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Optimization of an Eco-Friendly Extraction to Obtain Monosaccharides from the Invasive Macroalga *Rugulopteryx okamurae*

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Abstract

Background: The invasive macroalga *Rugulopteryx okamurae* poses severe environmental challenges but presents an opportunity as a sustainable source of valuable carbohydrates. This study develops an eco-friendly method to extract monosaccharides from this biomass. Methods: Ultrasound-assisted extraction (with sonotrode) using water as the solvent was optimized with Design of Experiments, and the time, power and mass:solvent ratios were evaluated. The monosaccharide composition was evaluated by gas chromatography coupled to high-resolution mass spectrometry (GC-QTOF). Results: The analytical method was validated for accurate monosaccharide quantification using oximes and silylation, achieving a fast time of analysis (<20 min). The optimized UAE (1:15 mass:solvent ratio, 70% power, 30 min) yielded a total monosaccharide concentration of 210 mg mL^{-1}, significantly outperforming traditional acidic hydrolysis. Compared to the extraction of monosaccharides from the well-known seaweed *Ulva ohnoi* (31 mg mL^{-1}), it is far superior. Mannitol comprised 95% of the total extract. Conclusions: This scalable and green UAE methodology valorizes this seaweed by achieving high yields of simple sugars, offering a sustainable resource for industrial applications while mitigating the environmental impact of this invasive species.

Keywords: gas chromatography; seaweed; ultrasound-assisted extraction; algae; green extraction

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1. Introduction

Rugulopteryx okamurae (R.o.) is an invasive Asian seaweed that has rapidly spread through the Mediterranean Sea, possibly because of global warming, and appeared for the first time in the Strait of Gibraltar in 2015, provoking numerous problems wherever it settles [1]. This seaweed reproduces very efficiently and tends to grow in dense mats on rocks and sea beds, displacing native algae and organisms and reducing local biodiversity. Furthermore, when it breaks off and washes up on shore in large quantities, it can make swimming difficult, affect fishing activities, and alter the usual appearance of beaches. In 2020, it was included in the Spanish legislation of invasive exotic species known as the Spanish Catalogue, and its revision continues steadily due to the rapid expansion [2].

In the summer of 2025, the Regional Ministry of Sustainability and Environment of the Regional Government of Andalusia published a Management Plan to combat this alga on the Andalusian coast, highlighting the regional and national concern that this alga poses to the environment [3].

R.o. is a species of brown algae, 10 to 20 cm long, belonging to the *Dictyotaceae* family. Current studies have shown that this type of seaweed is rich in certain biomolecules, such as fucoxanthin, phlorotannin, fucoidan, alginate and laminarin, with demonstrated activity against oxidation, inflammatory and cancer models, among others [4]. However, in terms of total dry weight (d.w.), structural carbohydrates constitute more than 50%, with alginate, laminarin, fucoidan or mannitol (in its free and polymerized form) constituting a high percentage of the total carbohydrates [5]. Alginate is the main carbohydrate and is a linear polymer composed of two monomers, guluronic and mannuronic acid, linked by (1 → 4) glycosidic bonds.

Beyond the composition of R.o. as well as its problematic effects on the environmental, the scientific literature has started to study this matrix as a sustainable source with diverse applications. There are authors that have investigated its potential as a biofilm, specifically because of its high content of alginate [6,7]. Other authors have evaluated the potential of this seaweed to be used in agronomic fields, including as a biostimulant or as a source for the extraction of carbohydrates and phenolics, increasing health-promoting phytochemicals in an eco-friendly way [8,9]. However, although works exist that extract specifically monosaccharides in other types of algae [10,11], to the best of our knowledge, no authors have investigated R.o. as a source of simple sugars. This targets molecules that have an important role in the food industry, pharmaceutical industry, cosmetics or in bioethanol production [12]. Thanks to their eco-friendly nature, low cost, renewability and flexibility, carbohydrates are a highly useful material in many industries [13]. For instance, specifically in the pharmaceutical industry, between 2000 and 2021, a total of 54 carbohydrate-based drugs were approved [14]. Specifically, R.o. has monosaccharides of 5 and 6 carbons (pentoses and hexoses, respectively) such as xylose (Xyl), rhamnose (Rha), glucose (Glu), galactose (Gal), and mannose/mannitol (Man).

The monosaccharide composition is considered relevant to characterize the structure of more complex carbohydrates. The hydrolyzation of polysaccharides to obtain simple sugars has been explored in numerous articles. This reaction consists of breaking down a complex's polymers into its monomers through the addition of molecules of water. With that objective, it has been widely used by employing strong acidic media, such as H_2SO_4 or HCl, for the rapid and complete breakdown of bonding [15]. However, this methodology involves non-eco-friendly solvents that generate acidic, dangerous residues. Some authors have studied the usage of ultrasound-assisted extraction (UAE) or enzymatic extraction to obtain depolymerized fractions of seaweed, obtaining high percentage yields and sugar characterization, by eliminating the strong acids that are traditionally used [16,17].

For the quantification of monosaccharides, due to their high polarity, several strategies have been developed over time. Typical equipment used for monosaccharide analysis includes high-performance anion-exchange chromatography and liquid chromatography (HPLC) coupled to pulsed amperometric detection [16,18]. Some scientists have tried to develop methodologies involving gas chromatography, studying the best way to derivatize these analytes or the instrumental parameters. Haas et al., in 2018, developed an advance derivatization protocol for the separation and identification of monosaccharides in complex mixtures related to the oximation of monosaccharides and posterior silylation. This methodology avoids the direct silylation of simple sugars, which provokes several types of isomers (open-chain, furanose, pyranose, anomers), by protecting the alcohol moieties [19].

This study represents the first report on water-based UAE for extracting monosaccharides from R.o., characterized via GC-QTOF analysis. The three main objectives of this work were as follows: (i.) to optimize a UAE methodology by both univariant optimization and design of experiments (DoE), using water as the solvent, to evaluate different parameters while being easily scalable to industry; (ii.) to properly set up the chromatographic method and validate it; and (iii.) to apply the method to real algae samples.

2. Materials and Methods

2.1. Samples

Samples of R.o. and Ulva ohnoi (U.o.) under study were kindly given by CTAQUA in the context of the MAcroalgae Project. R.o. samples were collected from the coasts of Andalucía, with a special permit required from the Andalusian Regional Government. U.o. samples were grown at the CTAQUA facilities. Both samples were cleaned and treated to reduce the burden of epibionts in CTAQUA installations and dries at room temperature for several days until the percentage of humidity was less than 8%. Samples arrived at the CIDAF laboratories in April 2024 and were maintained in a dark, dry place until the experiments began. An image of the samples can be seen in the Supplementary Material (Figure S1).

2.2. Chemicals and Reagents

For the extraction of monosaccharides, absolute ethanol (EtOH) from PanReac AppliChem ITW Reagents Division® Co. (Castellar del Vallès, Barcelona, Spain) was used. Sulfuric acid was bought from PanReac. For the derivatization, the following reagents were used: hydroxylamine hydrochloride (HACl) (99% purity) from Sigma Aldrich, pyridine (Pyr) from VWR Chemicals (Suwon, Republic of Korea), BSTFA containing 1% TMCS (Thermo Scientific Co., Waltham, MA, USA), and ethyl acetate (AcOEt, HPLC grade, ≥99.5% purity) from Fisher Chemical Co. (Pittsburgh, PA, USA). Water in all experiments was taken from a Milli-Q Plus® system from Merck Millipore Co. (Billerica, MA, USA).

The standards used were D-Rha (≥99% purity), D-Xyl (≥99% purity), D-Gal (≥99% purity), D-Glu (≥99% purity), D-Man (≥98% purity) and myo-inositol (Ino) as internal standards, and all of them were from Sigma-Aldrich (Co., St. Louis, MO, USA).

2.3. Instrumentation and Software

For the pretreatment, samples were milled with a RETSCH centrifugal mill model Ultra ZM 200 (Haan, Germany) and agitated with a MaxQ™ 600 model shaker from Thermo Scientific. For the extraction optimization, a sonotrode VCX 750 from Sonics&Materials (Newtown, CT, USA)was used. For the separation and identification of target analytes, gas chromatography coupled to high-resolution mass spectrometry-QTOF (GC-QTOF) from Agilent Technologies was utilized. The GC instrument was a model 7890B, and the detector was an Accurate-Mass Q-TOF model 7200. The system also had a CombiPAL Autosampler model 120 from Agilent Technologies. The column used for the separation was an HP-5ms UI (5–phenyl-methylpolysiloxane phase, 30 m × 0.25 mm and 0.25 µm film thickness). Other instruments used were a Speedvac Concentrator Plus from Eppendorf (Hamburg, Germany), a Sorvall ST 16R centrifuge (Thermo) and a PCMT HC24 model thermoshaker from Grant Instruments TM (Royston, UK).

Data treatment was performed with Microsoft Excel®. Statgraphics plus 5.0 (Statpoint Technologies, Warrenton, VA, USA) was used for experimental design, and MassHunter Qualitative Analysis 10.0 (Agilent Technologies, Santa Clara, CA, USA) was used for GC-QTOF data treatment.

2.4. Experimental Procedure

2.4.1. Sample Pretreatment

Samples were dry when used. Stones and other debris that were not part of the alga matrix were manually removed. R.o. was milled with a 4 mm sieve to obtain a homogeneous powder. To eliminate phenolic compounds, carotenoids and other more apolar compounds from carbohydrates, samples were washed with 80% EtOH at different mass:solvent ratios for 1 h in an orbital shaker at 80 °C and 230 rpm. This fraction, which is rich in phenolic compounds and other bioactive molecules, can be used in various applications, such as agricultural bioproducts due to its biostimulant or biocidal effects, thereby promoting the full valorization of this seaweed and maximizing its bioactive potential. Thereafter, the samples were centrifugated for 10 min at 13,000 rpm, and the supernatant was eliminated. The resulting pellet was subjected to extraction.

2.4.2. Sample Treatment

Firstly, once the pretreatment was completed and prior to the optimization, two different extraction methodologies were compared for monosaccharides: traditional acidic hydrolysis and UAE. With this objective, 1 g of washed alga was extracted with 20 mL of 1 M H_2SO_4 (mass:solvent ratio 1:20) for 3 h at 80 °C. By contrast, 5 g of alga was mixed with 100 mL of alga (mass:solvent ratio 1:20) for 3 min at 50% power.

Once UAE was selected for optimization, the base protocol was as follows: the washed alga was extracted with 100 mL of milli-Q water at 70% power for 30 min in an ice bath working in "pulse on" mode for 30 s and in "pulse off" mode for 10 s to prevent overheating of samples. After that, it was transferred to Falcon tubes for centrifugation at 13,000 rpm for 10 min, and the supernatant was filtered with a regenerated cellulose filter (0.45 µm). From this solution, 10 µL was taken and inserted into a vial, evaporated in a *speedvac* at 35 °C until dryness, and derivatized as indicated in Section 2.4.3.

2.4.3. Gas Chromatography–High-Resolution Mass Spectrometry Analysis Derivatization with Oximes

For the oxime derivatives, a solution of HACl in pyr at 2.5% was prepared. For the silylation of compounds, a mixture of BSTFA:pyr:AcOEt 50:25:25 (*v*/*v*/*v*) was used. For the derivatization, 50 µL of HACl mixture was mixed with the evaporated quota and heated for 30 min at 70 °C. Next, after letting it cool down, 50 µL of BSTFA mixture was added, and the vial was carefully sealed to prevent ambient moisture from entering. This closed vial was heated again for 30 min at 70 °C to obtain silyl oxime monosaccharides. With a final volume of 100 µL, after letting it cool, the vial was ready to inject into the chromatographic system.

Monosaccharides were separated with an optimized chromatographic ramp: started at 110 °C and maintained for 1 min; then, ramped up at 25 °C/min until 170 °C, maintained for 2 min; then, 20 °C/min until 280 °C, maintained for 1.5 min (total run: 19.6 min). Figure S2 in the Supplementary Material shows the chromatographic ramp. A total of 0.8 µL of the sample was injected, the temperature of the injector was 250 °C, the carrier gas flow (He) was established at 1 mL min^{-1}, the interphase temperature was 270 °C, and the solvent delay was 5 min. The electronic impact of the mass detector was 70 eV.

2.4.4. Optimization of the Extraction

For UAE optimization, the mass:solvent (g:mL) ratio was evaluated in a univariant way at 4 levels from 1:15 to 1:30, using a constant time and power, namely, 70 min and 70%, respectively.

The optimization of ultrasound parameters was carried out with DoE. To evaluate the maximum and minimum values, a 3^2 factorial design was selected, with power and time as the studied parameters. The range of power was evaluated with 20% and 70% as the minimum and maximum, respectively. Meanwhile, the time of extraction was evaluated in the range of 30 to 120 min as the minimum and maximum, respectively. The total experimental runs were 12: 9 from the experimental design and 3 from the central points. The experimental matrix obtained can be seen in Supplementary Material, Table S1. This type of design allows for the exploration of two variables at three different levels, minimum, medium and maximum. In this case, the response variables were the concentration of each monosaccharide separately and the sum of monosaccharides. In all cases, two experimental replicas and two instrumental replicas were injected, with 4 data points per experimental run. ANOVA was used to determine the significance level of each variable.

2.4.5. Method Validation

All the methodology was validated according to the European Medicines Agency following the ICH Q2(R2) Validation of analytical procedures [20]. As it is not possible to obtain algae without carbohydrates to use as a blank matrix, the selectivity, sensibility, accuracy and linearity were evaluated with analytical standards. In all cases, Ino was utilized as an internal standard (IS) at 10 μg mL^{-1} in both standards and samples, with reference of the data results to the relative area, to consider the extraction yield. This IS was selected because it is a carbohydrate that is not found in seaweeds. For selectivity (or specificity), an experimental blank that underwent the entire experimental procedure was carried out. As selectivity is defined as the absence of interference, the experimental blank chromatogram was compared to one that was spiked. Calibration curves were evaluated in the range of 0.1 to 15 μg mL^{-1} to evaluate the working range. The injections were performed in consecutive small concentrations to study the detection and quantification limits (LOD and LOQ, respectively). For the evaluation of accuracy and trueness, standards at the LOQ, medium (5 μg mL^{-1}) and high (15 μg mL^{-1}) concentrations were injected on different days, in order to study the inter- and intra-day laboratory assay variability. Accuracy was evaluated by interpolating from the calibration curve of known concentrations and was expressed in terms of recovery. The precision analysis evaluated the homogeneity of reproducibility by calculating the Relative Standard Deviation (RSD) of these measurements, representing the trueness.

3. Results

3.1. Identification of Monosaccharides from R.o.

The monosaccharides Rha, Xyl, Gal, Glu, Man and Ino were injected separately to identify the retention time of each one. Furthermore, a test was performed to check whether the use of HACl was necessary and whether heating was required to achieve complete derivatization of the monosaccharides. A chromatogram of a standard mixture at 10 μg mL^{-1} is shown in Figure 1 as mentioned in Section 2.4.3., with the two isomers for each compound except for Man (5) and IS (Ino). Figure S3 (Supplementary Material) shows the spectral data for each monosaccharide, and Table 1 shows the retention time as well as the main m/z with GC-MS conditions as described before.

After adjusting the ramp, a correct separation of monosaccharides under study in less than 20 min of analysis was achieved. In the cases of Rha, Xyl, Gal and Glu, two peaks are shown per analyte, corresponding to the different E and Z isomers formed after the chosen derivatization. Hereafter, Table 1 shows the retention time and peaks for the spectra of each isomer analyte, as well as the most intense peaks in the spectrum.

All the analytes under study showed intense peaks at *m*/*z* 204, 191, 217, 73 and 147, which were slightly different for Man and Ino.

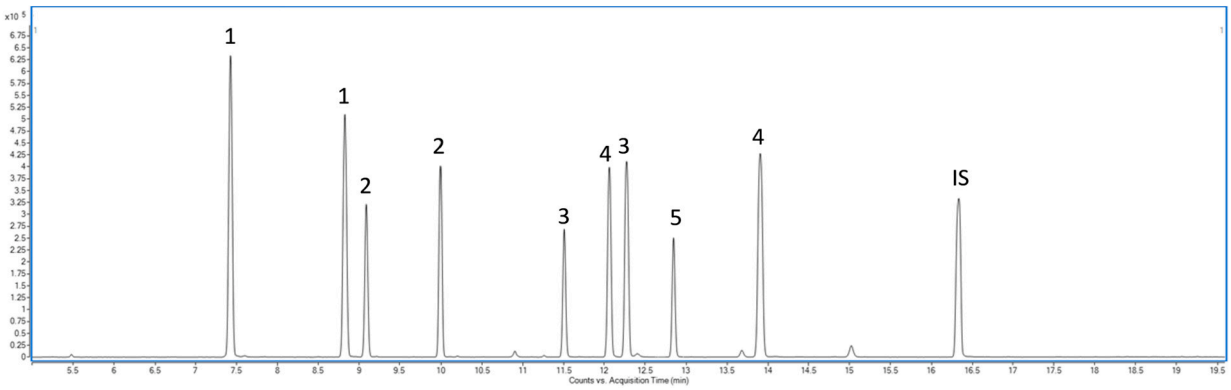


Figure 1. Chromatogram of a mixture of target derivatized monosaccharides. 1: Rhamnose; 2: Xylose; 3: Galactose; 4: Glucose; 5: Mannitol. IS: Internal Standard (Inositol).

Table 1. Monosaccharide chromatographic and spectral parameters.

Compound	tR (min)	*m*/*z* Peaks (Relative Volume)
Rha 1	7.4	204.0988 (100), 73.0467 (62.3), 191.0877 (42.6), 205.0968 (35.73), 189.0725 (27.08), 147.0632 (26.25), 204.0988 (14.71), 217.1028 (13.92)
Rha 2	8.8	204.0959 (100), 73.0466 (39.85), 191.0876 (36.29), 147.0633 (19.79), 189.0726 (17.31), 205.0968 (17.15), 217.1024 (8.9), 206.0933 (6.92)
Xyl 1	9.1	204.0962 (100), 73.0467 (55.6), 271.1033 (51.86), 191.0881 (43.87), 147.0634 (29.71), 189.0723 (17.96), 205.0969 (17.46), 218.1048 (8.61)
Xyl 2	9.9	204.0965 (100), 73.0468 (66.26), 217.1034 (59.79), 191.0879 (54.41), 147.0635 (35.2), 189.0724 (22.83), 205.0968 (20.69), 218.1044 (11.13)
Gal 1	11.5	204.0957 (100), 191.0880 (56.97), 73.0466 (42.65), 217.1029 (27.04), 147.0631 (19.41), 205.0970 (16.98), 189.0721 (8.34), 192.0891 (8.34)
Glu 1	12.1	204.0962 (100), 191.0879 (52.73), 73.0466 (42.53), 147.0632 (21.9), 217.1031 (19.97), 205.0967 (19.09), 189.0718 (10.02), 192.0892 (8.03)
Gal 2	12.3	204.0963 (100), 217.1038 (86.65), 191.0885 (72.49), 73.0468 (64.84), 147.0635 (34.16), 205.0975 (17.94), 218.1053 (15.88), 192.0894 (11.6)
Man	12.8	319.1823 (100), 73.0580(81.73), 147.0801 (72.11), 217.1242 (70.83), 205.1102 (49.86), 320.1647 (40.11), 205.1413 (36.98), 157.0705 (36.96)
Glu 2	13.9	204.0963 (100), 191.0881 (59.01), 73.0468 (44.12), 147.0632 (22.02), 217.1033 (19.23), 205.0968 (18.54), 189.0726 (10.59), 192.0892 (8.68)
Ino	16.4	318.1807 (100), 217.16380 (99.92), 305.1821 (99.92), 73.0616 (99.39), 191.1119 (96.27), 147.0836 (95.27), 306.1670 (89.59), 319.1747 (68.55), 218.1248 (59.55)

Rha: Rhamnose; Xyl: Xylose; Gal: Galactose; Glu: Glucose; Man: Mannitol; Ino: Inositol; tR: Retention time.

3.2. Validation

For the evaluation of selectivity, an experimental blank was injected in order to identify interference. Figure S4 in the Supplementary Material shows the resulting chromatogram, which indicates that there is no interference in the retention times of the analytes and that the only clearly visible peak is that of Ino. Therefore, it can be said that the method is selective.

Table 2 shows the parameters for the validation. For the evaluation of sensibility and linearity, calibration curves from low concentration to 15 μg mL^{-1} were generated, also defining the work range. Calibration was performed with the sum of the two peaks for each monosaccharide. Linearity was evaluated with the R^2 of the calibration curves obtained. In all cases, R^2 was 0.99 except for Gal, whose value was 0.9865. Thus, the method can be considered linear. For the LOD and LOQ, in all cases, the analytical standards ranged from concentrations of 0.1 to 0.5 μg mL^{-1} that were injected to visually determine both values. Table 2 shows that in all cases, the LOD was established at 0.1 μg mL^{-1}, because at this concentration, a detectable peak appeared corresponding to each monosaccharide. The LOQ was established at 0.5 μg mL^{-1}, since at this value, it was possible to correctly quantify the target analyte. In that sense, it can be said that the method is sensitive, and the working concentration range of the equipment is, in all cases, from LOQ (0.5) to 15 μg mL^{-1}.

Table 2. Validation parameters.

Analyte	Calibration Curve	R^2	LOD (μg mL^{-1})	LOQ (μg mL^{-1})	% Recovery (%RSD)		
					0.5 μg mL^{-1}	5 μg mL^{-1}	15 μg mL^{-1}
Rha	$y = -0.0176x^2 + 0.9891x - 0.0937$	0.9923	0.15	0.5	94.9 (1.8)	80.5 (2.6)	105.5 (6.4)
Xyl	$y = -0.017x^2 + 0.451x - 0.0101$	0.9939	0.15	0.5	74.9 (12.8)	111.8 (2.0)	101.3 (3.1)
Gal	$y = 0.0024x^2 + 0.3768x + 0.0186$	0.9865	0.15	0.5	80.0 (12.4)	116.2 (3.8)	102.5 (2.6)
Glu	$y = -0.0028x^2 + 0.6614x - 0.1159$	0.9909	0.15	0.5	112.9 (3.5)	100.4 (6.5)	98.7 (4.2)
Man	$y = 0.053x - 0.0056$	0.9938	0.15	0.5	82.3 (14.2)	95.0 (12.0)	98.9 (1.3)

Rha: Rhamnose; Xyl: Xylose; Gal: Galactose; Glu: Glucose; Man: Mannitol; LOD: Limit of detection; LOQ: Limit of quantification; RSD: Relative Standard Deviation.

For recovery, injections were made at low (LOQ, 0.5 μg mL^{-1}), medium (5 μg mL^{-1}) and high (15 μg mL^{-1}) concentrations on different days. In all cases, the % of recovery (accuracy) ranged between 75 and 116 in all analyses, with an RSD (precision) of less than 15% in all cases. For that reason, this method has been demonstrated to be accurate, precise and, therefore, truthful.

3.3. Comparison Between Traditional Hydrolysis and UAE

Figure 2 shows the Total Ion Chromatogram (TIC) for R.o. treated in different ways. As can be seen, the most predominant peak occurs in the UAE extraction (in green) at 12.8 min, which corresponds to Man. Moreover, in the H_2SO_4 extraction, more interference was extracted, which was not related to carbohydrates. As 3 min in the sonotrode with water as a solvent gave us a larger area than traditional hydrolysis, the optimization was continued with UAE.

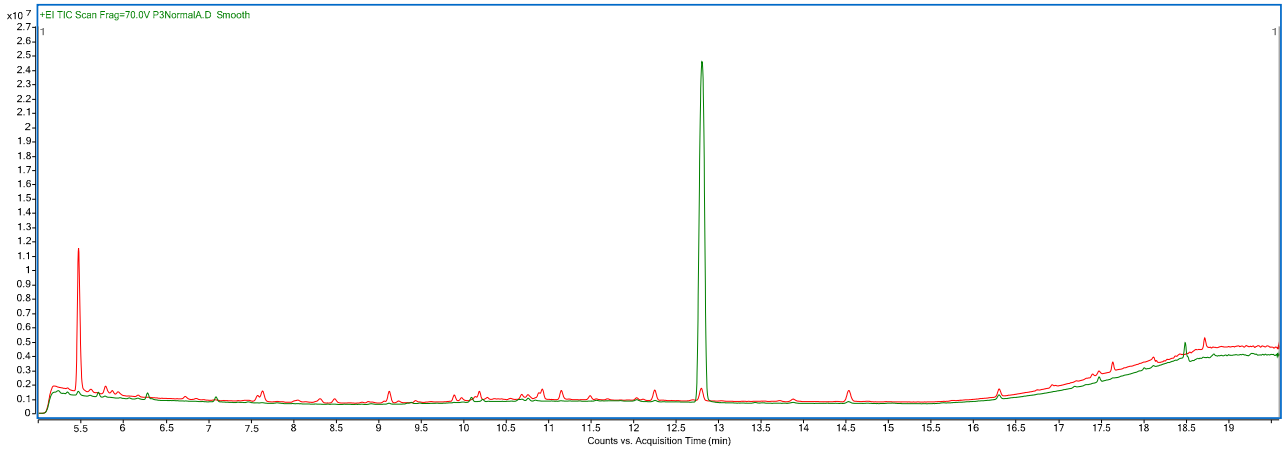


Figure 2. Comparison of TICs from traditional hydrolysis (red) and UAE (green).

3.4. Optimization of Monosaccharide Hydrolysis by UAE

In the first experiment, that is, the mass:solvent ratio test, the power was maintained at 70% for 70 min. Due to technical specifications for the sonotrode, 100 mL of water being the setting, and the mass of algae changing for the extraction, four ratios were evaluated: 1:15 (6.67 g alga), 1:20 (5 g of alga), 1:25 (4 g alga) and 1:30 (3.33 g alga). Each ratio was experimentally repeated twice, and two instrumental replicas of each extract were injected. The results can be seen in Figure 3.

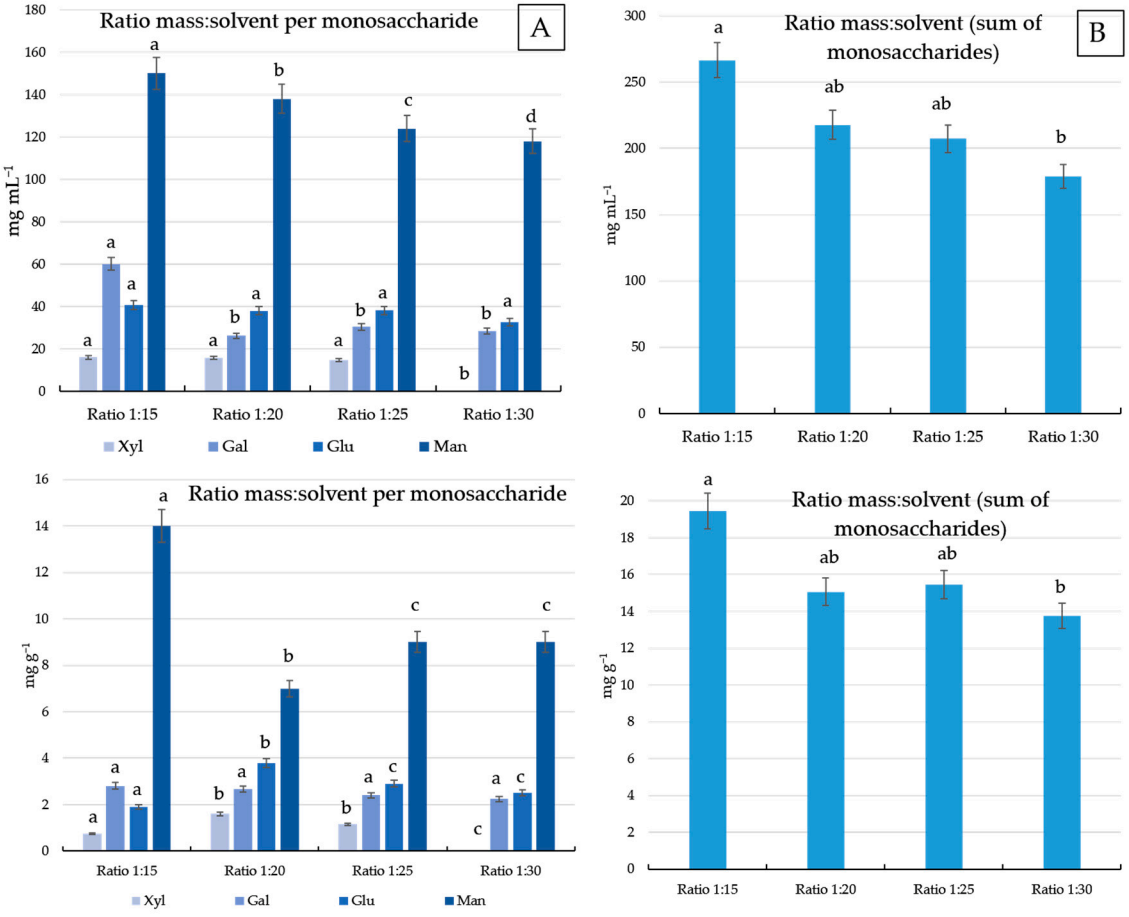


Figure 3. Optimization of mass:solvent ratio. (**A**): Monosaccharides individually; (**B**): sum of monosaccharides. Different letters for the same monosaccharide denote significant differences ($p < 0.05$).

The best ratio for obtaining the maximum concentration of monosaccharides was a 1:15 ratio. In that ratio, the concentrations for Xyl, Gal, Glu and Man were 16.0, 60.0, 40.8 and 150.8 mg mL^{-1}, respectively, and their sum was 266.8 mg mL^{-1} extract. Additionally, the yield extraction for each experiment (in mg yield per g of sample) was 160.4, 178.0, 160.0 and 189.2 for 1:15, 1:20, 1:25 and 1:30, respectively. The extract concentrations in mg of monosaccharide per g of extract followed a similar trend, with the highest values observed at a 1:15 ratio. For these reasons, it was decided to continue the multivariant optimization using 6.67 g of alga.

3.5. Optimization of Time and Power by UAE. Design of Experiments

Using the previous optimized ratio, DoE was executed with 12 experimental runs in a 3^2 factorial design, with power from 20 to 70% and time ranging from 30 to 120 min. In that sense, the results for each monosaccharide, for their sum and for the yield of the extraction for each experiment are shown in Figure 4, as well as the multi-response optimization for the sum of monosaccharides and yield.

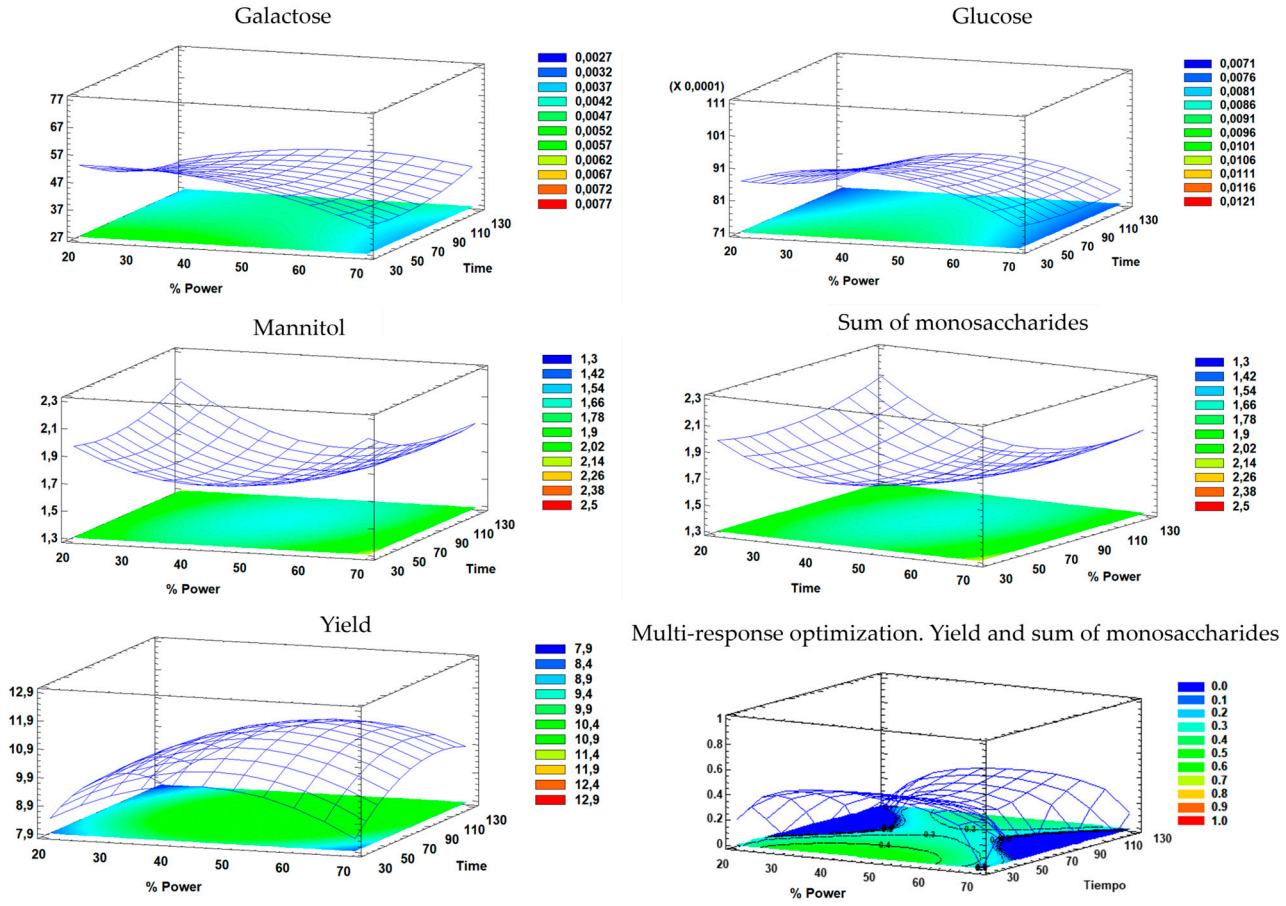


Figure 4. Optimization of the UAE by DoE. The mesh pattern indicates the trend for each response variable evaluated.

The Xyl results are not shown because it could only be quantified in two of the experimental runs, and its quantification was the lowest compared with the rest of the analytes. All the experiments had a *p*-value greater than 0.05, and no significant lack-of-fit was detected. Table S2 in the Supplementary Material shows the equation fitted model for each monosaccharide, the yield and their sum, and Figure S5 shows the standardized Pareto chart for each monosaccharide. In all cases, Man was the monosaccharide with the highest concentration, as it was 99% of the total sum of monosaccharides. Gal and Glu had the

same tendency in DoE, obtaining their maximum points with medium–low power of the sonotrode and 30 min, although none of the parameters were statistically significant according to the standardized Pareto chart, which indicates significance if the data points fall to the right of the blue line (Figures 4 and S5). By contrast, the maximum concentration of Man was obtained at maximum power (70%) for 30 min, being the percentage of power that was statistically significant. When considering the total amount of monosaccharides, since Man accounts for 99% of simple sugars, the same trend is observed as for this monosaccharide. In the yield, the tendency of the model is the opposite of the sum of monosaccharides: maximum yield was obtained at 70 min and 55% power. As it is considered that this methodology is appropriate for its scalability in industry, the optimization of multiple responses was performed with the sum of monosaccharides and yield.

As shown in Figure 4, maximum desirability was established at 65% in a range between 22 and 60% power for 30 min. However, since both Man and the sum of monosaccharides exhibit maximums at 70% power for 30 min, and less power produced a decrease in the concentration of monosaccharides, that sonotrode parameter was selected as desirable.

3.6. Application of the Method for Algae Samples

R.o. was evaluated under optimized conditions. In addition, a sample of U.o. was also evaluated with same the parameters as R.o. to observe differences in the monosaccharide composition and yield percentage of extraction in both types of algae. The results can be seen in Table 3.

Table 3. Concentration of simple sugars in R.o. and U.o. Parenthesis: Standard deviation.

Sample	Rha (mg mL^{-1})	Xyl (mg mL^{-1})	Gal (mg mL^{-1})	Glu (mg mL^{-1})	Man (mg mL^{-1})	Total Monosaccharides (mg mL^{-1})	Total Monosaccharides per g of Extract (mg g^{-1})	Yield (%)
R.o.	NQ	NQ	2.7 (0.2)	7.1 (0.3)	201 (18)	210.5	131.9	13.2
U.o.	2.7 (0.5)	7.2 (0.6)	9 (2)	13 (1)	ND	31.7	269.9	30.3

Rha: Rhamnose; Xyl: Xylose; Gal: Galactose; Glu: Glucose; Man: Mannitol; R.o.: *Rugulopteryx okamurae*; U.o.: *Ulva ohnoi*.

It was not possible to quantify Rha and Xyl in R.o., as expected, after all of the optimization processes. Man was the monosaccharide that was most abundant in R.o., constituting 95% of the total sum of monosaccharides. On the other hand, in U.o., a different type of seaweed, an extract with Rha, Xyl, Gal and Glu was obtained, whereas Man was not detected. The total concentration of monosaccharides was 31.7 mg mL^{-1}, with Glu accounting for 41% of the total sum of simple sugars.

4. Discussion

In the present work, an eco-friendly methodology was optimized to valorize the invasive alga R.o. by extracting simple sugars and establishing an unequivocal detection method via GC-HRMS. Key parameters, including the solvent:mass ratio and UAE conditions, were optimized. Furthermore, a rapid analytical methodology involving oxime derivatization was developed, which was achieved in a total analysis time in the equipment of less than 20 min.

Research interest in R.o. has surged across multiple disciplines since 2020. Using the search query [TITLE-ABS-KEY (rugulopteryx AND okamurae)] in the Scopus database, only 121 related documents appear, including both articles and reviews (data as of March 2026). Approximately 65% of this research falls within Agricultural and Biological Sciences and Environmental Sciences. Figure S6 in the Supplementary Material shows the statistics

and data. This trend suggests that while scientific and governmental efforts to manage this invasive species are intensifying, the field remains in its early stages.

Notably, although R.o. exhibited a lower gravimetric yield compared to U.o. (13.2% vs. 30.3%), it demonstrated a superior capacity for monosaccharide solubilization. The optimized extract of R.o. reached a concentration of 210.5 mg/mL, nearly sevenfold higher than that of U.o. (31.7 mg/mL). This high sugar density, which was particularly rich in mannose, highlights the efficiency of the UAE method for this invasive species, providing a highly enriched feedstock while minimizing solvent consumption. Nevertheless, U.o. also demonstrated a high monosaccharide content per gram of sample without prior optimization, proving to be an excellent feedstock for the recovery of simple sugars.

To the best of our knowledge, no previous studies have specifically investigated UAE for the recovery of simple sugars from R.o. While some literature characterizes this alga, the methodologies and objectives differ significantly. For instance, Paulo et al., in 2025, used an ultrasonic bath, among other techniques, to compare different strategies with the objective of measuring different bioactivities (antioxidant capacity, yield of extraction, total polyphenols), quantifying them spectrophotometrically. They observed that, with an ultrasonic bath, the major percentage recovered was phenolics [21]. Similarly, Lanzas et al. (2025) used different approaches to obtain bioactive compounds from R.o., where they used an ultrasonic bath at 55 °C for 30 min, assisted by enzymatic treatment [22]. Among the bioactivities, they analyzed the total content of carbohydrates and acid sulfated polysaccharides (fucoidans) by traditional methods involving acidic media. The authors observed that using UAE, they achieved an extraction of carbohydrates of 71.92 mg per g of sample. In the present work, 131.9 mg per g extract was obtained, achieving almost double the yield using the same methodology of extraction and only water as the solvent. In 2024, León-Marcos et al. evaluated the reducing sugars from R.o. and optimized several parameters by UAE, but it was used as a pretreatment. After that, they proceeded with enzyme hydrolysis assays and studied the reducing sugars by spectrophotometry. The authors obtained a concentration of 90 mg per L of extract [23]. The results from our work are widely superior, as we obtained a concentration of 210.5 mg mL^{-1} after all of the optimization of parameters for UAE.

Other authors have also evaluated R.o. as a source of bioactive compounds by using alternative extraction methods. Rincón-Cervera et al., in 2024, optimized the extraction of alginate, Man and phlorotannin [9]. Man was extracted with acidified water (HCl) at 60 °C for 6 h, and an estimated concentration of 117 mg per gram of R.o. was obtained. Rincón-Cervera et al. were the first to report data exclusively for the carbohydrate Man. Furthermore, this result is in concordance with ours, although our estimation of Man is slightly superior (131.9 mg per g) with the use of ultrasound, the requirement of less time and the avoidance of acid. These results make sense, since UAE is characterized by cavitation processes that break down cell walls, allowing bioactive compounds to be extracted from plants more effectively than with conventional extraction methods [24]. In this paper, the authors also investigated the use of R.o. as a source of alginate, and they used acidic water, alkaline treatment and other organic solvents to obtain the alginate. In fact, research on R.o. has focused on obtaining alginate for different applications, including as a hydrogel for the encapsulation of orange peel polyphenols [6], to enhance the printability of soy protein-based bioinks [7] and to prepare biomaterials by extracting it with microwave-assisted extraction [25]. However, to the best of our knowledge, and based on the available literature, the characterization of this macroalga—and specifically of its carbohydrates—remains limited compared to other seaweeds, such as *Pyropia* with a concentration of Gal of 77.9 mg 100 g^{-1}; *Sargassum*, *Ulva* and *Laminaria* (data not reported) with application of the optimized extract in cosmeceuticals; or *Sargassum fusiforme*, whose concentration

of galacturonic acid and fucose comprise about 80% of the total monosaccharides [26–28]. However, considering the significant alginate levels in R.o., the residue from the UAE process can be repurposed for alginate production, thereby ensuring the full exploitation of this invasive seaweed for various downstream applications.

This work is focused on R.o. and its valorization, but once the optimization was carried out, U.o. from the same financial project was evaluated to observe differences and obtain conclusions. In this sense, it is valuable to test the versatility of this optimized UAE process. The present paper reveals a concentration of monosaccharides of 31.7 mg per mL of extract, which is much lower than in R.o. This type of macroalga has been studied more than R.o. In 2017, Glasson et al. extracted ulvan with a predominant composition of Rha, glucuronic acid, iduronic acid and Xyl, although other monosaccharides were detected. The authors used a methanolic HCl for the extraction for 18 h [29]. Since the present article was focused on R.o., optimization was carried out directly on this alga, without consideration of U.o. However, given the structural diversity between the species, these results underscore the necessity of species-specific optimization to maximize the recovery of target bioactive compounds.

Regarding the industrial scalability of the proposed UAE method, some factors should be considered. Firstly, the use of water as a solvent significantly reduces costs and environmental impact compared to organic solvents, simplifying downstream purification and waste management. Regarding energy consumption, the use of a high-power sonotrode in continuous flow has been shown to improve energy efficiency at larger scales, especially with the time involving the extraction of several compounds compared to solid–liquid extractions. In 2025, Cauduro et al. reviewed the state of art of the scale up of UAE and observed that several authors have provided different solutions to some problems regarding UAE scalability, such as the localized heating and decrease in energy density with increasing distance from the probe tip. Moreover, in their review, they observed that there have been different types of reactor configurations and, in general, that scalability is a success due to the high percentage of yields obtained by the authors. However, they point to a lack of information regarding process design and reactor configurations, as well as a significant shortage of studies focusing on continuous flow UAE for large-scale implementation [30].

From a logistical and regulatory perspective, the use of R.o. presents unique challenges and opportunities. As an invasive species in the Mediterranean, its collection is subject to specific environmental regulations; however, its industrial valorization aligns with regional strategies for biomass control and environmental remediation. Furthermore, the resulting aqueous extract, free of toxic solvents, facilitates the subsequent recovery of high-value compounds such as Man and the further utilization of the solid residue for alginate production, supporting a circular biorefinery model.

Finally, it should be highlighting that Man, the main monosaccharide detected in this work, is used in several industries. For example, in the food industry, due to its sweetness, mannitol is widely used in products designed for people with diabetes. It also serves as a food additive to extend shelf-life by reducing the crystallization tendency of other sugars. Furthermore, in the pharmaceutical industry, it is used as an excipient to mask the unpleasant taste of drugs, or as a dehydrating agent and diuretic in injections. In 2017, Y. Dai et al. evidenced the necessity of further optimizing high-level Man production, emphasizing that research into downstream processes should be strengthened in the future [31].

5. Conclusions

A new methodology for the extraction of monosaccharides from R.o. using UAE and for their detection and quantification with GC-QTOF has been optimized. This procedure involves water and requires 30 min, so it is green and GRAS (Generally Recognized as Safe). Furthermore, as the industry is expanding extractions with non-traditional procedures for the extraction of bioactive compounds, this process is suitable for scaling up to pilot or industrial levels, provided the necessary adjustments are made to account for scale-related losses. Furthermore, this process can serve as an intermediate step in the complete recovery of R.o. waste: the washes pretreatment can be used to recover phenolic compounds and other removed pigments, and the final pellet residue after the UAE can be used to produce alginate. In this sense, it is possible to fully recover this invasive species. In addition to generating various products for different applications, this approach helps the environment and offers a potential solution to the accumulation of marine debris worldwide. However, the scientific literature on this alga is still limited, and its comprehensive characterization is not conclusive—at least not to the same extent as for other, more extensively studied algae. As this alga is considered to be a growing concern, increasing funding and reducing administrative hurdles are essential when applying for acquisition permits.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/appliedchem6020036/s1, Figure S1: Samples of *Ulva ohnoi* (left) and *Rugulopteryx okamurae* (right); Figure S2: Chromatographic ramp of the optimized method; Figure S3: Spectra for each target analyte; Figure S4: Blank chromatogram; Figure S5: Standardized Pareto Chart for each monosaccharide; Figure S6: Documents (Scientific literature) that contain the words *Rugulopteryx okamurae* in Scopus database (March 2026); Table S1: Experimental matrix employed for the Design of Experiment; Table S2: Equation of the fitted model for each monosaccharide.

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Abbreviations

The following abbreviations are used in this manuscript:

R.o.	*Rugulopteryx okamurae*
Gal	Galactose

Glu	Glucose
Man	Mannitol
Rha	Rhamnose
Xyl	Xylose
ACN	Acetonitrile
UAE	Ultrasound-assisted extraction
EtOH	Ethanol
LOQ	Limit of quantification
LOD	Limit of detection
RSD	Relative Standard Deviation
GC	Gas chromatography
HPLC	High-performance liquid chromatography
HRMS	High-resolution mass spectrometry
HACl	Hydroxylamine
Pyr	Pyridine
U.o.	*Ulva ohnoi*
AcOEt	Ethyl acetate

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