Evaluation of Quantitative Methods for the Determination of Polyphenols in Algal Extracts

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Marine brown algae such as *Ascophyllum nodosum* and *Fucus vesiculosus* accumulate polyphenols composed of phloroglucinol units. These compounds are of ecological importance and, due to their antioxidative activity, of pharmacological value as well. In this study four methods for the quantitative determination of phlorotannins are compared: spectrophotometric determinations using Folin−Ciocalteu's phenol reagent or 2,4-dimethoxybenzaldehyde (DMBA), quantitative ^{1}H NMR spectroscopy (qHNMR), and gravimetrical measurements. On the basis of the relative standard deviation and the F-test, the determination using Folin−Ciocalteu's phenol reagent and qHNMR proved to be the most reliable and precise methods.

Phlorotannins are oligomeric or polymeric phloroglucinol (1,3,5-trihydroxybenzene) derivatives in which phloroglucinol units are connected by aryl−aryl bonds (fucols, Figure 1), ether bonds (phlorethols, hydroxyphlorethols, fuhalols, Figures 2 and 3), or both (fucophlorethols, Figure 4). From *Fucus vesiculosus* several low molecular weight compounds were isolated, including 15 fucophlorethols and four fucols containing 3–8 units and 2–4 units of phloroglucinol, respectively.[1] These compounds were isolated after derivatization as acetylated derivatives[2–4] and also as free phenols.[5] High molecular weight compounds ($>10^4$) were investigated after calcium−liquid ammonia degradation using the method of Ragan,[6] and before and after methylation or acetylation following the procedure of McInnes et al.[7] For *Ascophyllum nodosum* only the structure of a single sulfated phlorotannin is described.[1,8] Brown algae, such as *F. vesiculosus* and *A. nodosum,* which were used for this study, contain phlorotannins with a large range in molecular weight (MW) including compounds with a small MW up to $>1 \times 10^5$.[1]

The concentration of polyphenols in several brown algae was determined using Folin−Denis reagent and yielded values of up to 15% of the dry mass.[1] This concentration was found to be dependent on the season of harvesting,[9–11] the habitat,[12] and other extrinsic factors such as light intensity and ambient nutrients.[12–15] Furthermore, phenolic content varied within an individual algal thallus[16,17] and between different indivuduals of an algal population.[18]

Phlorotannins were shown to be of ecological importance to the producing organisms. They protect brown algae against UV irradiation,[19,20] and are also discussed as a chemical defense to deter herbivores.[21,19,1,22,20] Like gallotannins or condensed tannins from terrestrial plants, phlorotannins are able to precipitate proteins or metal ions out of solution.[1,23] The polymeric phlorotannins inhibit enzymes such as phospholipase A, lipoxygenase and cyclooxygenase-1,[24] hyaluronidase,[25] and tyrosinase.[26] Antioxidative effects have been observed using the DPPH (α,α-diphenyl-β-picrylhydrazyl) assay.[27–31] Additionally, antibacterial activities were described for some tannins from the algae *Ecklonia kurome, E. cava*, and *F. vesiculosus.*[32,33] Some polyphenols from the alga *E. cava* were reported to exhibit inhibitory effects on HIV-1 reverse transcriptase and protease.[34]

In order to correlate bioactivity data (ecology and pharmacology) with the presence of phlorotannins in algal extracts, reliable methods for the quantitative determination of the latter are necessary.

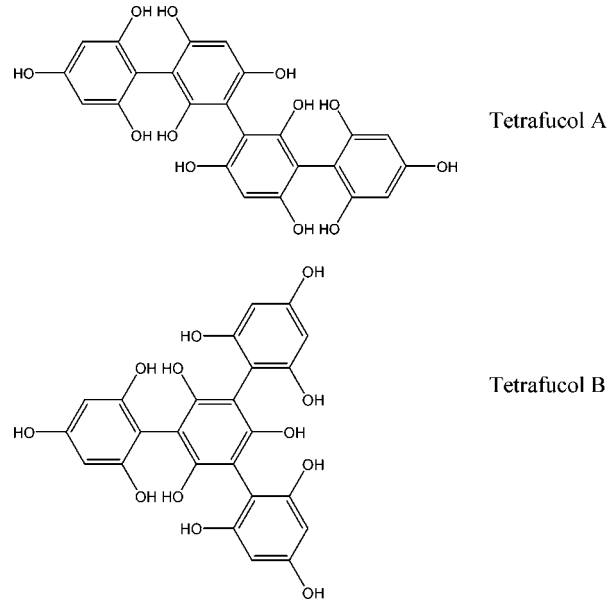

Figure 1. Fucols of *Fucus vesiculosus* (examples; Ragan and Glombitza, 1986).

Although phlorotannins have different structures and molecular weights, their chemical properties are extremely similar. Consequently, the quantitative determination of single phlorotannins in a complex algal extract is hardly feasible and in many cases not useful. Hence, determination methods are used that yield the total polyphenolic content of an extract.

In this study four methods for the quantitative determination of phlorotannins in extracts of *F. vesiculosus* and *A. nodosum* were compared. The determination using Folin−Ciocalteu's (FC) phenol reagent is a well-known and documented photometric method for the quantification of phenolic substances (Figure S1).[10,21,35–39] The reaction of phenols with Folin−Ciocalteu's phenol reagent is unspecific and based on the reductive potential of these compounds. The second photometric determination investigated used the reaction of phlorotannins with 2,4-dimethoxybenzaldehyde (DMBA), which yields a colored pigment (Figure S2).[40,41] Both spectrophotometric methods are conventional determination methods and referenced to phloroglucinol. The third method is a gravimetric determination[1] and is based on the ability of phlorotannins to bind to polyvinylpyrrolidone (PVPP, Figure S3). Finally, the quantification of phlorotannins was evaluated by means of quantitative ^{1}H NMR spectroscopy (qHNMR), making use of characteristic resonance

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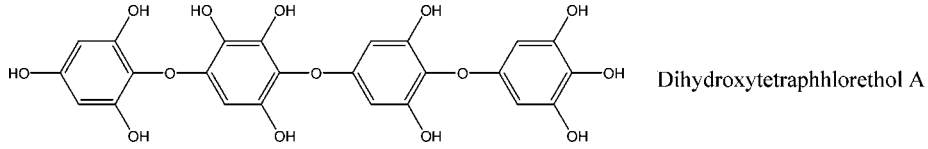

Figure 2. Dihydroxytetraphlortethol A of *Sargassum spinuligerum* (Keusgen, 1993[47]).

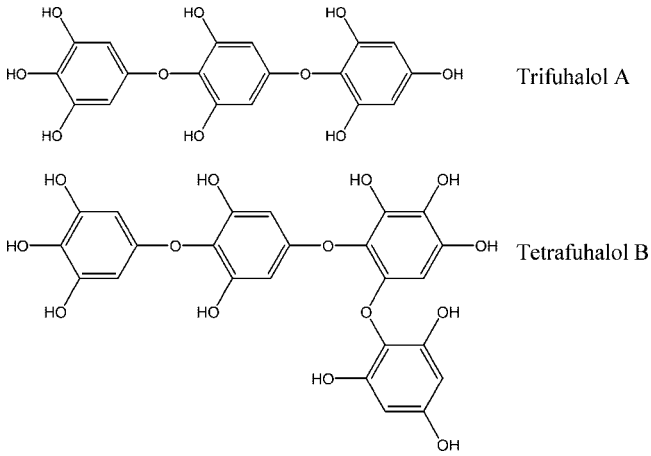

Figure 3. Fuhalols of *Bifucaria bifurcate* (examples; Ragan and Glombitza, 1986).

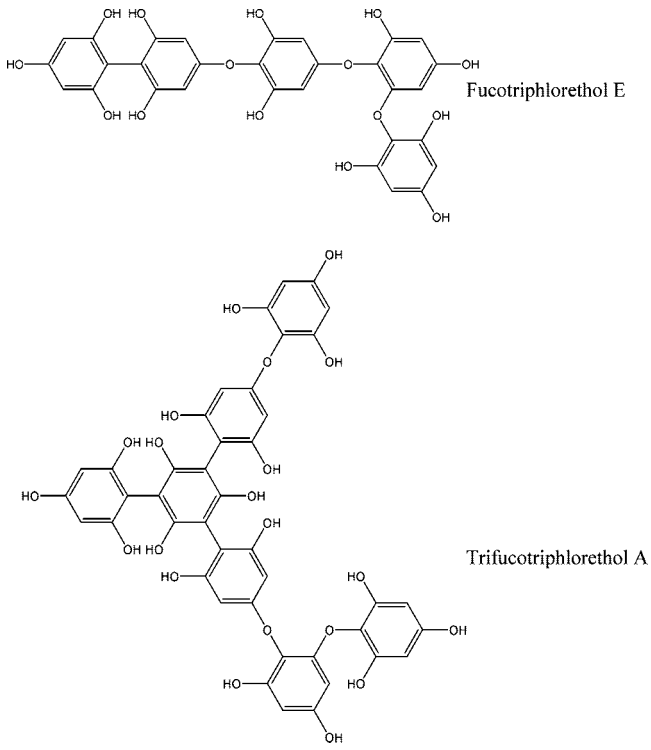

Figure 4. Fucophlorethols of *Fucus vesiculosus* (examples; Ragan and Glombitza, 1986).

signals in the aromatic region of ^{1}H NMR spectra.[42,43] In contrast to the spectrophotometric methods, qHNMR was referenced to trimesic acid as the internal standard.

Results and Discussion

Polyphenols such as phlorotannins are very sensitive toward oxidation. Hence, algal material was extracted on ice and under gassing with N_2. In principle, after a first extraction with EtOH, lipophilic compounds were removed by liquid–liquid partition between petroleum ether/CH_2Cl_2 and the aqueous phase (extract I). In a second step the residue of the first extraction was treated in the same manner and yielded extracts II (Figure S4). Frozen fronds of *F. vesiculosus* (520 g) resulted in 19 g of freeze-dried extract termed Fucus I and 19 g of freeze-dried extract termed Fucus II. To obtain the extracts Asco I and Asco II, 509 g of deep frozen fragments of *A. nodsosum* were used. The extracts were freeze-dried to yield 29 g (Asco I) and 13 g (Asco II).

For some of the methods to determine polyphenolic content, PVPP was used to remove such phenols that were able to interact with amide bonds. Therefore, the number of treatments necessary to quantitatively remove phenols was investigated using the standard compound phloroglucinol. After two successive additions of PVPP, greater than 94% of the phloroglucinol was removed from solution, and further additions did not result in a further decrease of phenols (Figure S5). Thus, for the FC method and the gravimetric determination, the appropriate solutions were treated twice with PVPP.

Folin−Ciocalteu's phenol reagent is composed of sodium tungstate and reduced to mixed oxides when reacting with phenolic compounds. Because the resulting mixed oxides are blue-colored ("tungsten blues"), they can be determined spectrophotometrically. The determination with Folin−Ciocalteu's reagent is unspecific and merely based on the reductive potential of the phenols. The FC method is made somewhat more specific by the fact that phenols like phloroglucinol, as well as more complex compounds based on it, bind under slightly acidic conditions to PVPP, mimicking the binding of phenols to proteins. In this way phenols that are able to interact with peptide bonds can be removed from the sample solution (Figures S1, S5). Other oxidizable substances, not able to interact with PVPP, remain in the test solution. The difference between the first and the second measurement is equivalent to the content of phenolic compounds (i.e., phlorotannins) able to bind to PVPP. Table 1 shows the results for this determination, calculated as phloroglucinol for extract Fucus I (15.88%) and Asco I (13.49%). Extracts Fucus II and Asco II had a lower content of phenolics with 10.18% and 7.85%, respectively.

DMBA reacts specifically with 1,3- and 1,3,5-substituted phenols such as phloroglucinol and phlorotannins, forming triphenylmethane pigments after electrophilic substitution (Figure S2). Various parameters, such as different temperatures and time intervals, were optimized during this study. It is important to note that reaction time and temperature have to be kept strictly constant. The polyphenol content calculated as phloroglucinol for extracts Fucus I and Asco I was found to be 8.92% and 9.45%, respectively. Lower values were again found for both Fucus II (5.60%) and Asco II (4.09%, Table 1).

Table 1 shows a ratio calculated from the results of the two colorimetric methods (FC method to DMBA). This index can be envisaged as a useful parameter to characterize algal extracts and is proposed to be similar for extracts of comparable composition regarding the type of phenols present.

qHNMR is based on the integration of resonance signals in ^{1}H NMR spectra and their comparison with an internal standard. This standard has to be stable, chemically inert, available in highly pure form, and completely soluble in the same deuterated solvent(s) as the sample. As the ^{1}H NMR resonances of phloroglucinol appear at approximately the same chemical shifts as those of phlorotannins, it could not be used as an internal standard. Trimesic acid, however, is an ideal standard, giving rise to only one resonance signal at δ 8.70 ppm (Figure S6). Before use, trimesic acid was recrystallized from a mixture of MeOH and demineralized H_2O and its final purity

Table 1. Comparison of the Spectrophotometric Determinations, the Gravimetric Determination, and qHNMR of the Phlorotannin Content [mean ± SD (RSD)] in Algal Extracts[d]

					qHNMR [%]	
extract	FC [%]	DMBA [%]	ratio: FC/DMBA	gravimetry [%]	$N_{Anl} = 1.7$	$N_{Anl} = 2$
Fucus I	15.88 ± 0.95 (5.96)	8.92 ± 1.05 (11.77)	1.4	15.58 ± 3.84 (24.65)[a,b]	18.02 ± 1.21 (6.69)	15.32 ± 1.02 (6.69)
Fucus II	10.18 ± 0.37 (3.60)	5.60 ± 0.56 (10.01)	1.9	11.65 ± 4.94 (42.40)[a,c]	14.10 ± 0.33 (2.37)	11.99 ± 0.28 (2.37)
Asco I	13.49 ± 0.31 (2.30)	9.45 ± 0.97 (10.25)	1.7	14.79 ± 2.43 (24.65)	29.82 ± 1.15 (3.87)	25.34 ± 0.98 (3.87)
Asco II	7.85 ± 0.71 (9.00)	4.09 ± 0.35 (8.47)	1.8	7.57 ± 3.53 (46.63)	13.74 ± 0.54 (3.94)	11.68 ± 0.46 (3.94)

[a] This series of measurement concludes one outlier. [b] Parameter calculated without outlier: 14.76% ± 2.28 (15.45). [c] Parameter calculated without outlier: 10.64% ± 3.12 (29.32). [d] DMBA = 2,4-dimethoxybenzaldehyde. FC = Folin−Ciocalteu's phenol reagent. DMBA and FC: phloroglucinol used as standard. qHNMR: trimesic acid used as standard.

analyzed by ^{1}H NMR and HPLC to be 99.2%. Analysis of linearity was done by mixing different volumes of phloroglucinol solution with a solution of the standard compound, trimesic acid. The linearity for concentrations of 0.5 to 4 mg/mL of phloroglucinol is shown in Figure S7. For every concentration three measurements were performed.

As the extracts of *A. nodosum* and *F. vesiculosus* contain a complex mixture of phlorotannins, a definite number of protons (N_{Anl}) on each aromatic ring for the calculation of the polyphenolic content cannot be determined. Thus, in order to estimate the average number of protons for each aromatic ring, ^{1}H NMR spectra were measured in DMSO-d_6, and the ratio of the resonances for hydroxyl protons and protons of aromatic moieties was determined. It was found to be 1:0.6 for all samples. This result was supported by calculating this ratio for some published structures of fucols and fucophlorethols. For example, in a mixture of tetrafucols A and B and of fucotriphlorethol E and trifucotriphlorethol A (Figures 1 and 4) the ratio between hydroxyl groups and aromatic protons is on average 1:0.6. The number of protons on each aromatic ring of these compounds varies between 1.5 and 2.0 on average. Because we expect that fucophlorethols are the dominant structural type in our extracts, the average number of protons on each aromatic ring was taken as $N_{Anl} = 1.7$ and $N_{Anl} = 2$.

The integration of resonances was problematic due to the fact that resonance signals of aromatic protons were broad and overlapping. Therefore, instead of integrating single signals all resonance signals in the aromatic region of the spectra were integrated as a whole. The full width at half-maximum height of the resonance of trimesic acid was used for the calculation of the integration limits for both trimesic acid and phlorotannins.

With qHNMR the polyphenolic content of *F. vesiculosus* extracts calculated as phloroglucinol with trimesic acid as internal standard ranges between 15.32% and 18.02% for Fucus I and between 11.99% and 14.10% for Fucus II depending on N_{Anl}. The extracts Asco I and Asco II contain polyphenols in a range between 25.34% and 29.82% and 11.68% and 13.74%, respectively (Table 1).

The gravimetric determination was performed by comparing the dry weight of an algal extract before and after treatment of the dissolved extract with PVPP (Figure S3). Thus, similar to the FC method the gravimetric determination measured only polyphenols able to bind to PVPP. Using this method the determination of polyphenolic content resulted in 15.58% for Fucus I, 11.65% for Fucus II, 14.79% for Asco I, and 7.57% for Asco II (Table 1).

The statistical parameters (mean, standard deviation (SD), and relative standard deviation (RSD)) for all methods and extracts are shown in Table 1 and Figure 5. RSD for the FC method is between 2.3% and 9.0% (average 5.2%), for DMBA assay between 8.4% and 11.8% (average 10.1%), for qHNMR between 2.4% and 6.7% (average 4.2%), and for gravimetry between 24.7% and 46.6% (average 34.6%). Our analysis shows that the RSD for qHNMR and for the FC method differ only slightly. Both the RSDs for qHNMR and the FC method were considerably lower than those for the DMBA method.

However, the F-test (p-value $\alpha = 0.05$) for DMBA versus qHNMR resulted in an insignificant difference in variances (=SD^2)

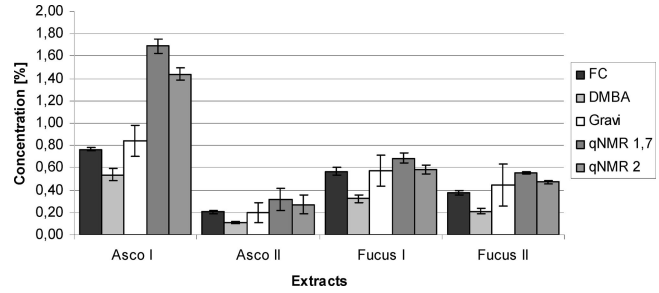

Figure 5. Phlorotannin content (mean ± SD) calculated as phloroglucinol for FC method, DMBA assay, and gravimetry, or as phloroglucinol with trimesic acid as internal standard for qHNMR based on the fresh weight of algae.

for all extracts, indicating an equal precision for both methods. In contrast the DMBA versus FC method resulted in an insignificant difference in variances for the extracts of *F. vesiculosus* and in significant differences for the extracts of *A. nodosum.* FC method versus qHNMR yielded an insignificant difference for extracts Asco II, Fucus I, and Fucus II, whereas for extract Asco I this comparison showed a significant difference in variances. Significant differences in variances signify an unequal precision. Hence, for extract Asco I the RSD for the determination procedures FC and qHNMR indicated their precision.

Comparison of the gravimetric determination with the spectrophotometric methods clearly revealed that gravimetry had the highest SD and RSD. Even though this method is ideally suited to determine the content of phenols reacting with proteins, the analytical procedure must be judged as not sufficiently precise. The F-test supports this result, suggesting that gravimetry versus spectrometric methods had an unequal precision. Further a confidence interval (two-sided, p-value $\alpha = 0.05$, degrees of freedom $= 14$, $t = 2.145$) was calculated for the gravimetric determination. The confidence interval includes every single value with a probability of 95%. The probability that not more than one of the 15 values lay outside is also 95%. In addition to that, a test for statistical outliers was performed for all extracts. Outliers or extreme values represent a random error, which has a great influence on the statistical parameters. Hence, outliers should be identified and removed. Therefore, mean and SD had to be calculated without values of possible outliers and a new interval (mean ± 4 × SD) was calculated. All values that lie outside this interval were identified as outliers and were not used for further statistical investigations. Outliers were identified for both extracts of *F. vesiculosus* but not for the extracts of *A. nodosum*. The calculation of the confidence interval and the test for statistical outliers demonstrated that gravimetry had the least repeatability and precision of all determination methods.

Table 1 shows the phlorotannin content in terms of percent calculated as phloroglucinol for the FC method, DMBA assay, and gravimetry. These values were compared with *t*- and Mann–Whitney U-tests. Both tests of significance resulted in a significant difference in polyphenolic content for the FC method versus DMBA and gravimetry versus DMBA (p-value $\alpha < 0.05$) according to the

Table 2. Comparison of Methods for the Quantitative Determination of Phlorotannins

method	principles	advantages	disadvantages
FC method	redox reaction before and after removal of phenols with PVPP	well-known and documented method; indirect measurement; very good repeatability; highest precision	unspecific reaction; difference in number of hydroxy groups results in a different reductive potential; extracts containing different structural types are not comparable; complex procedure
DMBA assay	reaction between phenols and aldehyde	specific reaction with 1,3- and 1,3,5- substituted phenols; indirect measurement; good repeatability; high precision	different maxima of absorbance for the adducts of the standard phloroglucinol and of algal phenols; complex procedure highly dependent on reaction conditions
gravimetric determination	precipitation of phenols with PVPP	well-known method; direct measurement; easy handling	only phenols binding to PVPP are measured; time-consuming method; low repeatability; low precision
qHNMR	integration of ^{1}H NMR signals	quick sample preparation; well-established for single substances; direct measurement; very good repeatability; highest precision	not well-established for mixtures of similar compounds; all phenolic compounds in the extract are measured; difficult integration of signals; phloroglucinol cannot be used as internal standard; not comparable to other methods; molecular mass not known, and calculation difficult since number of protons on phenols can only be estimated

different chemical reactions taking place. The comparison of mean contents obtained with the FC method and the gravimetric determination demonstrated that the analysis of polyphenol content with these methods resulted in no significant difference in content. Both the FC method and the gravimetric determination measured the concentration of phlorotannins binding to PVPP; these are the phlorotannins that are responsible for the tanning effect (=tanning part). Hence, both methods yielded approximately the same content of polyphenols in the extracts (Table 1). For qHNMR trimesic acid was used as the standard instead of phloroglucinol. Therefore, the comparison of the mean polyphenolic content with *t*- and U-tests was not done for this method.

For the quantitative analysis of phlorotannins in algal extracts four different methods (FC method, DMBA assay, gravimetric determination, and qHNMR) were optimized and the results compared. The four methods used are based on different principles, namely, redox reaction, reaction between phenols and aldehydes, precipitation of phenols, and integration of ^{1}H NMR signals (Table 2). Due to the fact that our determination methods exploit different physical and chemical properties of phlorotannins, the calculated polyphenolic contents vary depending on the method used (Table 1).

The determination with Folin−Ciocalteu's phenol reagent is based on the reductive potential of the phenols. Structural types like fuhalols (Figure 3) and hydroxylated phlorethols (Figure 2) show a different reductive potential because of their difference in numbers of free hydroxyl groups. The calculated content of phlorotannins may thus vary depending on the structural type of phenols in the sample.[1,41] Hence, the polyphenol contents of extracts containing different types of phlorotannins are not comparable.

It also has to be noted that other compounds like simple phenols, polypeptides, urea, and even diethyl ether[1] react with Folin−Ciocalteu's phenol reagent. This makes the second step in the quantitative determination of polyphenols necessary wherein PVPP-binding phenols are removed from solution (Figure S1).

Toth and Pavia analyzed the use of insoluble PVPP for the removal of polyphenols from dissolved algal extracts.[44] These investigations showed that the removal of phlorotannins using insoluble PVPP was especially dependent on the number of PVPP treatments (3 × 10 min) and the amount of PVPP (10 mg/mL) under slight acidic conditions. In contrast to Toth and Pavia,[44] in the current study the algal extracts were treated only twice for 10 min with insoluble PVPP (10 mg/mL). Our investigations showed that after the second treatment >94% of the phloroglucinol was removed from solution (Figure S5). The third addition of PVPP did not result in a further decrease of the phloroglucinol concentration in solution. Therefore, for both the FC method and gravimetry the aqueous algal solutions were treated twice with insoluble PVPP. The polyphenol concentration in extracts of the algae *F. vesiculosus* and *A. nodosum* can be underestimated if not all phenolics have been removed from solution by PVPP.

The DMBA assay is dependent on the reaction between phenols and aldehydes. Both the FC method and the DMBA assay are indirect determination methods using the formation of a colored chromophore for a spectrophotometric determination. In contrast to the reaction with Folin−Ciocalteu's phenol reagent, the DMBA assay is not based on a difference measurement (i.e., PVPP binding phenols are not removed). The biggest disadvantage (Table 2) of this method, as determined in this study and also by Stern et al.,[41] is the fact that the reaction is both time- and temperature-dependent. Additionally, DMBA adducts of standard phloroglucinol versus phenols in algal extracts have different UV/vis maxima (Figure S8). As a consequence, standard and sample solutions are measured at 494 and 515 nm, respectively, which could lead to mistakes in the calculation of phlorotannin content. As noted for the FC method, the chromophore formed with DMBA depends on the chemical properties of the analytes (i.e., ether bonds or aryl−aryl bonds affect the reaction between DMBA and polyphenols). Carbon−carbon bonds between aromatic moieties especially influence the reaction between the DMBA and phlorotannins because this bond type eliminates aldehyde reactive sites.[1]

Gravimetry is a direct determination method and is based on the precipitation of phenols with insoluble PVPP. Similar to the FC method the gravimetric method thus only determines the polyphenol content binding to PVPP. The biggest disadvantages of this method are its low repeatability and precision. However, it is a well-known and long-practiced method that is easily performed (Table 2).

The fourth method, qHNMR, is also a direct measurement of phenols. ^{1}H NMR resonance signals of all phenolic compounds contained in the algal extracts are integrated and compared with those of the internal standard. In contrast to gravimetry and the FC method, however, no removal of phenols with PVPP is done. Hence, the phlorotannin content determined with qHNMR is possibly higher than that determined with the other methods. Since trimesic acid instead of phloroglucinol was used as the internal standard, the average phenolic content obtained with this method is not comparable with those of the other methods. One of the biggest advantages of qHNMR is the very short sample preparation time. Furthermore, besides the FC method, qHNMR is the most precise method

according to the RSD. The biggest disadvantage is the difficult integration of resonance signals and the calculation of polyphenolic concentration since the molecular mass and the number of protons on an aromatic ring can only be estimated (Table 2).

Statistical evaluation (SD, RSD, and F-test) showed that in terms of precision the FC method, DMBA assay, and qHNMR were more precise than gravimetry. Thus, these methods were regarded as more accurate. The *t*- and Mann–Whitney U-tests indicate that the polyphenol content determined with methods based on different chemical reactions, e.g., colorimetric methods (FC method, DMBA assay), are significantly different. As proven statistically (*t*-, Mann–Whitney U-test), the results obtained with the FC method and gravimetry do not significantly differ. Both of these methods use PVPP to remove phenols.

None of the four methods for the quantitative determination of brown algal polyphenols are without problems. The advantages and disadvantages of all methods described above are shown in Table 2. Statistical parameters were consulted to identify differences between determination methods. The comparison resulted in the conclusion that the colorimetric methods (FC method, DMBA assay) and qHNMR had comparable precision as shown with the F-test. It is necessary for the determination using Folin−Ciocalteu's method as well as DMBA to keep reaction conditions strictly constant; otherwise the calculated polyphenolic contents are not reliable. Based on the F-test and the RSD, qHNMR and the FC method were the most precise procedures. The two methods, however, measure different properties of phlorotannins in algal extracts.

Experimental Section

General Experimental Procedures. Spectrophotometric measurements (Folin−Ciocalteu's method and DMBA assay) were done with a Perkin-Elmer Lambda 40 UV–vis spectrometer with UV WinLab software Version 2.80.03. The NMR spectra were recorded on a Bruker Avance 300-DPX spectrometer operating at 300.13 MHz (^{1}H NMR).

Algal Material. The algae *A. nodosum* and *F. vesiculosus* were harvested in February 2003. The collecting site was Corniche Armorique, St. Efflam, France. The samples used in this study were identified by one of us (K.-W.G.). The fresh algal material was deep frozen and stored at −20 °C. Voucher specimens of *A. nodosum* and *F. vesiculosus* have been deposited at the herbarium of the Institute for Pharmaceutical Biology, University of Bonn, Germany.

Extraction. For quantitative analysis, extracts obtained from *A. nodosum* (Asco I/II) and *F. vesiculosus* (Fucus I/II) were prepared according to the extraction procedure shown in Figure S4. For this purpose, deep frozen algal fragments (500 g) were pulverized and extracted on ice and under N_2-gassing with 96% EtOH (800 mL, extract I) employing an Ultra Turrax (Ika T 25) for 2 h. The solid residue was removed by centrifugation and extracted a second time with 60% EtOH (1000 mL, extract II). After evaporation of the EtOH under reduced pressure, chlorophyll and lipophilic substances were removed by liquid–liquid partitioning three to eight times between PE or CH_2Cl_2 (each 300 mL) and the residual aqueous phase until the organic layer was slightly yellow. Subsequently, the aqueous phases were freeze-dried to yield extracts Asco I and Fucus I. Extracts II (Asco II and Fucus II) were obtained in the same manner as described for extracts I.

Folin−Ciocalteu's Method (FC method). Freeze-dried algal extract (50.0 mg) was dissolved in 25.0 mL of demineralized H_2O, and 10.0 mL of this solution was diluted with H_2O up to 25.0 mL. From this diluted solution, 2.0 mL was mixed with 10.0 mL of H_2O, 1.0 mL of Folin−Ciocalteu's phenol reagent (Merck, Germany), and 12.0 mL of $Na_2CO_3 \times 10\ H_2O$ (29% m/V) to a final volume of 25.0 mL (=reaction volume). The mixture was incubated for 30 min in the dark at room temperature. Thereafter the absorbance was measured at 760 nm against a blank (measurement 1). The blank was prepared in the same way as described above and contained H_2O, Folin−Ciocalteu's phenol reagent, and Na_2CO_3 solution. After 30 min in the dark, the blank remained colorless.

To prepare a calibration curve 200.0 mg of phloroglucinol (Merck, Germany) was dissolved in 100.0 mL of demineralized H_2O. The following concentrations of phloroglucinol were used: 0.0, 3.0, 4.0, 5.0, and 6.0 mL of solution of phloroglucinol were each diluted with H_2O up to 100.0, and 2.0 mL of this solution was treated in the same way as described above.

In a second experiment 10 mg/mL insoluble polyvinylpolypyrrolidone (PVPP; Sigma-Aldrich, Germany) was added to the sample or the phloroglucinol solutions in order to remove all polyphenolics able to be adsorbed on PVPP. Sample or phloroglucinol solutions were stirred for 10 min with a magnetic stirrer. Afterward PVPP was removed by centrifugation and the supernatant carefully pipetted into another reaction vessel. The pH of this solution was in the range between 6 and 7. The sample was treated once again with PVPP in the same manner. After this, the remaining supernatant was treated as described for the freeze-dried algal extract (measurement 2). The difference in absorbance between measurement 1 and 2 is equivalent to the content of phenolic compounds able to interact with PVPP (Figure S1).

DMBA Assay. Equal volumes of a stock solution of 2,4-dimethoxybenzaldehyde (2.0 g/100.0 mL glacial acetic acid; Merck, Germany) and HCl (16.0 mL concentrated HCl per 100.0 mL glacial acetic acid) were mixed just prior to use (working reagent).

An aqueous solution of the dry extract (10.0 μL of a 20.0 mg/mL solution) was mixed with 2.5 mL of the working reagent and 10 μL of DMF. The mixture was incubated for 60 min at a temperature between 30 and 33 °C. Thereafter the absorbance was measured at 515 nm against the blank (Figure S2). The calibration curve was prepared by dissolving 5.0 mg of phloroglucinol in 10.0 mL of H_2O. Volumes of 0.0, 4.0, 5.0, 6.0, and 8.0 μL of this solution were treated in the same way as described above except that the absorbance of standard solutions was measured at 494 nm.

qHNMR. Extracts (20.0 mg) were dissolved in 0.5 mL of standard solution. Standard solution was prepared by dissolving 2.0 mg of trimesic acid (after two recrystallizations; Merck, Germany) in a mixture of 0.8 mL of MeOH-d_4 (Deutero GmbH 99.8%) and 0.2 mL of D_2O (Deutero GmbH 99.9%).

For validation 20.0 mg of phloroglucinol was dissolved in 2.0 mL of D_2O or 1.0 mL of MeOH-d_4. Afterward 500 μL of standard solution was added to the phloroglucinol solution, obtaining concentrations of 0.5, 1, 2, 3, and 4 mg/mL of phloroglucinol. Each sample solution was transferred to a 5 mm NMR tube. The measurements were done at room temperature. The following acquisition parameters were used: number of scans 256, no spinning, acquisition time 9.1 s, relaxation delay (d1) 30 s, pulse angle 30°, pulse width 7.8 μs, spectrum data point and time domain 64 k. All ^{1}H NMR spectra were referenced to the resonances of the residual solvent signals (HDO in D_2O at δ 4.68, CH_3OD in CD_3OD at δ 3.35).

The data were processed with Bruker XWin-NMR software 3.5, using a line-broading parameter of 0.2 Hz, manual phasing, and Bruker "basl" command for baseline correction.

Calculation of polyphenol content:[45]

$$K_{An1} = \frac{m_{An1}}{m_{matrix}} = \frac{I_{An1} N_{Std} M_{An1} m_{Std}}{I_{Std} N_{An1} M_{Std} M_{matrix}} \times f_{Std}$$

where m = weighed sample [mg], I = intensity, N = number of ^{1}H/ring $N_{Std} = 3$, $N_{Anl} = 1.7$ or 2, M = molar mass, $M_{Std} = 210.14$, $M_{Anl} = 124$, f = grade of purity, Anl = analyte, and Std = standard.

Gravimetric Determination. Algal extract (100.0 mg initial weight) was dissolved in 25 mL of demineralized H_2O. The aqueous sample was supplemented with 10 mg/mL PVPP and treated as described for the FC method. After this the supernatant was filtrated twice. The filters were washed 2× with demineralized H_2O, and the washing H_2O was added to the filtrate. Afterward the aqueous sample was concentrated under vacuum. The remaining H_2O was removed by freeze-drying. The weight of tannins in the extract was calculated by the difference between the weight after removal of phenols with PVPP and the initial weight (Figure S3).[1]

Statistical Evaluation. The repeatability of both colorimetric methods and the gravimetric determination was analyzed using 15 independent samples from each extract. For the validation of qHNMR 15 independent samples were used for extracts Asco I and Fucus I and five independent samples for Asco II and Fucus II. This allowed the calculation of statistical parameters [mean, standard deviation (SD), and relative standard deviation (RSD)]. Furthermore the linearity, sensitivity, and robustness of the two colorimetric methods were determined for the standard compound phloroglucinol. The same

determinations were done for qHNMR using trimesic acid as internal standard and phloroglucinol.

The two-sided Mann–Whitney U-test was performed. This nonparametric test of significance estimates whether two independent samples of observation are the same (hypothesis H_0). The Mann–Whitney U-test also can be used for the evaluation of a normal distribution of samples; however, this rank test has an efficiency of only 95% compared to the *t*-test.[46] Further the F-test (analysis of variances) was done in order to determine if the variances of the four methods were significantly different (hypothesis H_1) or not (hypothesis H_0). Afterward the (nonpaired) two-sided *t*-test was performed. All tests of significance were done with a probability value (*p*-value) of $\alpha = 0.05$.

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Supporting Information Available: Schemes of quantitative determination methods and extraction, removal of phloroglucinol, example of a qHNMR spectrum, determination of linearity of qHNMR, table with original data of the FC method, DMBA assay, qHNMR, and gravimetry as well as tables with results of the F-, *t*-, and Mann–Whitney U-test are available free of charge at http://pubs.acs.org.

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