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Review article

Comprehensive evaluation of Folin-Ciocalteu assay for total phenolic quantification in algae (Chlorophyta, Phaeophyceae, and Rhodophyta)

Priscila Torres *, Sayuri Osaki, Elielson Silveira, Deborah Y.A.C. dos Santos, Fungyi Chow *

Department of Botany, Institute of Biosciences, University of São Paulo, 277 Rua do Matão, São Paulo 05508-090, São Paulo, Brazil

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ABSTRACT

The Folin-Ciocalteu assay is a tool widely employed to measure total phenolic content (TPC) in various applications across different fields, including food industry, agriculture, medicine, and environmental sciences. However, despite its popularity, there are limitations and an ongoing debate concerning its accuracy in TPC measurement. This review addresses these concerns within the context of phycology. A comprehensive database was compiled to quantitatively and qualitatively analyze the use of the Folin-Ciocalteu assay in this field. The quantitative approach led to the creation of an activity scale, indicating that brown algae, with their phlorotannins, and green microalgae stand out within the algal groups. Moreover, the qualitative approach indicates that the assay has proven to be a sensitive tool for studying algal ecology and physiology, and it has been widely employed to evaluate the biotechnological potential. However, a critical analysis reveals concerns related to interfering non-phenolic compounds and insufficient information on data reporting, including imprecise language and undefined terms, which hinders comparison with literature. Despite the growing interest in the Folin-Ciocalteu assay, most articles (98.7 %) do not take into account the effect of interfering compounds on TPC determination. Thus, the Folin-Ciocalteu assay is used as a measure of reducing power or antioxidant capacity in phycology rather than a measure of TPC. Nevertheless, with proper consideration of limitations, the Folin-Ciocalteu assay remains a valuable tool due to its simplicity, cost-effectiveness, and sensitivity.

1. Introduction

Algae are a diverse group of photosynthetic organisms that lack vascular tissue and are classified into major clades based on their molecular, biochemical, morphological, and reproductive features. This diversity includes green algae (Chlorophyta), red algae (Rhodophyta), and brown algae (Phaeophyceae, Ochrophyta) [1]. Algal morphology varies greatly, encompassing unicellular and colony forms as well as complex multicellular forms with diverse shapes, such as motile and non-motile coccoids, branched and unbranched filaments, articulated calcareous, coarsely branched, sheet, leathery and thick leathery, pseudoparenchymatous and parenchymatous and others [2–4].

Green, red, and brown algae are commonly found in aquatic habitats, such as estuaries, freshwater environments, wetlands, and marine domains, as well as in moist terrestrial environments like soils, rocks, and tree trunks [5]. Each major group contains numerous species that exhibit distinct biochemical, metabolic, physiological, and ecological features [3]. In aquatic ecosystems, especially in marine environments, algae play a significant role as primary producers, supporting and nourishing diverse species ranging from zooplankton to marine mammals such as whales [6]. Furthermore, representatives of these three clades of algae are consumed as a dietary staple in various cultures and serve as a potential source of chemicals and biofuels in diverse industrial applications [7,8].

Phenolic compounds are a diverse group of secondary metabolites found in various organisms. The composition and concentration of phenolic compounds in algae can vary significantly between different species and even within the same species under different environmental conditions. For example, brown algae are particularly rich in phlorotannins [9]. On the other hand, the occurrence of phenolic compounds in red and green algae is not as prevalent as it is in brown algae.

To measure total phenolic content (TPC) in algae, phycologists often use the Folin-Ciocalteu assay, which is also widely used in other biological samples such as land plants. In this review, the relationship between the Folin-Ciocalteu assay and phycology was examined through a comprehensive analysis of the available literature. A background on the history of the assay was provided to aid understanding of its application. We then evaluated the limitations of the assay as a tool for studying TPC

* Corresponding authors.
E-mail addresses: priscila.torres@alumni.usp.br (P. Torres), fchow@ib.usp.br (F. Chow).

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and critically discussed its potential for future research in the field of phycology.

2. The history of the Folin-Ciocalteu assay

The history of the Folin-Ciocalteu assay began when Folin and Macallum [10] reported that adding phenolic compounds to a phosphotungstic acid solution produced a blue color upon subsequent addition of an alkali. Based on this reaction, Folin and Denis [11] developed a phenolic reagent for colorimetric quantification. This reagent, known as the Folin-Denis reagent, consists of a mixture of phosphomolybdic acid, phosphoric acid, sodium tungstate, and water. Due to its high reactivity to several phenolic compounds (e.g., tannic acid, salicylic acid, catechin, vanillin, and eugenol), the Folin-Denis (FD) reagent was quickly proposed to quantify vanillin in flavoring extracts [12], tyrosine in protein [13], and total phenolics in urine [14].

In the coming years, Otto Folin and his co-workers made continuous improvements to the reagent. The main changes included the addition of hydrochloric acid and sodium molybdate, with this latter compound replacing phosphomolybdic acid [15–17]. These modifications increased the sensitivity and stability of the phenolic reagent, apparently by the increase in the proportion of molybdate [17,18]. However, the occasional production of a white precipitate remained a problem for the method. Finally, Folin and Ciocalteu [19] reported that the addition of lithium sulfate to the phenolic reagent prevents this precipitation. Thus, the phenolic reagent that includes the proposed improvements has widely become known as the Folin-Ciocalteu reagent.

A comparison of the Folin-Ciocalteu (FC) reagent with that of Folin and Denis revealed that the former is more sensitive and reproducible. Both phenolic reagents are believed to have the same chemical bases, which consist of a mixture of heteropoly-compounds of molybdenum and tungsten [18]. These heteropoly-compounds act as the active oxidizing agents in the reagent, with the molybdates more easily reduced to heteropoly-blue species than the tungstates [18].

The chemistry behind FC and FD assays involves electron-transfer reactions from reducing agents to the heteropoly-compounds, resulting in a blue color that varies with reductant concentrations [20]. Phenolic compounds react with the phenolic reagents only by the subsequent addition of an alkali due to basic conditions (pH ~10) that favor the dissociation of phenols into phenolate ions. These phenolate ions have higher reducing power compared to phenols. However, since the early studies by Otto Folin and his co-workers, the specificity of the phenolic reagents has been questioned because many non-phenolic compounds, including ascorbic acid and some amino acids, are reactive in the assays. Given the increasing popularity of the assays for the determination of TPC in different samples, the effort to limit the interference of non-phenolic compounds is a crucial strategy for the success of these analyses. Notwithstanding, in cases where diminishing the effects of interfering compounds is not possible or unpractical, these assays should be used as an estimate of the reducing power [18,20].

Initially, most studies using phenolic reagents in plant samples aimed to quantify TPC, notably to estimate tannins in fruits and beverages. However, there was no standardized protocol, and unfortunately, the FD reagent was the most used. Only at 1965, Singleton and Rossi [21] proposed a standardized protocol to quantify TPC in plant samples using the FC reagent rather than the FD reagent. Several reaction parameters were optimized, such as the volume ratio of alkali and reagent, reaction time, temperature, and optimum wavelength for absorbance measurements. Gallic acid was suggested as the reference standard once the target samples were wine tannins.

The standardized protocol proposed by Singleton and Rossi [21] increased reproducibility and allowed researchers to compare their results with greater accuracy. As a result, the FC assay became widely used to quantify TPC. In subsequent years, the instrumentation developments for chemical analysis allowed the use of small volumes of solvents and reagents in the analytical assays. Consequently, the Singleton and Rossi protocol has been adapted for use with a microplate reader (e.g., [22]). However, most adaptations in the protocol are primarily made to make the assay feasible within the context of the article and not necessarily to optimize its performance. Overall, the FC assay remains popular among researchers due to its simplicity and sensitivity. The commercial availability of the FC reagent has further enhanced its convenience and ease of use, saving researcher's time and contributing to the growing interest in the assay.

3. Methodology for evaluating the use of the Folin-Ciocalteu assay in phycology

A bibliographic survey was conducted from 1900 to 2022 on the Google Scholar platform to identify relevant literature on the utilization of phenolic reagent assays in phycological research. The search terms 'Folin' and 'algal genus' were used in combination to ensure comprehensive coverage of the topic. Articles were included if they met the following criteria: written in English, indexed in Scopus, or published in reputable journals by Elsevier, Springer, or MDPI. The focus was on the clades of brown, green, and red algae. The genus accepted taxonomically were obtained from Guiry and Guiry [23] (consulted in 2022) for each algal clade. The initial screening process involved evaluating titles and abstracts to identify potentially relevant articles, followed by a reading of their methodology sections. Fig. 1 summarizes the process of obtaining data.

Articles were excluded if they did not pertain to the utilization of phenolic reagent assays in phycological research or if they were not accessible through the selected databases and publishers. The search provided a comprehensive list of 1147 publications from 1976 to 2022 that used these assays in phycological research, forming the total database of articles. Data extraction was performed by one reviewer for each clade and subsequently reviewed by two others. Extracted data included country, order, genus, methodology details, and results. Furthermore, abstracts, titles, and keywords from the selected publications underwent additional analysis using VOSviewer software (version 1.6.9). This software facilitated the generation of a network map illustrating the relationships between words commonly occurring in the literature. A minimum threshold of 10 occurrences per word was established to ensure the inclusion of only relevant terms in the network map.

Within this total database, articles that adequately reported their findings were included in the filtered database, which formed the basis for quantitative analysis. Exclusion criteria for articles in the filtered database included a lack of differentiation between algal biomass and extract mass, failure to specify the standard used in assays, and expression of results in mass per volume units. Quantitative analysis of the extracted data was conducted utilizing appropriate statistical methods to facilitate meaningful comparisons and identify significant trends. The Mann–Whitney test and Kruskal-Wallis test with multiple comparisons using Dunn's tests ($p < 0.05$) were employed to compare data across different groups. Additionally, statistical outliers were identified employing the ROUT method (Q = 0.1 %) to mitigate the influence of potential anomalies on the overall analysis. The compiled database allowed for a qualitative approach (item 4), followed by a quantitative approach (item 5), evaluation of extraction methods (item 6), and a critical analysis of the assay (item 7).

4. Applications of the Folin-Ciocalteu assay in phycology: a historical perspective

The data presented in Fig. 2 provides insights into the utilization patterns of the phenolic reagents assay over time in phycology. Only 6 % of the research used the FD reagent. However, these values refer to almost all studies from the 1970s to the late 2000s (Fig. 2A). From 2010 onwards, the FC reagent only not surpassed the FD reagent, but also there has been an exponential increase in the number of articles (Fig. 2A). The number of publications per year confirms that the interest

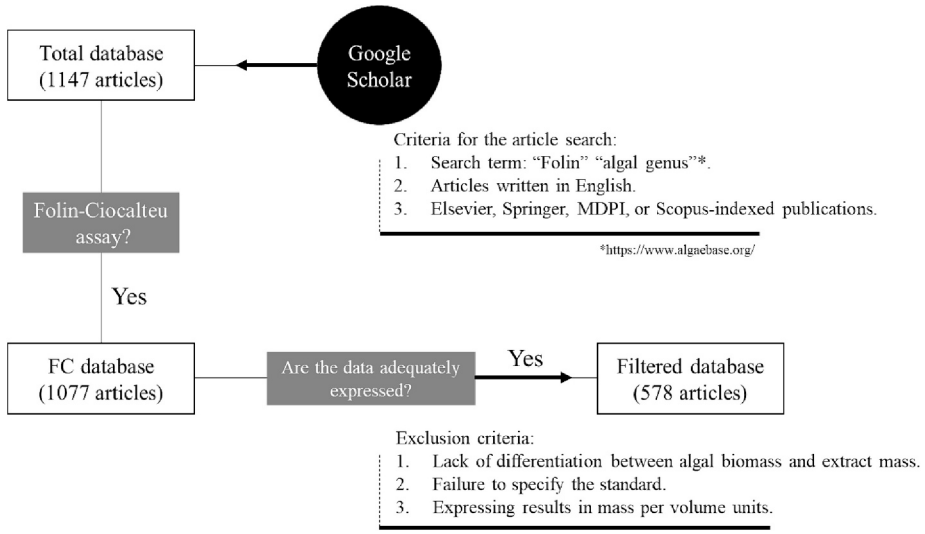

Fig. 1. Diagrammatic representation of the process for obtaining the databases. The search was executed using the Google Scholar, utilizing the search terms 'Folin' and 'algal genus'. The survey was restricted to articles written in English, indexed in Scopus, or published in Elsevier, Springer, or MDPI. Bibliographic survey yielded 1147 publications from 1976 to 2022. The 'total database' and the 'filtered database' represent two distinct sets of articles, with the former encompassing all articles gathered, while the latter consists solely of articles that adequately reported their findings, enabling a quantitative comparison. Both databases are in the supplementary material. FC: Folin-Ciocalteu.

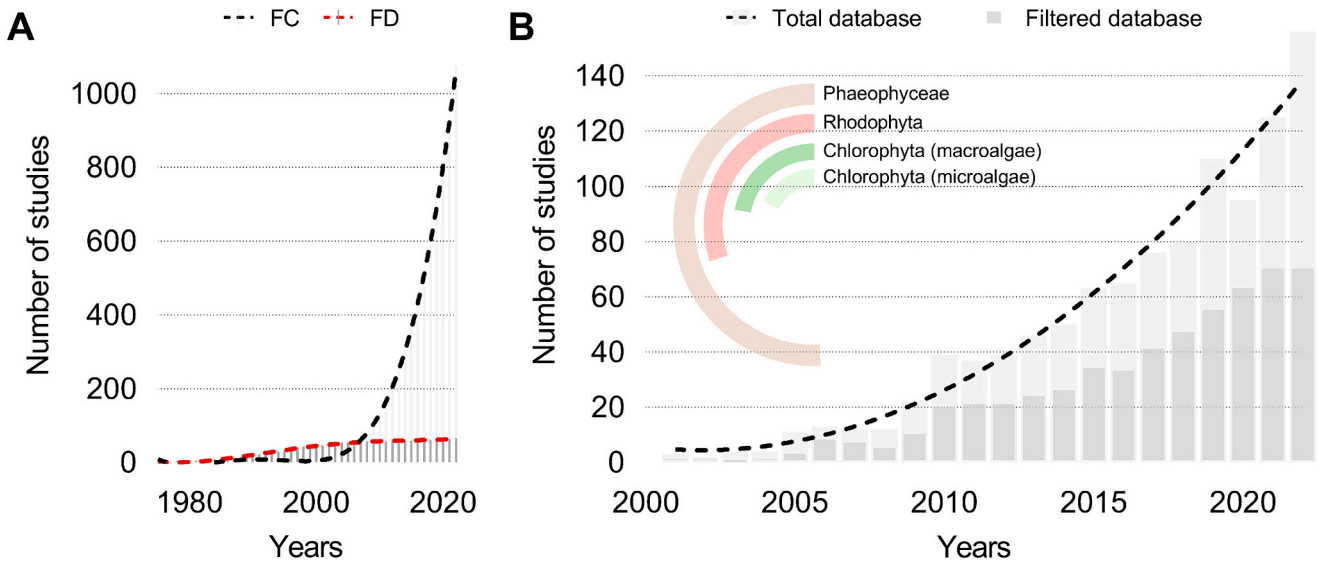

Fig. 2. Overview of the number of publications that have analyzed algal samples using phenolic reagents. (A) Cumulative number of publications utilizing the Folin-Denis (FD) and Folin-Ciocalteu (FC) reagents. (B) Total number of publications per year employing the FC reagent and the distribution of publications for algal groups (represented by a radial bar chart).

has grown in the FC assay in the field of phycology, with about 90 % of publications occurring in the last 12 years (Fig. 2B).

Algae belonging to the Phaeophyceae are the most studied species, followed by those in the Rhodophyta and Chlorophyta (Fig. 2B, insert chart). To accommodate the significant diversity and distinct biological characteristics of microalgae and macroalgae within Chlorophyta, we chose to analyze these two morphological groups of green algae separately. Up until 2010, the study of phenolic-reagent assays focused on the analysis of total phenolic compounds in Phaeophyceae. Other algal groups were not studied in relation to this assay. Phenolic compounds from Phaeophyceae are known since the nineteenth century, with Berthold [24] reporting the presence of tannin-like compounds in the physodes, small cell vesicles unique to brown algae [25]. Later, Crato [26] concluded that the physodes contained phloroglucinol and its derivatives. However, it was only in the 1970s that the structure of these compounds was elucidated as polymers based on phloroglucinol through studies conducted by Ragan and Glombitza (for more details [27]). These compounds are now commonly known as phlorotannins [27].

Phlorotannins are a type of tannin unique to brown algae and are the most known phenolic compounds found in algae [28]. Today, growing knowledge about their biological activities (e.g., anti-aging, antibacterial, anticancer, antidiabetic, and antioxidant) and ecological roles (e.g., defenses against herbivores, desiccation tolerance, and photoprotection against solar UV radiation) can be found in the literature [29,30]. However, even today, studying phlorotannins from ecological, physiological, and phytochemical perspectives remains challenging due to their structural complexity. Thus, the search by colorimetric methods to quantify phlorotannins in brown algae was encouraged.

The studies by Mark A. Ragan and his co-workers were pioneers in

the use of the FD assay to quantify phenolic compounds in brown algae [31,32]. Compared to other methods for quantifying phenolic compounds, the phenolic reagent assay was relatively simple and fast, making the research more efficient [27]. These studies provided the basis for subsequent studies that contributed to a wealth of information about the ecological significance of phenolic compounds in brown algae, including research on spatial and seasonal variation [33–36], defenses against herbivores [37–40], and photoprotection against solar UV radiation [41,42].

The FD assay has been a crucial tool for studying the ecology of Phaeophyceae, and until recently, it was the main application of phenolic-reagent assays in phycology. However, given the changes in research trends, we used the VOSviewer software (version 1.6.9) to analyze abstracts and titles of publications that utilize the FC assay (excluding the FD assay). The results revealed seven prominent themes in which the FC assay is more commonly used, and these themes are represented by different colors in the network map (Fig. 3).

The first four themes focus on the biological applications of algal biomass and extracts, encompassing screening of antioxidant activities, evaluating the bioactivities, mainly antimicrobial and cytotoxic, assessing the nutritional potential of seaweeds as a source of vitamins, minerals, and other nutrients, and development of efficient methods for the use and preservation of biomass and extracts. The next two themes delve into the study of how algal species interact physiologically and ecologically with each other and their environment. The last theme focused on research into green microalgae, which are cultivated for biotechnological purposes, such as biorefinery and biofuel production.

All clusters have a common link to the keyword 'TPC', indicating that the primary use of the FC assay is to estimate TPC (Fig. 3). Furthermore, the keyword 'antioxidant' is closely associated with 'TPC' and is also linked to all the clusters (Fig. 3). These two keywords are frequently mentioned in the articles likely because many studies evaluated the antioxidant properties and correlated them with TPC. In fact, based on the reviewed articles from total database, approximately 50 % of them

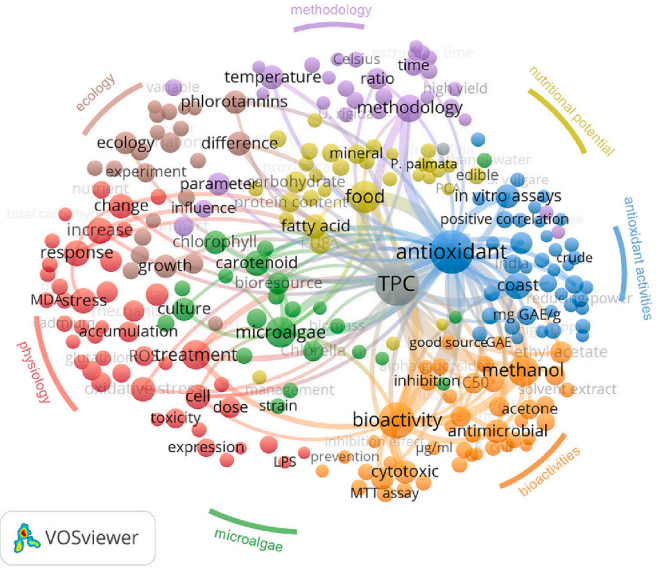

Fig. 3. Network map created with the use of VOSviewer software (version 1.6.9). The map illustrates the relationship between words (with a minimum of 10 occurrences) in the abstracts and titles publications that reported the analysis of algal samples using the Folin-Ciocalteu reagent. The seven clusters in the map represent the following research topics: screening of antioxidant activities, bioactivities, nutritional potential, methods for efficient extract and biomass use, physiological interactions, ecological interactions, and research on green microalgae. All clusters are linked to the keyword 'TPC' (Total Phenolic Content) represented in gray, as well as the keyword 'antioxidant'. The size of the bubbles represents the frequency of the words.

evaluated antioxidant activities, and among those, a correlation was possible to be determine in about 70 % of the cases. These correlations are often performed because phenolic compounds play a crucial role in providing antioxidant activities to photosynthetic organisms, particularly land plants [43]. The main assays used for establishing these correlations were DPPH (2,2-diphenyl-1-picrylhydrazyl) in 80 % of the publications, ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) in 60 % of the publications, and FRAP (ferric ion reducing antioxidant power) in 40 % of the publications.

According to Chan [44], correlation coefficients between 0.6 and 0.8 are considered moderately strong, and values above 0.8 are considered very strong. Regarding the strength of the correlation, 80 % of the articles that reported a correlation found a moderately strong to very strong correlation with an absolute value of the correlation coefficient ($|r| > 0.6$. On the other hand, 40 % of the articles reported a weaker correlation ($|r| < 0.6$) (Fig. 4A). These correlations were observed between extracts from different variables or solvents of the same species, as well as within of a set of algae. For example, Harb et al. [45], who studied Brazilian macroalgae, and Meng et al. [46], who studied Chinese brown macroalgae, found a strong correlation between FC assay and antioxidant activity. Another example includes Connan et al. [47], who observed that the correlation between the FC and DPPH assays varied depending on the species. They observed a strong correlation for the extracts from *Ascophyllum nodosum* ($|r| = 0.8$) and a weaker correlation for the extracts from *Laminaria hyperborean* ($|r| = 0.2$), both brown algae.

In addition to its correlation with antioxidant activities, about 20 % of the articles used the FC assay to evaluate physiological and ecological responses. This widespread usage can be attributed to the pivotal roles played by phenolic compounds in ecology and physiology, including defense mechanisms against herbivores and antioxidant properties that help mitigate stress. The findings from this review suggest that the assay is highly sensitive, with 75 % of the articles detecting variations in the algae responses to different factors, such as biogeographical patterns, herbivory, and distinct stresses.

Regarding biogeography, the FC assay has been used to study seasonal and spatial variations in algal populations, with 90 % of the articles reporting significant changes among variables (Fig. 4B). One example is the study by Roleda et al. [48], which reported that brown algae (*Saccharina latissima* and *Alaria esculenta*), and the red alga *Palmaria palmata* along the Atlantic coast of Europe responded to seasonality, with the highest values of TPC being observed in winter for brown algae and in spring for red alga. However, there was no significant spatial variation. In contrast, Tanniou et al. [49] found that the brown alga *Sargassum muticum* responded spatially along the Atlantic coast of Europe. Furthermore, van Hees et al. [50] found that brown algae had higher TPC values at higher latitudes in coastal Western Australia.

The results of the FC assay concerning herbivory have been inconsistent, reflecting the complex interactions between marine herbivores and algae, as well as the limitations of the FC assay in measuring TPC. Some studies found a positive relationship between TPC values and herbivore deterrence [51–53], while others have found no significant relationship [37,54] or even a herbivory beneficial relationship [55]. On the other hand, the FC assay has shown to be highly sensitive in detecting responses to toxicity, particularly from heavy metal pollutants. In fact, 90 % of the articles showed changes in the assay values in response to toxicity (Fig. 4B). For example, the values showed significant increases or decreases based on the concentration and the exposure time of the algae to cadmium [56–59]. The effects of other stressors on the FC assay have been studied less frequently. However, it is possible to say that exposure to UV radiation has either increased or not affected the assay level [60,61]. Conversely, stress from nutrient imbalances such as nitrogen, phosphorus, and iron has primarily been shown to increase the values obtained from the FC assay. For example, nitrogen starvation in the green microalga *Acutodesmus dimorphus* [62] and nitrogen enrichment in the green macroalga *Ulva rigida* [63] have increased the FC

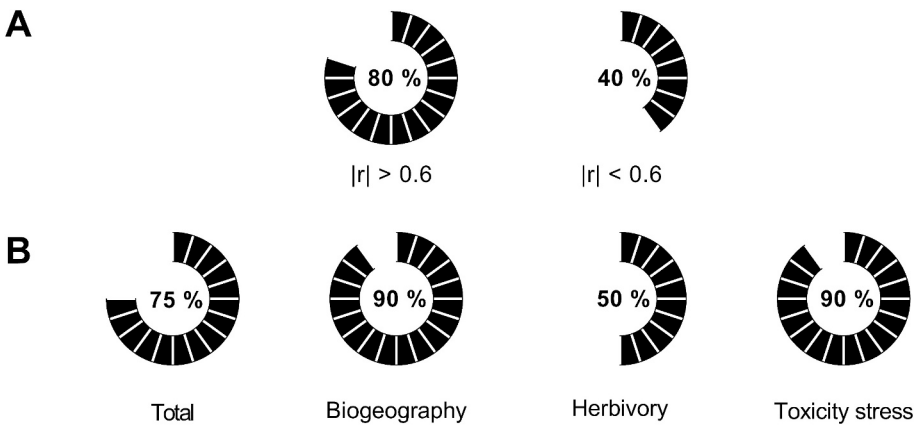

Fig. 4. Evaluating the responses of the phenolic-reagent assays. (A) Distribution of articles that reported strong ($|r| > 0.6$) or weak ($|r| < 0.6$) correlation coefficients (r) between the assays and antioxidant activities. (B) Percentages of physiological and ecological studies where the assay was sensitive to study variables, highlighting the factors: biogeography, herbivory, and toxicity stress.

assay levels. However, the response of *A. dimorphus* was influenced by the concentration of nitrogen, suggesting that different concentrations of the nutrient may trigger different response levels.

In addition to the above applications, the FC assay has been used to evaluate the correlation between TPC and various biological activities. However, the number of available studies reporting these values for each bioactivity is currently limited. Consequently, the existence of a definite trend in the correlation between bioactivities and the FC assay in algae remains unclear. Another application concerning biological activities is the utilization of the assay as a selection criterion of samples or methods based on higher yields of TPC. For example, Kang et al. [64] used the FC assay to choose the brown alga *Ecklonia cava* for a diabetes study, assuming that higher TPC values might indicate greater biological potential.

Therefore, the FC assay is widely employed across various fields in phycology, spanning from basic to applied research (as seen in Fig. 3). The use of the assay in these fields provides insights into the chemical composition, biological potential, and physiological and ecological significance of algae.

5. A quantitative approach to the use of the Folin-Ciocalteu assay in phycology

In addition to the qualitative approach previously discussed (item 4), the 'total database' was screened to obtain a 'filtered database' (Fig. 2B), which allowed a quantitative approach to some variables. A quantitative approach can reveal patterns and trends between various algal groups. A description of the composition of the database used for the quantitative analysis is provided in Fig. 5. The dominant orders in each group are as follows: Gigartinales, Ceramiales, and Gracilariales for red algae; Chlorellales, Sphaeropleales, and Chlamydomonadales for green microalgae; Ulvales, Bryopsidales, and Cladophorales for green macroalgae; and Fucales, Laminariales, and Dictyotales for brown algae (Fig. 5A). The composition by algal groups is similar to that in Fig. 2B.

The distribution of the algae in the database was analyzed based on marine ecoregions following Spalding et al. [65], revealing a disproportionate representation. The majority of the samples were obtained from the Temperate Northern Atlantic Ocean (Portugal, Spain, France, Ireland, and Norway), followed by the Western Indian Pacific Ocean (India) for green macroalgae, Central Indo-Pacific for red algae, and the Temperate Northern Pacific Ocean for brown algae (South Korea, Japan,

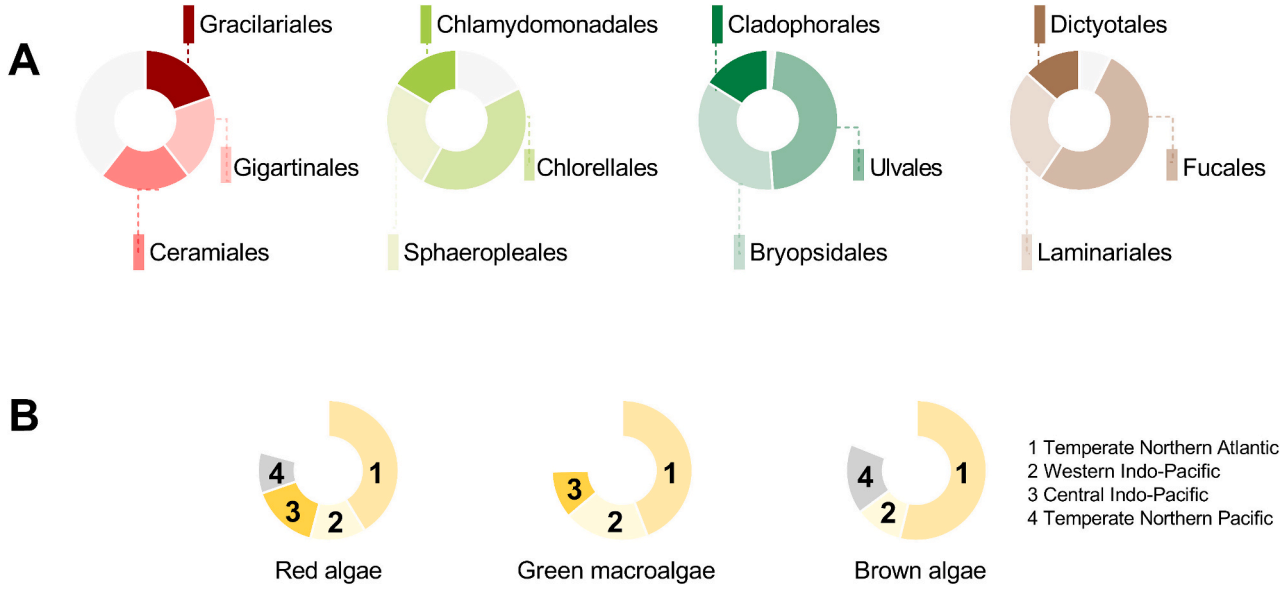

Fig. 5. Composition and distribution of the database used for quantitative analysis. (A) Dominant orders within each algal group: red algae, green microalgae, green macroalgae, and brown algae, respectively. (B) Geographical distribution of the samples in the database based on marine ecoregions by Spalding et al. [65].

and China) (Fig. 5B). The database lacks representation from the tropical and polar regions. Additionally, the green microalgae in the database were obtained from culture, so a discussion of their origin is not relevant in this article.

Gallic acid was the most used reference standard, with 53 % of the articles reporting its use for brown algae and 80 % reporting its use for green and red algae. However, due to its chemical similarity with phlorotannins, phloroglucinol was specifically used in brown algae, with 41 % of articles focusing on brown algae and <10 % on other algae. The results from the filtered database were predominantly expressed in dry weight (DW) units. To enable comparison, units such as μg mg^{-1} and % were converted to mg g^{-1}, which represents the majority of the data. Researchers expressed the results from the filtered database in two main ways: some calculated the results based on the weight of algal biomass (i.e., the algal tissue) used in the extraction process, while others use the weight of the extract (i.e., the total extractable components) obtained after evaporating the liquid extract. In general, the results are expressed as milligrams of gallic acid equivalent per gram (mg GAE g^{-1} DW) or milligrams of phloroglucinol equivalent per gram (mg PE g^{-1} DW) of algal biomass or extract mass.

At group level, the quantitative analysis is in Fig. 6. The mean results for algal biomass in GAE were 5.2 mg GAE g^{-1} DW for brown algae, 2.3 mg GAE g^{-1} DW for green macroalgae, 4.6 mg GAE g^{-1} DW for green microalgae, and 3.2 mg GAE g^{-1} DW for red algae. For algal biomass in PE, the mean results were 22 mg PE g^{-1} DW for brown algae, 11 mg PE g^{-1} DW for green macroalgae, and 8 mg PE g^{-1} DW for red algae. Regarding extract mass, the mean results in GAE were 26 mg GAE g^{-1} DW for brown algae, 12 mg GAE g^{-1} DW for green macroalgae, 13 mg GAE g^{-1} DW for green microalgae, and 12 mg GAE g^{-1} DW for red algae. The mean results for extract mass in PE were 99 mg PE g^{-1} DW for brown algae, 29.4 mg PE g^{-1} DW for green macroalgae, and 24.5 mg PE g^{-1} DW for red algae.

Brown algae had significantly higher values in the FC assays than other algal groups, regardless of whether the analysis was based on extract mass or algal biomass and whether gallic acid or phloroglucinol was used to express the data (Fig. 6A and B), except for biomass (GAE), which was similar to that of green microalgae (Fig. 6A). The other algal groups generally exhibited similar values to each other (Fig. 6A and B).

The findings are in line with the algal screening analysis. Although not all brown algae have the highest values in the FC assays, they tend to show superior results compared to other macroalgae when several species are evaluated simultaneously. For instance, Guinea et al. [66] reported that the highest FC activities were found in *Ascophyllum nodosum*, *Lessonia vadosa*, and *Fucus vesiculosus*, while other brown algae showed similar activities to red algae. Similarly, Zubia et al. [67] observed that most of the highest values in the FC assay were found in brown algae. Additionally, Tibbetts et al. [68] reported that all brown algae had higher FC values than other algae. Although green microalgae exhibit high values in the FC assay for biomass a more in-depth discussion is not possible due to the lack of comparison with other algal groups in the literature, especially considering that microalgae are cultivated. It is not known whether cultivation is responsible for these higher values.

The quantitative data differed when comparing the values expressed as GAE to their counterparts expressed as PE (Fig. 7). The results for phloroglucinol are significantly higher than those for gallic acid. The FC activity varies according to the structure of the compounds being analyzed. Phloroglucinol has a lower molar absorptivity in the FC assay than gallic acid [18]. In fact, Harb et al. [45] (supplementary material provided by authors) reported higher angular coefficients for gallic acid than for phloroglucinol ($y = 0.0763\times + 0.0534$ vs. $y = 0.0125\times + 0.029$ in mg mL^{-1}), obtaining results in GAE to 16 % of those expressed in PE. These results indicate that Gallic acid will exhibit higher absorbance than phloroglucinol, considering equal concentrations of both standards. Therefore, when using phloroglucinol as a reference standard for quantification in the FC assay, the values obtained should be higher than gallic acid as a reference standard, helping explain the results in Fig. 7. Hence, it is crucial to make comparisons using the same reference standard.

At the group level, there is a high variation in the results, likely due to environmental and interspecific-genetic characteristics. To investigate these differences, we were able to separate the data by order (Fig. 8). Comparisons among red algae and their main orders show that

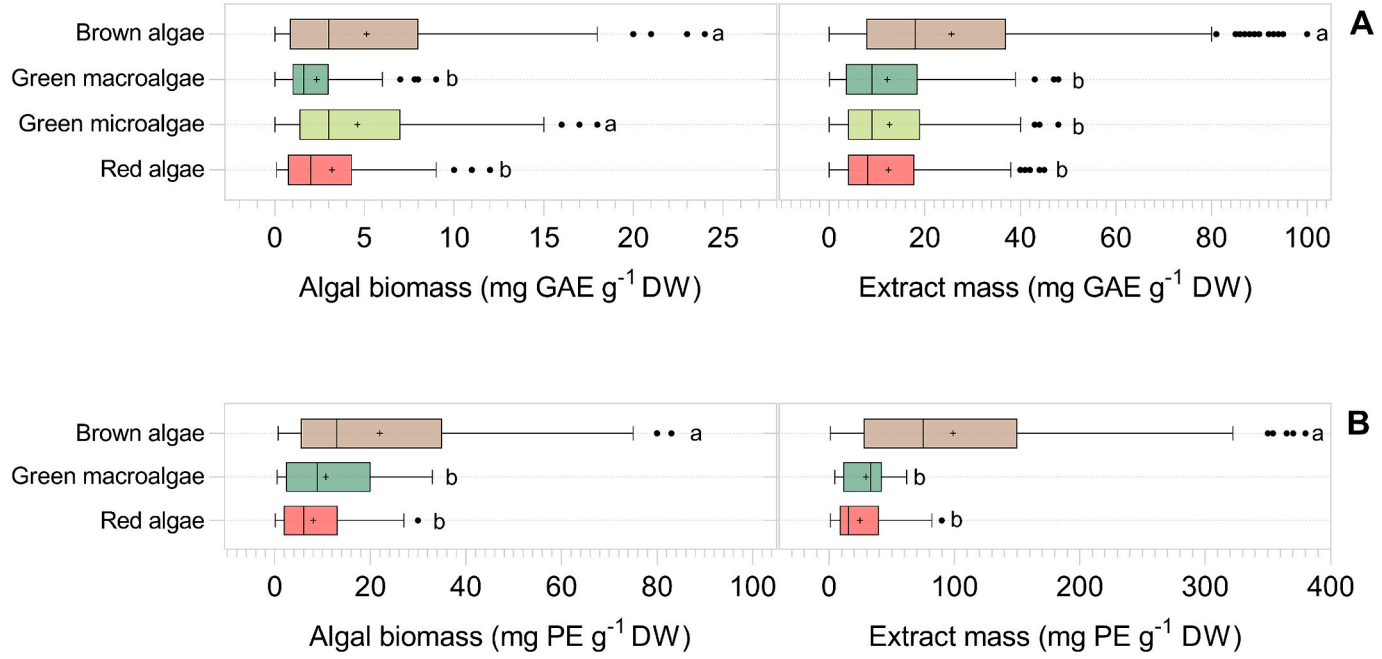

Fig. 6. Comparison of values in the Folin-Ciocalteu assays for algal biomass or extract mass using gallic acid (A) or phloroglucinol (B) as the standard. Data expressed as mg GAE g^{-1} DW or mg PE g^{-1} DW. The data are presented as boxplots using the Tukey method. Each dot represents a value from the filtered database. The mean of the data is represented by a plus symbol (+), while a horizontal bar within the box represents the median. Statistical comparisons were performed using Kruskal-Wallis tests with multiple comparisons using Dunn's tests ($p < 0.05$). Statistical outliers were identified using the ROUT method (Q = 0.1 %).

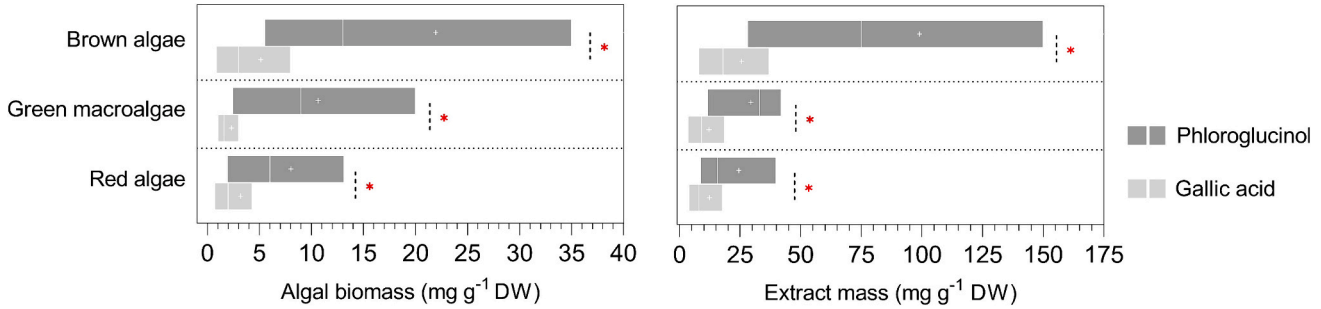

Fig. 7. Comparison between the values of FC assays expressed as gallic acid equivalents – GAE to their counterparts expressed as phloroglucinol equivalents – PE in different algal groups. The data are expressed in mg GAE g^{-1} DW and mg PE g^{-1} DW. The boxplots were generated using the Tukey method, where the horizontal bar within the box represents the median and the plus symbol (+) represents the mean. For better data visualization, only the interquartile ranges are shown. Mann–Whitney test was used to compare each standard within the same algal group, with statistical significance indicated by asterisks (*$p < 0.0001$). Statistical outliers were identified using the ROUT method (Q = 0.1 %).

Gracilariales had the lowest FC values, while Ceramiales exhibited the highest, regardless of whether the analysis was performed on the extract mass or algal biomass (Fig. 8A). Comparing green microalgae and their main orders, we find that Sphaeropleales exhibit the highest FC values for algal biomass, while Chlorellales exhibit the highest FC values for algal extract (Fig. 8B). No significant differences were found between the green macroalgae and their orders Ulvales and Bryopsidales (Fig. 8C).

The brown algae were compared with Fucales and Laminariales (Fig. 8D and E). The biomass data for both standards (gallic acid and phloroglucinol) showed that Fucales had significantly higher FC values than brown algae, while Laminariales had the lowest values compared to brown algae. Similar trends were observed for the extracts (Fig. 8D and E). However, only Fucales, as expressed by GAE, showed a significant difference, with FC values higher than brown algae (Fig. 8D). This finding is consistent with previous research conducted by Steinberg [69]. Although Magnusson et al. [70] did not discuss this trend in their review, they found similar results from data collected in temperate regions. However, Laminariales from subtropical regions showed results similar to Fucales, while tropical Fucales had low values. Therefore, the separation of data by order reveals that there is specificity in the results. However, further separation of the data by genus or region was hindered due to the low representation of some variable groups. More specific patterns could likely be uncovered with a larger representation of data.

The development of a scale of activity for the FC assay represents a significant achievement of this study (Table 1). We opted to develop the scale based on the boxplots in Fig. 6 at the group level. By utilizing algal groups, the scale of activity for the FC assay is a more practical approach due to the limitations of available data. Values within the interquartile range were considered moderate activity, the lower whiskers as low activity, and the higher whiskers as high activity. To improve the accuracy, we suggest comparing results using gallic acid for Chlorophyta and Rhodophyta, given the scarcity of results using phloroglucinol for these groups (<10 %). This scale has the potential to facilitate meaningful comparisons and interpretations across studies. However, further research is needed to validate and expand the utility of this scale, particularly by adding data from a wider diversity of regions and different ecological niches.

6. Evaluating extraction methods for the Folin-Ciocalteu assay in phycology

The extraction of algal material is a critical step that can affect the yield and composition of the final extract. Some protocols commonly referenced in algal physiology and ecology include the method developed by Koivikko et al. [29] and its modified version by Gómez and Huovinen [71] for analyzing soluble and insoluble phlorotannins in brown algae as well as the method developed by Félix Figueroa and his co-workers for analyzing TPC [72–78]. However, our database reveals that there are multiple procedures for algal extraction. By analyzing the frequency of each step in these protocols from the filtered database, we can gain valuable insights into the most used approaches for extracting algal biomass (Fig. 9). This information can be an initial point to help optimize and standardize algal extraction protocols in various phycology fields.

The extraction process usually involves drying, choosing solvents, identifying the optimal solvent-to-solid ratio, and selecting extraction methods. The main drying methods in the database included sun drying, shade drying, oven drying, and others. Freeze-drying was the most used method for drying brown algae (36 % of articles), red algae (34 %), and green microalgae (50 %). Shade drying was the second most common method used for brown and red algae (about 20 %), while for green macroalgae, it was the most used (33 %) (Fig. 9A). Drying at temperatures above 30 °C was reported in about 20 % of articles, and fresh mass was reported in between 10 % to 16 % of the articles (Fig. 9A). In these studies, which reported fresh mass, a portion of the algal material is taken and dried to determine the dry mass percentage before extraction.

In recent years, several methodological studies have evaluated the effect of drying methods on algal material using the FC assay as a research tool. However, the value of the FC assay from such studies can vary depending on the algal samples. For example, Silva et al. [79] discovered that only freeze-drying had a less impact on the brown alga (*Fucus vesiculosus*), while the values of the FC assay for red alga (*Gracilaria* sp.) and green alga (*Ulva rigida*) were not affected by drying methods. Similarly, Amorim et al. [80] observed that the values of the FC assay for brown alga (*Sargassum stenophyllum*) were more affected by drying methods, with freeze-drying being the most effective method. Ruiz-Medina et al. [81] showed that drying methods did not significantly affect FC assay results for most of the 24 studied algae species. However, two green algae species (*Dasycladus vermicularis* and *Cladophora liebetruthii*) were extraordinarily affected, showing a decrease in TPC when samples were dried in the air compared to freeze-drying.

The solid-to-solvent ratio used for extraction can affect the efficiency, selectivity of the extraction process, and compound concentration. Around 20 % of articles did not report this ratio (Fig. 9B). Among articles that reported do, the most frequently reported ratio ranged from 1:100 to 5:100 (algal weight: solvent volume) (Fig. 9B). Several extraction methods are available, including conventional methods like Soxhlet extraction and maceration extraction (with or without agitation or temperature above 30 °C), as well as non-conventional methods such as ultrasound-assisted extraction, microwave-assisted extraction, and pressurized liquid extraction. Maceration under agitation was the most used method, accounting for between 24 % to 36 % of articles, while simple maceration without agitation and elevated temperature (> 30 °C) was used in about 20 % of articles (Fig. 9C). Ultrasound-assisted extraction was used especially for green microalgae (Fig. 9C). Other

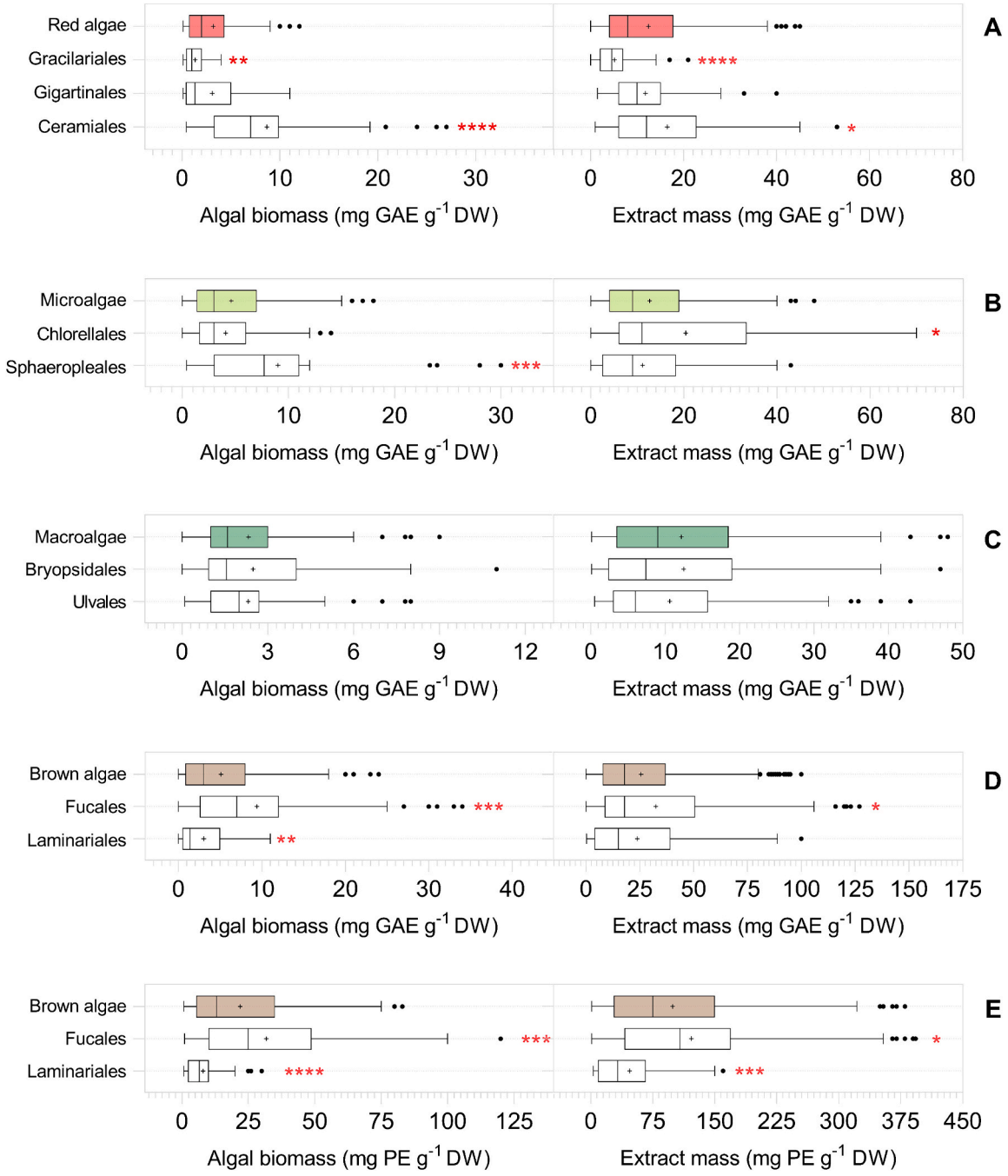

Fig. 8. Comparisons of values in the Folin-Ciocalteu assay among major algal orders and their corresponding groups: red algae (A), green microalgae (B), green macroalgae (C), and brown algae (D and E). Results are expressed as mg GAE g^{-1} DW (A to D) and mg PE g^{-1} DW (E) by algal biomass or extract mass. Each dot represents a value from the filtered database. The Tukey method was used to construct the boxplots, with the horizontal bar within the box representing the median and the plus symbol (+) representing the mean. Mann–Whitney test was used for comparisons of each order and its corresponding algal group, with statistical significance indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$). Statistical outliers were identified using the ROUT method (Q = 0.1 %).

extraction methods, such as microwave-assisted extraction and pressurized liquid extraction, were reported less frequently but more and more studies are exploring their potential for algal extraction, reporting potential advantages such as higher extraction efficiency and shorter extraction time [70,82–85].

The choice of solvent used for algal extraction varied between studies and included both aqueous solvent mixtures and pure solvents, with the latter being more commonly used. In our analysis, the most common solvents used for algal extraction were methanol, water, ethanol, and acetone, with methanol being the most frequently reported (Fig. 9D). Some studies also reported the use of non-polar solvents, such as ethyl acetate, dichloromethane, and hexane, as well as mixtures of organic solvents (e.g., [86–88]). However, these were reported less frequently compared to the more used solvents.

Unfortunately, quantitative approaches for extraction parameters were only possible for the solvents used in the studies (Fig. 10). The effectiveness of solvents varied depending on the algal group and the purpose of the extraction, whether it is aimed at maximizing the effectiveness of results for biomass or extract. Methanol was the most effective solvent for extracting biomass (Fig. 10A and B). In the case of

Table 1
Activity scale for the Folin-Ciocalteu assay based on a filtered database from brown algae, green macroalgae, green microalgae, and red algae. Data expressed as either milligrams of gallic acid equivalent or phloroglucinol equivalent per gram of dry weight (DW) (mg GAE g^{-1} or mg PE g^{-1}, respectively).

Activity level	Brown algae	Green macroalgae	Green microalgae	Red algae
Algal biomass (mg GAE g^{-1} DW)				
High activity	>8	>3	>7	>4
Moderate activity	1 to 8	1 to 3	1.5 to 7	1 to 4
Low activity	<1	<1	<1.5	<1
Extract mass (mg GAE g^{-1} DW)				
High activity	>37	>18.5	>19	>18
Moderate activity	8 to 37	3.5 to 18.5	4 to 19	4 to 18
Low activity	<8	<3.5	<4	<4
Algal biomass (mg PE g^{-1} DW)				
High activity	>35	–	–	–
Moderate activity	6 to 35	–	–	–
Low activity	<6	–	–	–
Extract mass (mg PE g^{-1} DW)				
High activity	>150	–	–	–
Moderate activity	28 to 150	–	–	–
Low activity	<28	–	–	–

extract, water and ethanol were found to be the best solvents for red algae, while methanol and ethanol were the most effective for both green algae (Fig. 10C). In the case of brown algae, the results expressed by mg GAE g^{-1} (Fig. 10C) suggest ethanol and water as the most effective for obtaining the extracts, while the results by mg PE g^{-1} (Fig. 10D) suggest only ethanol. In terms of biomass extraction from brown algae, acetone was also possible to be evaluable but was found to be less efficient compared to methanol and ethanol (Fig. 10B).

In general, methanol was a suitable solvent for extracting algal biomass, while ethanol appears to be a versatile and efficient solvent for obtaining algal extracts, as it performs well across different groups. This difference arises from the selection of the solvent for each situation. The ideal solvent for biomass extraction should be capable of extracting all active components, regardless of purity. On the other hand, the optimal solvent for obtaining extract aims to ensure maximum purity by extracting the active compounds with minimum interference. Due to the limited number of articles available using aqueous solvents, a separate analysis of solvent mixtures containing water and those without was not feasible for both ethanol and methanol. Nevertheless, ethanol and methanol, without water, were more used as solvents for extraction, followed by 80 % (*v*/v) methanol or ethanol.

In summary, the more frequent choice for extraction of the algal material for analysis in the FC assays involves grinding the lyophilized biomass into a fine powder, and then macerating the powder (at a ratio of 1:100 to 5:100 *w/v*) using either methanol for biomass extraction or ethanol for obtaining extract mass on agitation at room temperature (Fig. 11). In general, the expression of data for biomass was utilized in the food, ecology, and physiology fields, while extract mass was often employed in studies related to bioactivity. Thus, the choice of reporting the data must be appropriately based on the intended use.

7. Challenges and future directions for the use of the Folin-Ciocalteu assay in phycology

Phenolic-reagent assays are used to estimate TPC, as seen in Fig. 3. Moreover, the high concentration of phlorotannins in brown algae led to the generalized use of these assays to measure phlorotannins as well. When using these assays for TPC and phlorotannin measurements, a crucial concern in the use of these assays should be the potential presence of interfering compounds, which could affect the accuracy and reliability of the results. In the case of phlorotannins, apart from the usual non-phenolic compounds, this approach raises the possibility that other phenolic compounds may interfere with the measurements. Several phenolic compounds, mainly phenolic acids (gallic acid, chlorogenic acid, caffeic acid, ferulic acid, and vanillic acid), as well as flavanols (catechin, epigallocatechin, and epicatechin), have been identified in brown algae [89,90]. It is still unknown if the effects of these phenolic compounds on assays are significant. These effects probably depend on environmental and genetic variations.

In turn, the interference of the non-phenolic compounds on the FC assay was studied by van Alstyne [91] using PVPP (polyvinylpolypyrrolidone), a polymer that selectively removes phenolic compounds from a solution. The study compared the values in the FC assay obtained with and without the addition of the PVPP to the algal sample and found that, at least in the samples from *Fucus gardneri*, non-phenolic compounds were insignificant, accounting for only 5 % of the results. However, subsequent studies by Cronin and Hay [92], Parys et al. [93], and Vega et al. [94] on different algal species yielded results that differ from Van Alstyne's findings, indicating that the values obtained without taking into account the effects of interfering compounds may be significantly overestimated. The results by Vega et al. [94], for example, suggested that some algal samples had no phenolic compounds after removing interfering compounds.

More recently, comparisons have been made between the FC assays with other analytical techniques more specific to phenolic compounds, such as NMR (nuclear magnetic resonance) and HPLC (high-performance liquid chromatography). However, these studies have produced mixed results, with Parys et al. [95] finding a positive correlation between the results of NMR and FC assay using extracts purified with PVPP, while other studies reported ambiguities [96–99]. These ambiguities may have been caused by the use of crude extracts for analysis in the FC assay without corrections for interfering compounds, which may have contributed to the discrepancies in results. Overall, the data suggest that the influence of the interfering compounds in the FC assays can be significant and can affect the accuracy and reliability of the results.

Despite the growing interest in the FC assay within the field of phycology, as seen in Fig. 2, a critical issue needs to be addressed. It is important to note that most articles (98.7 %) do not take into account the effect of interfering compounds on TPC or phlorotannin determination. This is a significant concern, especially for Chlorophyta and Rhodophyta, as most of the research on chemical characterization and interfering compounds focused on Phaeophyceae. Of the known interfering compounds [100], it is expected that vitamins and its derivatives (e.g., ascorbic acid), inorganic ions, and amino acids can affect the accurate estimation of TPC in algae, as these substances can be present in these organisms [101]. Moreover, recent research has revealed that mycosporine-like amino acids (MAAs) (non-phenolic compounds) can also act as reactive compounds in the FC assay [102]. This issue suggests that MAAs can interfere with the TPC measurements, particularly in red algae, which can contain significant amounts of these compounds [103].

Another concern is the lack of standardization of the FC assays in phycology, which can have significant implications for the comparability of results. While the protocol developed by Singleton and Rossi [21] is the most cited reference (about 20 % of articles reference it), other well-cited references, such as Velioglu et al. [104] and Farvin and Jacobsen [105], use a modified version of the Singleton and Rossi protocol. Even so, no papers followed the exact steps of the original protocol. Generally, most researchers use a modified protocol that involves using small volumes of solvents and reagents. These modifications are often made in an attempt to make the assay faster, more adaptable to available resources, and reduce the amount of waste generated during the analysis. However, the use of modified protocols can make it difficult to compare results between studies, particularly if the modifications are

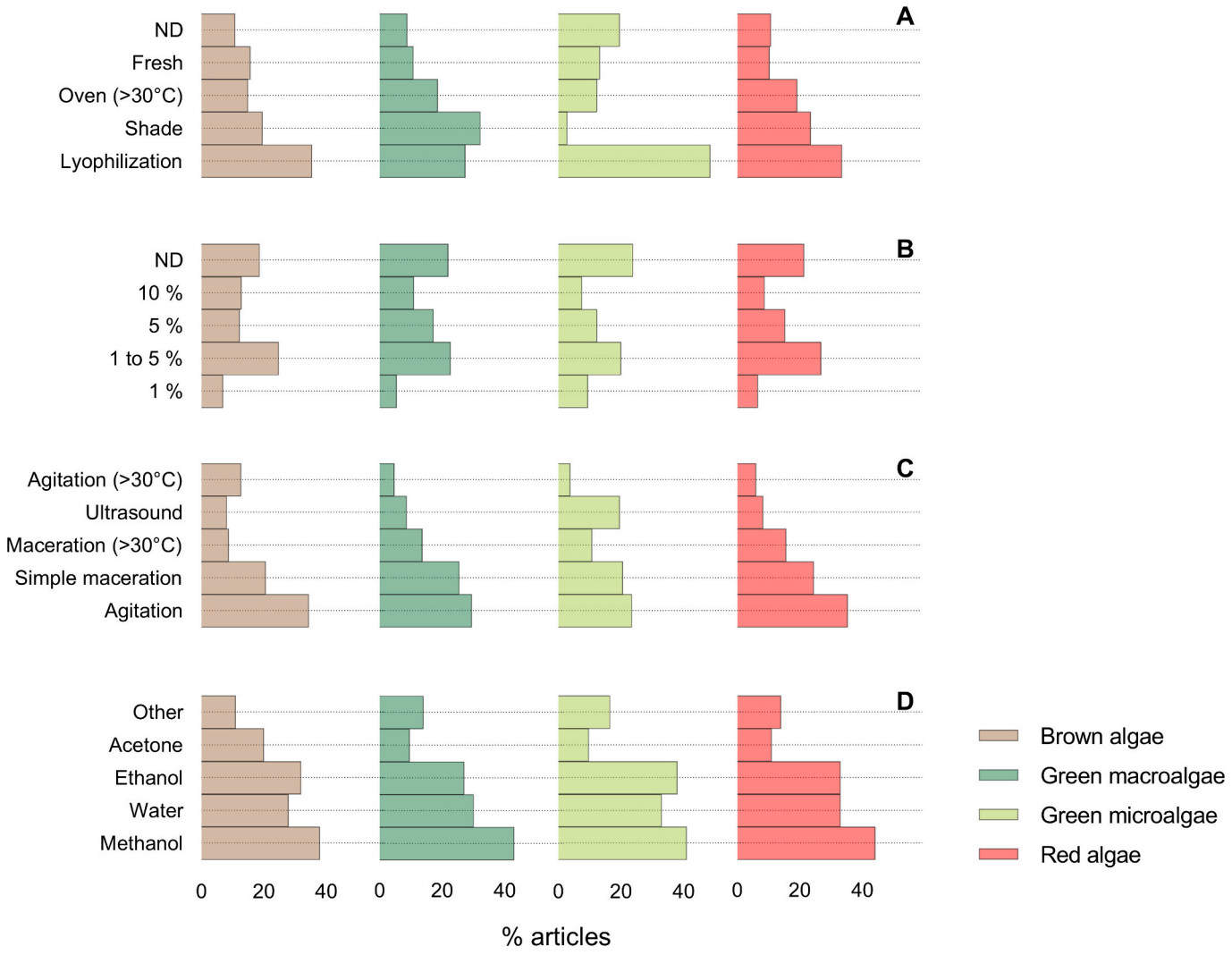

Fig. 9. Frequency of each step, expressed as a percentage of articles, for algal extraction, showing the most used methods for drying (A), solid-to-solvent ratios (B), extraction methods (C), and solvent types (D) for different algal groups. 'ND' means 'not determined'.

not described or standardized.

Although the presence of interfering compounds and the lack of standardization in the assays are serious methodological concerns for determining the TPC and comparability of the results, the primary issue in this field is to express the final data clearly. This ensures accurate comparisons between studies. However, three issues often arise that hinder the comparison of results. Firstly, imprecise language and undefined terms can lead to confusion. For example, it is not always clear whether the results are based on algal biomass or extract mass and whether fresh weight (FW) or dry weight (DW) was used for algal biomass. The words 'samples', 'material', or 'dry weight' alone can refer to both algal biomass and extract mass, leading to confusion and a lack of clarity.

Secondly, expressing results for algal biomass or extract mass in mass per volume units (e.g., mg L^{-1}) makes the data incomparable since these units are typically used for liquid samples such as wine and broth medium because they reflect the activity in a given volume of liquid. For solid samples, such as algal biomass or extract mass, it is more appropriate to use mass per mass units (e.g., mg g^{-1}), which express the activity per unit mass of the solid sample, allowing comparison between different solid samples. Thirdly, many studies do not specify the standard used, which can lead to misinterpretation of results. Unfortunately, 47 % of the articles did not adequately report their findings, and these details are crucial for making accurate comparisons between studies. Fig. 1B shows that excluding non-comparable data significantly affected the 'total database' compared to the 'filtered database'.

As results from this review, we can conclude that the FC assay critically is used as a measure of reducing power or reducing antioxidant capacity in phycology rather than as a measure of TPC because corrections for interference compounds are not made. This discussion is not exclusive to the field of phycology. For example, Otto Folin and his coworkers developed the FD protocol [13] for the quantification of tyrosine (a phenolic amino acid), but tryptophan (a non-phenolic amino acid) shows similar reactivity in the assay. Several attempts were made to correct the assay, but Folin and Ciocalteu [19] abandoned the use of the assay for the measurement of only tyrosine and proposed a measure for tryptophan as well. Therefore, the original protocol measures reducing power for the estimation of amino acids. Moreover, Vernon L. Singleton, one of the developers of the Singleton and Rossi protocol, suggested that the assay should be considered a measure of reducing power rather than TPC for unknown samples [18], which is consistent with most studies in phycology.

Although the FC assay is not used in most phycological studies as a measure of TPC, it remains a valuable tool for several reasons. It is relatively simple and cost-effective method accessible to many researchers, resulting in a substantial and expanding database. The high correlation of the FC assay with established assays commonly used to measure antioxidant potential further supports its reliability in assessing

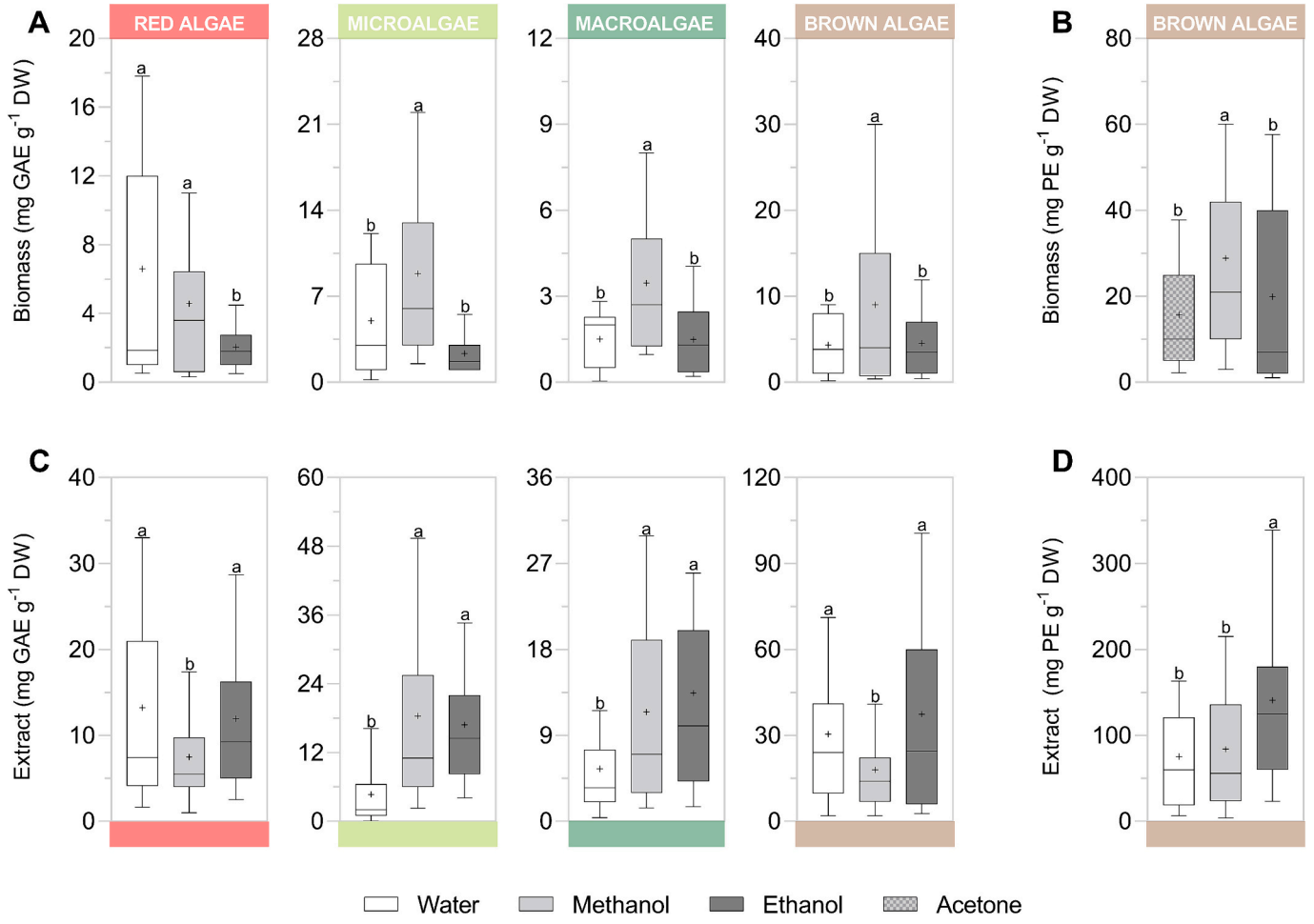

Fig. 10. Comparisons of values from Folin-Ciocalteu assays for different solvents used in extracting algal biomass and obtaining extract mass from red, green, and brown algae. Algal biomass expressed as mg GAE g^{-1} DW (A) and mg PE g^{-1} DW (B), and extract mass expressed as mg GAE g^{-1} DW (C) and mg PE g^{-1} DW (D). Box plots show the distribution of data with whiskers indicating minimum and maximum values. The mean of the data is represented by a plus symbol (+), while a horizontal bar within the box represents the median. Statistical comparisons were performed using Kruskal-Wallis tests with multiple comparisons using Dunn's tests. Statistical outliers were identified using the ROUT method (Q = 0.1 %).

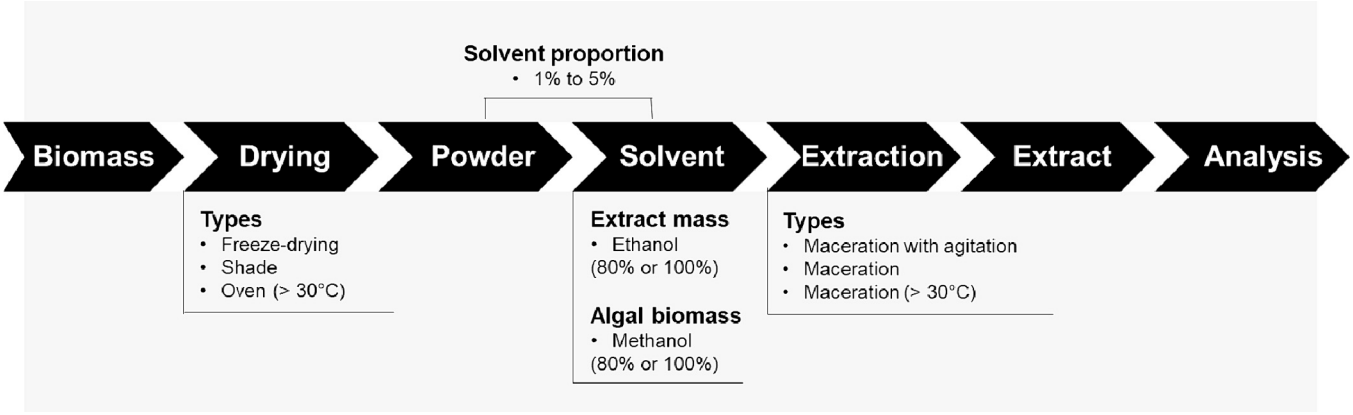

Fig. 11. Summary of the extraction steps most frequently used to obtain algal extracts for analysis in Folin-Ciocalteu assays. The steps analyzed include drying methods, solid-to-solvent ratio, solvent types, and extraction methods for different types of algae.

the reducing antioxidant capacity. Moreover, the assay is a sensitive tool for studying algal ecology and physiology. Its consistent reproducibility, as evidenced by the results expressed as GAE and PE, further strengthens its reliability. As with any assay, it is crucial to understand its limitations and potential sources of error, but with the proper interpretation, the FC assay can provide valuable information.

CRediT authorship contribution statement

Priscila Torres: Conceptualization, Data curation, Formal analysis,

Investigation, Methodology, Writing – original draft, Writing – review & editing, Validation. **Sayuri Osaki:** Data curation, Formal analysis, Validation, Writing – original draft, Writing – review & editing. **Elielson Silveira:** Data curation, Validation, Writing – original draft, Writing – review & editing. **Deborah Y.A.C. dos Santos:** Conceptualization, Validation, Writing – review & editing. **Fungyi Chow:** Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2024.103503.

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