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Short communication

NMR use to quantify phlorotannins: The case of *Cystoseira tamariscifolia*, a phloroglucinol-producing brown macroalga in Brittany (France)

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ABSTRACT

Among the most renowned natural products from brown algae, phlorotannins are phloroglucinol polymers that have been extensively studied, both for their biotechnological potential and their interest in chemical ecology. The accurate quantification of these compounds is a key point to understand their role as mediators of chemical defense. In recent years, the Folin–Ciocalteu assay has remained a classic protocol for phlorotannin quantification, even though it frequently leads to over-estimations. Furthermore, the quantification of the whole pool of phlorotannins may not be relevant in ecological surveys. In this study, we propose a rapid ^{1}H qNMR method for the quantification of phlorotannins. We identified phloroglucinol as the main phenolic compound produced by the brown macroalga *Cystoseira tamariscifolia*. This monomer was detected *in vivo* using ^{1}H HR-MAS spectroscopy. We quantified this molecule through ^{1}H qNMR experiments using TSP as internal standard. The results are discussed by comparison with a standard Folin–Ciocalteu assay performed on purified extracts. The accuracy and simplicity of qNMR makes this method a good candidate as a standard phlorotannin assay.

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1. Introduction

Phlorotannins are secondary metabolites produced by Phaeophyceae (brown algae). These phenolic compounds are polymers of phloroglucinol (1,3,5 trihydroxybenzene). The degree of polymerization of these molecules varies from one to several thousand [1]. Several classes are usually distinguished according to the chemical linkage between phloroglucinol units: fucols consist of aryl–aryl linked phloroglucinol units while aryl–ether bonds characterize phlorethols and fuhalols. In eckols and carmalols, phloroglucinol units are associated through dibenzodioxin bonds [2–4].

Phlorotannins have been extensively studied since 1960s. Beyond the structural elucidation of phenols from various seaweeds, many experiments have investigated their implication in the chemical defense of brown algae. Multiple ecological roles have been demonstrated, including herbivore deterrence, antimicrobial activity and sunscreen effect (see for review [5]). The variability of phlorotannin content in seaweeds has also been assessed. It is now clear that the phlorotannin levels vary between tissues in one individual [6,7], between species [8–11], populations [9,11,12], and through several geographic scales [13]. Furthermore, the phlorotannin content is depending on the techniques used for extraction, especially when comparing "classic" solvent extraction with alternative methods [14].

The quantification of these polyphenols is not an easy task. In most experiments, phlorotannin levels are assessed as a whole. For this purpose, the Folin–Ciocalteu (FC) assay is the most common method used to determine the phenol content of brown algae [15,16]. The protocol is based on the oxidation of phenol rings by phosphotungstic and phosphomolybdic acids. Depending on the quantity of phenolic molecules, this reaction generates blue tungsten and molybdene oxides that can be determined by spectrophotometry. The phenolic content is then expressed as an equivalent quantity of phloroglucinol. Unfortunately, this method is subject to interferences as the Folin–Ciocalteu reagent also oxidizes several non-phenolic compounds [17–19]. Other

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ways to quantify phenols have been proposed, such as the DMACA–HCl assay [20]. However, it is also a colorimetric test adapted to condense tannins (proanthocyanidins), which can be limited regarding the quantification of algal compounds.

Furthermore, quantifying phlorotannins as a whole can be ambiguous for chemical ecology studies. Several phlorotannins are implicated in various phenomena, but not necessarily all at the same time. Consequently, the quantification of the whole mixture of phlorotannins may not be relevant to study their role. Instead of the FC assay, Koivikko et al. [21,22] used liquid chromatography (UV detection) to quantify phenols in several populations of the brown macroalga *Fucus vesiculosus*. This approach gave more reliable results compared to the FC assay. A similar chromatographic method (MS detection) was used by Goo and co-workers [23] to assess the phlorotannin composition of another brown macroalga, *Ecklonia stolonifera*. Chromatography was a precise and accurate method to quantify eckol, dieckol, and phlorofucofuroeckol-A. However, it requires standards for quantification, and pure phlorotannins are most of the time absent in the catalogs of chemical suppliers. In this context, quantitative nuclear magnetic resonance experiments (qNMR) have also proven themselves trustworthy in the quantification of natural products [24]. To our knowledge, this approach has only once been used to quantify polyphenols in brown algae [25]. Besides, as part of our study of the genus *Cystoseira* we demonstrated the usefulness of solid-state NMR (High Resolution-Magic Angle Spinning HR-MAS) spectroscopy to detect secondary metabolites in brown algae [26]. In Brittany, five species of *Cystoseira* occur on rocky shores: *Cystoseira baccata*, *Cystoseira foeniculacea*, *Cystoseira humilis*, *Cystoseira nodicaulis* and *Cystoseira tamariscifolia* [27]. Among them, *C. tamariscifolia* is an interesting species as it produces the monomer phloroglucinol, as a major phenolic compound [15,27].

In recent years, HR-MAS spectroscopy has furnished new insights in the taxonomy, chemistry and physiology of various living forms, prokaryotes and eukaryotes. It was notably used for the disambiguation of closely-related taxa in brown algae [26,28] and lichens [29]. HR-MAS experiments have also been used in food chemistry, notably to discriminate samples from diverse geographical origin [30,31] and/or corresponding to different varieties of the same product [32,33]. The non-destructivity of this technique has also allowed the *in vivo* investigation of bacterial cells [34,35] and from a general point of view, new developments in metabolomics. Finally, new applications of HR-MAS have yielded a better understanding in several physiological processes, such as the accumulation of ceramide related with obesity in a mutant drosophila [36] and the loss of a capsular component of *Campylobacter jejuni* associated with a greater resistance of the pathogen towards human serum [37].

In the present study, NMR experiments were used to identify and quantify phloroglucinol from the brown macroalga *C. tamariscifolia*. ^{1}H High Resolution Magic Angle Spinning (HR-MAS) NMR experiments were applied on small algal fragments to detect phloroglucinol in the algae. ^{1}H qNMR experiments (using TSP as an internal standard) were performed to determine the evolution of phloroglucinol levels among tissues and throughout seasons in a 3-year period. The accuracy of this method was assessed using a standard solution of phloroglucinol. qNMR and Folin–Ciocalteu assay results were also compared to validate the qNMR method. Our paper presents then for the first time an innovative and rapid method to quantify phloroglucinol in a brown macroalga, which can be extended to all metabolites clearly identified on a NMR spectrum, for any algal species.

2. Material and methods

2.1. Biological material and sampling

C. tamariscifolia (*Sargassaceae*, *Phaeophyceae*) is an iridescent brown alga that settles in the North-Eastern Atlantic Ocean and in the Western Mediterranean Sea. In Brittany, it occurs notably in intertidal rock pools [38]. This alga shows a seasonal development, with an active growth during spring and summer that ends with the apparition of reproductive organs [27]. Previous investigations on European brown algae revealed that it produces phenols like phloroglucinol, phlorethols and fuhalols [39].

From February 2009 to July 2010, *C. tamariscifolia* samples were collected in Penmarc'h (Brittany, France) after identification according to unambiguous criteria well described in the literature, such as the clear purple-to-green iridescence and the presence of spine-like appendices [40]. The samples prepared for HR-MAS NMR experiments were composed of small apical fragments (1 cm long). They were dedicated to phloroglucinol detection using HR-MAS NMR. Larger apical fragments were collected for phlorotannins extraction and qNMR analyses. Each month this sampling was performed in triplicates. However, *C. tamariscifolia* enters a dormancy-like phase during autumn and winter in Brittany [27]. The main parts of its thallus naturally fall and hence, no suitable material could be collected for quantification from October 2009 to February 2010. In the laboratory, the biological material was rinsed several times with deionized water, immediately freeze-dried and then ground to powder.

2.2. Identification of phloroglucinol

One algal sample (1 g of dry weight) was ground and submitted to extraction using methanol/water (1:1 V/V) at 40 °C in the dark. After centrifugation, the extract was evaporated under vacuum, freeze-dried and dissolved in D_2O. It was submitted to a ^{1}H NMR acquisition on a Bruker DRX 500. Afterwards, pure phloroglucinol (Sigma-Aldrich, France) was added to the NMR tube and another spectrum was acquired. The comparison of both spectra was used to identify phloroglucinol.

2.3. Detection of phloroglucinol by ^{1}H HRMAS NMR

The presence of phloroglucinol in *C. tamariscifolia* was investigated using solid-state NMR experiments, according to the procedure described in our previous study [26]. The spectra were acquired on a Bruker DRX 500 spectrometer equipped with an indirect HR-MAS ^{1}H/^{31}P probe-head with gradient Z at 25 °C. Each spectrum was a collection of 64 scans, with presaturation of the water signal. Approximately 5 mg of algal freeze-dried fragment was placed in the rotor, and then 30 μL of D_2O was added to lock the ^{2}H field. The rotor turned on an axis placed at the "magic angle" (54.7°) to the magnetic field B_0, and the spinning frequency was set at 5000 Hz. As described in other brown algae [41,42], mannitol signals were the most intense ones. Consequently, they were used as a reference: for each spectrum, the phloroglucinol singlet, *i.e.* at 6.02 ppm with 3 aromatic protons detected per molecule when dissolved in D_2O, was compared to these signals to evaluate relative intensities. Hence, we obtained an overview of the evolution of phloroglucinol content in *C. tamariscifolia* according to the seasons.

2.4. Phloroglucinol quantification using the Folin–Ciocalteu assay

In parallel to NMR experiments, a Folin–Ciocalteu (FC) assay was undertaken on semi-purified extracts of *C. tamariscifolia*. However, previous experiments revealed that the use of the FC assay on crude extracts could lead to an over-estimation of phenols in *C. tamariscifolia* [27]. Consequently, we used an ethyl-acetate/water partition to purify phloroglucinol before quantification. Such an ethyl acetate extraction is a classic phlorotannin purification step, well-described in the literature [42–44]. For each sample, phenolic compounds were extracted twice, successively from the same 200 mg of powdered algae with a methanol/water (1:1) mixture during 2 h, at 40 °C in the dark. The two extracts were pooled, methanol was evaporated and the

volume of the resulting crude extract was set to 10 mL of aqueous solution. Furthermore, 5 mL of ethyl acetate was added and the resulting 15 mL was mixed and centrifuged (5000 rpm, 4 °C). The organic phase was isolated while the remaining aqueous phase was re-extracted once with 5 mL ethyl acetate. The two organic phases were combined and the solvent was removed using a rotary evaporator. The organic molecules were dissolved in 10 mL of water (containing less than 1% ethanol for a better solubility). The recovery of phloroglucinol in this organic extract was checked by ^{1}H NMR. The phloroglucinol content in this organic phase was evaluated using the FC assay procedure slightly adapted from Le Lann and co-workers [28]. Briefly, 100 μL of diluted organic phase was mixed with 50 μL of Folin–Ciocalteu reagent, 200 μL of Na_2CO_3 (15%) and 650 μL of distilled water. This mixture was heated during 20 min at 70 °C and put on ice for 10 min to stop the reaction. The absorbance was measured at 750 nm. Standard phloroglucinol solutions were also submitted to this FC assay to get a calibration curve. The results were expressed as phloroglucinol content in % DW.

2.5. Accuracy of the ^{1}H qNMR method for phloroglucinol quantification

In the present study, phloroglucinol levels were also evaluated using ^{1}H NMR analyses of liquid extracts using sodium trimethylsilylpropionate-d4 (TSP) as the internal standard. The accuracy of this approach was evaluated using a freshly-prepared 3.1 mg/mL solution of pure phloroglucinol (Sigma-Aldrich, France). For this, 1 mL of the phloroglucinol stock solution was evaporated to dryness, then dissolved in 700 μL D_2O in a NMR tube. 5 μL of a TSP solution (14.0 mg/mL) was added and the content of the tube was mixed before ^{1}H NMR analysis. A typical spectrum consisted in 64 scans and a long inter-scan delay (d1 = 10 s) was set in order to let all nuclei relax, resulting in an optimal correspondence between intensity of the signal on the spectra and quantity of ^{1}H. The intensity of the phloroglucinol signal was obtained by integration of the singlet at 6.02 ppm. The proportionality between the quantity of protons and the intensity of a signal was deduced thanks to TSP, *i.e.* by integration of the singlet at 0.00 ppm, 9 protons per molecule, value set at 9000 units. The absolute quantity of phloroglucinol in the NMR tube was determined according to the following formula:

$$n_{phloroglucinol\ in\ tube}(mol) = n_{TSP\ added\ in\ tube} \times \frac{Area_{phloroglucinol}}{Area_{TSP}} \times \frac{9}{3}$$

The "9" and "3" coefficients correspond to the number of ^{1}H detected in one molecule of TSP and phloroglucinol, respectively. The quantity of phloroglucinol in the tube assessed using qNMR was compared to the actual value (3.1 mg) to check the validity of the method. The accuracy was calculated as the ratio (%) of phloroglucinol quantified/phloroglucinol in tube.

2.6. Quantification of phloroglucinol by ^{1}H qNMR

The sample set used for quantification covered one year and a half, with 13 months of active sampling. For each month, three different samples were collected and analyzed. Phenolic compounds were extracted using two successive methanol/water (1:1) extractions from the same 200 mg of powdered algae during 2 h, at 40 °C in the dark. The two extracts were pooled, methanol was evaporated and the volume of the resulting crude extract was set to 10 mL of aqueous solution. The procedure for qNMR analysis is the same as described before. Hence, 1 mL of aqueous extract was sampled, evaporated to dryness and dissolved in exactly 700 μL of D_2O in a NMR tube. Moreover, 5 μL of a TSP stock solution was subsequently added in the NMR tube and mixed before analysis. After determination of the phloroglucinol content of the tube, the phloroglucinol level of the alga was determined as % DW (dry weight).

3. Results and discussion

3.1. Identification of phloroglucinol

Fig. 1 introduces two ^{1}H NMR spectra, the upper one is a spectrum of a crude extract of *C. tamariscifolia* collected in May 2009. One can clearly notice the water peak (4.7–4.9 ppm), the mannitol signals (3.6–3.9 ppm) and 3 signals characteristic of aromatic ^{1}H (6.02, 6.33 and 6.43 ppm). The lower spectrum is an analysis of the same extract after enrichment using phloroglucinol. One can observe exactly the same signals, with a much higher intensity of the singlet at 6.02 ppm, and the apparition of ^{13}C–^{1}H coupling satellite signals on both sides of

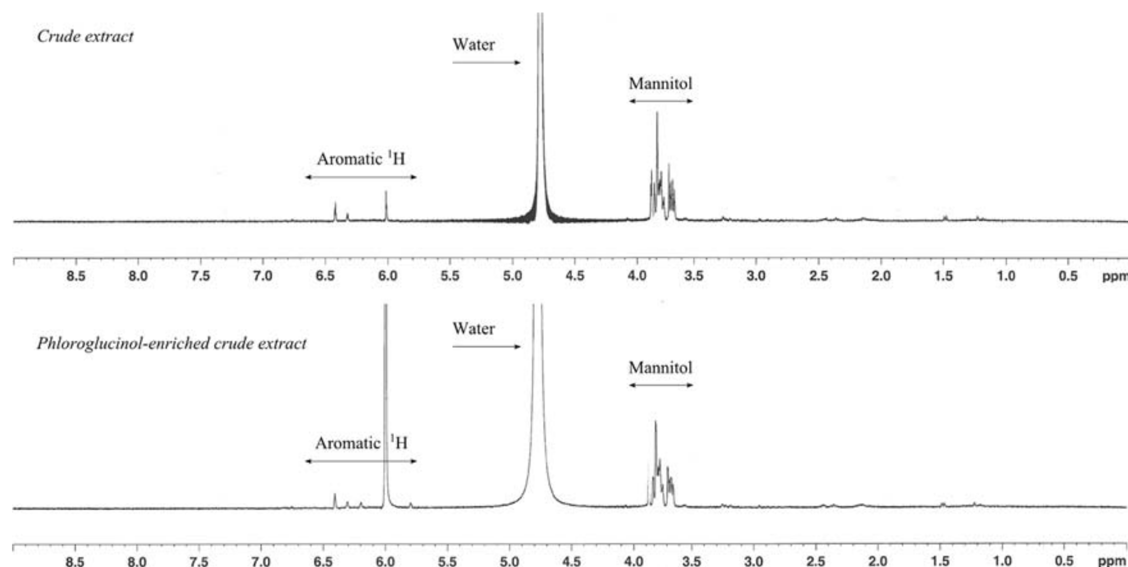

Fig. 1. ^{1}H NMR spectra of crude extract of *Cystoseira tamariscifolia* (upper spectrum), and the same extract enriched with phloroglucinol (lower spectrum); water peak around 4.7 ppm, mannitol signals between 3.6 and 3.9 ppm.

this peak. Hence, phloroglucinol was identified in the extracts of *C. tamariscifolia*. The acquisition of 2D NMR spectra also supported the consistency with phloroglucinol [15].

3.2. Detection of phloroglucinol by ^{1}H HR-MAS NMR

The evolution of the relative intensity of the phloroglucinol singlet on HR-MAS NMR spectra of *C. tamariscifolia* is indicated in Table 1. According to these data, phloroglucinol was detected in the algal tissues throughout the sampling period, from February 2009 to July 2010. Among these 19 months of sampling, the singlet at 6.02 ppm could not be retrieved only during one month (November 2009). We could also detect large amounts of phloroglucinol from February to July, for both years, and a decrease of these levels during autumn.

3.3. NMR analyses during the purification prior to Folin–Ciocalteu assay

Fig. 2 provides two ^{1}H NMR spectra of the aqueous phase and the organic (ethyl acetate) phase which were used for quantification *via* the FC assay.

These results indicate that phloroglucinol is the major component of the ethyl acetate phase, owing to the presence of its characteristic singlet. Alternatively, it is absent from the aqueous phase as no signal could be distinguished from baseline. Based on NMR data, the liquid–liquid extraction using ethyl acetate is an effective step to purify phloroglucinol from *C. tamariscifolia* extracts.

3.4. Accuracy of the ^{1}H qNMR method for the quantification of phloroglucinol

After analyses of standard solutions, we obtained at least 94.2% accuracy result between the real quantity of phloroglucinol present in the NMR tube and the one deduced using the integration of both phloroglucinol and TSP signals (qNMR). This result is in accordance with the good linearity observed in experiments carried out by Parys and coworkers [25] for a phloroglucinol concentration range from 0.5 to 4 mg/mL.

3.5. Quantification of phloroglucinol by ^{1}H qNMR and Folin–Ciocalteu assay

Fig. 3 gives an example of a typical spectrum used for the quantification of phloroglucinol levels in *C. tamariscifolia*. It illustrates quite well all the samples since most of the spectra had intense signals of water (solvent), mannitol (main carbohydrate of brown algae) and phloroglucinol (major phenolic compound, frequently the only one in *C. tamariscifolia*) as displayed in Fig. 2. After enrichment in TSP, an intense characteristic singlet is present at 0.00 ppm. The monitoring of phloroglucinol content in *C. tamariscifolia* tissues is presented in Fig. 4. It is in accordance with the results of the ^{1}H HR-MAS NMR results of solid sample. Each year, maximal phloroglucinol content was observed around May. In 2010, the maximum was 0.46% DW according to the qNMR method, or 0.63% according to the FC in year 2010. A dramatic decrease occurred in summer, before the dormancy-like period. During late winter and spring for both years, the phloroglucinol content increased up to May, and the cycle started again. For all samples, phloroglucinol levels were less than 0.53% DW (qNMR data) or 0.83% DW (FC data).

It should be noted that the FC always indicated superior results, when compared to qNMR. This phenomenon is particularly true when, according to qNMR results, the phloroglucinol levels drastically decrease before autumn. For example, the FC assay indicated a 30 times greater phloroglucinol level than qNMR in September 2009 (0.070% *versus* 0.0023, respectively). This over-estimation is

Table 1
In vivo phloroglucinol content determined with ^{1}H HR-MAS spectra. (−): absence; (+): signal barely distinguished from the baseline; (++): signal clearly noticeable; (+++): highest intensity of phloroglucinol.

Month		02/09	03/09	04/09	05/09	06/09	07/09	08/09	09/09	10/09	11/09	12/09
Intensity		+++	+++	+++	+++	+++	+++	++	+	+	−	+++
Month	**01/10**	**02/10**	**03/10**	**04/10**	**05/10**	**06/10**	**07/10**					
Intensity	+++	+++	+++	+++	+++	+++	+++					

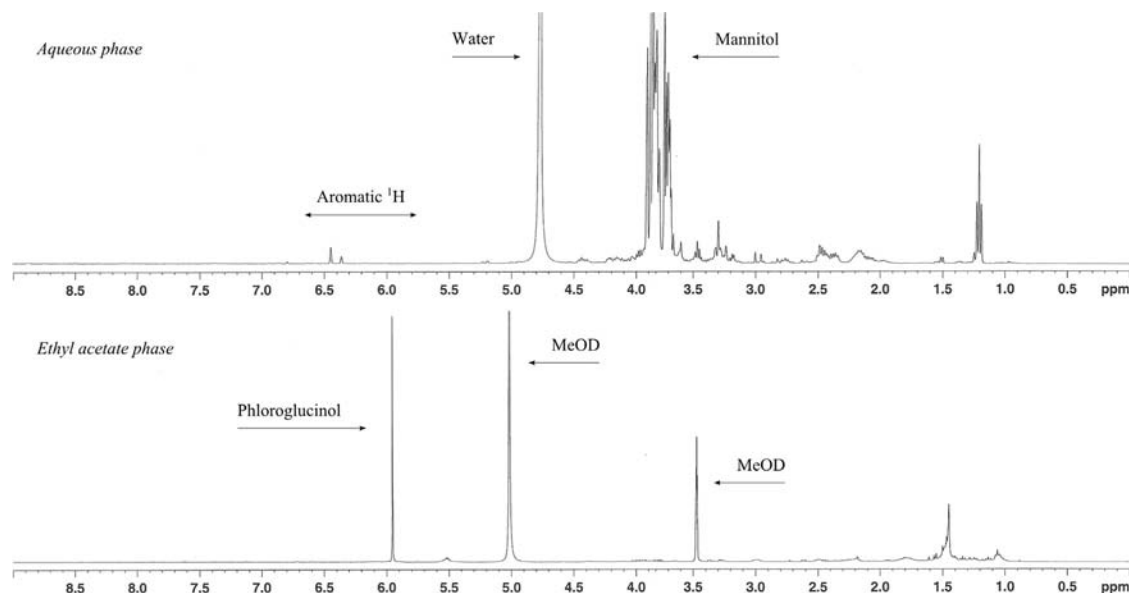

Fig. 2. ^{1}H NMR spectra of the aqueous phase of the crude extract of *Cystoseira tamariscifolia* (upper spectrum, in D_2O) and the organic phase (lower spectrum, in MeOD).

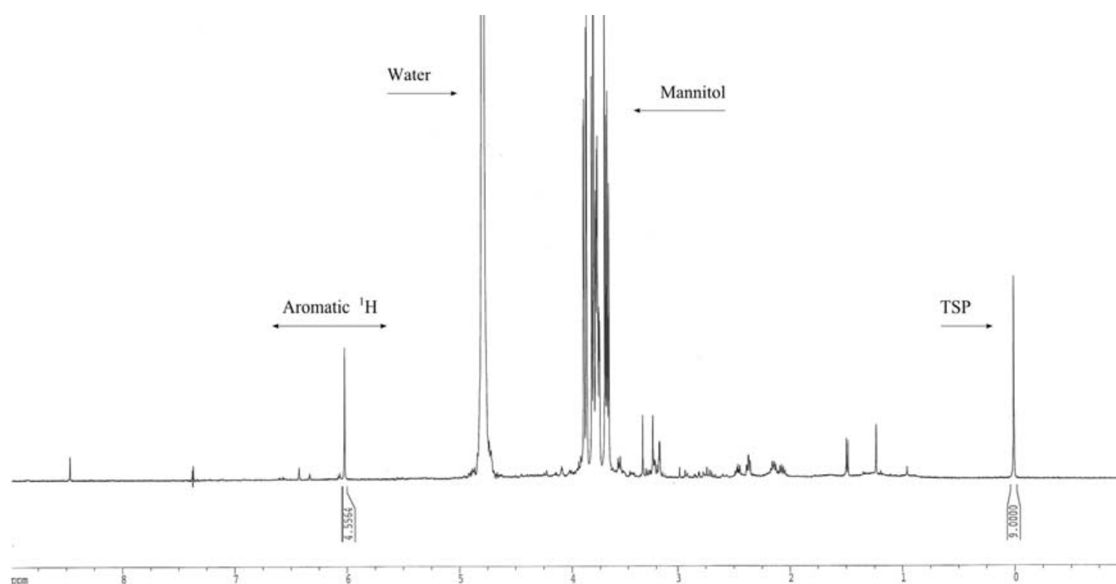

Fig. 3. ^{1}H NMR spectra of crude extract of *Cystoseira tamariscifolia* (apical parts of the thallus) with TSP as an internal standard (signal at 0.00 ppm); phloroglucinol signal is at 6.02 ppm.

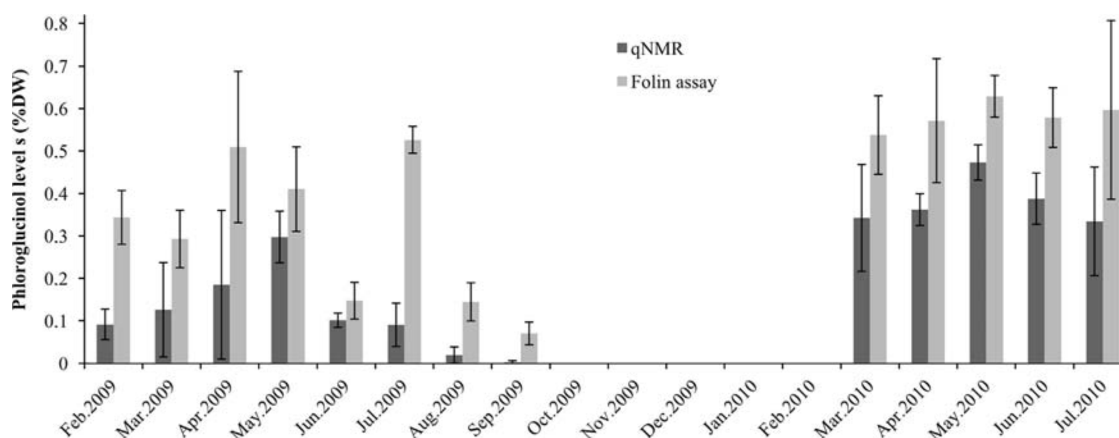

Fig. 4. Monthly evolution of phloroglucinol contents between February 2009 and July 2010 (mean ± standard deviation, expressed in % relative to dry weight), as determined by NMR quantification using TSP as internal standard (dark gray) or *via* Folin–Ciocalteu assay (light gray). The autumn/winter periods for which no data are presented are due to a dormancy-like period of *Cystoseira tamariscifolia* in Brittany.

even greater when the FC assay is used directly on crude extracts, with resulting levels up to 2.3% DW (data not shown).

As mentioned before, the qNMR method used here is a direct quantification of the phloroglucinol, as it relies on the unique resonance signal of this molecule on ^{1}H NMR spectra (in D_2O). On the other side, the FC reagent can oxidize many reductive molecules beyond phlorotannins, so for our model the exactness of this method can be doubtable. During their experiments, Parys and co-workers [25] observed that FC and qNMR methods had comparable accuracies. However, they analyzed extracts that had been purified before (using liquid–liquid partitioning to remove lipophilic substances). Furthermore, they used PVPP to precipitate phlorotannins, and the absorbance of such phlorotannin-free samples was used as a blank to take into account the interferences of non-phenolic molecules. Considering this, our results suggest that for our model *C. tamariscifolia*, the FC assay shall not be performed without prior purification of the crude extract and precipitation of phenols as a blank. This necessity probably depends on the species investigated. In fact, Connan [45] observed that the FC method could be applied directly on crude extracts from several Fucaceae species, after verification using HPLC. Nevertheless, our results point out that qNMR is the most reliable method for the determination of phloroglucinol levels in *C. tamariscifolia*.

3.6. Interests of the qNMR method for chemical ecology

Phlorotannins are known to be accumulated in brown algae according to seasonal patterns. However, most of the published studies use simple FC assays on crude extracts, sometimes providing contradictory results. The main drawback of this approach is that phlorotannins are quantified as a whole, which can limit our understanding of the chemical defense of the algae.

Many hypotheses have been proposed to explain the temporal variation of phlorotannin levels in brown algae, especially in Sargassaceae species [15]. In our study, we give the results of the quantification of a single molecule, *i.e.* phloroglucinol, the most abundant phenolic compound, produced by *C. tamariscifolia*. Stiger and co-workers [9] highlighted that the maximal phenolic content in two sargassacean species from tropical areas, *Sargassum mangarevense* and *Turbinaria ornata*, occurred in austral summer, during their reproductive period. Other studies showed maximal content before or around the fertile period in several populations of *Sargassum muticum* and *Cystoseira baccata* from Brittany [11,46]. The same tendency was observed for eight species of Fucales, assuming a photoprotective role of phlorotannins [8]. Steinberg [47] reported a negative correlation between phlorotannins production and growth rate; nevertheless, the

author noticed the co-occurrence of high phlorotannins levels and high growth rates in spring. In Brittany, *C. tamariscifolia* grows intensively from May to August, and all individuals get mature in May. In August, the apical parts of the thalli fall off and the macroalga begins a dormancy-like period until next February [27]. We cannot assume a photoprotective role of phloroglucinol, as its concentration dramatically decreased at the beginning of summer in 2009 and 2010 (Fig. 4). Nevertheless, one should hypothesize that this monomer could be used by the algae to produce more complex compounds (polymeric phenolic compounds) as demonstrated by Meslet-Cladière and co-workers [48] in the brown macroalgal model *Ectocarpus siliculosus*. Our observations tend to indicate that the energetic cost of growth could prevent phloroglucinol production in summer. Besides, phloroglucinol can be regarded as a phenological status indicator of *C. tamariscifolia*, with the highest levels occurring when receptacles begin to develop, possibly in order to protect them.

The main advantage of qNMR, compared to the FC assay, lies in its specificity towards the targeted molecules. To our knowledge, this is the first time that the precise quantification of one phlorotannin is achieved using this method. Furthermore, qNMR requires only few preparation of the sample and leads to accurate and specific results. By reducing the number of manipulations after the extraction step, the qNMR method limits losses of the targeted molecule and therefore, is particularly relevant for accurate quantification. Compared to chromatography, qNMR does not require the experimenter to possess the quantified molecule in pure form. For this reason, this method seems relevant for the analysis of “exotic” molecules, for which no analytical standard is commercially available. Actually, the only limitation of our qNMR method lies in that it requires signal(s) (singlet, doublet, *etc.*) to be unambiguously attributed to one and only one compound. Hence, overlapping signals between several compounds can lead to interferences and over-estimations. In such cases, isolated signals could be obtained after a minor purification procedure.

As indicated before, qNMR can be used to determine unambiguously the variation of phlorotannins in algal extracts and hence, it could become a standard method for chemical ecology purposes. Furthermore, qNMR can be really helpful to discover the role of phlorotannins when a high accuracy is required, like for the investigation of their biosynthetic pathways, which is at its early stage [48].

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