JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Structural, Physical, and Chemical Modifications Induced by Microwave Heating on Native Agar-like Galactans

Ana M. M. Sousa,[†] Simone Morais,[‡] Maria H. Abreu,[§,#] Rui Pereira,[§] Isabel Sousa-Pinto,[§] Eurico J. Cabrita,[⊥] Cristina Delerue-Matos,[‡] and Maria Pilar Gonçalves*[,†]

[†]REQUIMTE, Faculdade de Engenharia, Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

[‡]REQUIMTE, Instituto Superior de Engenharia do Porto, Rua Dr. António Bernardino de Almeida 431, 4200-072 Porto, Portugal

[§]CIMAR/CIIMAR – Centro Interdisciplinar de Investigação Marinha e Ambiental, Rua dos Bragas, 289, 4050-123 Porto, Portugal, and Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, Porto, Portugal

[#]Daithi O'Murchu Marine Research Station, Gearhies - Bantry, Ireland

[⊥]REQUIMTE, CQFB, Departamento de Química Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal

ABSTRACT: Native agars from *Gracilaria vermiculophylla* produced in sustainable aquaculture systems (IMTA) were extracted under conventional (TWE) and microwave (MAE) heating. The optimal extracts from both processes were compared in terms of their properties. The agars' structure was further investigated through Fourier transform infrared and NMR spectroscopy. Both samples showed a regular structure with an identical backbone, β-D-galactose (G) and 3,6-anhydro-α-L-galactose (LA) units; a considerable degree of methylation was found at C6 of the G units and, to a lesser extent, at C2 of the LA residues. The methylation degree in the G units was lower for MAE^{opt} agar; the sulfate content was also reduced. MAE led to higher agar recoveries with drastic extraction time and solvent volume reductions. Two times lower values of $[\eta]$ and M_v obtained for the MAE^{opt} sample indicate substantial depolymerization of the polysaccharide backbone; this was reflected in its gelling properties; yet it was clearly appropriate for commercial application in soft-texture food products.

KEYWORDS: *native agars, microwave irradiation, conventional heating, structural analysis, physicochemical properties, Gracilaria vermiculophylla*

INTRODUCTION

Native agar-like galactans have gained much industrial interest in recent years as gelling, stabilizing, and thickening agents in soft-texture food products. For these type of applications gel strengths in the range 30−200 g/cm^2 are considered the most appropriate.[1] Traditionally, these polysaccharides are hot water-extracted during several hours from selected marine red algae under conventional heating. No alkali pretreatment is used in the process as is the case for agars with higher gel strengths (i.e., alkali-treated agars; >700 g/cm^2) which are used industrially to increase the viscosity of aqueous solutions, to form gels (jellies) with several degrees of firmness, and to stabilize some products, such as ice cream. However, conventional heating is a time-consuming process that requires high solvent consumption and generates large amounts of waste disposal. Therefore, recycling of water is necessary, which restricts the factory location.[2]

Typically, native agars present a complex structure composed mainly of two linear polysaccharides, agarose and agaran. Agarose, considered the ideal structure, is built of repeating units of 3-linked β-D-galactose (G) and 4-linked 3,6-anhydro-α-L-galactose (LA), and is responsible for agars' gelling properties. When the anhydride bridge of the 4-linked unit is absent, the polysaccharide is called agaran.[3,4] Several substituents such as sulfate ester, methyl ether, pyruvate acid acetal, and other monosaccharides can replace the hydroxil groups of these polysaccharides. For instance, α-L-galactose 6-sulfate units (L6S; percursor units of LA), commonly observed in native agars, are responsible for their low gelling ability.[4]

Gracilaria vermiculophylla (Gracilariales, Rhodophyta) is a nonindigenous seaweed species currently dominant in Ria de Aveiro, Portugal (40° 38″ N, 8° 43″ W). Because of the negative impact that it may have in the recipient community it is important to manage its abundance.[5] Accordingly, intensive joint efforts of Portuguese research groups of different expertise areas have been carried out to explore the potentialities of this seaweed.[6−8] Among the several tested strategies the use of *G. vermiculophylla* after cultivation in integrated multi-trophic aquaculture (IMTA) systems, as raw material for agar extraction using a greener process, is by far the most promising.[6] The use of this seaweed as a biofilter component in a sustainable ecosystem-like farming approach, such as IMTA, was recently tested with success.[8] Subsequently, the cultivated biomass (of no apparent commercial value) was used in a pioneer study concerning the isolation of alkali-treated agars using microwave-assisted extraction (MAE).[6] As observed in many other cases, the use of MAE allowed the process to become less laborious, using less energy and solvent volume, at the same time improving the reproducibility and extraction

Received: December 28, 2011
Revised: April 27, 2012
Accepted: April 28, 2012
Published: April 28, 2012

yields when compared with the traditional method.[6,9,10] Moreover, agars obtained by MAE revealed enhanced gelling properties compared to the ones extracted using the conventional method.[6] Yet, some crucial aspects related to the microwave influence in the structure of the polysaccharides were not covered in this first work. For instance, the great accelerations caused by the heating of the whole material simultaneously (as long as the solvent−matrix system has the ability to absorb microwaves) and the use of elevated temperatures/pressures which promote the rapid mass transfer of target compounds from the sample matrix[9] can lead to significant modifications in the extracted polysaccharides. Although the structural analysis of seaweed galactans extracted using conventional heating is well documented,[4,7,11−16] no report was found focused on the structural and physicochemical characterization of red seaweeds polysaccharides obtained by MAE.

Therefore, in the present study, we focused on the study of native (i.e., non-alkali-treated) agar extracted from low value biomass (*G. vermiculophylla* produced in IMTA systems) using the MAE technique. The process was first optimized in terms of yield and gel strength; then, a detailed physicochemical characterization of the optimal agar extract was conducted. The aim was to show possible chemical and structural differences in the optimal MAE native agar with respect to the native agar extracted by the conventional method (TWE). Changes in the backbone structure of the polysaccharides were followed through Fourier transform infrared (FTIR) and NMR spectroscopy. Also, gelling and melting temperatures, intrinsic viscosities, as well as sulfate and LA contents, of the MAE and TWE native agars were determined.

MATERIALS AND METHODS

Reagents. The reagents used in this experiment included hydrochloric acid, resorcin, acetaldehyde diethyl acetal, fructose, sodium thiocyanate, and trichloroacetic acid, all of analytical and/or HPLC grade. The standards used in chemical analysis were sodium sulfate from Fluka (puriss p.a. ACS ≥ 99.0%) and agarose, type II from Sigma-Aldrich Co., St. Louis, MO. 3-(Trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSP) added as an internal chemical shift reference (δ_H = −0.017 ppm; δ_C = −0.18 ppm) in NMR experiments and commercial agar (A-7002) used as reference in structural analysis were purchased from Sigma-Aldrich as well.

Seaweed Preparation. *Gracilaria vermiculophylla* samples were obtained after a cultivation period of four weeks in an IMTA system located at A. Coelho & Castro aquaculture, Rio Alto, northwestern Portugal. Biomass of 12 different tanks was mixed, washed with freshwater, and dried in an oven at 60 °C during 72 h. The algal material was then ready for extraction. Two mixtures of IMTA *G. vermiculophylla* were used in this work: the first to obtain the optimal MAE conditions of native agars and the second to perform the structural and physicochemical characterization of optimal MAE and TWE agars. Both batches of seaweeds resulted from a mixture between the several IMTA tanks differing only in the season of the cultivation period.

Apparatuses and Operational Parameters. TWE of native agar was performed in a Binder GmbH heating oven (FD 53, Tuttlingen, Germany) following a previously optimized procedure[7] with some adjustments: four grams of dried sample were hot water extracted (200 mL) at 85 °C during 2 h with no agitation. Contrary to the previous work, no steps prior to TWE were performed as the main goal of the current study was to infer the induced differences in native agars extracted under different heat sources (microwave irradiation or conventional heating). MAEs were carried out in a MARS-X 1500 W (Microwave Accelerated Reaction System for Extraction and Digestion, CEM, Mathews, NC, USA) configured with a 14 position carousel of Teflon extraction vessels and following a procedure described elsewhere.[6] Optimal extracts from both processes were purified through the centrifugation of 0.2% (w/w) agar solutions using a Beckman Coulter centrifuge (model Alegra 25R) at 40 °C, 21000*g*, 9 ACC for 1 h.

The NMR spectroscopic measurements of samples were carried out nonspinning at 80 °C on a Bruker Avance III spectrometer operating at 400.15 MHz for protons and 100.62 MHz for carbon, equipped with pulsed gradient units, using a 5 mm QNP probe equipped with a *z* gradient coil capable of producing magnetic field pulsed gradients in the *z*-direction of 56.0 G/cm. Agar was dissolved in D_2O to a final concentration of 30 mg mL^{-1} and TSP was added as reference. Standard 1D ^1H NMR experiments were performed with 30° pulses for excitation (typical 90° degree pulse width of 12.5 μs), acquisition time 3.98 s, relaxation delay 1 s; 16 transients of a spectral width of 8000 Hz were collected into 64 K time domain points. For the full range broad-band proton decoupled ^{13}C NMR spectra, the following acquisition parameters were used: experiments with 30° excitation pulses (90° degree pulse width of 9 μs), acquisition time of 0.8 s, relaxation delay of 2 s, spectral with 24 000 Hz, 32 K time domain points, and 5K transients were collected. Two-dimensional gradient-selected $^1H/^{13}C$ heteronuclear single quantum coherence (HSQC) spectra were recorded using the standard Bruker software via double inept transfer, using sensitivity improvement and decoupling during the acquisition. A total of 1024 data points in F2 over a spectral width of ca. 6000 Hz for ^1H and 256 data points in F1 over a spectral width of 16 000 Hz for ^{13}C were collected, with eight scans per increment and a relaxation delay of 1.5 s between scans. The delay for the INEPT transfer was set to 1.73 ms corresponding to a C−H coupling constant of 145 Hz. All the NMR spectra were processed in MNova Software version 7.1.1. The chemical nomenclature of residues followed the shorthand notation system defined by Knutsen et al.[3] (see Abbreviations Used for details). The degrees of methylation at C6 of G and at C2 of LA units were estimated from the ^1H NMR spectral data by the ratio between 1/3 of the area of the peak at ∼3.41 and areas of low intensity singlets in the range ∼3.54−3.44 ppm, respectively, and the peak area of H1 of the 4-linked residues [LA (5.13 ppm) + L6S (5.28 ppm)].[7] FTIR analysis seemed to indicate some kind of sulfation in LA units, but it was clearly below the detection limit of the NMR analysis and so it was not considered in the calculation. The FTIR spectra of native agars films were recorded using a Bomem MB-series FTIR spectrometer (ABB Bomem, Inc., Quebec) and following a previous procedure.[7] Commercial agar was used as a reference in both spectroscopic measurements.

Gel strength (GS) of 1.5% (w/w) agar gels was determined using a texture analyzer (Stable Micro Systems model TA-XT2, Surrey, England) with a cylindrical probe (10 mm diameter and 0.2 mm/s penetration rate). The gelling (T_g) and melting (T_m) temperatures of native agars were obtained through dynamic rheological measurements in a stress-controlled rheometer (ARG2, TA Instruments, USA). The sulfate and LA contents of optimal extracts were determined, by turbidimetric (Jackson & McCandless)[17] and colorimetric (Yaphe & Arsenault)[18] methods, respectively. The intrinsic viscosities ([η]; mL/g) were determined by dilute solution viscometry using a Cannon-Fenske viscometer (size 50; Comecta S.A., Barcelona), at 35.0 ± 0.1 °C. Agar dilute solutions were prepared using 0.75 mol/L NaSCN as solvent as it inhibits the aggregation between agar molecules.[20] Average flow times were used for the calculations, and [η] was obtained by extrapolation to zero concentration of Huggins (eq 1) and Kraemer (eq 2) relations, respectively:

$$\eta_{red} = [\eta](1 + K_H[\eta]C) \quad (1)$$

$$\frac{\ln(\eta_{rel})}{C} = [\eta](1 - K_K[\eta]C) \quad (2)$$

where η_{red} and $(\ln \eta_{rel})/C$ are the reduced and inherent viscosities, C is the concentration of the polymer solution, and K_H and K_K are the Huggins and Kraemer constants, respectively. The viscosity-average

Table 1. Analysis of Variance (ANOVA) for the Regression Models[a]

	yield, Y_1 (%)					gel strength, Y_2 (g/cm^2)				
source	SS	DF	MS	*F*-value	*p*	SS	DF	MS	*F*-value	*p*
model	104.03	14	7.43	9.21	<0.0001[b]	8132.43	14	580.89	7.99	<0.0001[b]
X_1	11.93	1	11.93	14.78	0.0010[b]	339.68	1	339.68	4.67	0.0429[b]
X_2	29.26	1	29.26	36.25	<0.0001[b]	3053.05	1	3053.05	42.00	<0.0001[b]
X_3	2.60	1	2.60	3.22	0.0878[c]	447.29	1	447.29	6.15	0.0221[b]
X_4	1.65	1	1.65	2.05	0.1679[d]	1281.85	1	1281.85	17.63	0.0004[b]
X_1^2	19.48	1	19.48	24.14	<0.0001[b]	143.19	1	143.19	1.97	0.1758[d]
X_2^2	11.28	1	11.28	13.97	0.0013[b]	3.36	1	3.36	0.05	0.8319[d]
X_3^2	10.00	1	10.00	12.38	0.0022[b]	499.43	1	499.43	6.87	0.0164[b]
X_4^2	6.23	1	6.23	7.72	0.0116[b]	78.23	1	78.23	1.08	0.3119[d]
X_1X_2	1.38	1	1.38	1.71	0.2057[c]	49.88	1	49.88	0.69	0.4173[d]
X_1X_3	2.81	1	2.81	3.48	0.0770[c]	18.43	1	18.43	0.25	0.6201[d]
X_1X_4	0.02	1	0.02	0.02	0.8864[d]	211.56	1	211.56	2.91	0.1035[d]
X_2X_3	0.08	1	0.08	0.09	0.7627[d]	6.14	1	6.14	0.08	0.7744[d]
X_2X_4	4.73	1	4.73	5.86	0.0251[b]	618.52	1	618.52	8.51	0.0085[b]
X_3X_4	0.68	1	0.68	0.84	0.3694[d]	317.91	1	317.91	4.37	0.0495[b]
residual	16.14	20	0.81			1453.92	20	72.70		
lack of fit	5.25	9	0.58	0.59	0.7816[d]	864.91	9	96.10	1.79	0.1784[d]
total	120.17	34				9586.35	34			
R^2 0.87			R^2 adjusted 0.77			R^2 0.85			R^2 adjusted 0.74	

[a] X_1 = extraction time (min); X_2 = temperature (°C); X_3 = solvent volume (mL); X_4 = stirring speed. SS = sum of squares; DF = degree of freedom; MS = mean square; R^2 = quadratic correlation coefficient. [b] Significant ($p < 0.05$). [c] Marginally significant ($p < 0.10$). [d] Not significant ($p > 0.10$).

molecular masses (M_v; Da) were determined using the Mark–Houwink equation (eq 3) for the described experimental conditions:[20]

$$[\eta] = 0.07M_v^{0.72} \quad (3)$$

All experiments from physicochemical characterization were performed, at least, in triplicate and details can be found elsewhere.[6,7]

Statistical Analysis and Optimization Using Design of Experiments. An orthogonal central composite design with four parameters (2^4) coupled with RSM was used to optimize native agar MAE.[19] The influence of extraction time (X_1; min), temperature (X_2; °C), solvent volume (X_3; mL), and stirring speed (X_4; four positions are available in modern apparatus: turned off, minimum, medium, and maximum speed) on response variables, yield (Y_1; %) and GS (Y_2; g/cm^2), was studied. Details of the adopted statistical strategy can be found elsewhere.[6]

RESULTS AND DISCUSSION

Native Agars MAE Optimization. The coefficient estimates of the regression models for the two studied response variables were determined according to experimental results (data not shown). By eliminating the non-significant parameters ($p > 0.10$ in Table 1), response surface regression gave the following model equations for the recoded factor values (± 1, 0, $\pm \alpha$):

$$Y_1 = 19.00 + 1.410X_1 + 2.208X_2 + 0.658X_3 - 1.683X_1^2 - 1.280X_2^2 - 1.205X_3^2 - 1.434X_4^2 + 0.838X_1X_3 + 1.088X_2X_4 \quad (4)$$

$$Y_2 = 31.36 + 7.524X_1 + 22.56X_2 + 8.634X_3 + 15.46X_4 - 8.519X_3^2 + 12.44X_2X_4 + 8.915X_3X_4 \quad (5)$$

As desired, yield second order model (eq 4) reached high statistical significance ($p < 0.0001$) contrary to its lack of fit ($p > 0.05$). The high *F*-value of the model (9.21) was indicative of the factor's significant effect in the response (Table 1). The significant ($p < 0.05$) effects assumed by the prediction equation were X_1, X_2, X_1^2, X_2^2, X_3^2, and X_4^2, while X_3 and X_1X_3 were marginally significant ($p < 0.10$). Clearly, a strong curvature effect of the factors in yield was observed. The value of $R^2 = 0.87$ and adjusted $R^2 = 0.77$ stated a good degree of correlation between observed and predicted values. R^2 was clearly above the minimum accepted for data of chemical nature (>0.8),[6] and so the model was selected. The software predicted a maximum agar yield of, $Y_1 = 20.4\%$, at critical values $X_1 = 19.5$ min; $X_2 = 114.9$ °C; $X_3 = 48.3$ mL; $X_4 = 2.9$ (maximum speed) which was considered.

GS second order model (eq 5) also reached high statistical significance ($p < 0.0001$), and the lack of fit was found to be insignificant ($p > 0.05$) meaning that the fitted model adequately represented the experimental results (Table 1). Again, the R^2 value (0.85) was above the minimum limit of acceptance for chemical data. All the main effects (X_1, X_2, X_3, X_4) were considered significant ($p < 0.05$) by the model as well as X_3^2, X_2X_4, and X_3X_4. The software predicted a saddle point close to a minimum and so it was not considered.

An accurate observation of experimental data, surface 3D plots, and ANOVA results was carried out in order to ensure an adequate interpretation of RSM. All parameters were considered in the design with the intent of minimizing the error. X_2 was the most influential parameter in the yield of native agars MAE ($p < 0.0001$; Table 1) with values in the range 110–120 °C clearly improving results, reaching its maximum at around 115 °C (e.g., Figure 1). Solubility of the target compound and diffusion rate to the sample-matrix are favored by high temperatures due to the decrease in surface tension and viscosity. These findings were in line with our previous study of alkali-treated agars extracted from the same IMTA biomass.[6] Additional experiments performed at higher temperatures (130 °C) showed that, after this point, polysaccharide degradation took place, decreasing the recovery (~30% less; results not shown). Therefore, operating with temperatures above 120 °C should be avoided. The negative effect of X_2^2 ($p < 0.01$) was in line with these findings. High

dx.doi.org/10.1021/jf2053542 | J. Agric. Food Chem. 2012, 60, 4977–4985

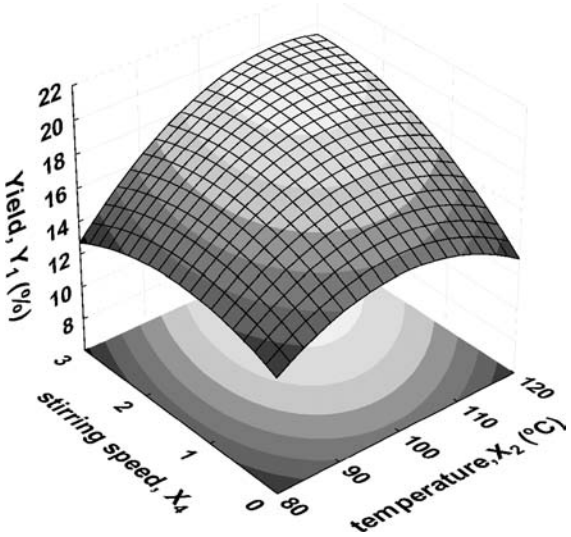

Figure 1. Response surface of MAE yield (Y_1; %) of native agars from IMTA *G.vermiculophylla* as a function of temperature (X_2; °C) and stirring speed (X_4) (extraction time (X_1; min) = 15 min and solvent volume (X_3; mL) = 40 mL).

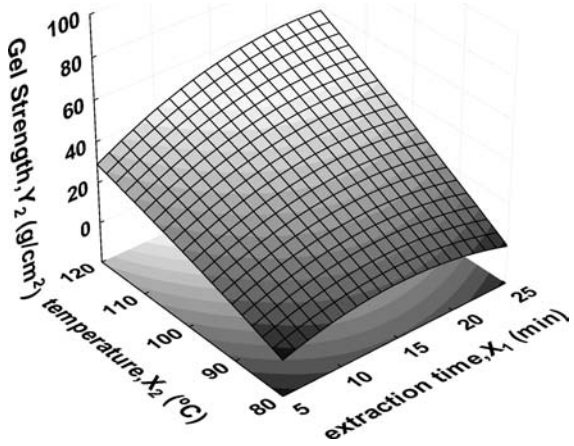

Figure 2. Response surface of gel strength (Y_2; g/cm^2) of MAE native agars from *G.vermiculophylla* as a function of extraction time (X_1; min) and temperature (X_2; °C) (solvent volume (X_3) = 40 mL and stirring speed (X_4) = medium).

speeds of agitation (medium and maximum) coupled with temperatures in the optimum range (110−120 °C) attained the best recoveries ($p < 0.05$; Figure 1). A positive relation was found between the solvent amount and the time used in the extraction process ($p < 0.10$; figure not shown). Besides the predicted maximum point, the best sets of experimental conditions (set 1: 20 min, 110 °C, 30 mL and minimum speed, Y_1 = 18.3%; set 2: 20 min, 110 °C, 50 mL and maximum speed, Y_1 = 19.8%; set 3: 15 min, 120 °C, 40 mL and medium speed, Y_1 = 19.7%) were tested. Replicates of each set of parameters showed that the response obtained with the critical point (19.3 ± 1.1%) was not significantly different ($p > 0.05$) from the remaining conditions (set 1: 18.3 ± 4.8%, set 2: 19.7 ± 2.2%, and set 3: 19.6 ± 1.0%). This could be attributed to the variability not accounted for by the regression equation.

X_2 has also the most influential effect on native agars GS ($p < 0.0001$; Table 1) with an increase in temperature clearly producing an improvement in agar gelling properties (e.g., Figure 2). A strong X_2X_4 interaction was found ($p < 0.01$), with high values of both parameters producing the best results (figure not shown). Highest GS was achieved with 15 min, 120 °C, 40 mL of solvent, and medium speed of agitation (set 3; Y_2 = 80.5 g/cm^2). Additional experiments proved that, above this temperature limit, native agars were degraded and their gelling properties were compromised. X_3 had a positive influence in GS ($p < 0.05$) with values in the range 40−60 mL being the best option. Outside these limits, a decrease in response was observed (X_3^2 negative effect; $p < 0.05$). The p-value of X_3X_4 (<0.05) stated a positive interaction between volume and speed of agitation in the GS of the polymer. Finally, extraction times in the range of 15−25 min favored GS ($p < 0.05$; Table 1 and Figure 2).

Considering energy and solvent saving concerns, lower range limits of solvent volume (40 mL) and extraction time (15 min) were chosen, as no significant differences were found in the response when considering lower/upper limit values. Remaining parameters were fixed at their maximum levels: 15 min of extraction, 120 °C, 40 mL of solvent, and maximum stirring speed (set 4 conditions). Experimental run that led to the highest GS (set 3) was also investigated. Replicates of the tested set of optimal conditions showed that, clearly, set 3 led to the best gelling properties (78.0 ± 2.3 g/cm^2 against 53.3 ± 1.0 g/cm^2 of set 4 conditions; $p < 0.05$). The observed differences could be related to problems with the model predictability (15.17% of unexplained variability in the data by the model); therefore, optimal GS conditions were considered to be set 3 conditions: 15 min of extraction, 120 °C, 40 mL of solvent, and medium stirring speed. As no significant differences were found between all tested set of parameters for agar recovery (Y_1), set 3 conditions were also chosen for Y_1. From this point on, native agar obtained using the conventional extraction (2 h, 85 °C, 200 mL of solvent and no agitation)[7] will be denoted as TWE^{opt} agar, while MAE^{opt} agar will be used for the optimal extract obtained using MAE (set 3 conditions).

Structural Analysis. Tables 2 and 3 show, respectively, the obtained ^{13}C and ^{1}H chemical assignments of native agars. Resonances found by other authors are also included in the tables for comparison purposes. The observed displacement (~2.2 ppm more downfield) between the obtained chemical shifts when compared with other reports[11,15] are attributed to differences in the chemical shift of the reference used by the authors relative to what was recently found when measured in polar solvent.[7] As temperature and stirring speed were the most influential parameters in the studied responses of MAE, an additional sample was considered in the structural analysis in order to better understand the changes induced by the microwave heating in agar structure (MAE^1 agar: 15 min, 100 °C, 40 mL of solvent and no agitation). HSQC spectra of MAE^{opt} (a), TWE^{opt} (b), and MAE^1 (c) agars are presented in Figure 3. The 12 characteristic signals of agarose were assigned in the ^{13}C NMR spectra of all native agars. This was positively compared with the commercial sample (spectra not shown). L6S residues were detected in MAE agars (Figure 3a,c) through the presence of a minor cross-peak at (~5.28; 103.5) ppm attributed to H1 and C1 of L6S, respectively.[7] This peak was absent in the commercial agar spectrum. Typical C1 signal of G (~105.6 ppm) in G-L6S diads was barely discernible in ^{13}C NMR spectra (Table 2).[4,11] The absence of the respective cross peak of L6S units in TWE^{opt} agar (Figure 3b) was expected since this sample needed to be diluted due to its high viscosity. Nevertheless, it was clear from its ^{1}H NMR spectrum the

Table 2. Assignments of Major Chemical Shifts (ppm) in the ^{13}C NMR Spectra of Native Agars from IMTA *G. vermiculophylla* Extracted Using Microwave (MAE[1] and MAE^{opt}) and Conventional (TWE^{opt}) Heating[a]

	carbon chemical shift (ppm)							
	C1	C2	C3	C4	C5	C6	M	ref
G	102.3	70.1	82.2	68.6	75.2	61.3		Usov et al., 1980
LA	98.2	69.7	80.0	77.2	75.5	69.7		
G6M	102.4	70.0	82.0	67.2	73.2	70.0	59.0	
LA	98.3	70.0	80.0	77.4	75.6	69.0		
G	102.6	70.2	92.7	68.7	75.6	61.4		
LA2M	98.7	78.8	78.4	77.6	75.3	69.8	59.1	
G	103.7	69.7	81.1	69.1	75.8	61.4		
L6S	101.2	69.7	70.9	79.0	73.5	67.6		
G (LA)	102.3	70.2	82.1	68.7	75.2	61.3		Falshaw et al., 1999
G(L6S)	103.4	70.5	80.9/81.0	69.0	75.2	61.6		
LA	98.1	69.8	80.0	77.2	75.5	69.3		
L6S	100.9	nd	70.9	78.9	nd	67.3		
G	104.8	72.3	84.6	71.2	77.8	63.8		MAE[1]
LA	100.7	71.8	82.5	79.8	78.1	71.4		
G6M	104.9	72.2	84.6	71.3	75.9	72.8	61.5	
LA	100.7	72.6	82.5	79.8	78.2	71.4		
G	104.9	72.3	nd	71.3	78.1	63.9		
LA2M	100.7	81.1	80.1	79.9	78.1	71.5	61.5	
G	105.9	71.6	84.4	71.3	77.8	64.1		
L6S[b]	103.5	71.3	73.0	81.1	72.5	69.8		
G	104.8	72.3	84.6	71.2	77.8	63.8		MAE^{opt}
LA	100.7	71.8	82.5	79.8	78.1	71.4		
G6M	104.6	72.2	84.6	71.2	76.0	72.8	61.5	
LA	100.7	72.6	82.5	79.8	78.2	71.4		
G	104.9	72.3	nd	71.3	78.2	63.9		
LA2M	100.7	80.5	80.1	79.8	78.1	71.5	61.5	
G	105.9[d]	71.6	84.4	71.3	77.9	63.9		
L6S[b]	103.5	71.3	72.8	80.5	72.5	69.9		
G	104.8	72.3	84.6	71.2	77.8	63.8		TWE^{opt}
LA	100.7	71.8	82.5	79.8	78.1	71.4		
G6M	104.9	72.6	84.6	71.2	76.0	72.5	61.5	
LA	100.8	72.5	82.5	79.9	78.2	71.4		
G	104.9	72.3	nd	71.3	78.2	63.9		
LA2M	100.8	81.1	80.0	79.9	77.8	71.5	61.5	
G	nd	71.6	84.5	71.3	77.8	64.1		
L6S[c]	103.5	71.4	72.8	81.1	72.5	70.0		

[a]Comparison with assignments of other reports. The displacement observed in the assignments results from differences in the reference compound used (see discussion section for details). nd = not discernible. [b]Corroborated by a signal in ^{1}H NMR spectra (~5.28 ppm) and FTIR analysis. [c]Other lower intensity peaks were observed in this region; see Discussion for details. [d]Two low intensity signals at 105.6 and 105.9 ppm; see Discussion for details.

presence of the typical signal of the LA precursor residues (5.28 ppm; horizontal trace in Figure 3b). An additional signal in the region of C1 of L6S residues (105.9 ppm) was observed for MAE^{opt} agar and could be attributed to different positions of the adjacent groups of the sulfate substituents. Considerable methylation at C6 of the G residue was detected through the presence of a cross peak at (3.41; 61.5) ppm in all spectra (Figure 3). The presence of M groups in the polysaccharides results in a peak at about 59 ppm due to an upfield shift of the carbon M signal when compared with remaining carbons of the sugar residues in ^{13}C NMR spectra.[12] The resonances observed at ~75.9 and ~72.8 ppm result of an upfield shift of 2 ppm of C5 and a downfield of almost 10 ppm of C6 in the G units, respectively, and are also associated with this type of substitution.[12,21] The splitting of the H signal of the M group (~3.43 ppm; almost imperceptible in the horizontal 1D spectra of Figure 3a,b) could be attributed to different configurations (axial and/or equatorial) of the −OH and CH_2OH.[11,12] Despite the different energy sources used in the extraction process, MAE[1] (15 min under microwave heat, 100 °C, 40 mL of solvent, no agitation) and TWE^{opt} (2 h under conventional heat, 85 °C, 200 mL of solvent, no agitation) agars revealed identical methylation degree at C6 of G units (~30% mol). On the contrary, more severe MAE conditions (MAE^{opt}: 15 min, 120 °C, 40 mL, medium agitation) led to a decrease of the methylation level of native agar (25.6% mol). This could be

Table 3. Assignments of Major ^{1}H Chemical Shifts (ppm) of Native Agars from IMTA *G. vermiculophylla* Extracted Using Conventional (TWE^{opt}) and Microwave (MAE^{opt} and MAE^1) Heating. Comparison with Assignments of Villanueva et al. (2010)[7]

	proton chemical shift (ppm)			
	MAE^1	MAE^{opt}	TWE^{opt}	Villanueva et al. (2010)
G1	4.54	4.55	4.55	4.55
G2	3.62	3.62	3.62	3.63
G3	3.75	3.76	3.76	3.76
G4	4.10	4.11	4.11	4.11
G5	3.71	3.71	3.71	3.69
G6	3.67^a/3.75^b	3.68^a/3.76^b	3.68^a/3.76^b	3.73^a/3.79^b
G6M	3.40	3.41	3.41	3.41
LA1	5.13	5.13	5.13	5.13
LA2	4.10	4.10	4.11	4.11
LA3	4.52	4.52	4.52	4.55
LA4	4.65	4.66	4.66	4.66
LA5	4.54	4.52	4.52	4.55
LA6	4.01^c/4.25^d	4.02^c/4.26^d	4.00^c/4.26^d	4.02^c/4.24^d
LA2M	3.49	3.50	3.50	3.52
L6S	5.28	5.28	5.28	5.28

^{a}G6 proton. ^{b}G6′ proton. ^{c}LA6$_{exo}$ proton. ^{d}LA6$_{endo}$ proton.

related to some instability of the M groups that are more likely to suffer degradation under more extreme heating conditions. Two additional minor cross-peaks at around (3.10; 56.0) and (3.21; 42.0) ppm were detected in both MAE samples (Figure 3a,c). Additional studies would be needed in order to clarify the nature of these signals. Another methylation occurring in lower extent was observed at C2 of the LA units through the appearance of small intensity peaks in the region (3.54−3.45) ppm in the ^{1}H spectra of all extracts. Distinct configurations of the adjacent groups were assumed to cause the presence of several chemical shifts.[11,12] MAE^1 agar evidenced a minor amount of this kind of substitution (6.4% mol) while TWE^{opt} and MAE^{opt} samples showed similar levels (7.7 and 7.9% mol). In this case, more extreme MAE conditions (MAE^{opt}) did not decrease the methylation level and this could be attributed to the high stability of the residues. The absence of a cross peak in 2D spectra was attributed to a concentration below the detection limit, as observed before for the same wild biomass.[7] G units with six-membered (4,6)-carboxylethylidene cyclic ketals[12] and more rarely five-membered cyclic ketals with O-3 and O-4,[14] can be found in algal polysaccharides. Typical signals of these pyruvate residues such as methyl, acetal, and carbonyl may be detected in the regions 15−60, 103.8−105.3/108.3, and 165−180 ppm, respectively.[14] No correlations in the HSQC spectra of the native polysaccharides were detected in the referred methyl region. Because of the typical low concentration of this type of residues in agars from *Gracilaria*[21] further 1D ^{13}C attached proton tests (APTs) were performed with more scans than the previous ^{13}C spectra in order to check for the existence of typical signals of acetal C. No methyl, acetal, or carbonyl carbons could be detected in the APT spectrum (data not shown). Therefore, we concluded that no traceable amounts of pyruvated G segments existed in the extracts and that the signal around (1.30; 30) ppm could be related with nongalactan signals.[22] No detectable starch contamination was found in native agars from IMTA seaweed (absence of typical C NMR signals of (1 → 4) (1 → 6)-α-D-glucan)[4] contrary to wild *G. vermiculophylla* from French waters.[23]

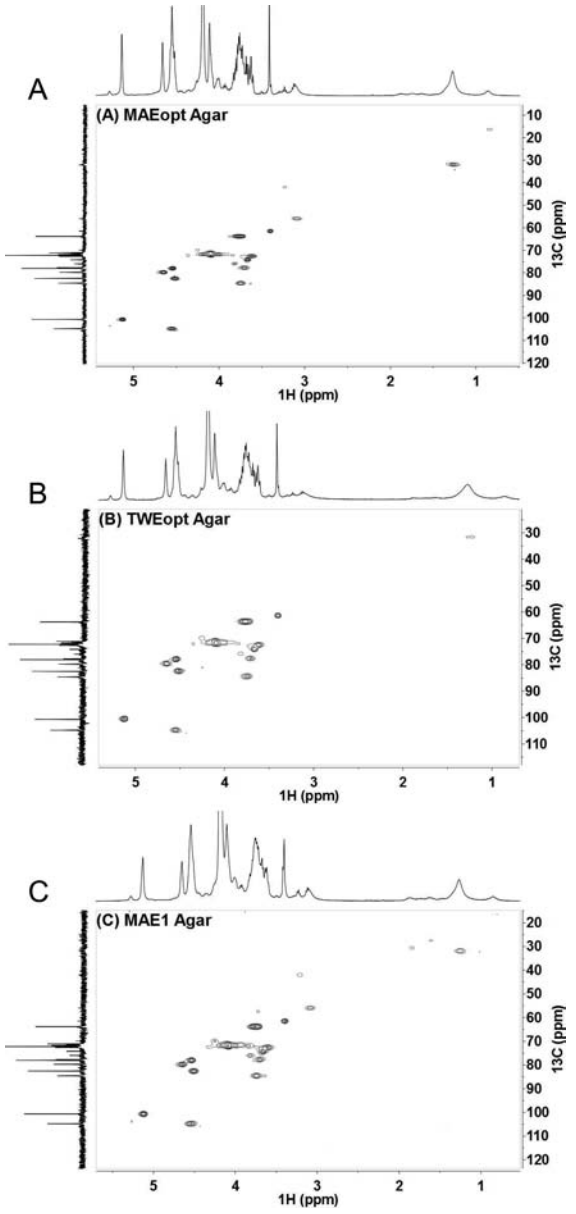

Figure 3. HSQC spectra of MAE^{opt} (A), TWE^{opt} (B), and MAE^1 (C) native agars from IMTA *G. vermiculophylla*. Outside the plots are the corresponding 1D NMR spectra of each nucleus (^{13}C on the left and ^{1}H on top). Other minor components resolved in individual spectra are presented in the text.

FTIR analysis showed typical bands of agar-like galactans and were well related with NMR results. All agars (including the commercial) showed similar spectra regardless of the extraction method used (Figure 4). A broad band at 930 cm^{-1} attributed to C−O vibration of LA units and diagnostic bands of total sulfate, at 1250 cm^{-1}, related to the S═O antysimmetric stretching vibration (used for calculating total S content)[13,16,23,24] and at 1370 cm^{-1}, related to ester sulfates,[23] were observed. As expected, the A_{930}/A_{1250} ratio was higher for the commercial sample (11.60 ± 0.41) and lower for native agars (MAE^1: 4.17 ± 0.47; MAE^{opt}: 4.76 ± 0.53; and TWE^{opt}: 3.86 ± 0.55). An intense absorption region centered at ∼1060 cm^{-1} and a band at ∼1150 cm^{-1} could be assigned to C−O and

dx.doi.org/10.1021/jf2053542 | J. Agric. Food Chem. 2012, 60, 4977−4985

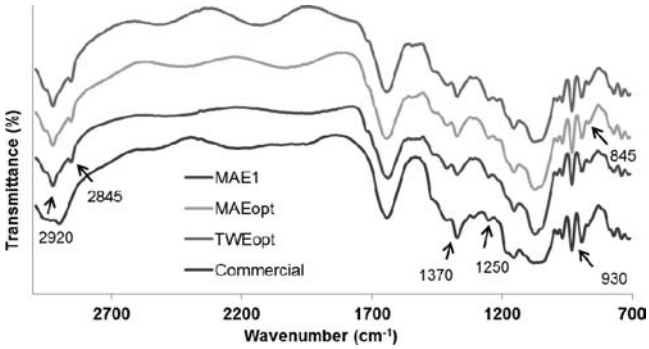

Figure 4. FTIR spectra of native agars from IMTA *G. vermiculophylla* extracted under microwave (MAE^1 and MAE^{opt}) and conventional (TWE^{opt}) heatings. Commercial agar was used as reference. Bands assigned to total sulfate (1250 and 1370 cm^{-1}), LA (930 cm^{-1}), sulfate at 4-position in G units (845 cm^{-1}, weak signal), total sugar content (C−H; 2920 cm^{-1}), and respective shoulder of CH_3 (2845 cm^{-1}) are marked accordingly.

C−C stretching vibrations of the pyranose ring common to all polysaccharides. Two bands were assigned to the C−O−C bending mode in glycosidic linkages at ∼712 and 740 cm^{-1}.[23] The first is specific of agar-like polysaccharides and refers to the L-configurations present throughout the polymer skeleton, while the latter is assigned to the skeletal bending of the pyranose rings.[25] Typical bands of S substitutions are assigned in the region 800−850 cm^{-1}. The peak at 845 cm^{-1} indicative of an axial S at C4 of G units was present in all samples but was higher in MAE agars (Figure 4), while 2-sulfate G moieties usually detected at 830 cm^{-1} [13,26] were absent. The same pattern of absorptions was found previously for the same wild species.[7,23] A low sulfation level at C2 of LA units was detected through the presence of a minor shoulder at 805 cm^{-1}. This result, not detected in wild *G. vermiculophylla*,[7] could not be confirmed by ^{13}C NMR spectra as no splitting of the C1 resonance of the LA moiety was observed probably due to the detection limit of the analysis. The shoulder at 810−820 cm^{-1} was indicative of a minor S substitution in L residues (L6S).[24,26] A broad band at 2920 cm^{-1} attributed to C−H groups was observed in all spectra. Additionally, agars from IMTA biomass presented a shoulder on this band at 2845 cm^{-1} due to M groups. This pattern, expected in FTIR spectra of highly methylated agars of *Gracilaria* species,[13] was not detected in the commercial sample.

Physical and Chemical Properties. Physical and chemical properties of native agars extracted using both processes are summarized in Table 4. Higher yield (15.8 ± 1.4%; $p < 0.05$) was obtained when using MAE instead of TWE (13.5 ± 0.7%). MAE allowed the recovery of higher agar amounts, while reducing drastically the extraction time (eight times less) and the achieved yield was within the range accepted worldwide for the agar industry (15−25%).[27] These results were in line with those obtained previously for MAE of alkali-treated agars.[6] Moreover, higher yields could be attained if processing of the thaw-water was carried out as some native polysaccharide is leached out during the freeze−thawing process. On the contrary, the GS for MAE^{opt} agar was significantly lower (115 ± 7 g/cm^2) compared with TWE^{opt} agar (227 ± 32 g/cm^2). (Note: We remind the reader that the yield and GS values displayed in Table 4 differ from the ones obtained during the optimization step because two different batches of seaweeds were used. Each batch was a mixture of the several IMTA seaweed tanks (thus minimizing differences of cultivation conditions) but originated from different cultivation periods. Since this cultivation system is outdoors, some influence from irradiance and/or temperature seasonal differences may be expected.) In our previous work regarding the study of MAE of alkali-treated agars,[6] no decrease in GS was observed when using microwave heating. In fact, a significant enhancement in agar gelling properties was found when using microwaves in opposition to conventional heating.[7] The inclusion of an alkali-treatment step prior to the extraction promotes the conversion of L6S in LA.[4] These anhydride structures are much more stable than their precursor units and for that reason are more unlikely to suffer degradation under microwave heating. Nevertheless, GS values for MAE^{opt} agar clearly fell in the range considered adequate for soft-texture food products applications (30−200 g/cm^2).[1] Nitrogen availability is known to affect phycocolloid synthesis and gel quality; generally, seaweeds grown in enriched nitrogen media will synthesize less phycocolloid but with higher gel quality. MAE^{opt} native agar from *G. vermiculophylla* with high tissue nitrogen, due to IMTA conditions,[8] showed this tendency in yield and GS when compared with the results from wild biomass of the same species (17.5 ± 1.9%; 72 g/cm^2).[7,28] Nevertheless, it is important to bear in mind that differences in results are due not only to differences in the extraction conditions used, but also to the dependence of agar properties upon species, season, environmental parameters, and stage of life cycle.[26] The $[\eta]$ obtained by extrapolation of Huggins (eq 1) and Kraemer (eq 2) equations was well related with structural analysis and GS values. TWE^{opt} agar was much more viscous (300 ± 2 mL/g) than the MAE^{opt} sample (179 ± 1 mL/g) proving a higher degree of degradation of the MAE^{opt} agar backbone. While in TWE the energy is transferred to the sample matrix through conduction and convection, in MAE the energy is transformed into heat through ionic conduction and dipole rotation. The polarized molecules rotate to align themselves with the electromagnetic field at a rate of 4.9×10^9 times per second, and so molecules are more likely to suffer degradation.[29] The K_H is used as an approximation of the state of aggregation of

Table 4. Physical and Chemical Properties of Native Agars from IMTA *G. vermiculophylla* Extracted Using Microwave (MAE^{opt}) and Conventional (TWE^{opt}) Heating

physical/chemical properties	MAE^{opt}[a]	TWE^{opt}[b]
yield (%)	15.8 ± 1.4	13.5 ± 0.7
gel strength (g/cm^2)	115.1 ± 7.3	227.2 ± 31.9
$[\eta]$ mL/g	178.9 ± 0.8	300.3 ± 2.1
$K_H \times 10$	4.7	4.5
$K_K \times 10$	0.8	1.1
M_v (kDa)	54.0 ± 0.4	110.9 ± 1.1
gelling temperature (°C)	38.4 ± 0.3	36.7 ± 0.7
melting temperature (°C)	81.9 ± 2.2	84.7 ± 0.5
LA content (%)	31.7 ± 0.8	27.8 ± 1.4
sulfate content (%)	2.7 ± 0.3	3.1 ± 0.9
% mol M (G6M)[c]	25.6	30.2
% mol M (LA2M)[c]	7.9	7.7
water content (%)	10.4	11.1

[a]15 min of extraction, 120 °C, 40 mL of water and medium stirring speed using microwave heating. [b]Extracted at 85 °C during 2 h with 200 mL of water and no agitation using conventional heating. [c]Estimated from the ^1H NMR spectral data (see Materials and Methods for details).

dx.doi.org/10.1021/jf2053542 | J. Agric. Food Chem. 2012, 60, 4977−4985

the polymer molecules as well as their interactions with the solvent.[30] For flexible molecules in a good solvent, $K_H \sim 0.35$, but due to aggregative phenomena between the molecules of the polymer, deviations from this ideal value are often observed.[30] When significant polymer−polymer aggregation occurs, K_H can be higher than 1.[31] Both samples showed K_H values in the same order of magnitude (0.47 and 0.45 for MAE^{opt} and TWE^{opt}, respectively) and stated a good polymer−solvent interaction. Also, the constraint $K_H + K_K \sim 0.5$ was satisfied regardless the extraction method. The $[\eta]$ values were used in the Mark−Houwink equation (eq 3) to determine the M_v of the native agars. As expected, agar extracted using microwave irradiation presented a significantly lower M_v (54 ± 0.4 against 111 ± 1 kDa for TWE^{opt} agar). Native agars from wild species previously studied[7] showed significantly lower M_v values (44−63 kDa) than TWE^{opt} sample from IMTA seaweeds. Yet, these could be attributed not only to the cultivation/growth process but also to several physiological factors as well as extraction conditions.

The gelation of agar takes place during cooling of the polymer's solution at temperatures below the gelation point (T_g), which allows the aggregation between the helical chains through hydrogen bonding. Contrary to what has been reported by other authors,[4,21] MAE^{opt} agar, with lower methylation degree, revealed slightly higher T_g (38.4 ± 0.3 °C) when compared with the conventional extract (36.7 ± 0.7 °C). The more draconian conditions of MAE did not lower the agar gelling point. Both extracts exhibited T_g that were appropriate for commercial purposes (32−43 °C).[32] MAE^{opt} agar, with significantly lower M_v, presented slightly lower T_m (81.9 ± 2.2 °C) when compared with TWE^{opt} agar (84.7 ± 0.5 °C). This finding could be related to the lower degradation suffered by the TWE^{opt} sample (higher M_v), as molecular chains of higher size are expected to promote the formation of more stable interactions with the consequent increase in T_m.[4] TWE^{opt} agar showed higher total sulfate (3.1 ± 0.9%) and lower LA (27.8 ± 1.4%) contents than MAE^{opt} sample (2.7 ± 0.3 and 31.7 ± 0.8%, respectively), and both chemical properties were inversely related as found in our previous investigations.[6,7] However, no direct relation between high LA/low sulfate levels and the gel quality of the polymer was observed. MAE^{opt} native agar with higher LA fraction showed lower GS. This could be explained by its significantly lower M_v as larger molecules have been associated with the formation of more rigid gel networks.[24] In spite of being extracted under more severe conditions, the LA units were found in a higher extent in MAE^{opt}, and this could be related to the high stability of these residues which was in line with the results of structural analysis. The opposite was observed for sulfate content (more labile character). Lower T_g and T_m were observed for native agars from the same wild species (26.4 ± 0.6 °C and 70.0 ± 2.6 °C).[7] Again, these differences can be explained by several geographic and physiological factors.

Given the advantages of MAE, future work focusing on the degradation process of native agars under microwaves must be conducted, since this type of agar seemed to be more sensitive to the microwaves than alkali-treated ones (higher yields were obtained but the resulting agars were more degraded which was reflected in their GS). A strict control of MAE conditions can produce agars with GS more appropriate for soft-texture food products applications while spending much less time, energy, and solvent in the process. Native agars from IMTA *G. vermiculophylla* exhibited higher methylation levels than the agar from the same wild species previously studied.[7] Presently, the cultivation conditions of the seaweeds in the IMTA tanks and its influence in the agar final quality are being carefully monitored with the intent of achieving a final product with enhanced and more controlled features. Considering the seasonal variation of the abundance and quality of the biomass harvested from wild populations, the production of seaweeds in IMTA systems can offer a continuous supply of raw material with reliable quality to the transformation industries.

AUTHOR INFORMATION

Corresponding Author

*Tel: +351 22 5081684; fax: +351 22 5081449; e-mail: pilarg@fe.up.pt.

Funding

This work has been supported by Fundação para a Ciência e a Tecnologia (FCT) through Grant No. PEst-C/EQB/LA0006/2011. A.M.M.S., M.H.A., and R.P. were also supported by FCT with the respective fellowships (SFRH/BD/64315/2009, SFRH/BD/21363/2005, and SFRH/BPD/36451/2007). The NMR spectrometers are part of the Portuguese National NMR Network and are supported with funds from FCT, as well.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors acknowledge the technical assistance of L. Martins and the company A. Coelho & Castro, Lda for supplying the raw material.

ABBREVIATIONS USED

Adopted nomenclature of agar residues:[3] G, 3-linked β-D-galactose; LA, 4-linked 3,6-anhydro-α-L-galactose; L, 4-linked α-L-galactose; M, O-methyl; S, ester sulfate. The carbon and/or proton in each residue were represented by the number of the position and the respective letter (e.g. carbon at position one C1); MAE, microwave-assisted extraction; TWE, traditional hot-water extraction; native agar obtained using TWE optimal conditions, TWE^{opt} agar; native agar obtained using MAE optimal conditions, MAE^{opt} agar.

REFERENCES

(1) Pereira-Pacheco, F.; Robledo, D.; Rodríguez-Carvajal, L.; Freile-Pelegrín, Y. Optimization of native agar extraction from *Hydropuntia cornea* from Yucatán, Mexico. *Bioresour. Technol.* **2007**, *98*, 1278−1284.

(2) McHugh, D. J. *A Guide to the Seaweed Industry*; FAO Fisheries Technical Paper 441; Food and Agriculture Organization of the United Nations: Rome, 2003; p 25 (http://www.fao.org/DOCREP/006/Y4765E/Y4765E00.HTM, accessed Sept 4, 2011).

(3) Knutsen, S. H.; Myslabodski, D. E.; Larsen, B.; Usov, A. I. A modified system of nomenclature for red algal galactans. *Bot. Mar.* **1994**, *37*, 163−169.

(4) Rodríguez, M. C.; Matulewicz, M. C.; Noseda, M. D.; Ducatti, D. R. B.; Leonardi, P. I. Agar from *Gracilaria gracilis* (Gracilariales, Rhodophyta) of the Patagonic coast of Argentina − Content, structure and physical properties. *Bioresour. Technol.* **2009**, *100*, 1435−1441.

(5) Abreu, M. H.; Pereira, R.; Sousa-Pinto, I.; Yarish, C. Ecophysiological studies of the non-indigenous species *Gracilaria vermiculophylla* (Rhodophyta) and its abundance patterns in Ria de Aveiro lagoon, Portugal. *Eur. J. Phycol.* **2011**, *46*, 453−464.

(6) Sousa, A. M. M.; Alves, V. D.; Morais, S.; Delerue-Matos, C.; Gonçalves, M. P. Agar extraction from integrated multitrophic aquacultured *Gracilaria vermiculophylla*: Evaluation of a microwave-

assisted process using response surface methodology. *Bioresour. Technol.* **2010**, *101*, 3258−3267.
(7) Villanueva, R. D.; Sousa, A. M. M.; Gonçalves, M. P.; Nilsson, M.; Hilliou, L. Production and properties of agar from the invasive marine alga, *Gracilaria vermiculophylla* (Gracilariales, Rhodophyta). *J. Appl. Phycol.* **2010**, *22*, 211−220.
(8) Abreu, M. H.; Pereira, R.; Yarish, C.; Buschmann, A.; Sousa-Pinto, I. IMTA with *Gracilaria vermiculophylla*: Productivity and nutrient removal performance of the seaweed in a Land-based Pilot Scale System. *Aquaculture* **2011**, *312*, 77−87.
(9) Dai, J.; Yaylayan, V. A.; Raghavan, G. S. V.; Paré, J. R. J.; Liu, Z.; Bélanger, J. M. R. Influence of operating parameters on the use of microwave-assisted process (MAP) for the extraction of azadirachtin-related limonoids from Neem (*Azadirachta indica*) under atmospheric pressure conditions. *J. Agric. Food Chem.* **2001**, *49*, 4584−4588.
(10) Kwon, J.-H.; Bélanger, J. M. R.; Paré, J. R. J. Optimization of microwave-assisted extraction (MAP) for ginseng components by response surface methodology. *J. Agric. Food Chem.* **2003**, *51*, 1807−1810.
(11) Usov, A. I.; Yarotsky, S. V.; Shashkov, A. S. ^{13}C-NMR spectroscopy of red algal galactans. *Biopolymers* **1980**, *19*, 977−990.
(12) Usov, A. I. NMR spectroscopy of red seaweed polysaccharides: Agars, carrageenans and xylans. *Bot. Mar.* **1984**, *XXVII*, 189−202.
(13) Rochas, C.; Lahaye, M.; Yaphe, W. Sulfate content of carrageenan and agar determined by infrared spectroscopy. *Bot. Mar.* **1986**, *XXIX*, 335−340.
(14) Bilan, M. I.; Vinogradova, E. V.; Shashkov, A. S.; Usov, A. I. Structure of a highly pyruvylated galactan sulfate from the Pacific green alga *Codium yezoense* (Bryopsidales, Chlorophyta). *Carbohydr. Res.* **2007**, *342*, 586−596.
(15) Falshaw, R.; Furneaux, R. H.; Pickering, T. D.; Stevenson, D. E. Agars from three Fijian *Gracilaria species*. *Bot. Mar.* **1999**, *42*, 51−59.
(16) Andriamanantoanina, H.; Chambat, G.; Rinaudo, M. Fractionation of extracted Madagascan *Gracilaria corticata* polysaccharides: Structure and properties. *Carbohydr. Polym.* **2007**, *68*, 77−88.
(17) Jackson, S. G.; McCandless, E. L. Simple, rapid, turbidimetric determination of inorganic sulfate and/or protein. *Anal. Biochem.* **1978**, *90*, 802−808.
(18) Yaphe, W; Arsenault, G. P. Improved resorcinol reagent for the determination of fructose, and 3,6-anhydrogalactose in polysaccharides. *Anal. Biochem.* **1965**, *13*, 143−148.
(19) Montgomery, D. C. Response Surface Methods and Designs. In *Design and Analysis of Experiments*, 3rd ed.; Wiley: New York, 1991.
(20) Rochas, C.; Lahaye, M. Average molecular weight and molecular weight distribution of agarose and agarose-type polysaccharides. *Carbohydr. Polym.* **1989**, *10*, 289−298.
(21) Murano, E. Chemical structure and quality of agars from *Gracilaria*. *J. Appl. Phycol.* **1995**, *7*, 245−254.
(22) Gordon-Mills, E.; Tate, M.; Hounslow, A. Use of solid and gel state ^{13}C NMR spectroscopy for differentiation between agarophytes and carrageenophytes. *Hydrobiologia* **1990**, *204/205*, 629−636.
(23) Mollet, J. C.; Rahaoui, A.; Lemoine, Y. Yield, Chemical composition and gel strength of agarocolloids of *Gracilaria gracilis*, *Gracilaria longissima* and the newly reported *Gracilaria* cf. *vermiculophylla* from Roscoff (Brittany, France). *J. Appl. Phycol.* **1998**, *10*, 59−66.
(24) Rupérez, P; Ahzarem, O.; Leal, J. A. Potential antioxidant capacity of sulfated polysaccharides from the edible marine brown seaweed *Fucus vesiculosus*. *J. Agric. Food Chem.* **2002**, *50*, 840−845.
(25) Matsuhiro, B. Vibrational spectroscopy of seaweed galactans. *Hydrobiologia* **1996**, *326/327*, 481−489.
(26) Romero, J. B.; Villanueva, R. D.; Montaño, M. N. E. Stability of agar in the red seaweed *Gracilaria eucheumatoides* (Gracilariales, Rhodophyta) during postharvest storage. *Bioresour. Technol.* **2008**, *99*, 8151−8155.
(27) Armisen, R. World-wide use and importance of *Gracilaria*. *J. Appl. Phycol.* **1995**, *7*, 231−243.
(28) Arvizu-Higuera, D. L.; Rodríguez-Montesinos, Y. E.; Murillo-Alvárez, J. I.; Muñoz-Ochoa, M.; Hernández-Carmona, G. Effect of alkali treatment time and extraction time on agar from *Gracilaria vermiculophylla*. *J. Appl. Phycol.* **2008**, *20*, 515−519.
(29) Srogi, K. A review: Application of microwave techniques for environmental analytical chemistry. *Anal. Lett.* **2006**, *39*, 1261−1288.
(30) Harding, S. E. The intrinsic viscosity of biological macromolecules. Progress in measurement, interpretation and application to structure in dilute solution. *Prog. Biophys. Mol. Biol.* **1997**, *68*, 207−262.
(31) Bastos, D. S.; Barreto, B. N.; Souza, H. K. S.; Bastos, M.; Rocha-Leão, M. H. M.; Andrade, C. T.; Gonçalves, M. P. Characterization of a chitosan sample extracted from Brazilian shrimps and its application to obtain insoluble complexes with a commercial whey protein isolate. *Food Hydrocolloids* **2010**, *24*, 709−718.
(32) Orduña-Rojas, J.; Suaréz-Castro, R.; López-Alvaréz, E. S.; Ríosmena-Rodriguez, R.; Pacheco-Ruiz, I.; Zertuche-González, J. A.; Meling-López, A. E. Agar properties of two species of Gracilariceae from the Gulf of California, Mexico. *J. Appl. Phycol.* **2008**, *20*, 169−175.