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Spectroscopic Characterization and Gel Properties of Agar from Two *Gelidium* Species from the Atlantic Coast of Morocco

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Abstract: The commercial value of agar in the phycocolloid market depends mostly on the agar yield and quality. The present study investigates the agar content and characterization of two Gelidiales *Gelidium corneum* and *Gelidium microdon* from the Moroccan Atlantic coast. Spectroscopic and rheological characterization of extracted agar without and with alkali pretreatments were evaluated. The highest agar yield was detected for mild alkaline pretreatment (N_2CO_3). The native agar content in *G. corneum* was 16.21%, while those pretreated with NaOH and N_2CO_3 ranged from 6.2 to 20.50 %. The agar yields of *G. microdon* showed values of 12.23%, 14.87%, and 17.73%, corresponding respectively to native agar, NaOH, and Na_2CO_3 pretreatments. Agar with alkali pretreatments depicted the better gelling property with higher gel strength and elevated gelling and melting temperatures. ^{13}C NMR spectroscopy showed that *G. Corneum* has a typical unsubstituted and weakly methylated agar pattern. However, *G. microdon* revealed the presence of methyl and sulfate groups at the C4 of 4-O-L-galactose residues, responsible for the low gelling ability of the native agar. The decline of sulfate groups after alkali pretreatments were proved by FTIR spectroscopy. This study demonstrates that *G. microdon* produces a quality of agar similar to that of *G. corneum*. Thus *G. microdon* could be regarded as a potential additional source of agar industry in Morocco.

Keywords: agar; ^{13}C NMR; FT-IR; Florideophyceae; Morocco.

1. Introduction

The seaweed hydrocolloids have attracted considerable interest in terms of their industrial commercialization [1–6]. The phycocolloids industry is incessantly rising at level of 2–3% per year [7], with an annual global production that recently achieved 100,000 tons and a gross market value of above US$ 1.1 billion [8]. According to Rhein-Knudsen *et al.* [9] and Veeragurunathan *et al.* [10], the agar gave the highest retail price per kg (18 US$/kg) compared to the alginates with value of 12 US$/kg and carrageenans with 10.4 US$/kg.

Agar is a strongly gelling hydrocolloid that occurs in the cell-matrix of marine red algae. Its main structure is chemically characterized by repetitive units of β-D-galactose and 3,6-anhydro-α-L-galactose [11–13]. Substitution with sulfates, methyl ethers, and/or pyruvate ketals can occur at various sites in the polysaccharide chain [14, 15]. The agar is used as a gelling agent in many foods and industrial applications[16–18]. The use of agar in foods was widespread throughout the Far East, including Japan, China, Taiwan, Korea, the Philippines, and Indonesia [14].

The commercial value of agars is mainly linked to their yield and quality. Some *Gelidium* species are globally used to extract agars [14,17,19]. The natural stocks represent the significant source for the agar industry [20]. The demand for agar from *Gelidium* has currently exceeded the offer [21]. The bacteriological agar demand from *Gelidium* shifted from 250 T to about 700 T [20].

Morocco is the world's fifth-largest agar exporter, following Chile, China, Indonesia, and Spain [22]. Between 2012 and 2016, the annual export weight of agar in Morocco varied from 905 tonnes to 1066 tonnes, respectively [22]. The authorized export quota of locally produced agar is 1247 tonnes annually [23]. *Gelidium corneum* is the main species exploited for the local agar production. The available data and statistics on monitoring the *Gelidium corneum* population reported that this species is overexploited [24]. Furthermore, the number of *Gelidium* collectors without a harvest license was out of control. The collect season was often not respected. To ensure the continuity of seaweed production at the local scale, as an environmentally and economically sustainable activity, an intensive effort in fundamental research is required to consider other algal resources from the Moroccan coast with potential industrial interest. In this context, this study aims to evaluate the yield and the spectroscopic and rheological characterization of the agars, under different types of extraction, from *Gelidium corneum* and *Gelidium microdon* harvested from the Moroccan Atlantic coast.

2. Materials and Methods

2.1. Sample collection.

Samples of *Gelidium corneum* and *Gelidium microdon* were harvested in March 2020 on the rocky coast of Sidi Bouzid, El Jadida, Morocco (33° 13'55.8" N 8° 33'24.8" W). The thalli were rinsed with tap water and distilled water to remove attached shells, sand, and other algae. Seaweed samples were sun-dried for 3 days and then dried in the oven at 50°C to constant dry weight.

2.2. Agar extraction.

Native agar extraction was done using dry algae (10 g) hydrated in 500 ml distilled water at ambient temperature for 2 h and then heated at 100 °C for 1 h. The mixture was filtered using a filter cloth. The filtrates were allowed to gel at room temperature, frozen overnight, and thawed. The thawed gel was then washed and dehydrated with ethanol (96%) and was oven-dried (50°C) to constant weight.

Alkali-pretreated agar extraction was performed according to two alkali pretreatment procedures: *i)* Alkaline pretreatment using NaOH according to the method described by Villanueva *et al.* [25], with modification. 10 g of dry algae soaked in 500 mL of 10% w/v NaOH solution and heated in a water bat at 90 ° C for 2 h. The algal material was washed thoroughly with distilled water and then soaked in acetic acid (0.5%) at room temperature for

1 h. The Acetic acid solution was thrown, and seaweeds were extracted with 500 mL distilled water (with a pH 6.5) at 100°C for 1 h. The other remaining processes were done the same way as the native agar extraction. *ii)* Alkaline pretreatment using Na_2CO_3 according to the method described by Freile-Pelegrin *et al.* [26] slightly modified. Before the extraction, 10 g of dried seaweeds were exposed to 0.5% solution of Na_2CO_3 (500 ml) at 90 °C for 30 min and then washed with distilled water 4 times. The agar was extracted with distilled water at pH 6.5 and 100 °C for 2 h.

2.3. Spectroscopic characterization.

2.3.1. FT-IR.

FTIR spectral measurements of the agars samples were performed using a Thermo Scientific Nicolet Impact 400D FT-IR Spectrometer (Nicolet Instrument Co., Madison USA). The spectra were scanned between 4000 and 500 cm^{-1} in attenuated total reflectance (ATR) mode. A total of 32 scans were averaged for each sample at a 4 cm^{-1} resolution, and subsequently, the IR spectra were processed using the OMNIC software (Nicolet, Madison, USA).

2.3.2. ^{13}C NMR.

The ^{13}C NMR spectroscopic measurements of agar samples dissolved in D_2O were carried out at 353 K on Spectrometer AV II, operating at 400 MHz equipped with pulsed gradient units, using a 5 mm Triple resonance Broadband Inverse probe at a base frequency of 100.62 MHz. Presaturation was applied during the relaxation delay and mixing time. The raw data were apodized in one dimension with 0.5 for line broadening prior to Fourier transformation.

2.4. Rheological analyses.

Solutions (1.5% w/v) of extracted and commercial agar (Bacteriological agar type E, Biokar diagnostics, A1012 HA) were prepared and allowed to gel overnight at room temperature. The Gel strength was evaluated by measuring the load (g/cm^2), causing a cylindrical plunger (1 cm^2 cross-section) to break the gel in the 20s [27]. Gelling and melting temperatures were determined according to the method described by Freile-Pelegrın and Robledo [28] with modification. 1.5 % of extracted and commercial agar each separately were stirred for 5 min at 95°C in the water bath. The gelling temperature was measured by cooling 20 mL of hot agar solutions placed in test tubes (15 mm diameter, 200 mm height), including iron bead (8 mm diameter). The tubes were tilted up and down at room temperature until the bead ceased moving. The gel temperature was immediately measured by introducing a precision thermometer (0.1 °C divisions) into the agar. Melting temperature was measured on the same tubes used for the gelling temperature by clamping the tubes test in a water-bath. The temperature rose from 50 to 100°C at 0.5°C/ min. The melting point was recorded with a precision thermometer when the bead sank into the solution.

3. Results and Discussion

3.1. Extraction yield.

The agar yields from *Gelidium* species without and with alkali pretreatment are presented in Table 1. *G. corneum* showed agar yield varying from 6.20 to 20.50% dw, while *G. microdon* depicted agar contents from 12.23 to 17.73% dw. The assessment of these extraction yields with values previously recorded in the literature is very complex due to various factors such as collection time and region, environmental and physical factors of the species, and extraction conditions [29,30]. The results showed that the samples pretreated with a mild alkaline solution of Na_2CO_3 (0.5%) give a high agar yield compared to native extraction and strong alkali pretreatment using 10% NaOH. It has been reported that the alkali pretreatment results in significantly decreased extraction yields [28,31,32]. However, other literature reports highlighted that pre-extraction treatment using alkali or acid promotes a rise in agar yield compare to untreated samples [33,34]. The alkali pretreatment using 10% NaOH prior to the hot water extraction may have been too harsh for the *Gelidium* species resulting marked decrease in the extraction yields. This could be explained by the fact that the agar undergoes degradation and diffuses towards the aqueous medium during the alkaline treatment, thus causing the extraction yield to decrease [30,35]. The alkali concentration, pretreatment time, and temperature need to be optimized to minimize agar losses and enhance the extraction yield. Future investigation should be performed to verify the optimum extraction parameters needed to maximize agar yield and properties from both studied *Gelidium* species.

Table 1. Agar yield of *G. corneum* and *G. microdon* without and with different alkali pretreatments.

	Agar yeild (% Dw)		
	Native Extraction	**Pretreatment NaOH 10%**	**Pre-treatment Na_2CO_3 0.5%**
G. corneum	16.21 ± 0.42	6.20 ± 0.9	20.50 ± 0.37
G.microdon	12.23 ± 0.74	14.87 ± 1.69	17.73 ± 1.34

3.2. FTIR spectroscopy.

The ATR-FTIR spectra (Figure 1) of the extracted agars were recorded to identify major compositional differences between species and the used extraction method. All analyzed samples presented the typical spectra of agar-like galactans (Figure 1). The most characteristic bands were located at the region 800-1400 cm^{-1}, typically recognized as agarocolloid [36]. The small band located at 852 cm^{-1}, detected for native and Na_2CO_3 pretreated agar from *G. microdon* (Figure 1b), could be related to the sulfate groups at the C-4 position in the D-galactose units [36–39]. This pic disappears in agar pretreated with 10% NaOH. The bands at 887 cm^{-1} correspond to the C-H bending at the anomeric carbon in β galactopyranosyl residues [30,38]. This is at 928 cm^{-1} assigned to the C−O vibration of 3,6-anhydro-galactose residue [39,40]. The intensity of the characteristic band around 928 cm^{-1} was relatively higher after alkaline pretreatment using NaOH. It has been reported that alkaline pretreatment could convert sulfate substitution to 3,6-anhydrogalactose [30,41]. An intense absorption region centered at 1030 cm^{-1} and a band at 1147 cm^{-1} could be assigned to C−O and C−C stretching vibrations of the pyranose ring common to all polysaccharides [38–40]. The bands detected at 1243 cm^{-1}, and 1365 cm^{-1} are attributed to the ester sulfate groups antisymmetric stretching vibration [38–40,42]. The decrease in amplitude at 1250 cm^{-1} was detected after alkali pretreatments with NaOH and Na_2CO3 (Figure 1a,b). It has been previously suggested that eliminating unstable sulfate was achieved upon alkaline pretreatment [41].

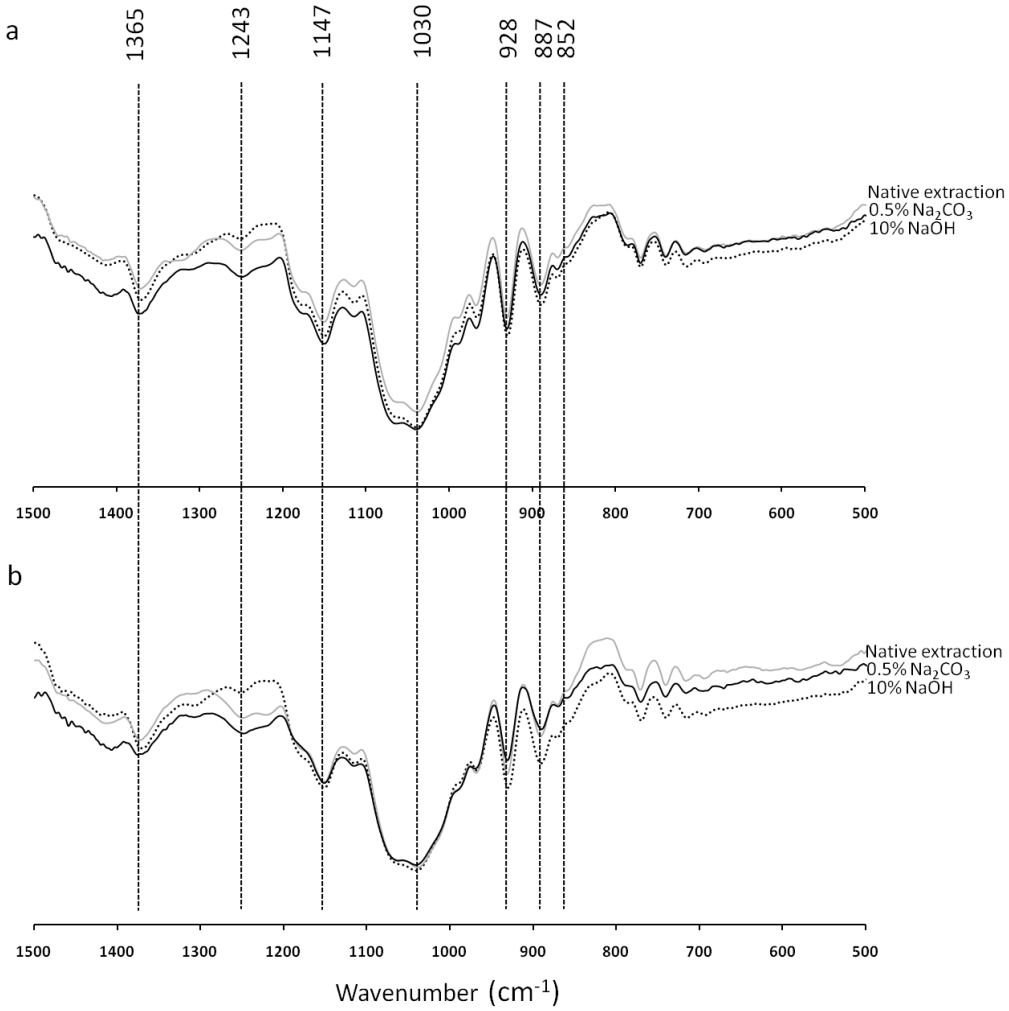

Figure 1. FTIR spectra of the agar extracted from (**a**) *Gelidium corneum;*(**b**) *Gelidium microdon.*

3.3. NMR spectroscopy.

Nuclear magnetic resonance (NMR) spectroscopy of ^{13}C is considered an efficient method to analyze red seaweed polysaccharides' structural features. The ^{13}C-NMR spectra of native and alkali-treated samples (Figures 2 and 3) showed an agar-type structure with 12 signals attributed to the carbons in agarobiose. The ^{13}C-NMR spectrum of the native agar from *G. corneum* (Figure 2a) was not significantly different from that shown in the alkali-treated agar (Figure 2b,c). All spectra in Figure 2 depicted signals at 102.6, 70.4, 82.4, 68.9, 75.5, and 61.6 ppm corresponding to C1, C2, C3, C4, C5, and C6 of β-D-galactopyranosyl 3-linked units [43], while the signals at 98.5, 80.3, 77.6, 75.8, 70.08, and 69.6 ppm attributed to C1, C3, C4, C5 C6, and C2, respectively, in 3,6-anhydro 4-linked-α-L-galactopyranosyl units [43–45]. The absence of the peak at 59.0 ppm corresponding to the O-methyl group indicates a lowly methylated agarose structure [44,46]. Similarly, O-methyl groups have not been detected in the agar of *G. corneum* from on the northern coast of Spain [39]. Nevertheless, the native agar from *G. microdon* (Figure 3a) had a partially methylated agarose structure. It showed minor resonance at 59.4 ppm typical of O-methylated agarobiose and another one at 98.9 ppm imputed to agarobiose containing 2-O-methyl-3,6-anhydro-L-galactose [43,46].

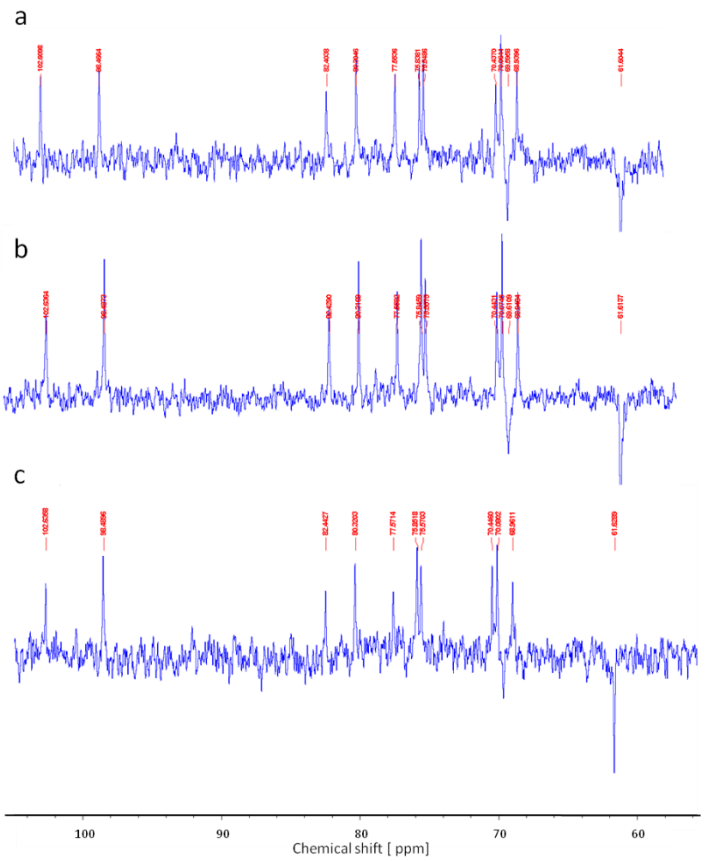

Figure 2. ^{13}C NMR spectra of agar extracted from *Gelidium corneum*; (**a**) native agar; (**b**) agar pretreated with 10% NaOH; (**c**) agar pretreated with 0.5% Na_2CO_3.

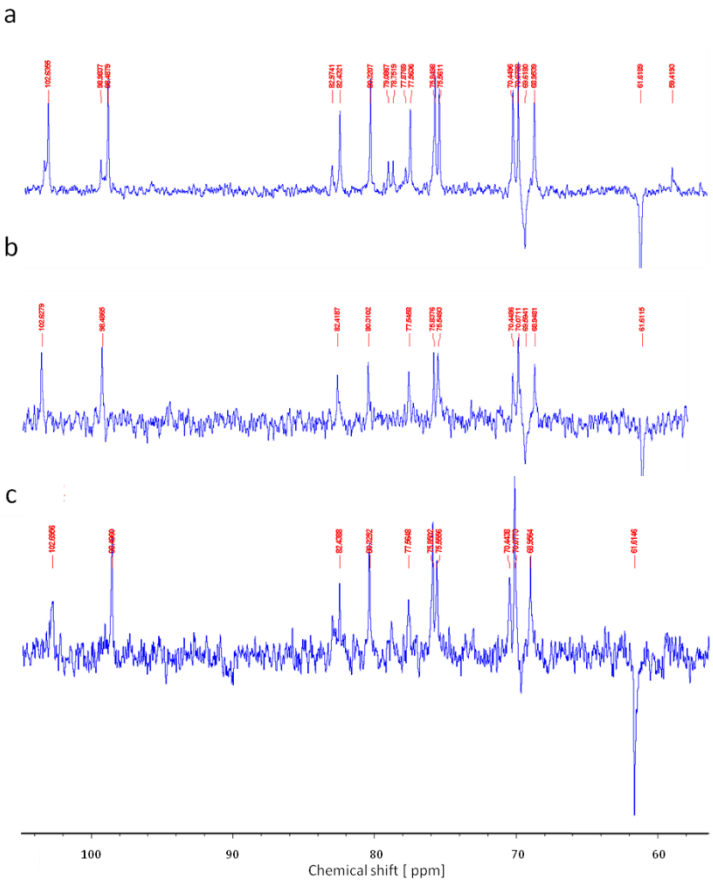

Figure 3. ^{13}C NMR spectra of agar extracted from *Gelidium microdon*; (**a**) native agar; (**b**) agar pretreated with 10% NaOH; (**c**) agar pretreated with 0.5% Na_2CO_3.

The comparison of the ^{13}C-NMR spectrum of native Agar from *G. microdon* (Figure 3a) and those of agar obtained after alkali pretreatment (Figure 3b,c) showed the disappearance of the signal at 78.7 ppm attributed to C4 in 4-O-L-galactose-6-sulfate [43,47].

3.4. Rheological properties.

3.4.1. Gel strength.

Gel strength is the main indicator of agar quality. It refers to the compressive force required to fracture an agar gel of a standard concentration of 1.5% (w/v). *G. corneum* showed higher gel strength than *G. microdon* (Table 2). The maximum value was detected for alkali treatment 10% NaOH with 528.55±11.08 g.cm^{-2} and 489.00±19.41 g.cm^{-2} in *G. corneum* and *G. microdon*, respectively. Alkali treatment's ability to reduce the sulfate content and improve the gel strength of agars has already been demonstrated for *Gelidium* species [35,48,49]. Fundamentally, agar gelation arises during the aggregation of helical conformation of agar polymers via hydrogen bonds. The charged groups (sulfate group) interfered with the intermolecular hydrogen bonding for double helices formation [50]. Thus, the decline of the sulfate group by alkaline hydrolysis contributes to increasing the ability to form double helices, strengthening the gel network [51]. The gels obtained from the commercial agar presented greater fracture strength values (Table 2). Nevertheless, the agars from both *Gelidium* species showed gel strength values within the range required by the international market. The latter, commonly, agree to the standard values appointed by the Japanese Specifications for Processed Agar (JSPA), which is 350 g.cm^{-2} for 1.5% (w/v) of the first-grade agar and 600 g.cm^{-2} for the higher grade agar, as measured by the Nikan-Sui method [52,53].

Table 2. Gel strength of agar from *G. corneum* and *G. microdon* without and with different alkali pretreatments.

	Gel strength (g.cm^{-2})		
	Native Extraction	**Pretreatment NaOH 10%**	**Pre-treatment Na_2CO_3 0.5%**
G. corneum	341.08 ± 16.91	528.55 ± 11.08	358.84 ± 15.30
G. microdon	350.00 ± 17.66	489.00 ± 19.41	364.55 ± 12.25
Commercial agar[1]	765.49 ± 48.06		

[1] type of extraction was not indicated

3.4.2. Gelling and melting temperatures.

Gelling and melting temperatures of agars without and with different alkaline pretreatments are presented in Table 3. Commercial agar had melting and gelling temperatures of 82.1and 32.8°C, respectively. The G. corneum's Gelling temperature ranged from 36 to 39°C, while *G. microdon* showed a gelling temperature of 35-38°C. These findings seem to corroborate the established negative relationship between methoxyl and sulfate contents and the gelling temperature [54,55]. As ^{13}C NMR highlighted, *G. microdon* spectra indicated a partially methylated agarose structure with the presence of sulfate groups in native agar. This could partially explain the lower gelling temperature recorded in *G. microdon* compared to *G. corneum*. The difference in gelling temperature between species may also be due to molecular weight, and molecular weight distribution may have an essential role in agar's gelation process [56]. Generally, *Gelidium* species have gelling temperatures in the range of 34-39°C [26,57]. It is evident that the extraction processes influenced both gelling and melting temperatures. The native agar formed gel at 35.12-36.01°C, and those with N_2CO_3 pretreatment became gel at a temperature range of 36.25-37.25°C, while agar with NaOH pretreatment formed gel at 38.27-39.16°C. The corresponding gels molt at 85.65-86.71°C, 86.32-87.13°C, and 88.31-

89.83°C, respectively. Higher melting temperatures of agar gels with alkaline pretreatment revealed that they were more stable than those of native agar [41]. It has been reported that pretreatment using NaOH concentration than 4% resulted in increases in gelling and melting temperatures [25].

Table 3. Gelling and melting temperatures of agar from *G. corneum* and *G. microdon* without and with different alkali pretreatments.

		Melting temperature (°C)	**Gelling temperature (°C)**
Gelidium corneum	Native Extraction	86.7 ± 0.6	36.0 ± 0.9
	Na_2CO_3 0.5%	87.1 ± 0.6	37.2 ± 0.9
	NaOH 10%	89.8 ± 0.1	39.2 ± 1.0
Gelidium microdon	Native Extraction	85.6 ± 0.2	35.1 ± 0.7
	Na_2CO_3 0.5%	86.3 ± 0.1	36.2 ± 0.5
	NaOH 10%	88.3 ± 0.7	38.3 ± 0.8
Commercial agar[1]		82.1 ± 0.4	32.8 ± 0.3

[1] type of extraction was not indicated

4. Conclusions

Spectroscopic and rheological characterization of agar extracted from the Moroccan Gelidiales *G. corneum* and *G. microdon* without and with alkali pretreatments (NaOH and N_2CO_3) were investigated. The highest agar yields (17.73-20.5%) of both species were obtained from mild alkaline pretreatment using 0.5% N_2CO_3. The native agar of *G. corneum* and *G. microdon* does not exceed 16.21% and 12.23%, respectively. The alkali pretreatments improved the gel strength, gelling, and melting temperatures. ^{13}C NMR spectra of *G. Corneum* demonstrated the characteristic pattern of unsubstituted and lowly methylated agar. Though, the native extraction of *G. microdon* gave agar substituted with methyl and sulfate groups at the C4 of 4-O-L-galactose residues, responsible for the low gelling ability of native agar. The FTIR spectroscopy revealed that both *Gelidium* species presented the typical spectra of agar-like galactans, attenuating sulfate groups after alkali pretreatment. *G. microdon* produces a quality of agar after alkaline treatment comparable to that of *G. Corneum*. Thus, *G. microdon* could be regarded as a potential additional source of agar industry in Morocco. Future investigation should verify the optimum extraction parameters needed to maximize the agar yield and properties considering each species' life cycle and the seasonal variation in agar yield.

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Conflicts of Interest

The authors declare no conflict of interest.

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