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Distribution of Phlorotannins in the Brown Alga *Ecklonia cava* and Comparison of Pretreatments for Extraction

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Abstract

The brown seaweed *Ecklonia cava* is known to be a rich source of phlorotannins that have diverse biological activities. Among the phlorotannins in *E. cava*, concentrations of dieckol and phlorofucofuroeckol-A, which were identified as major active components, were determined in different parts of the tissue. We compared the efficacy of different pretreatments for their extraction. A high-performance liquid chromatography (HPLC) method to determine phlorotannin concentrations showed good accuracy (92.64 and 94.02%, respectively), precision (3.92 and 3.94%, respectively), and linearity (r>0.996). Mature thalli contained 1.5-fold more dieckol (1.82 mg/g-dry tissue) than young thalli. In the tissues of *E. cava,* blade tissue contained more phlorotannins than the stipe or holdfast. Among differently dried thalli, approximately 90% or more dieckol and phlorofucofuroeckol-A were extracted from shadow-dried tissue as compared with lyophilized tissue. In sun-dried and oven-dried thalli, approximately 60% of the phlorotannins were extracted. Thalli washed with fresh water, boiled thalli, and steamed thalli showed reduced extraction of the compounds.

Key words: Dieckol, *Ecklonia cava*, HPLC, Phlorofucofuroeckol-A, Phlorotannins

Introduction

The brown seaweed (Laminariaceae) *Ecklonia cava* is distributed in temperate coastal areas of the Korean peninsula and Japan (Li et al., 2008). It is found in sub-littoral deep water (4-25 m or more) and grows attached to a solid substratum, anchored in place by fibrous holdfasts. *E. cava* grows to 1-3 m in height in a dense population, which has been called a marine forest (Maegawa, 1990). It grows abundantly on Jeju Island, Korea, and is used as a food ingredient, animal feed, fertilizer, and medicine (Li et al., 2008). *E. cava* contains a variety of compounds, including carotenoids, fucoidans, and phlorotannnins, that play diverse biological and ecological roles. Marine algal polyphenols, known as phlorotannins, are major metabolites of *Ecklonia* species; they have only been found to exist within brown algae and are formed by the polymerization of phloroglucinol (1,3,5-trihydroxybenzene) (Ragan and Glombitza, 1986). In brown algae, phlorotannins function as defense against herbivore (Steinberg, 1984; Targett and Arnold, 1998), microbes (Waterman and Mole, 1994; Pavia and Toth, 2000), and the harmful effects of ultraviolet (UV) radiation (Pavia et al., 1997). Phlorotannins also have allelopathic activity against epibionts (Davis et al., 1989) and serve as structural compounds in cell wall hardening (Schoenwaelder and Clayton, 1998; Schoenwaelder, 2002; Arnold and Targett, 2003). During the past decades, it has been reported that *Ecklonia* species, by virtue of the phlorotannin contents, exhibit antioxidant and anti-inflammatory activities (Kim et al., 2009), radical scavenging activity (Li et al., 2009), anti-allergic activity (Li et al., 2008), anti-plasmin activity (Nakayama et al., 1989), bactericidal activity (Nagayama et al., 2002), HIV-1 reverse transcriptase and protease inhibitory

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activity (Artan et al., 2008), acetyl cholinesterase inhibitory activity (Myung et al., 2005; Yoon et al., 2008), and tyrosinase inhibitory activity (Heo et al., 2009).

Quantitative measurement of phlorotannins in seaweed tissues by colorimetric method has been uncertain (Stern et al., 1998) and crude phlorotannins have been measured simply on a dry weight basis (Shibata et al., 2004). In both methods, total amount of whole phlorotannins is measured. Such a total quantification of phlorotannins provides no information on the biological and pharmacological properties of individual phlorotannins. Indeed, phlorotannins are highly soluble in mixtures of water and organic solvent, and high-performance liquid chromatography (HPLC) can act as a suitable tool for their qualitative and quantitative analysis. Koivikko et al. (2007) used HPLC to analyze phlorotannin from *Fucus vesiculosis*. Among the phlorotannins from *E. cava*, dieckol and phlorofucofuroeckol-A were selected in this study because they have been demonstrated to be potential biologically active components (Yoon et al., 2008; Kim et al., 2009). A study on the local distribution of phlorotannins revealed that they tend to be concentrated within the vegetative cells of the outer cortical layers, and the mitotic, meristematic, and meiotic sporogenous tissues of Laminariaceous brown algae (Shibata et al., 2004). However, the distribution of each phlorotannin compound in mature and non-mature tissues of different body parts, such as the thalli, stipe, and holdfast, of *E. cava* has not been previously reported. After collection from the sea, seaweeds are usually dried before being used in any nutritional evaluation, industrial processing, or storage. Drying treatment is an essential processing method for prolonging the self life, as well as for conserving optimum nutrient content. Thus, the drying process would seem to be a critical factor affecting the quality of seaweed samples. Sun-drying, shadow-drying, oven-drying, and lyophilization (Hamdy and Dawes 1988; Carrillo et al., 1992; Mabeau and Fleurence, 1993) are common drying methods used in seaweed treatment. Thus, the objectives of our study were to develop a state-of-the art analytical method for phlorotannin quantification from *E. cava* by HPLC, to determine the chemical distribution of dieckol and phlorofucofuroeckol-A in the different tissues of *E. cava*, and to determine the contents of dieckol and phlorofucofuroeckol-A after various drying treatments.

Materials and Methods

Collection of seaweed materials

The brown alga *E. cava* Kjellman was collected from the coast of Busan, South Korea, in 2009. A voucher specimen was deposited in the author's laboratory (Y. K. Hong). To study chemical distribution of dieckol and phlorofucofuroeckol-A, both young (≤30 cm long) and mature (≥ 50 cm long) thalli, and blades, stipes and holdfasts from mature thalli were separately collected.

Drying methods

The seaweed thalli were cleaned with filtered seawater. Epiphytes were removed by sonication for 1 min. The samples were then dried using different drying methods: sun-dried at room temperature for 3 days; shadow-dried at room temperature for 3 days with an electric fan; oven-dried at 60ºC for 24 h; and lyophilized for 24 h. For lyophilization, a vacuum freeze dryer (SFDSM 24L; Samwon Freezing Engineering Co., Busan, Korea) was used. To remove salt from the thalli, samples were washed with tap water. The mature blades of *E. cava* were boiled (1 min or 5 min) and steamed (5 min or 30 min) with distilled water to determine the effects of heat treatments on the phlorotannin extraction yield from *E. cava*. Dried seaweed (average 10% moisture content) was pulverized in a grinder (HMF-340; Hanil Co., Seoul, Korea) for 5 min. Samples were stored under nitrogen gas in the dark at 20°C until use.

Extraction of crude phlorotannins

Crude phlorotannins were extracted from the algal powder according to the method of Folch et al. (1957), with some modifications. The algal powder (0.5 g) was rotated on a rotator (Rototorqe 7637-10; Cole-Parmer, Vernon Hills, IL, USA) with methanol (2 mL) at room temperature for 2 h. Chloroform (4 mL) was added and the mixture was shaken for 5 min and filtered with defat-cotton. After filtration, the methanol-chloroform extract was partitioned by adding deionized water (1.5 mL) with shaking for 5 min. The upper layer (non lipid fraction) was collected and extracted by ethyl ether (3 mL). The ethyl ether fraction was then evaporated with a nitrogen generator (G 4510E; Domnick Hunter Ltd., Dukesway, England). The crude phlorotannin residue was collected and measured with a digital electronic balance (Mettler AG245; Toledo GmbH, Greifensee, Switzerland). The crude phlorotannin was dissolved in 100% methanol (1 mg/mL) and stored at -20°C until use.

Isolation of dieckol and phlorofucofuroeckol-A

To isolate the pure dieckol and phlorofucofuroeckol-A from the crude phlorotannin extracted from *E. cava*, a C18 column (250 mm×10.0 mm) (Altech Associates Inc., Deerfield, IL, USA) was used. The isolation was performed by HPLC, consisting of a Waters 486 Turnerable Absorbance Detector (Waters Associate Inc., Milford, MA, USA). HPLC elution was performed at a flow rate of 1.0 mL/min using a linear gradient of 30-100% methanol. The UV detector was set at 290 nm. The column temperature was ambient. The two phlorotannins, dieckol and phlorofucofuroeckol-A, were isolated on the basis of retention time and the UV-VIS spectra. The retention times

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of dieckol and phlorofucofuroeckol-A were approximately 32 and 39 min, respectively. The isolated dieckol and phlorofucofuroeckol-A were analyzed by ^{1}H nuclear magnetic resonance (NMR) spectra using a JNM-ECP 400 NMR spectrometer (JEOL, Tokyo, Japan) with methanol-*d* (CD_3OD). The structure of each compound was verified by comparison with previously published spectral data (Kim et al., 2009).

Quantification of dieckol and phlorofucofuroeckol-A

To measure the amounts of dieckol and phlorofucofuroeckol-A from *E. cava*, the extracted crude phlorotannin from different treatments were assessed. Each 100-μL aliquot of 100% methanol-dissolved crude phlorotannin (1 mg/mL) was injected into the HPLC using the same HPLC conditions for the isolation procedure. The amount of each compound was assessed by measuring the dimensions of HPLC peaks and using the standard curve of each isolated pure compound. Validation of the HPLC quantification was performed by assessing accuracy, precision, linearity, limit of detection (LOD), and limit of quantification (LOQ). Accuracy was determined by analyzing each standard sample at different concentrations. Three replications were performed for each concentration. The % recovery was calculated from the mean concentration of the three replications. Peak height was used for quantification purposes, using the equation of Snyder et al. (1997): response factor (RF)=standard peak height/standard concentration. Sample concentration was calculated as: sample concentration=sample peak height/RF. Accuracy (or % recovery) was calculated with the following formula: accuracy=(actual concentration of analyte/theoritical concentration of analyte)×100. Linearity of the quantification for each compound was assessed with three standards, from 0 to 750 μg/mL. Calibration curves for each compound were constructed separately by plotting peak height (*y* axis) versus the concentration (*x* axis) of the standards. Regression analysis was used to relate each isolated pure phlorotannin standard concentration individually as the dependent variable with peak height as the independent variable. The precision of the method was expressed by the standard deviation (SD) and related standard deviation (RSD). SD and RSD were calculated using equation described by Snyder et al. (1997). LOD and the lower LOQ were determined by analyzing pure compound solutions that were sequentially diluted in a series with 100% methanol to obtain the lowest level of analyte that gave a measurable response with a signal-to-noise ratio of 3 and 10, respectively (Snyder et al., 1997).

Statistical analysis

All data are presented as the mean±SEM. Statistical comparisons of the mean values were performed by an analysis of variance (ANOVA), followed by a Duncan's multiple test using SPSS software version 12.0 (SPSS Inc., Chicago, IL, USA). *P*-values<0.05 were considered to indicate statistical significance. The results shown in each of the figures are representative of at least four independent experiments.

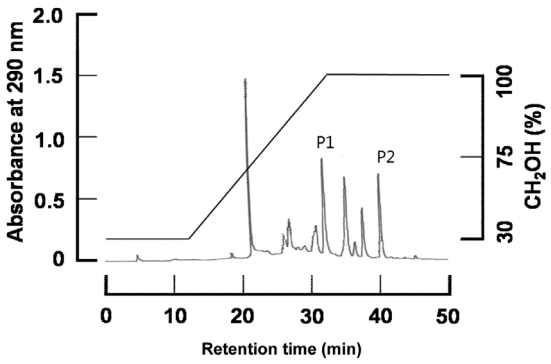

Fig. 1. High-performance liquid chromatography profile of phlorotannins from the brown seaweed *Ecklonia cava*. P1 indicates a peak of dieckol at 32 min of retention time and P2 indicates a peak of phlorofucofuroeckol-A at 39 min of retention time. 100 μL aliquot of crude phlorotannin (1 mg/mL) dissolved in 100% methanol was separated on C18 column, using the same isolation procedure.

Results

Validation of HPLC quantification

To quantitatively evaluate dieckol and phlorofucofuroeckol-A from the *E. cava*, HPLC analysis was conducted (Fig. 1). Two major peaks were detected for the crude phlorotannin at approximate retention times of 32 and 39 min. ^{1}H-NMR data revealed that the two compounds, P1 and P2, were dieckol and phlorofucofuroeckol-A, respectively (data not shown). The amounts of the two phlorotannins in *E. cava* were measured, by the dimension of the HPLC peaks and standard curve for each isolated pure compound. The HPLC quantification method was validated by accuracy, linearity, precision, LOD and lower LOQ. For dieckol and phlorofucofuroeckol-A, the accuracy was calculated to be 92.64% and 94.02%, and the precision was 3.92% and 3.94%, respectively. The LOD was 7.27 μg/mL and 5.82 μg/mL, and the lower LOQ was 24.15 μg/mL and 19.41 μg/mL, respectively. Standard curves for each pure compound up to 750 μg/mL were generated. The calibration plots of peak height versus concentration of the pure compound exhibited a straight linear line. Results of the regression analysis for the correlation coefficient (*r*) are presented in Table 1. Correlation coefficients (*r*) were >0.996, indicating a positive linear relationship between peak height and concentration.

Distribution of phlorotannins in *E. cava* tissue

To determine the chemical distribution of phlorotannins in different tissue parts of *E. cava,* young and mature thalli were

Table 1. Validation parameters for the determination of phlorotannin compounds in *Ecklonia cava* extract

	Retention time (min)	Accuracy (%)	Precision (%)	LOD (μg/mL)	Lower LOQ (μg/mL)	Correlation coefficient (*r*)
Dieckol	32	92.64±6.95	3.92	7.25	24.15	0.996
Phlorofucofuroeckol-A	39	94.02±5.70	3.94	5.82	19.41	0.999

LOD, limit of detection; LOQ, limit of quantification.

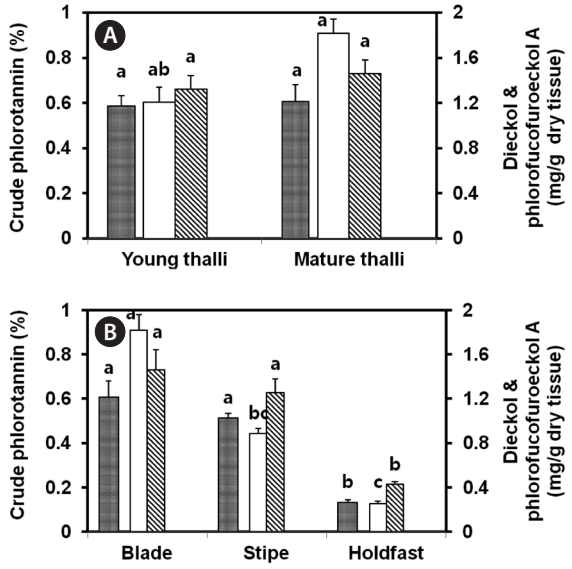

Fig. 2. Comparison of phlorotannnins from different tissues of *Ecklonia cava*. Amounts of crude phlorotannin (▩), dieckol (□) and phlorofucofuroeckol-A (▨) from young and mature thalli (A) and from blade, stipe and holdfast parts of mature thalli (B). Values are mean±SE (*n*=4). Means with different letters are significantly different by Duncan's multiple range test ($P<0.05$). a, b and c indicate statistical relation among samples.

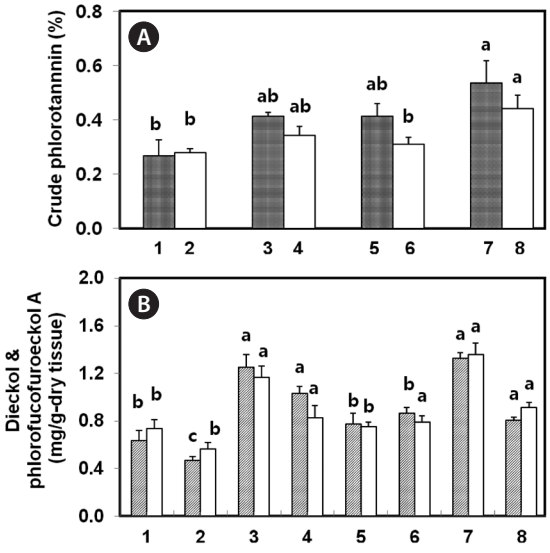

Fig. 3. Comparison of dry pretreatments for the extraction of phlorotannins from *Ecklonia cava*. (A) Content of crude phlorotannin on dry matter basis from natural (▩) and tap water-washed tissues (□). (B) Amount of dieckol (▨) and phlorofucofuroeckol-A (□). According to treatments: 1, natural and sun dried tissue; 2, washed and sun dried tissue; 3, natural and shadow dried tissue; 4, washed and shadow dried tissue; 5, natural and oven dried tissue; 6, washed and oven dried tissue; 7, natural and lyophilized tissue; 8, washed and lyophilized tissue. Values are mean±SE (*n*=4). Means with different letters are significantly different by Duncan's multiple range test ($P<0.05$). a, b and c indicate statistical relation among samples.

compared for the amounts of crude phlorotannin, dieckol, and phlorofucofuroeckol-A. Blade, stipe, and holdfast from mature thalli were also compared with regard to the amounts of phlorotannins. All samples were lyophilized. As compared with young thalli, mature thalli contained 1.5-fold more dieckol (1.82 mg/g-dry tissue). In the case of crude phlorotannin and phlorofucofuroeckol-A, mature thalli showed marginally higher amounts than young thalli (Fig. 2). Among the blade, stipe, and holdfast of mature thalli, the blade portion generally showded the highest amount of crude phlorotannins (0.61±0.07%), dieckol (1.82±0.12 mg/g-dry tissue), and phlorofucofuroeckol-A (1.46±0.18 mg/g-dry tissue). The sum of these two compounds represented about 54% of the crude phlorotannins. The holdfast contained a low level of phlorotannins.

Drying pretreatments on phlorotannin extraction

E. cava tissues prepared by the different drying pretreatments, such as sun-drying, shadow-drying, oven-drying, and lyophilization, of both natural and tap water-washed thalli, were analyzed to compare different drying methods with regard to the extraction of phlorotannins. Among the samples, natural non-washed lyophilized tissue yielded the highest amount of crude phlorotannin (0.53%) on a dry weight basis, whereas sun-dried tissues showed the lowest yield (0.27%). Overall, tap water-washed tissue was found to have lower crude phlorotannin than non-washed tissue (Fig. 3A). Compared with natural tissues for crude phlorotannin, shadow- and oven-dried tissues were extracted at almost 80% of lyophilized tissue. Meanwhile, sun-dried tissues of both natural and tap water-washed thalli showed only half the yield of lyophilized tissue. When considered dieckol and phlorofucofuroeckol-A contents in *E. cava* tissue, no significant difference ($P<0.05$)

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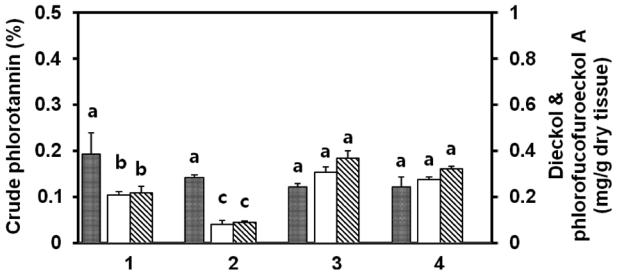

Fig. 4. Amount of phlorotannins remained in blades after boiling or steaming treatment. Crude phlorotannin (▨), dieckol (□) and phlorofucofuroeckol-A (▧). According to treatments: 1, 1-min boiled and lyophilized tissue; 2, 5-min boiled and lyophilized tissue; 3, 5-min steamed and lyophilized tissue; 4, 30-min steamed and lyophilized tissue. Values are the mean±SE (n=4). Means with different letters are significantly different by Duncan's multiple range test (P<0.05). a, b and c indicate statistical relation among samples.

was observed between lyophilized and shadow-dry tissues of both natural and salt-removed thalli (Fig. 3B). Approximately 90% or more dieckol and phlorofucofuroeckol-A was extracted from shadow-dried tissue as compared with lyophilized tissue. In the case of sun-dried and oven-dried thalli, approximately 60% of the phlorotannins were extracted. Washing with fresh water decreased the compounds of compounds extracted from thalli down to 61-82% of that from natural tissues, even when shadow-dried or lyophilized. In the case of natural non-washed lyophilized and shadow dried tissues, the sum of dieckol and phlorofucofuroeckol-A represented 51% and 59% of the crude phlorotannins, respectively.

Effect of boiling and steaming on phlorotannin yield

Boiling or steaming before tissue drying and extraction is commonly used to denature enzymes that are involved in the decomposition of useful compounds. To determine the effects of boiling and steaming before drying pretreatment on the yield of phlorotannins from *E. cava*, fresh mature blade was boiled or steamed with distilled water. After the boiling or steaming treatment, samples were dried in the lyophilizer. Crude phlorotannin yields after boiling and steaming dropped to half of the yield of directly lyophilized tissue (Fig. 4). The amounts of dieckol and phlorofucofuroeckol-A were also reduced to 30-50% after boiling or steaming.

Discussion

Marine brown algae have recently become a subject of interest due to their bioactive secondary metabolites, such as phlorotannins. *E. cava*, an edible marine brown alga with a long history of use as a folk medicine in Korea (Donguibogam Committee, 1999), is widely distributed in Korea and Japan. This species of seaweed has already been reported to be a rich source of phlorotannin derivatives with diverse bioactivities, which is why we attempted to determine the chemical distribution of phlorotannins in different parts of *E. cava* and the effects of drying pretreatment on the extraction yield. An HPLC method for the determination of phlorotannins was also validated. The validation process involves challenging the method and determining the limits of allowed variability for the conditions needed to run the quantification. For the assessment of low-level impurities, precision of 5-10% RSD is usually accepted (Snyder et al., 1997), which is higher than our precision range. Thus, the quantification of dieckol and phlorofucofuroeckol-A from *E. cava* seaweed using the HPLC method described here is a simple and reliable method and does not depend on the degree of multiplicity of seaweed compounds.

More amount of crude phlorotannin was present in mature thalli as compared with young thalli (Fig. 2). Similar to other phenolic compounds, phlorotannins are produced extensively in the cytoplasm or chloroplasts of growing tissues, and are subsequently stored in physodes (Schoenwaelder and Clayton, 1998). Generally, young thalli produce more primary metabolites with faster growth, whereas mature thalli have multiple mechanisms to modify biomolecules, thus supporting diverse cellular functions (Chen et al., 1998). Phlorotannin-rich cells were found to be localized only within the outer cortical layer of the blades of *E. cava, E. kurome,* and *Eisenia bicyclis* (Shibata et al., 2004). In this study, we found the presence of phlorotannins not only in blade tissue, but also in the stipe and holdfast to some degree. Shibata et al. (2004) and Sugiura et al. (2007) reported that *E. cava* and *Eisenia arborea* contained about 3.1% and 0.08% crude phlorotannin, respectively. We found 0.6% of crude phlorotannin from *E. cava* thalli on a completely dry weight basis. Phlorotannin contents in brown algae have been found to be highly variable, both among and within species (Targett and Arnold, 2001). While young thalli contained large amounts of crude phlorotannin and phlorofucofuroeckol-A, mature thalli contained 1.5-fold more dieckol (1.82 mg/g-dry tissue) than young thalli. Dieckol may be one of the key metabolites in understanding the function of mature thalli.

In this study, four dry pretreatments were applied. Among them, approximately 90% or more dieckol and phlorofucofuroeckol-A could be extracted from shadow-dried tissue as compared with lyophilized tissue, whereas sun-dried thalli yielded the lowest amount. Lyophilization or cryodesiccation are processes used for the dehydration of almost all heat-sensitive materials. The materials are frozen and then the surrounding pressure is reduced. Just enough heat is then added to allow the frozen water in the material to sublime directly from the solid to the gas phase (Oetgen and Haseley, 2004). However, the equipment and operating costs for freeze-drying are higher, and the drying capacity is lower, than for sun-, shadow-, or oven-drying. Loss of phlorotannins during sun-drying might be due to photo-oxidation processes. Some phenolic compounds are decomposed rapidly when exposed to

direct sunlight or dried at elevated temperature (Mueller-Harvey, 2001). Oven-drying at 60°C may not immediately inactivate degradative enzymes, and thus phlorotannin compounds may be degraded before complete drying. Washing, boiling, and steaming procedures with fresh water likely released some water-soluble phlorotannins from tissues. Considerable amounts of phlorotannins were observed in the water after boiling or even dipping in seawater at room temperature (data not shown). One explanation could be the presence of certain very polar phlorotannins in *E. cava* seaweed, which can be extracted with a very polar solvent, such as water. Identification of these water-soluble compounds is in progress. The thalli washed with fresh water, boiled thalli, and steamed thalli showed decreased compound extraction. In conclusion, with respect to the most appropriate drying method for *E. cava*, shadow-drying is recommended for the extraction of considerable amounts of phlorotannins and due to its cost effectiveness.

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