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Research Article

Spectrophotometric Determination of Chlorophyll - A, B and Total Carotenoid Contents of Some Algae Species Using Different Solvents

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Abstract: In our study, chlorophyll a, chlorophyll b and the total amounts of carotenoids in *Cladophora glomerata* (L.) Kuetzing, *Ulva rigita* L., *Codium tomentosum* (Huds.) Stackh and *Cladostephus verticillatus* Ag. species were determined. Methanol, acetone and diethyl ether were used as solvents. The chlorophyll amount in *Cladophora glomerata L. Kuetzing,* species and carotenoids in *Cladostephus verticillatus Ag.* were found the highest. It was observed that sonication had no much contribution to the extraction.

Key Words: Algae, Chlorophyll, Carotenoid, Solvents

Farklı Çözücüler Kullanılarak Bazı Alg Türlerinde Klorofil - A, B ve Toplam Karotenoid İçeriğinin Spektrofotometrik Saptanması

Özet: Çalışmamızda *Cladophora glomerata* (L.) Kuetzing., *Ulva rigita* L., *Codium tomentosum (Huds.) Stackh ve Cladostephus verticillatus* Ag. türlerinde klorofil a, klorofil b ve toplam karotenoid miktarları saptandı. Çözücü olarak metanol, aseton ve dietil eter kullanıldı. *Cladophora glomerata L. Kuetzing,* türünde klorofil a miktarı, *Cladostephus verticillatus Ag.* da ise karoten miktarı en yüksek bulunmuştur. Sonikasyonun ektraksiyona fazla bir katkısının olmadığı gözlenmiştir.

Anahtar Sözcükler: Alg, Klorofil, Karotenoid, Çözücüler

Introduction

The spectrophotometric definition of photosyntethic pigments that cause light energy to turn into chemical energy in all photosyntethic organisms was first determined by Stokes in 1864 (1). Later, the examples obtained from *Fucus* L. and *Laminaria* L. were classified as blue chlorophyll (chlorophyll a), green chlorophyll (chlorophyll b), chlorofucin (chlorophyll c1, chlorophyll c2) and orange-yellow (xanthophyll) according to the pigment colors (2).

The absorbance properties of pigments facilitate the qualitative and quantitative analysis of them. In the definition of the pigment content of freshwater and sea algae various methods and solvents have been used (3,4) and it was determined that the pigment level was influenced by limiting factors such as highlight, lack of nitrogen and limited nutrient (5,6).

In earlier studies, it was suggested that the content of chlorophyll a relating to the pigment level was almost the same in all algae groups, but chlorophyll b and c changed, and so did the carotene level depending on the algae species and environmental conditions, and especially there was an increase in the carotene level in stress conditions (7, 8, 9). Although the role of carotene pigments in algae was not exactly known, it has been suggested that they functioned as a passive light protecting filter and have got the role of accessory pigments transferring energy and oxygen (10, 11, 12). Vechetel and et al. (1992) in their studies determined that carotene pigments were the most

important photosyntethic pigments, and they prevented chlorophyll and thylakoid membrane from the damage of absorbed energy by photooxidation (13).

In our study, the pigment levels of four algae species were determined by using various solvents (methanol, acetone, diethyl ether) and the contribution of these solvents to the extraction in various species was examined comparatively. In addition, after homogenization it was examined whether sonication had any contribution to the extraction. Furthermore, the determination of suitable methods and solvents that could be used in studies on pigments was aimed.

Material and Method

Of the samples we used for the studies in the laboratory, freshwater form *Cladophora glomerata* (L.) Küetzing *(Chlorophyta)* was collected from the Yeşilırmak (Tokat), sea forms *Codium tomentosum* (Huds.) Stackh *(Chlorophyta), Ulva rigita* L. *(Chlorophyta)* and *Cladostephus verticillatus* Ag. *(Phaeophyta)* were collected from the Aegean sea (Narlıdere-İzmir). The samples were brought to the laboratory in the natural water in which they had lived, and one day later the extraction processes were carried out.

Extraction Processes

The weighed samples, having been put separately in 95% diethl ether, 96% methanol and 100% acetone (50 ml for each gram), were homogenized with the B-Brawn type homogenizer at 1000 rpm for one minute. The homogenate was filtered through two layer cheese cloths, and was centrifuged using the Nüve Füj 650 model centrifuge at 2500 rpm for ten minutes. The supernatant was separated and the absorbances were read at 400-700 nm on Schimadzu UV-260 spectrophotometer. It was recorded that Chlorophyll a showed the maximum absorbance at 662 nm, chlorophyll b at 646 nm and total caroten at 470 nm and the amount of these pigments was calculated according to the formulas of Lichtentaler and Wellburn (1985) (14). The formulas were showed in Table 1.

Table 1. The formulas that used in the calculation of chlorophyll a, chlorophyll b and total caroten levels

Diethyl ether	$C_a = 10.05\ A_{662} - 0.766\ A_{644}$ $C_b = 16.37\ A_{644} - 3.140\ A_{662}$ $C_{x+c} = 1000\ A_{470} - 1.280\ C_a - 56.7\ C_b/230$
Methanol	$C_a = 15.65\ A_{666} - 7.340\ A_{653}$ $C_b = 27.05\ A_{653} - 11.21\ A_{666}$ $C_{x+c} = 1000\ A_{470} - 2.860\ C_a - 129.2\ C_b/245$
Acetone	$C_a = 11.75\ A_{662} - 2.350\ A_{645}$ $C_b = 18.61\ A_{645} - 3.960\ A_{662}$ $C_{x+c} = 1000\ A_{470} - 2.270\ C_a - 81.4\ C_b/227$

C_a = Chlorophyll a, C_b = Chlorophyll b, C_{x+c} = Total carotene

Instead of the wave-lengths determined by these researchers, 662, 646 and 470 nm at which we observed maximum absorbance were used. The experiments were repeated three times. The results of the above experiments were analysed statistically using Snedecor's F-test for analysis of variance and to determine statistically significant differences between means "Multiple Range Test" was applied (15, 16).

Results

Chlorophyll a content in four algae species whose pigment levels were studied was determined the highest in *Cladophora glomerata species* (Table 2). Although chlorophyll a levels in the other three sea forms were very close to each other, it was found a little higher in *Ulva rigita* in comparison with the other two species (Table 2). In terms of chlorophyll b content no significant difference was observed between the species ($P<0.01$), but no absorbance could be de-

Table 2. Pigment levels of the species in different solvents (µg/gfw)

Species *	Methanol			Diethyl ether			Acetone		
	Cl_a	Cl_b	Carotene	Cl_a	Cl_b	Carotene	Cl_a	Cl_b	Carotene
Cladophora glomerata	60.7±0.05x	23.0±1.05x	19.2±0.95x	53.8±2.50x	20.1±0.65x	18.8±0.34x	56.2±0.46x	20.3±0.80x	20.1±1.05x
Ulva rigita	54.6±1.40y	24.1±0.80x	20.8±1.15x	48.6±0.72y	21.2±0.10x	21.6±0.72x	48.9±0.72y	23.3±0.95x	20.6±0.95x
Codium tomentosum	49.0±2.60y	21.8±0.30x	24.7±0.45x	46.2±0.05y	20.1±2.50x	20.5±0.96x	47.4±0.45y	20.2±1.05x	22.4±0.60x
Cladostephus verticillatus	51.3±1.00y	-	33.8±1.30y	47.9±0.65y	-	28.7±0.25y	48.2±0.20y	-	29.5±0.65y

* Data shown with the same symbols in the vertical column are not different from each other on 0.01 statistical levels
± Standard error

termined because of the absence of chlorophyll b in *Cladostephus verticillatus* species. The total carotene content was determined the highest in *Cladostephus verticillatus* and the lowest in *Cladophora glomerata* (Table 2). It was observed that the pigments were not extracted completely from *Cladostephus verticillatus* species. The total carotene content was determined the hiphest in *Cladostephus verticillatus* species and *Codium tomentosum* species as a result of the extraction, and particularly these two species contined pigments in certain proportions after homogenization.

It was determined that the solvents used were important in the pigment extraction, and the best solvent was methanol (Table 4). It was observed that the extraction with methanol was nearly complete in *Cladophora glomerata* and *Ulva rigita* because of the diversity of the cell wall structures, (Table 2, 3). Although it was seen that acetone was a better solvent in comparison with diethyl ether, no significant difference was determined between two solvents.

It was observed that the 5 minute sonication carried out in addition to homogenization was not very efficient on the isolation of the pigments during the extraction process. It was determined that it increased the extraction only a little in *Cladostephus verticillatus, Codium tomentosum* (Table 3).

Discussion

Although it was suggested in earlier studies that the chlorophyll a level was the same in all algae groups (7, 8, 9), in the present study it has been found that the level of chlorophyll a in fresh water form *Cladophora glomerata* was rather high in comparison with the other three species. The chlorophyll a level in *Ulva rigita* was also found higher than the other two species. Because of the variety of the cell wall structures of those two species, a well performed homogenization and a complete extraction facilitated the isolation of the pigments. In addition we have got an opinion that there was no much change at the pigment level in connection with the term when the samples were taken because *Cladophora glomerata* lives in freshwater and in an environment where much light and temperature stratifications are not seen (17). In previous studies it was determined that there were changes at the pigment level of algae species that live in an environment where light stratification is seen (5, 6). That is why we have got an opinion that the reason for the chlorophyll a level to be lower in other species is linked with light and temperature stratification during the term when the examples were taken.

Furthermore, because the accumulation of $CaCO_3$ was high on the cell walls of *Codium tomentosum* and *Cladostephus verticillatus* species that we used in our study (18), it was observed that homogenization was difficult and all of the pigments were not extracted. So we believe that the use of more suitable methods in the studies for breaking up cell walls will be useful for extraction to be complete.

It is known that carotene pigments are at various amount in algae groups. In the earlier studies it was proved that there were more carotene and xanthophyll pigments in *Phaeophyta* (19, 20). In the samples studied the carotene content in *Cladostephus verticillatus*, only species belonging to Phaeophyta group, was found higher in comparison with the other three species. The absence of chlorophyll b in this species may be due to more carotene pigments that have short wave-length and can absorb the light. It was pointed out that in the studies with *Chlorella* Beyerinck the carotene pigment level might be different even in the same species and it depends on various environmental conditions (21). According to these results we suggest that the comparison of the caroten levels of species used is not accurate because each one of the species examined in this study was collected from different mediums.

The solvents used in the pigment extraction have an important effect. In the studies with *Scenedesmus quadricauda* (Turp) de Bre'bisson in de Bre'bisson and

Table 3. Effect of sonication on extraction of pigmens (µg/gfw)

Species *	Methanol			Diethyl ether			Acetone		
	Cl_a	Cl_b	Carotene	Cl_a	Cl_b	Carotene	Cl_a	Cl_b	Carotene
Cladophora glomerata	61.4±1.05	23.6±0.72	19.4±1.05	53.7±1.65	20.8±1.67	19.1±0.05	57.0±2.67	20.4±1.45	19.7±2.45
Ulva rigita	54.7±1.40	24.1±0.30	21.1±0.65	49.0±0.25	21.3±0.95	21.5±0.65	49.3±1.95	24.2±0.30	21.4±0.95
Codium tomentosum	50.9±0.95	20.9±1.40	27.9±0.45	48.2±0.95	21.2±1.25	21.4±1.67	49.9±0.95	21.6±3.20	22.4±0.72
Cladostephus verticillatus	53.1±0.25	-	36.2±1.40	50.8±0.65	-	30.6±1.12	51.8±0.25	-	31.9±2.60

± Standard error

Table 4. Effect of solvents on pigment levels

Species	Pigment	Methanol*	Diethyl Ether	Acetone
	Cl_a	60.7±0.05a	53.8±2.50b	56.2±0.46b
Chladophora glomerata				
	Cl_b	23.0±1.05a	20.1±0.65b	20.3±0.80b
	Carotene	19.2±0.95a	18.8±0.34a	20.1±1.05a
	Cl_a	54.6±1.40a	48.6±0.72b	48.9±0.72b
Ulva rigita				
	Cl_b	24.1±0.80a	21.2±0.10b	23.3±0.95a
	Carotene	20.8±1.15a	21.6±0.72a	20.6±0.95a
	Cl_a	49.0±2.60a	46.2±0.05b	47.4±0.45b
Codium tomentosum				
	Cl_b	21.8±0.30a	20.1±2.50b	20.2±1.05b
	Carotene	24.7±0.45a	20.5±0.96b	22.4±0.60c
	Cl_a	51.3±1.00a	47.9±0.65b	48.2±0.20b
Chladostephus verticillatus				
	Cl_b	-	-	-
	Carotene	33.8±1.30a	28.7±0.25b	29.5±0.65b

* Data shown with the same symbols in the horizontal column are not different from each other on 0.01 statistical levels
± Standard error

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Godey and *Selenastrum capricornutum* Reinsch ethanol, methanol and acetone were used, and it was shown that the best solvent was ethanol (22). Yet in our study it was determined that the best solvent used for algae species was methanol. We think that this difference depends on the kind of the plant and its cel wall structure. It was observed that methanol was a better solvent for these species because the cell wall structures of *Codium tomentosum* and *Cladostephus verticillatus* we used were in porous structure and there was to much $CaCO_3$ accumulation (18). It has been seen that acetone, chloroform and diethyl ether had less effect on homogenization.

In fact, acetone, chloroform, diethyl ether, dimethyl formamid and methanol were used in the studies with high plant leaves, and it was determined that the extraction rate was various in every solvent (23). That is why we believe that the selection of the method and the solvent to be used in the studies in connection with pigments according to the species will be more useful. Although the methanol was a good extractant for some algae species, it should not be forgetten that it was toxic.

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