	0.002
Av.	**0.055**	**0.101**	**0.003**	**0.038**	**0.058**	**0.005**	**0.021**	**0.032**	**0.002**	**0.014**	**0.020**	**0.005**	**0.005**	**0.010**	**0.004**
D.	*0.0008*	*0.0007*	*0.0015*	*0.0008*	*0.0008*	*0.0015*	*0.0005*	*0.0007*	*0.0013*	*0.0013*	*0.0005*	*0.0010*	*0.0012*	*0.0007*	*0.0015*

Ultrasonic methode															
	1a	**1b**	**1c**	**2a**	**2b**	**2c**	**4a**	**4b**	**4c**	**5a**	**5b**	**5c**	**10a**	**10b**	**10c**
	653 nm	**666 nm**	**750 nm**	**653 nm**	**666 nm**	**750 nm**	**653 nm**	**666 nm**	**750 nm**	**653 nm**	**666 nm**	**750 nm**	**653 nm**	**666 nm**	**750 nm**
Var1	0.075	0.125	0.005	0.050	0.068	0.005	0.025	0.041	0.006	0.018	0.028	0.007	0.010	0.016	0.005
Var2	0.071	0.123	0.006	0.051	0.069	0.005	0.026	0.040	0.005	0.019	0.027	0.006	0.009	0.016	0.007
Var3	0.072	0.124	0.005	0.049	0.068	0.005	0.024	0.040	0.007	0.017	0.026	0.005	0.011	0.017	0.007
Var4	0.073	0.125	0.006	0.050	0.067	0.003	0.026	0.041	0.005	0.018	0.027	0.005	0.010	0.017	0.006
Var5	0.073	0.124	0.006	0.049	0.066	0.003	0.025	0.042	0.008	0.018	0.026	0.004	0.011	0.017	0.007
Avg.	**0.073**	**0.124**	**0.006**	**0.050**	**0.068**	**0.004**	**0.025**	**0.041**	**0.006**	**0.018**	**0.027**	**0.005**	**0.010**	**0.017**	**0.006**
Dev.	*0.0015*	*0.0008*	*0.0005*	*0.0008*	*0.0011*	*0.0011*	*0.0008*	*0.0008*	*0.0013*	*0.0007*	*0.0008*	*0.0011*	*0.0008*	*0.0005*	*0.0009*

Based on the equation in the previous chapter, the concentrations of the two methods were these (Table 3).

Table 3. Chlorophyl-a concentration in mg/l, boiling and ultrasonic methods and in case of different methods (calculated)

Dilution	Boiling	Ultrasonic	Method 1	Method 2	Method 3	Method 4
	chlorophyl-a, mg/l		chlorophyl-a, mg/l			
1	244.3	289.4	242.2	239.3	249.1	223.8
2	125.9	137.9	125.9	123.7	141.1	113.5
4	69.8	85.5	69.1	64.75	79.0	58.7
5	37.5	51.4	37.8	35.9	45.6	38.3
10	18.1	28.3	18.5	16.7	22.5	12.6

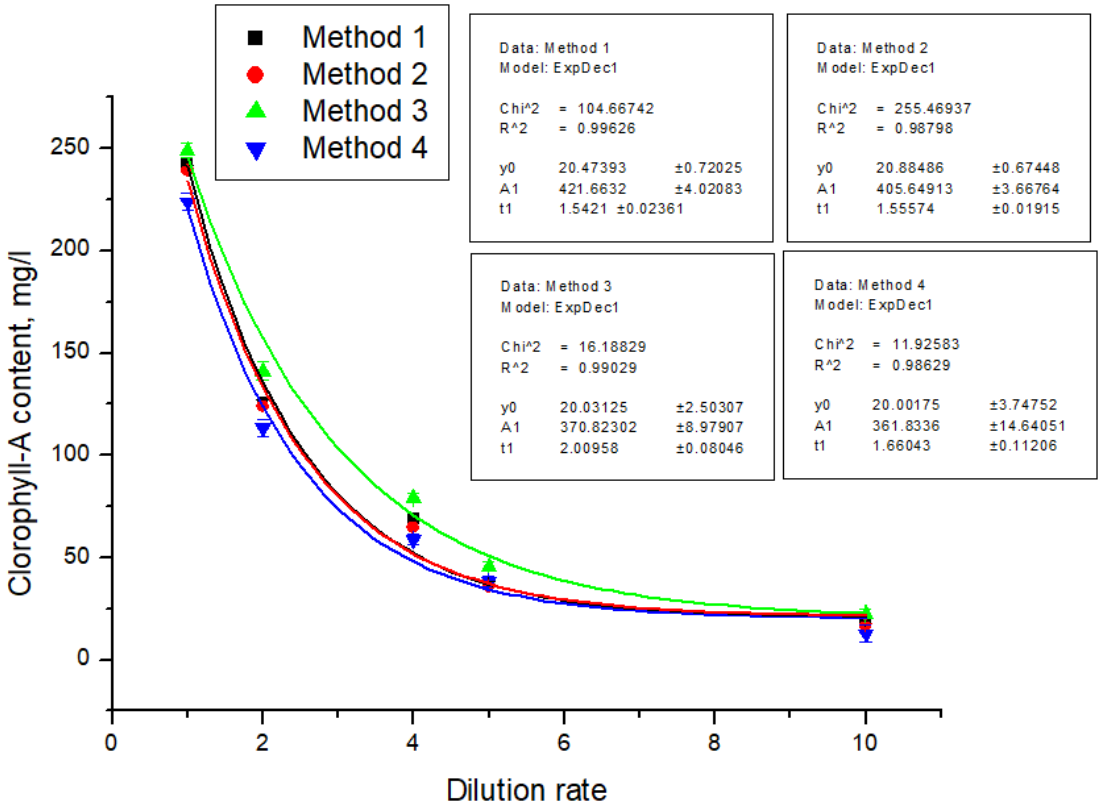

Figure 1a. The Chlorophyl-a concentration (mg/l) in case of dilution calibration curve, The comparsion of the four method

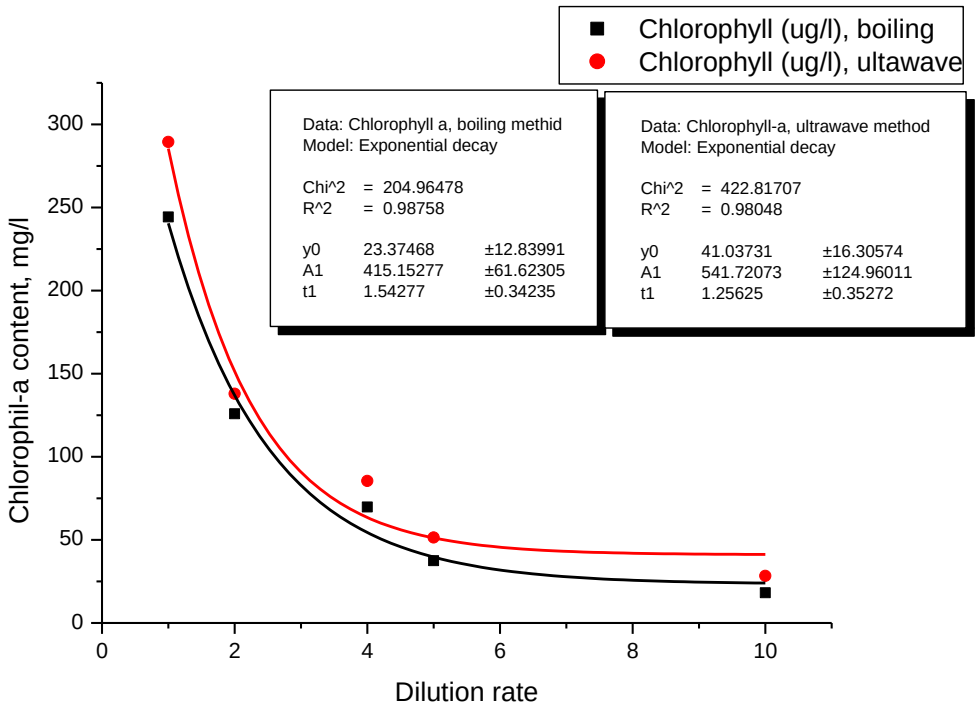

Figure 1b. The Chlorophyl-a concentration (mg/l) in case of dilution calibration curve, Boiling – black (Method 1), and ultrasonic – red compensation

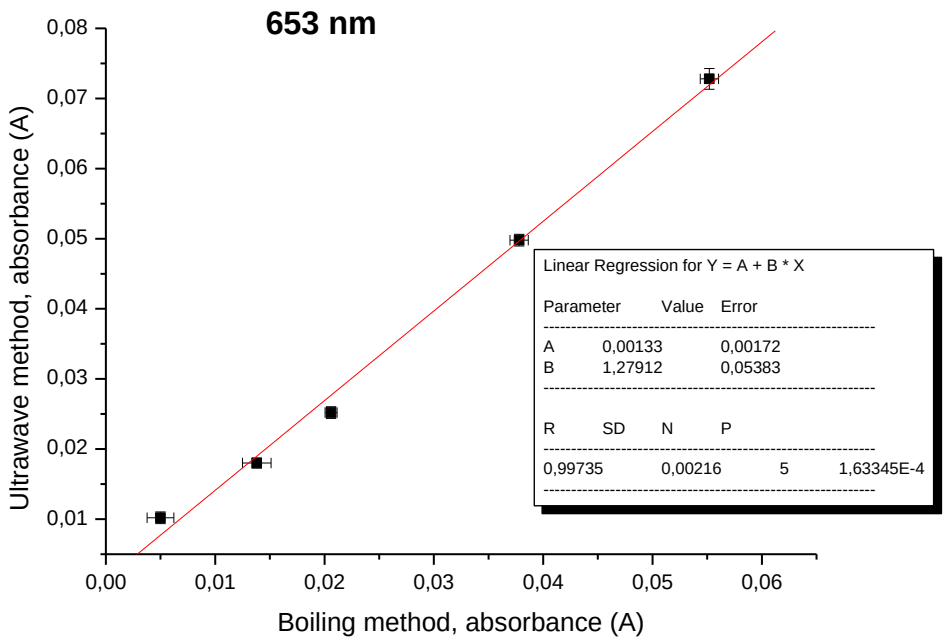

Figure 2. Comparison of the boiling methode (X axis) to ultrasonic methode (Y axis) in case of 653 nm wavelengths

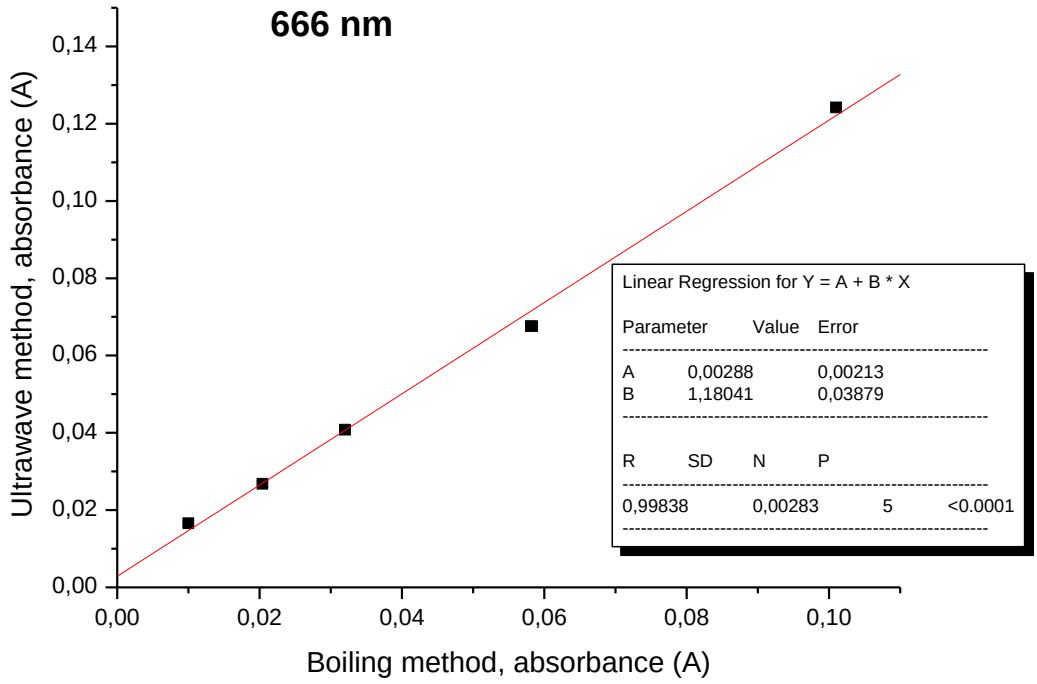

Figure 3. Comparison of the boiling methode (X axis) to ultrasonic methode (Y axis) in case of 666 nm wavelength

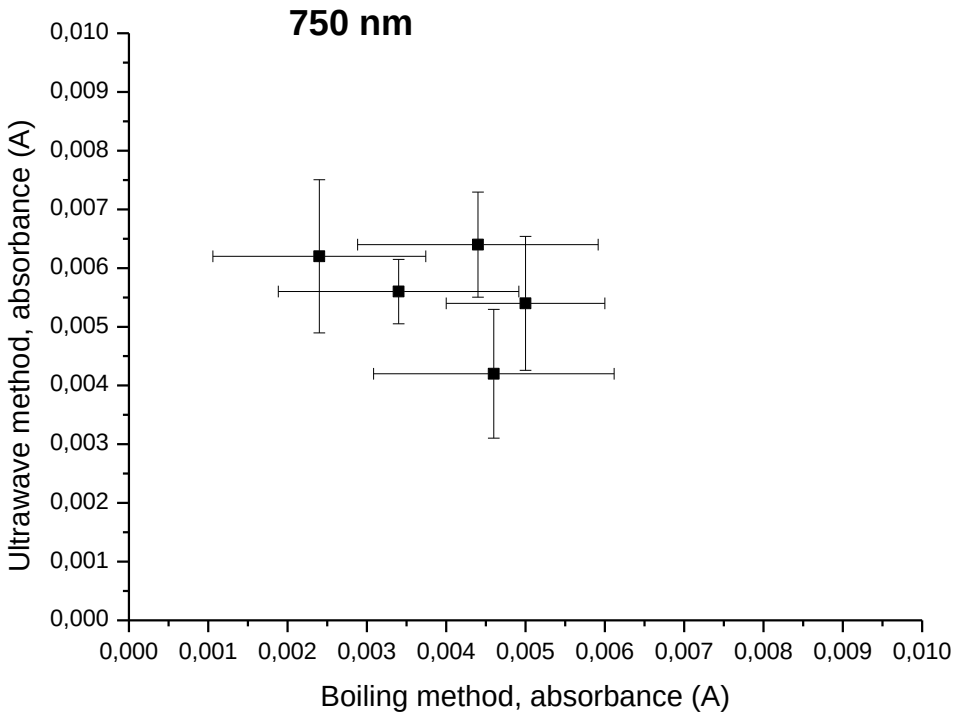

Figure 4. Comparison of the boiling methode (X axis) to ultrasonic methode (Y axis) in case of 750 nm wavelength

Conclusion

As can be seen in the Figure 1 (top), and as can be seen from the data in Table 3, the four methods give similar results. The first and second methods produced almost identical results, both in terms of variance and quantified chlorophyll-a content. Method 3 was the weakest. From the results in the first figure (bottom), it can be seen that a modified version of the Felföldy method (ethanol ultrasonically treated without boiling). The results obtained here are also comparable.

In general, all methods show an exponential decrease with a similar slope. The chlorophyll concentrations obtained are in the same order of magnitude and could be well comparable. This is particularly striking if one observes Figures 2 and 3: the values of the Felföldy method and the ultrasonic method show an almost perfect linear regression with each other (R^2 values 0.99).

The best correlation is between the original Felföldy method and the ISO standard. It should be noted that the other methods do not differ significantly from the values of the original Felföldy method (Method 1).

Taking into account the environmental and human health considerations, the use of ethanol has become justified and it is worth rethinking the methods based on the use of methanol. Measurement problems during boiling can be avoided by using a purely ultrasonic treatment, which not only gives relevant results but also simplifies the handling of the test.

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