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Rapid Microwave-Assisted Phloroglucinolysis in the Determination of Oligomeric Procyanidin Average Size in Fiber Extracts of Two *Cocos nucifera* L. Varieties

Floroglucinólise Rápida Assistida por Micro-ondas na Determinação do Tamanho Médio de Procianidinas Oligoméricas em Extratos de Fibra de Duas Variedades de Cocos nucifera L.

Gabriel R. Martins,[a] Ricardo M. Borges,[a] Celuta S. Alviano,[b] Daniela S. Alviano,[b] Antonio Jorge R. da Silva[a,]*

[a]Universidade Federal do Rio de Janeiro, CCS, Instituto de Pesquisas de Produtos Naturais, Bloco H, Avenida Carlos Chagas Filho 373, Cidade Universitária, . CEP 21941-902, Rio de Janeiro-RJ, Brazil.

[b]Universidade Federal do Rio de Janeiro, CCS, Instituto de Microbiologia Paulo de Góes, Bloco I, Avenida Carlos Chagas Filho 373, Cidade Universitária, CEP 21941-902, Rio de Janeiro-RJ, Brazil.

***E-mail:** ajorge@nppn.ufrj.br*

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The coconut tree (*Cocos nucifera* L., Arecaceae) is a palm tree distributed in coastal vegetation across the tropics. Its fibers are the main agro-industrial residue from coconut production. Extracts obtained from the fibers contain procyanidins with biological and pharmacological potentials. The main analytical methods used to characterize procyanidins are based on the depolymerization reaction by acid catalysis in the presence of nucleophile in excess. This study aimed to optimize a modified depolymerization reaction using microwave irradiation and to apply this method to characterize the procyanidins from the fiber extracts of two coconut varieties. Fibers of *C. nucifera* var. *typica* A and var. *typica* were extracted with acetone:water 6:4 (v/v). After acetone was evaporated, the extracts were lyophilized and and then partitioned partitioned with ethyl acetate:water (1:1, v/v). The resulting aqueous fractions, rich in polymeric procyanidins, were analyzed by HPLC, in triplicate, after depolymerization. A factorial design was applied in duplicate, using irradiation power (50 to 350 W) and reaction time (10 to 50 s) as independent variables, and the product yield as the dependent variable. The optimized independent parameters were 200 W of irradiation power and 30 s of reaction time, reducing the reaction time by ca. 40 times when compared with the standard protocol. The aqueous fractions exhibited a mean degree of polymerization of 7.06 and 9.98, instead of 4.49 and 4.87 using microwave-assisted phloroglucinolysis compared to the conventional protocol. Epicatechin was the only extender unit found for both varieties and catechin was the preferred terminal unit (78.51% and 51.55%) as terminal unit. A significant increase in the mean degree of polymerization was found, indicating higher efficacy in the depolymerization of procyanidins. The coconut fibers are a potential source of procyanidins with a high degree of polymerization, making this agro-industrial waste suitable for industrial applications.

Keywords: Phloroglucinolysis; microwave-assisted reaction; factorial design; agro-industrial residue; proanthocyanidin; average molecular size

1. Introduction

Brazil has the most extensive biodiversity in the world.[1] Such diversity and abundance of natural resources must be explored rationally and sustainably since it is a valuable source of natural products with chemical and biological potential for drug discovery innovations and new technological applications.[2,3] It is regrettable that Brazilian biomes are being threatened under the current environmental policies, noticeable with the return of deforestation, numerous fires and mining activities with significant impact on the environment.[4]

Brazil is also one of the main agricultural producers in the world. The agro-industrial activities generate over 600 million tons of waste per year, which is a growing industrial processing problem.[5] Waste and byproduct residues exploitation as raw materials in the production of various high valued chemicals prevents their accumulation, lowers their environmental impact and aims at a circular green economy.[5,6] Brazil has excellent potential for using renewable raw materials in the sustainable production of bio-products,[5,7,8] and this utilization becoming a reality would be a crucial step towards the agreement with global priorities and assessments of the United Nations (UN) 2030 Sustainable Development Agenda.[9]

The Arecaceae family has great importance in landscaping[10] and globally comprises about 2600 species, many of them with economical applications.[11] In Brazil, entire families and communities are involved in the management activities of palm crops, such as *Cocos nucifera* L. (coconut),

Euterpe oleracea Mart. (açaí), and *Elaeis oleifera* [Kunth] Cortés (oil palm), with high socio-economic importance for local populations.[12] Other relevant species, especially for the exploitation of oil, are *Acrocomia aculeata* (Jacq.) Lodd. ex Mart. (macaúba) for biofuel production[13], *Mauritia flexuosa* L.f. (buriti) and *Attalea speciosa* Mart. (babaçu), to name a few.[10,13]

The coconut palm (*Cocos nucifera* L.), also known as the "tree of life", is a subsistence crop for many populations, and it is widely distributed over coastal vegetation across the tropics. The coconut production worldwide is about 62 million tons per year, and Brazil is the 5th highest global coconut producer (FAO, 2020; http://www.fao.org/statistics).

Water, oil, "milk," and copra are the industrialized products derived from the coconut[14]. This agroindustry generates a staggering amount of residue since the coconut fibers (the seed's surrounding tissue) corresponds to 25%–35% of the coconut's biomass.[8,15] Even though coconut fibers have low-value applications in the production of doormats and rugs, mattresses, and other products[14], it is estimated that up to three million tons of coconut husk are discarded every year in Brazil. This waste may take up to ten years to degrade, causing urban and environmental problems and becoming foci for mosquitoes breeding and disease transmission.[16]

Our research group described, based on ethnopharmacological studies, the coconut's husk fibers as a source of bioactive extracts. Previous studies displayed antiviral, antibacterial, anti-inflammatory, leishmanicidal, and other biological activities for procyanidins of *Cocos nucifera typica* A, popularly known as "olho-de-cravo". However, this variety is relatively rare in Brazil. A more common variety, known as *C. nucifera typica* or "Coco-da-Bahia", is more resistant and diffuse among the Brazilian coast that also exhibited vast biological activities[17–22], illustrating how it could serve as a low-cost medicine for several therapies.

In terms of byproduct valorization, the exploitation of proanthocyanidins from the coconut fibers can be considered highly sustainable since it reduces the accumulation of an agro-industrial residue.[23] However, a comprehensive chemical characterization, especially the degree of polymerization (DP) is necessary when targeting any industrial use. For instance, for health-promoting applications, proanthocyanidins with a low degree of polymerization (DP≤4) have better absorption profile[24] while higher oligomers and polymers display other significant green industrial applications as biosourced foams, wood preservatives, corrosion inhibitors, polyurethane surface coatings, epoxy adhesives, binders for Teflon coatings, and many others.[25]

Proanthocyanidins, also called condensed tannins, are defined as oligomers or polymers of flavan-3-ol units.[26] They are ubiquitous in the Plant Kingdom and are the most abundant natural polyphenols type after the lignins[27]. Proanthocyanidins display structural diversity to an appreciable extent, which depends upon:

(a) the hydroxylation pattern, which produces at least 16 different monomeric units; [28]
(b) the interflavan bond connectivity. B-type procyanidins are characterized by a single interflavan bond between carbon-4 of an upper unit to either carbon-6 or carbon-8 of the A-ring of a lower unit, while A-type procyanidins have a additional linkage between A-ring hydroxyl group of the lower unit and the carbon-2 of C-ring of the upper unit.
(c) the stereogenic centers at C-2 and C-3. The C-2 configuration is almost always R, but with exceptions in nature[30], flavan-3-ols with 2S type configuration are distinguished by a prefix *enantio* (ent-).[31] Monomeric units have 2R,3R configuration, also identified as (2,3-*cis*) or 2R,3S configuration, also classified as (2,3-*trans*) monomeric units; [32]
(d) the degree of polymerization (DP), which characterize oligomers (DP=2-9) and polymers (DP≥10);
(e) substitutions in C3-hydroxyl group like gallic acid esterification.

Various analytical methods are used to characterize proanthocyanidins since their identification can be arduous and time-consuming. For example, at 25 ºC, ^{1}H-NMR of procyanidins shows broadening of signals due to atropisomerism. Even with derivatization, steric interactions in the vicinity of the interflavanoid bond affect the free rotation of flavan-3-ol units, difficulting clear correlations.[31,33–35]

One of the analytical methods used for proanthocyanidins characterization is the depolymerization under acid-catalysis with an excess of phloroglucinol, known as phloroglucinolysis. This protocol provides information on their composition and the Interflavan bond location. Under acidic conditions, the Interflavan bond breaks, releasing terminal (as neutral monomers) and extension units (as electrophilic intermediates). Phloroglucinol acts as a nucleophile generating adducts with the extension units. This method allows the determination of the mean degree of polymerization (mDP) of the sample and its composition by RP-HPLC-DAD. [31,36,37] Other structural information provided by this method are ratios between types of proanthocyanidins (like procyanidin/prodelphinidin), *cis*-/*trans*-flavan-3-ol ratios, molar percentages of galloylation, and A-type linkage when present in the sample. Kennedy & Jones (2001) devised this protocol which has 540 citations up to date (Web of Science). In the original communication, the reaction occured at 50 ºC for 20 min.

The fact that microwave-assisted reactions bring the advantage of enhanced reaction rates, higher yields, higher selectivity when applied to a large number of organic reactions has encouraged us to switch from conventional heating method to microwave-assisted phloroglucinolysis.[38] Thus, the purpose of this study was to improve the depolymerization methodology by using microwave irradiation.

A 2-factor, 3-level full factorial design (3^2) was employed for optimizing the reaction conditions using irradiation power (levels from 50 to 350 W) and time (levels from 10 to 50 s) as independent variables. The optimized conditions were then applied to characterize the proanthocyanidins from fibers of two Brazilian coconut varieties.

2. Experimental

2.1 Materials

HPLC grade acetone, ethyl acetate, acetic acid, and methanol were obtained from Tedia (Rio de Janeiro, Brazil). 2,5-dihydroxybenzoic acid (DHB), phloroglucinol, hydrochloric acid 12N, ascorbic acid, sodium bicarbonate, trifluoroacetic acid (TFA), sodium chloride, (+)-catechin, and (-)-epicatechin were purchased from Sigma-Aldrich (USA). Deionized water was obtained from a Milli-Q water purification system (Millipore Corporation, UK).

2.2 Botanical samples

Samples of *C. nucifera* var. *typica* A (CCR) were collected in Aracajú, Sergipe, Brazil and authenticated by Dr. Benedito Calheiros Dias from Centro de Pesquisas do Cacau, Bahia, Brazil, where a voucher specimen was deposited (CPC 2190). *C. nucifera* var. *typica* (CCO) samples were collected in the Campo Experimental de Itaporanga, Embrapa Tabuleiros Costeiros, Sergipe, Brazil. The plant material was authenticated by Humberto Rollemberg Fontes and a voucher specimen was deposited in the Herbarium of Universidade Federal de Sergipe (ASE 13.631).

2.3 Extraction and purification procedures

The dried grounded husk fibers of both varieties (1.0 g, in triplicate) were extracted exhaustively with acetone:water 6:4 (v/v). The extractions were made in three cycles of 10 min in an ultrasound bath. The obtained extracts were vacuum filtered, the acetone was evaporated at 35 ºC in a rotary evaporator, and the material was later lyophilized. Part of the extract was dissolved in 100 mL of water and then partitioned with the same volume of ethyl acetate three times. The organic phase was evaporated in a rotary evaporator, and the aqueous fraction was lyophilized.

2.4 Phloroglucinolysis

The reaction followed the protocol published by Kennedy (2002)[37] with slight modifications. The phloroglucinol solution was constituted of phloroglucinol (5.0 g), ascorbic acid (1.0 g), 0.84 mL of 12N HCl, and MeOH for 100 mL solution. Sodium bicarbonate (0.33 g) was dissolved in water Milli-Q for 100 mL solution forming the 40 mM final concentration. The lyophilized aqueous fraction (5.0 mg) was weighted in a sealed caped tube and added 1 mL of the phloroglucinol solution in a water bath at 50 ºC for 20 min. Following the end of the reaction, an aliquot of 200 µL was transferred to a 2 mL vial and neutralized with 1 mL of the sodium bicarbonate solution.

2.5 HPLC-DAD analysis

HPLC-DAD analysis followed the protocol published by Kennedy (2002)[37] in an Agilent 1200 Series HPLC. The neutralized samples were injected in a ReproSil-Pur C-18 (250 x 4.6 mm, 5 µm, Dr. Maisch GmbH) and guard column with the same material. The flow of 1 mL/min, λ= 280 nm, and injected volume of 20 µL. Mobile phase A was 1% aqueous acetic acid, and phase B was MeOH. The elution occurred in gradient mode, starting with 5% of B for 10 min, 5-20% of B in 20 min, 20-40 % of B for 25 min. The column was then washed (90% of B for 10 min) and reequilibrated. All samples were analyzed in triplicate. Identification occurred using previously published retention factors[36,37] and (+)-catechin and (-)-epicatechin analytical standards. The mean degree of polymerization (mDP) was calculated as the following equation.

$$mDP = \frac{extension + terminal\ units\ (in\ moles)}{terminal\ units\ (in\ moles)}$$

2.6 Optimization of microwave-assisted phloroglucinolysis

A 3^2 full factorial design with center points were used to optimize the reaction conditions with power and reaction time as independent factors. The levels evaluated are displayed in **Table 1**, the results were obtained in duplicate, and the response area integral (HPLC analysis) of (-)-epicatechin-(4β→2)-phloroglucinol was chosen as the dependent variable for the optimization. All the experiments used CCR aqueous lyophilized fraction and were carried out in random order. The software Statistica® 10.0 (Statsoft Inc, USA) was used for response surface analysis of factors.

2.7 Microwave-assisted phloroglucinolysis

All reactions occurred in a Biotage® initiator+ in glass vials sealed with caps and magnetic stirring. The lyophilized samples were weighted (6.0 mg) inside the vials. The phloroglucinol solution was prepared with 10 g/L phloroglucinol and 0.1 N HCl in Milli-Q water and the 40 mM aqueous sodium bicarbonate solution. The phloroglucinol solution (2 mL) was added to the glass vial, vortexed for 30 s before starting the reaction time. Afterward, 4 mL of the $NaHCO_3$ solution was added to neutralize the acid, and the inner pressure was reduced with a syringe.

Table 1 - 3^2 factorial design used to evaluate the microwave-assisted optimized conditions

		Level		
	Factors	-1	0	+1
	A: microwave irradiation (Watts)	50	200	350
	B: reaction time (seconds)	10	30	50
Exp.	A	B	(-)-epicatechin-(4β→2)-phloroglucinol peak area (mAU)	
1	-1	-1	83.2	123.9
2	-1	0	102.5	92.1
3	-1	+1	178.6	355.3
4	0	-1	132.2	205.6
5	0	0	2586.3	2426.2
6	0	+1	761.6	233.9
7	+1	-1	705.0	874.3
8	+1	0	295.2	391.7
9	+1	+1	0[a]	0[a]

[a]data not acquired

2.8 HPLC-DAD-ESI-TOF after phloroglucinolysis

After the microwave-assisted reaction, the samples were analyzed in an Agilent 1200 Series Gradient HPLC System with a quaternary pump, autosampler, and column oven. All analyses occurred in a Poroshell 120 C-18 (100 x 2.1 mm, 2.7 µm, Agilent) with a guard-column of the same material, a flux of 0.3 mL/min, oven temperature of 40 ºC, λ= 280 nm, injection volume of 2 µL. All samples were injected in 1 mg/mL concentration. Mobile phase A was a 0.5% (v/v) of aqueous formic acid, and phase B was MeOH. The elution occurred in a gradient mode, with 10% of B for 5 min, 10-40% of B in 10 min. The column was washed (90% of B for 10 min) and reequilibrated (for 10 min). The mass spectrometry analysis occurred in a Bruker MicrOTOF II equipped with a Z-spray electrospray interface. The results were obtained with the following settings: in negative mode, with a range of 100 to 1500 Da and collision energy of 10 eV. The drying-gas flow rate of 10.0 L min^{-1}; drying gas temperature of 200 ºC; set capillary of 3000 V; set endplate offset of 500 V; nebulizer pressure of 4.0 bar. All analytes were acquired using formic acid and sodium hydroxide as the internal standard. The samples were analyzed in triplicates with identification using (+)-catechin and (-)-epicatechin analytical standards and the MS data. The mean degree of polymerization (mDP) was calculated with the equation described in 2.4.

2.9 MALDI-TOF analysis

A MALDI-TOF Autoflex Speed Bruker spectrometer was used with a nitrogen laser (337 nm). Aqueous fractions (2 mg) were dissolved with 0.1 % aqueous trifluoroacetic acid and diluted to 1:10 with DHB matrix solution (0.1% TFA). An aliquot of 1 µL aqueous sodium chloride (1 mg/mL) was added to the solution and applied to the MALDI plate (duplicate). After drying was analyzed in positive mode with a mass range from *m/z* 600-3,000 was recorded using linear mode with a delay of 104 ns and an acceleration voltage of +20 kV. For the DHB matrix, final spectra were generated by summing 2 laser shots accumulated per profile and 100 profiles per sample (200 laser shots total per spectrum) using a Peptide Calibration Standard (Bruker). Data were processed using mMass 5.5.0 software (Strohalm M.®).

3. Results and Discussion

In this study, acetone:water 6:4 (v/v) was used for the first time to extract husk fibers of *C. nucifera*. In previous reports, water decoction was the extraction method used, resulting in a 5% yield.[19] Ultrasound-assisted extraction provided four times higher yield, as shown in **Table 2**. After acetone evaporation and water lyophilization, a liquid-liquid partition with ethyl acetate:water 1:1 (v/v) allowed the separation of the low weighted polyphenols, soluble in EtOAc, from the high weighted proanthocyanidins, soluble only in the aqueous fraction. The yield for both varieties was almost 75%, showing that oligomeric proanthocyanidins represent most of the extract.

All reactions used the lyophilized aqueous fraction to reduce any interference in the mDP caused by (+)-catechin and (-)-epicatechin present in the husk fibers extracts.[18,19,39] The original phloroglucinolysis method relies on the acid-catalyzed cleavage of proanthocyanidins and the subsequent attack by strong nucleophiles. After the cleavage of the interflavan bond, flavan-3-ol units are converted into extender units corresponding to phloroglucinol adducts, whereas the terminal units are released as monomeric flavan-3-ols.[31,36,37] Since the product is formed after these steps, the peak area figures for the extension units in the RP-HPLC analysis is an appropriate dependent variable to evaluate the factorial design. Accordingly, the RP-HPLC analysis ((-)-epicatechin-(4β→2)-phloroglucinol) were taken as dependent variable values in the factional design.

Table 2 - General information of *C. nucifera* husk fibers.

Sample		CCR	CCO
Extraction yield (%)		20.45±2.44[a]	16.90±0.66[a]
Liquid-Liquid Partition	Crude extract (mg)	300.3	380.5
	EtOAc fraction (mg)	54.4	89.3
	Yield (%)	18.1	23.5
	Aqueous fraction (mg)	225.4	281.9
	Yield (%)	75.1	74.1
mDP by phloroglucinolysis		4.49±0.19[a] [4.16][b]	4.87±0.14[a] [2.81][b]
Overall *cis/trans* molar ratio		78.51±0.04[a] [5.22][b]	51.55±0.04[a] [7.59][b]
mDP by microwave-assisted phloroglucinolysis		7.06±0.49[a] [6.93][b]	9.98±0.41[a] [4.06][b]
Overall *cis/trans* molar ratio		81.30±0.02[a] [2.53][b]	23.36±0.02[a] [7.17][b,c]

[a] mean ±SD [b][RSD] [c]Data obtained in duplicate

The RP-HPLC quantitative phloroglucinolysis products analysis allows the determination of proanthocyanidins nature and proportion of constitutive units. It also enables the calculation of their mean degree of polymerization (mDP). Therefore, these parameters were also used in the appraisal of the microwave-assisted method for this reaction.

In the microwave-assisted phloroglucinolysis, the factors chosen were the power of microwave irradiation (50-350 W) and reaction time (10-50 s). All experiments used CCR aqueous fraction in duplicate. However, before the factorial design experiments were executed, a few modifications in the protocol were necessary. Methanol (dielectric constant, ε=32.5) was replaced by water (ε=80.1) as this solvent has a more favorable interaction with microwave energy[38]. As phloroglucinol is less soluble in water, its concentration was reduced by 5-fold, but keeping its concentration in molar excess. Ascorbic acid used in the original protocol to preserve product integrity overtime was removed to facilitate the proanthocyanidin depolymerization set up.

Table 1 displays the factorial design and the main results. In the experiment, the microwave irradiation of 350 W and 50 s produced excess energy, and Biotage® initiator+ safety dispositive interrupted the reaction due to increased pressure inside the reaction vial. Other energic conditions (200 W, 50 s) and (350 W, 30 s) also exhibited side reactions and a decrease of (-)-epicatechin-(4β→2)-phloroglucinol peak area (Figure S1). After evaluating the interaction effects, the ANOVA table (Table S1) indicated that factors do not display a linear response, with the error calculated using all duplicate points instead of the central point triplicate. The results show that microwave irradiation was a significant variable, while the irradiation time had no statistical significance. Therefore 30 s was chosen as enough reaction time. The central point of 200 W and 30 s were the optimized conditions for the microwave-assisted phloroglucinolysis (Figure 1). Under optimized conditions, the reaction time was reduced by 40-fold, and no peaks related to epimerization were noticed. Notoriously, C-2 epimerization can be a significant side reaction under some conditions and influence the total amount of terminal flavan-3-ols, especially (-)-epicatechin.[31,40] A 20 min reaction time was established in the original protocol to avoid (-)-epicatechin epimerization.[36] The microwave-assisted phloroglucinolysis was also able to respond better with less phloroglucinol and water as the reaction solvent. Additionally, the use of a 100 mm Poroshell 120 C-18 reduced the chromatographic analysis time by 2-fold using less mobile phase.

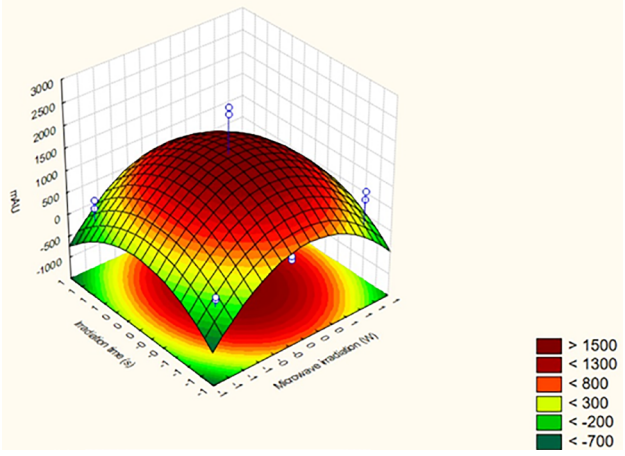

Figure 1. Response surface plot showing interaction effects of microwave irradiation and reaction time in CCR aqueous fraction

Firstly, both varieties were characterized by applying conventional phloroglucinolysis (Table 2, Figures S2 and S3). The results found revealed similar composition in extension and terminal units, with (-)-epicatechin as the only extension unit detected and a mixture of (+)-catechin and (-)-epicatechin as terminal units for both varieties. The percentage of (-)-epicatechin (78.51±0.04 %), as terminal unit, found for CCR was slightly higher than for CCO (51.55±0.04 %). The mDP, calculated in triplicate, was higher for the CCO aqueous fraction with an average of 4.9 (CCR displayed an average value of 4.5). (-)-Epicatechin is the most common flavan-3-ol unit in nature.[37] However, the higher amount found in CCR may be significant in terms of its biological activities. For example, a recent study identified antitumorigenic activity against human prostate cancer cells exclusively for epicatechin oligomers.[41]

Both varieties displayed the same constitutive units after the microwave-assisted phloroglucinolysis with (-)-epicatechin as the only extender unit (Table 2, Figures S4 and S5). (-)-Epicatechin was found as the major component as terminal unit in the CCR aqueous fraction (81.30±0.02%), while for CCO, the *cis*/*trans* molar ratio displayed a decreased value for this flavan-3-ol (23.36±0.02%), indicating (+)-catechin as the most abundant one (76.64±0.02%). Additionally, both exhibited significantly higher mDP values than the values obtained by applying the conventional protocol so that microwave irradiation may, hypothetically, increase the cleavage of higher proanthocyanidins leading to a more accurate determination of mDP values. The two most used chemical degradation of proanthocyanidins are thiolysis (using benzyl mercaptan as a nucleophile) and phloroglucinolysis (that uses phloroglucinol). Phloroglucinol is odorless and provides a better selectivity for the formation of 3,4-*trans* adducts, while benzyl mercaptan has an unpleasant odor and requires special handling with specialized fume hoods.[36] Benzyl mercaptan is a more powerful nucleophile, tending to generate higher yields than phloroglucinol.[42] However, there are reports on the presence of side reactions when the mercaptan is used, particularly epimerization rates and product instability, making the composition estimation difficult.[43]

The aqueous fiber extracts from CCR and CCO were also analyzed by positive MALDI-TOF. Table 3 displays the assignments of the labeled peaks of the mass spectra (Figures S6 and S7). All masses correspond to sodium ion adducts (+23 Da). A repetitive pattern of peaks was associated with specific oligomer series for B-type procyanidin with repetition unit masses of 288 Da. A B-type procyanidin sequence was identified for both varieties with DP = 3-7 for CCR and DP = 3-9 for CCO aqueous fraction. The MALDI-TOF data thus supports the microwave-assisted mDP values found for both samples.

Previous reports on proanthocyanidins from *C. nucifera* showed its fruit and its inflorescences displaying B-type procyanidin oligomers and heterogeneous oligomers with only (-)-epicatechin and (-)-epiafzelechin as the monomeric units.[44,45]

Also, to our knowledge, this is the first time *C. nucifera* procyanidins were identified using the methodology reported here. In addition, this is the first report of phloroglucinolysis assisted by microwave irradiation as a way to assess procyanidins structural parameters.

4. Conclusion

An improved phloroglucinolysis protocol used for determining structural parameters of procyanidins was optimized and applied in samples of *C. nucifera* fiber extracts. The conventional phloroglucinolysis protocol takes 20 min for the reaction to occur and an additional 70 min run in the HPLC analysis of the products. Our improved microwave-assisted phloroglucinolysis provides shorter reaction time and faster runs in the HPLC analyses.

The new protocol application to two varieties of *C. nucifera* produced better results with a more accurate mDP determination and similar procyanidin composition as the conventional protocol. CCR and CCO husk fibers extracts exhibited a similar chemical characterization with a small variation to CCO which displayed a higher mDP value.

These results indicate the *C. nucifera* fibers as a potential source of proanthocyanidins with industrial applications and an alternative raw material to be exploited sustainably in a more circular production chain.

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Table 3. CCR and CCO aqueous fractions MALDI-TOF $[M+Na]^+$ mass spectra

		CCR	CCO	
DP	Predicted $[M+Na]^+$(Da)	Observed $[M+Na]^+$ (Da)	Observed $[M+Na]^+$ (Da)	Monomeric units (epi)catechin
3	889.8	889.9	889.8	3
4	1178.0	1178.2	1178.1	4
5	1466.3	1466.5	1466.4	5
6	1754.5	1754.7	1754.7	6
7	2042.8	2044.1	2042.0	7
8	2331.0	-	2330.1	8
9	2619.3	-	2618.0	9

Previous reports on proanthocyanidins from *C. nucifera* showed its fruit and its inflorescences displaying B-type procyanidin oligomers and heterogeneous oligomers with only (-)-epicatechin and (-)-epiafzelechin as the monomeric units.[44,45]

Author Contributions

Conceptualization: AJRS; Methodology: GRM and RMB; Validation: RMB and AJRS; Formal analysis: GRM and RMB; Investigation: AJRS, CSA, and DSA; Resources: AJRS, CSA, and DSA; Writing - Original Draft Preparation: GRM and RMB; Writing - Review & Editing: AJRS, CSA, DSA; Supervision: AJRS, CSA, and DSA; Project administration: AJRS, CSA, and DSA; Funding acquisition: CSA, and DSA.

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