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# Determination of sulfate in algal polysaccharide samples: a step-by-step protocol using microplate reader

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## ABSTRACT

Algal polysaccharides exhibit a wide range of biological activities and potential applications. Many of these bioactivities correlate positively with the presence of sulfate groups on the polysaccharides. The most common method used for sulfate quantification in algal samples is the turbidimetric method using the barium chloride-gelatin reagent. However, the original procedure is difficult to adapt for routine analysis since it is laborious and time-consuming. An optimized method was established using 96-well microplates, with the advantage of reducing waste and discrimination between organic and inorganic sulfates. This proposed method produced the same accuracy as the original.

**Descriptors:** Sulfate, Polysaccharides, Step-by-step protocol, Microplate, Seaweed.

Sulfated polysaccharides are the major structural components of the cell wall of seaweeds. These compounds exhibit a wide range of biological activities with potential medicinal, agricultural, and industrial applications (Bouissil et al., 2019; Hentati et al., 2020).

Activity studies have suggested that there can be a positive correlation between sulfate groups and the biological activities of these polysaccharides (Jiao et al., 2011). Therefore, the dosage of organic sulfates (i.e., bonded sulfate groups) helps to clarify the mechanisms of action of these macromolecules.

Several methods are used to measure sulfate in samples, including protocols that require expensive and specialized equipment, such as infrared spectroscopy using the ratios of absorbance of characteristic bands (e.g., the method for carrageenans and agars by Rochas et al., 1986). However, the most common analytical protocols for sulfate dosage include gravimetric (e.g., the method of precipitation and weighing of sulfate as barium sulfate by AOAC, 1990), turbidimetric (e.g., the barium chloride-gelatin method for plant materials by Tabatabai and Bremner, 1970), and colorimetric (e.g., the method of Azure A, a dye able to bind to sulfate groups, by Torode et al., 2015) methods. In general, the turbidimetric methods are less laborious than the gravimetric method and include cheaper chemical reagents.

Dodgson and Price (1962) described a turbidimetric method widely used for sulfate dosage in polysaccharides and polysaccharide-rich samples (e.g., aqueous extracts and their fractions) from seaweeds. In this method, the sulfate ion ($SO_4^{2-}$) reacts with the barium ion ($Ba^{2+}$) to form barium sulfate ($BaSO_4$), a water-insoluble precipitate at low pH. The turbidity generated by the precipitate is commonly established by gelatin. Despite its popularity, the method proposed by Dodgson and Price is laborious and time-consuming. For example, the gelatin solution preparation requires 16 h of incubation, a higher reagent consumption (1 to 5 mL) is necessary, and

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two different acids (trichloroacetic – TCA, and hydrochloric acid – HCl) are used during the assay.

Therefore, there is not a simple and optimized protocol for sulfate analysis from algal samples. In this context, the main purpose of our study was the establishment of a fast and environmentally-friendly turbidimetric method, with the same accuracy as the traditional method (Dodgson and Price, 1962). The result is a protocol as accurate as and more practical than the original method. The proposed protocol is given below, providing step-by-step guidance.

1. Barium chloride-gelatin reagent: add 75 mg of gelatin powder and 25 mL of ultrapure water into a screw cap tube. Incubate at 80 °C for 10 min and vortex until complete homogenization. Add 250 mg of $BaCl_2$ to the gelatinous solution and homogenize under stirring. Note: Barium chloride-gelatin reagent can be stored at 4 °C for at least one week. However, the reagent should be allowed to reach room temperature and homogenized thoroughly before use.

2. Preparation of the hydrolyzed sample: transfer 1 mg of dry aqueous extract to a microtube, add 250 µL of 0.5 mol.L$^{-1}$ HCl (concentration: 4 mg.mL$^{-1}$), and vortex for 60 s. Incubate the sample in a dry bath incubator at 105 ± 5 °C for 3 h. After the incubation period, cool down the microtube to room temperature. Centrifuge the sample (13,400 *g* for 15 min at room temperature) and transfer the supernatant to a new microtube.

3. Preparation of the non-hydrolyzed sample: weigh 5 mg of dry aqueous extract of algae into a microtube. Add 500 µL of ultrapure water and vortex. Centrifuge the sample (13,400 *g* for 15 min at room temperature) and transfer 100 µL of the supernatant to a new microtube. Just before performing step 5, add 125 µL of 1 mol.L$^{-1}$ HCl and 25 µL of ultrapure water (concentration: 4 mg.mL$^{-1}$). Homogenize quickly and place the microtube into an ice-bath. Note: This step is unnecessary for purified samples.

4. Preparation of the calibration curve: prepare a sulfate stock solution that contains 10 mg.mL$^{-1}$ of sulfate ion by dissolving a sulfate salt in 0.5 mol.L$^{-1}$ HCl. For example, a stock solution of $Na_2SO_4$ at 14.79 mg.mL$^{-1}$ corresponds to 10 mg.mL$^{-1}$ of sulfate ion. Prepare sulfate ion standard solutions with concentrations between 0.2 to 2 mg.mL$^{-1}$ (well concentrations: 20 to 200 µg.mL$^{-1}$). Note: The figure 1 shows an example of standard curve plots.

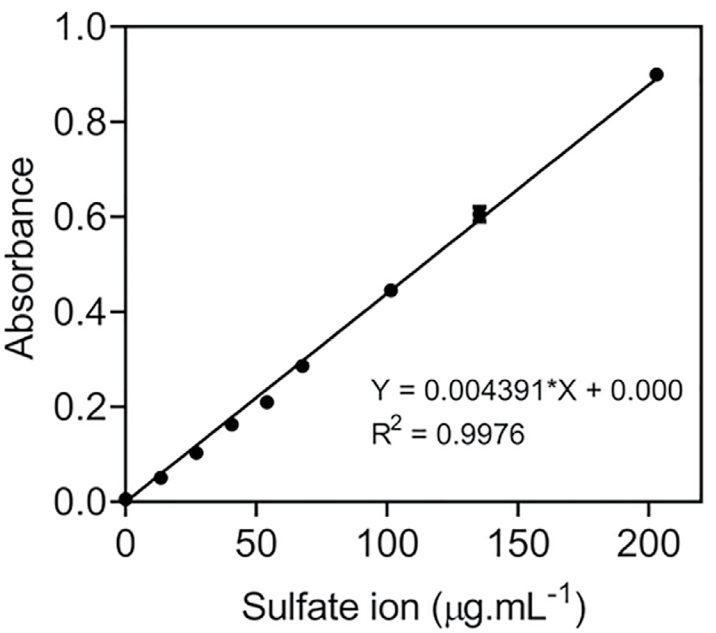


**Figure 1.** An example of a standard curve showing the absorbance of different concentrations of sulfate ion per well.

5. Analysis in microplate reader: add 20 µL of a sample (hydrolyzed or non-hydrolyzed), 0.5 mol.L$^{-1}$ HCl (negative control), or sulfate ion standards into a well of the 96-well clear polystyrene microplate already containing 140 µL of 0.5 mol.L$^{-1}$ HCl. Mix the contents of the wells. After mixing, read the absorbance of each well at 405 nm in a microplate reader (BioTek™; Synergy™ H1). These values correspond to the first reading and serve as a reference point. Add 40 µL of the barium chloride-gelatin reagent. Mix the contents of the wells. Incubate the microplate for 20 min. Again, mix the contents of the wells, and measure the absorbance at 405 nm. These values correspond to the second reading. Note: To mix the content of the wells, it is possible to use the shaking function of the plate reader (linear mode for 1 min at 567 cpm - 3 mm).

6. Sulfate calculation: correct the absorbance values of each sample subtracting the first reading from the second reading. Set up the calibration curve by plotting the corrected absorbances (y-axis) *versus* sulfate ion concentrations (x-axis). Use the regression equation to estimate sulfate ion concentration in each sample, and calculate the sulfate percentage. The organic sulfate percentage (OS) is estimated with the following formula: $OS = (\mu_H - \mu_N) \pm \sqrt{\sigma_H^2 + \sigma_N^2}$, where, µH and σH are, respectively, the mean and standard deviation of the total sulfate percentages, obtained with the hydrolyzed samples, and µN and σN are, respectively, the mean and standard deviation of the free sulfate percentages, obtained with the non-hydrolyzed samples. Note: Total and free sulfate dosages for the same sample must be performed simultaneously in the same microplate.

A comparison between total sulfate contents obtained using the proposed method and that from Dodgson and Price (1962) is presented in Table 1, using hot aqueous extracts of five algae species - *Crassiphycus caudatus* (Gracilariales, Rhodophyta), *Gracilaria domingensis* (Gracilariales, Rhodophyta), *Palisada flagellifera* (Ceramiales, Rhodophyta), *Sargassum vulgare* (Fucales, Ochrophyta), and *Ulva lactuca* (Ulvales, Chlorophyta). The extraction methods were described in detail by Torres et al. (2018) for *C. caudatus* and *G. domingensis* and Santos et al. (2019) for the other three species.

The total sulfate contents obtained with the Dodgson and Price (1962) method did not differ from those obtained with the proposed method (Table 1). No statistical differences were observed (Multiple t-test with Holm-Sidak correction, α = 5 %). Besides, the proposed protocol allows estimating both organic and inorganic sulfates, because it includes a step for the analysis of the non-hydrolyzed samples. Since the biological activities of sulfated polysaccharides are related to sulfate groups (Jiao et al., 2011), the distinction between free and esterified sulfate content might be crucial information, unavailable with the original method. For example, although the aqueous extract from *C. caudatus* exhibited the highest total sulfated content, most of this sulfate is in the free form (Table 1) and not associated with the polysaccharides. In conclusion, the proposed protocol presents some important advantages in comparison to the method of Dodgson and Price (1962):

i Time reduction: The preparation of the barium chloride-gelatin reagent does not need gelatin solution incubation of 16 h.

ii Reagents saving: The replacement of trichloroacetic acid (TCA) by HCl ensure low pH and reduces unnecessary reagent use. Besides, because all of the protocol is adapted to 96-well microplates, the volume of all reagents is reduced and, consequently, the disposal quantity is lower.

**Table 1.** Organic, inorganic, and, total sulfate contents of the aqueous extracts (% in dry mass) and comparisons between the results obtained in the conventional method with the proposed method. Values were expressed as mean ± SD (n = 4). There are no statistically significant differences between mean values of line for the total sulfate contents (multiple t-test with Holm-Sidak correction, α = 5 %).

| Algae | Proposed method | | | Conventional Method | Adjusted *p* value |
|---|---|---|---|---|---|
| | Organic (%) | Inorganic (%) | Total (%) | Total (%) | |
| *Crassiphycus caudatus* | 1 ± 1 | 19 ± 1 | 20 ± 1 | 21 ± 2 | 0.99 |
| *Gracilaria domingensis* | 6.2 ± 0.8 | 1.4 ± 0.5 | 7.6 ± 0.8 | 7.0 ± 0.3 | 0.99 |
| *Palisada flagellifera* | 13 ± 0.5 | 0.5 ± 0.2 | 13.5 ± 0.5 | 13.1 ± 0.5 | 0.99 |
| *Sargassum vulgare* | 3.6 ± 0.3 | 2.5 ± 0.1 | 6.1 ± 0.3 | 7.0 ± 0.3 | 0.94 |
| *Ulva lactuca* | 16 ± 1 | 0.9 ± 0.2 | 17 ± 1 | 17.5 ± 0.4 | 0.99 |

iii Increase of sample analysis capacity: The use of the 96-well microplate increases the number of assays carried out at the same time and reduce experimental time.

iv More information: The proposed protocol allows estimating both organic and inorganic sulfates, once it includes a step for the analysis of the non-hydrolyzed samples. Only the total sulfate content is estimated by the conventional method since only hydrolyzed samples are used.

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## AUTHOR CONTRIBUTIONS

P.T.: Conceptualization; Formal Analysis; Investigation; Writing – original draft; Writing – review & editing.

A.N.: Formal Analysis; Investigation; Writing – review & editing.

C.E.P.J.: Formal Analysis; Investigation; Writing – review & editing.

J.P.S.: Investigation; Writing – review & editing.

F.C.: Formal Analysis; Funding acquisition; Resources; Writing – review & editing.

D.Y.A.C.S.: Conceptualization; Formal Analysis; Resources; Supervision; Writing – review & editing.

## REFERENCES

AOAC (Association of Official Agricultural Chemists). 1990. Sulfate in water by gravimetric method. *In:* AOAC International (ed.). *Official methods of analysis.* Rockville: AOAC International.

BOUISSIL, S., PIERRE, G., EL ALAOUI-TALIBI, Z., MICHAUD, P., EL MODAFAR, C. & DELATTRE, C. 2019. Applications of algal polysaccharides and derivatives in therapeutic and agricultural fields. *Current Pharmaceutical Design,* 25(11), 1187-1199.

DODGSON, K. S. & PRICE, R. G. 1962. A note on the determination of the ester sulphate content of sulphated polysaccharides. *Biochemical Journal,* 84(1), 106-110.

HENTATI, F., TOUNSI, L., DJOMDI, D., PIERRE, G., DELATTRE, C., URSU, A.V., FENDRI, I., ABDELKAFI, S. & MICHAUD, P. 2020. Bioactive polysaccharides from seaweeds. *Molecules,* 25(14), 3152.

JIAO, G., YU, G., ZHANG, J. & EWART, H. S. 2011. Chemical structures and bioactivities of sulfated polysaccharides from marine algae. *Marine Drugs,* 9(2), 196-223.

ROCHAS, C., LAHAYE, M. & YAPHE, W. 1986. Sulfate content of carrageenan and agar determined by infrared spectroscopy. *Botanica Marina,* 29, 335-340.

SANTOS, J. P., TORRES, P. B., SANTOS, D. Y. A. C., MOTTA, L. B. & CHOW, F. 2019. Seasonal effects on antioxidant and anti-HIV activities of Brazilian seaweeds. *Journal of Applied Phycology,* 31, 1333-1341.

TABATABAI, M. A. & BREMNER, J. M. 1970. A simple turbidimetric method of determining total sulfur in plant materials. *Agronomy Journal,* 62(6), 805-806.

TORODE, T. A., MARCUS, S. E., JAM, M., TONON, T., BLACKBURN, R. S., HERVÉ, C. & KNOX, J. P. 2015. Monoclonal antibodies directed to fucoidan preparations from brown algae. *PLoS One,* 10(2), e0118366.

TORRES, P., NOVAES, P., FERREIRA, L. G., PIRES, J., MAZEPA, E., SANTOS, J. P., MAZEPA, E., DUARTE, M. E. R., NOSEDA, M. D., CHOW, F. & SANTOS, D. Y. A. C. 2018. Effects of extracts and isolated molecules of two species of *Gracilaria* (Gracilariales, Rhodophyta) on early growth of lettuce. *Algal Research,* 32, 142-149.