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Research Article

OBTENTION OF PROTEIN CONCENTRATE AND POLYPHENOLS FROM *MACADAMIA* (*MACADAMIA INTEGRIFOLIA*) WITH AQUEOUS EXTRACTION METHOD

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ABSTRACT

Objective: The aim of this study was to obtain protein concentrates from *Macadamia* using alkaline pH at different pHs of precipitation with water to analyze the protein isolates using the native-polyacrylamide gel electrophoresis (PAGE), sodium dodecyl sulfate-PAGE (SDS-PAGE) electrophoresis, and reversed-phase high-performance liquid chromatography (RP-UHPLC) methods.

Methods: *Macadamia* protein concentrates were obtained using the isoelectric precipitation method at different pHs using water as solvent. Proteins were analyzed using the native-PAGE, SDS-PAGE electrophoresis, and RP-UHPLC methods.

Results: A yield of 36.57±0.17[a] of protein concentrate of defatted *Macadamia* flour at pH 6.0 with a 51.564% of protein was obtained using the Dumas method. Polypeptides profile was identified in the 11-63 kDa range. Total polyphenols content was high at pH 5.0 with a value of 367,340 mg gallic acid equivalent equivalents/100 g.

Conclusions: *Macadamia* seed is a good source of proteins. Native-PAGE, SDS-PAGE, and RP-UHPLC are good methods to identify the *Macadamia* protein isolate in the presence of water.

Keywords: *Macadamia* protein concentrate, Polyphenols, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Reversed-phase high-performance liquid chromatography.

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INTRODUCTION

Tree nuts are dry fruits with one seed, in which the ovary wall becomes hard at maturity. Usually, consumable tree nuts include almond, Brazil nut, cashew, hazelnut, *Macadamia*, pecan, pine nut, pistachio, and walnut; the consumer definition also includes peanut, which is botanically legume, but has a nutrient profile similar to those of tree nuts and it is thus identified as part of the nut food group [1-3].

Macadamia is a genus of flowering plants in the family Proteaceae and is cultivated for its edible kernels. There are four species of *Macadamia*, and all occur in subtropical rainforests along the East coast of Australia. Only two of the species, *Macadamia integrifolia* and *Macadamia tetraphylla*, generate edible nuts and are of commercial importance. *M. integrifolia*, commonly known as the smooth-shell *Macadamia*, provides kernels with higher quality, whereas *M. tetraphylla*, known as the rough-shell *Macadamia*, is more adaptable and can grow more easily at low temperatures or over a wider range of temperatures [4,5]. The other two species, *Macadamia ternifolia* and *Macadamia jansenii*, are inedible, as they contain cyanogenic glycosides which are toxic [6].

The *Macadamia* is the only Australian plant that has been domesticated on a commercial scale as a food crop. *Macadamia* is cultivated mainly in Australia, the USA (Hawai and California), and South Africa. There are also expanding industries in Brazil, Guatemala, and Kenya, and smaller industries in New Zealand, Malawi, Paraguay, Ecuador, and other countries [7-9]. The worldwide production of *Macadamia* sp. is approximately 44,000 metric tons (kernel), 86% of which come from Australia, South Africa, Kenya, the United States, and Malawi. Australia is the world's largest producer, with approximately 14,100 metric tons [10]. *M. integrifolia* contains approximately 70% of oil and its oil is the most highly monounsaturated fatty acids, which possibly help lower blood cholesterol, and reduce the risk of heart disease and also containing 7.9% of protein [11,12]. Its defatted flours contain between 30.40% and 36.45% of protein. The *Macadamia* kernel is a rich source of lipids, proteins, and important micronutrients. However, its chemical composition can vary considerably depending on the variety, seed maturity, location, and growth conditions [7]. The aim of this study was to obtain *Macadamia* protein concentrates using the isoelectric precipitation method and phenolic component using water as solvent.

METHODS

Protein concentrates from *Macadamia* nuts

Commercial *Macadamia* nuts (Rey *Macadamia*) were purchased at the supermarket in Ecuador. *Macadamia* protein concentrate was prepared according to Martınez and Añón (1996) [13] with modifications. The defatted flour was suspended in water in a 1:10 w/v, and the suspension was adjusted at pH 8.0 by adding 2M NaOH. The suspension was stirred during 1 hrs and then centrifuged at 4500 g for 30 minutes at 25°C. The supernatant was adjusted at pHs 2.0; pH 3.0; pH 4.0; pH 5.0, and pH 6.0 with 2 NHCl and centrifuged for 20 minutes at 4500 g. The pellet was suspended in a small volume of water, neutralized with 0.1 M NaOH, lyophilized and then frozen at −20°C. The content of protein isolate was determined using the Dumas method (VELP NDA 701 Dumas Nitrogen Analyzer). The factor used to calculate the percentage of protein was (% Nx pf=% PROT): 5.70 [14].

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Native-PAGE and SDS-PAGE electrophoresis of *Macadamia* protein concentrates were carried out according to the method proposed by Laemmli (1970) [15] using 4-8% and 4-12% polyacrylamide gel in a Mini-PROTEAN electrophoresis system (Bio-Rad, Hercules, CA, USA). Polypeptide bands were stained in Coomassie Brilliant Blue G-250 for 12 hrs. Relative molecular masses of protein were determined by a comparison to molecular weight markers (Bio-Rad, Hercules, CA, USA).

Extraction of polyphenols

After the precipitation of proteins from *Macadamia* using water at different pHs, the supernatants were lyophilized during 48 h. Then, the dry samples were stored at −20°C.

Determination of total polyphenols

Total phenolics in the obtained extracts were estimated by a colorimetric assay based on the procedures described by Singleton and Rossi (1965) [16] with some modifications. Briefly, 1 mL of sample was mixed with 1 mL of Folin and Ciocalteu's phenol reagent. After 3 minutes, 1 mL of saturated sodium carbonate solution was added to the mixture and adjusted to 10 mL with distilled water. The reaction was kept in the dark for 90 minutes. Then, the absorbance was read at 725 nm using a spectrophotometer (Thermo Scientific Evolution 200). Gallic acid was used for constructing the standard curve (0-0.075 mg/mL). The results were expressed as mg of gallic acid equivalents (GAEs)/100g of dried sample.

Analysis of concentrate *Macadamia* protein using reversed-phase high-performance liquid chromatography (RP-UHPLC)

All concentrate *Macadamia* proteins were analyzed using RP-UHPLC on Agilent 1200 infinity series UHPLC System (Agilent Technologies, Waldbronn, Germany). The variable wavelength detector was 280 nm. The column used was EC C18 (Agilent Poroshell 120, 4.6 × 50 mm × 2.7 µm of particle size). Samples were eluted at 1.0 mL/minutes with a linear gradient from 0% to 70% of solvent B (acetonitrile and trifluoroacetic acid [TFA], 1000:0.270 v/v) in solvent A (water and TFA, 1000: 0.370 v/v) during 10 minutes. The injection volume was 100 µL for each duplicated sample.

Statistical analysis

Results are presented as means±standard deviation (SD) from the three replicates of each experiment. Differences between mean values were determined using the analysis of variance (ANOVA). The *post-hoc* analysis was performed with the Tukey's test. All tests were considered significant at $p<0.05$. Statistical analyses were performed using the software package Prism 4 for Windows, version 4.3 (GraphPad Software Inc., www.graphpad.com).

RESULTS

Nuts of *Macadamia* were used to obtain the defatted flour of *Macadamia*. The defatted *Macadamia* flour was used to obtain *Macadamia* concentrate protein using the isoelectric precipitation method at different pHs (pH 2.0; pH 3.0; pH 4.0; pH 5.0, and pH 6.0) with water as solvent. The highest yield was obtained at pH 6.0 with 36.57±0.17%. Yields for pH 2.0; pH 3.0; pH 4.0, and pH 6.0 presented no statistical differences. Only at pH 5.0, the treatment presented statistical differences with $p<0.05$ (Table 1). The contents of protein in the *Macadamia* concentrates protein were analyzed using the Dumas method. The treatments at pH 3.0; pH 4.0; pH 5.0, and pH 6.0 presented a higher content of protein. The best treatment identified was at pH 5.0 with a value of 52.962%. At pH 4.0 and pH 6.0, there are no statistical differences with $p<0.05$ (Table 1).

Values are expressed in grams per 100 g of protein. Values are means±SD of three determinations. Different letters show statistical differences between the groups ($p<0.05$) ANOVA and Tukey's test.

The profile proteins from *Macadamia* were analyzed using the electrophoresis native and SDS-PAGE methods.

Native-PAGE

In the gel of polyacrylamide, we can observe one band at all pHs assayed with molecular weight higher than 198 kDa. The gel shows bands between 6.5 and 41 kDa. These bands were strongly tinged with the solution Blue Coomassie (Fig. 1).

SDS-PAGE

In the presence of the reductor agent 2-β-mercpatoethanol, the gel shows that at pHs 3.0; pH 4.0; pH 5.0, and at pH 6.0, there are higher contents of proteins as all bands were strongly tinged with the solution of Blue Coomassie used in this assay. Two bands can also be observed, one corresponding at 50 kDa approximately and the second one with a molecular weight of 45 kDa approximately. These bands correspond to the globulin fraction from *Macadamia*. Two bands of 25 kDa were identified as the albumin fraction of *Macadamia*. At pH 2.0, the bands were stained lower, and this result is in accordance with results of protein content obtained using the Dumas method (Fig. 2). The analysis of the SDS-PAGE electrophoresis method in the absence of the reductor agent (2-β-mercaptoethanol) shows two bands with higher intensity at pH 3.0; pH 4.0; pH 5.0, and pH 6.0. Only at pH 2.0, we can observe a lower intensity of these bands. The molecular weight of these bands ranges between 45 and 50 kDa. Moreover, it was possible to observe two bands with lower molecular weights of 25 kDa (Fig. 3).

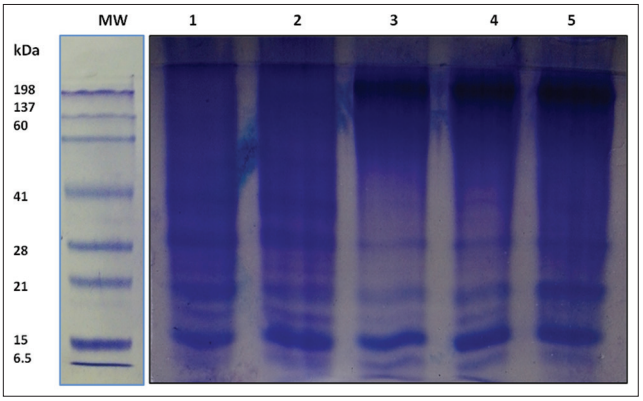

Fig. 1: Native-polyacrylamide gel electrophoresis of proteins from *Macadamia*. MW: Molecular weight. Lane 1: Concentrate of *Macadamia* obtained at pH 2.0; Lane 2: Concentrate of *Macadamia* obtained at pH 3.0; Lane 3: Concentrate of *Macadamia* obtained at pH 4.0; Lane 4: Concentrate of *Macadamia* obtained at pH 5.0 and Lane 5: Concentrate of *Macadamia* obtained at pH 6.0

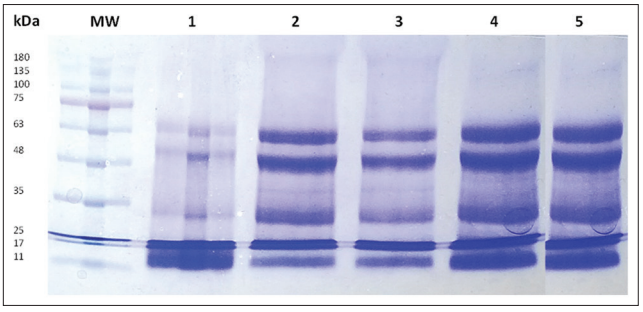

Fig. 2: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins from *Macadamia* without the reductor agent (2-β-Mercaptoethanol). MW: Molecular weight; Lane 1: Concentrate of *Macadamia* obtained at pH 2.0; Lane 2: Concentrate of *Macadamia* obtained at pH 3.0; Lane 3: Concentrate of *Macadamia* obtained at pH 4.0; Lane 4: Concentrate of *Macadamia* obtained at pH 5.0 and Lane 5: Concentrate of *Macadamia* obtained at pH 6.0

RP-UHPLC

All *Macadamia* protein concentrates were analyzed with the RP-UHPLC method during 12 minutes. The chromatograms show the profile of proteins obtained from *Macadamia* nuts. Fig. 4a and b show two peaks with high capacity of absorbance at 280 nm with low hydrophobicity. The peaks have the same time of retention. These proteins are acid proteins as proteins were obtained at low pH of precipitation. At pH 4.0; pH 5.0, and pH 6.0, the peaks present less intensity with 280 nm (Fig. 4c-e).

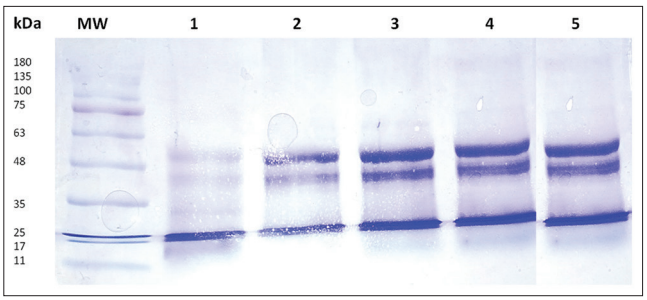

Fig. 3: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins from *Macadamia* with the reductor agent (2-β-Mercaptoethanol). MW: Molecular weight; Lane 1: Concentrate of *Macadamia* obtained at pH 2.0; Lane 2: Concentrate of *Macadamia* obtained at pH 3.0; Lane 3: Concentrate of *Macadamia* obtained at pH 4.0; Lane 4: Concentrate of *Macadamia* obtained at pH 5.0 and Lane 5: Concentrate of *Macadamia* obtained at pH 6.0

Content of polyphenols

The content of polyphenols presents in the supernatant after separation of *Macadamia* concentrate protein was evaluated. The content of polyphenols was determined using a colorimetric assay with the Folin and Ciocalteu's phenol reagent. The highest content of polyphenols was obtained at pH 5.0 with a value of 367,340 mg GAE/100 g protein of sample. At pH 6.0, the content was 289,150 mg GAE/100 g protein sample. At acid pHs, the content values of polyphenols were low (Table 2).

Values are means±SD of three determinations. Different letters show a statistical difference between the groups (<0.05) ANOVA and Tukey's test.

DISCUSSION

Protein concentrates are considered with a protein content of 35-80% on a dry basis. Protein isolates are defined to have protein content higher than 85%. Whey protein concentrate and Whey protein isolate are used in the food industry to obtain pure proteins, hydrolysates, and ingredients in many foods such as the production of infant formula [17,18]. In this study, it was possible to obtain *Macadamia* protein concentrates at pH 3.0; pH 4.0; pH 5.0, and pH 6.0. Only at pH 2.0, the *Macadamia* protein concentrate was difficult to obtain. The gel SDS-PAGE confirms that at pH 2.0 bands were very little stained. The best treatment was at pH 5.0 with a value of 52.962% protein content on a dry basis using the Dumas method.

Legumins, vicilins, and 2S albumins represent major seed storage of protein components of nuts. Vicilins, also called 7S globulins, comprise one well-known class of storage proteins and can constitute as much

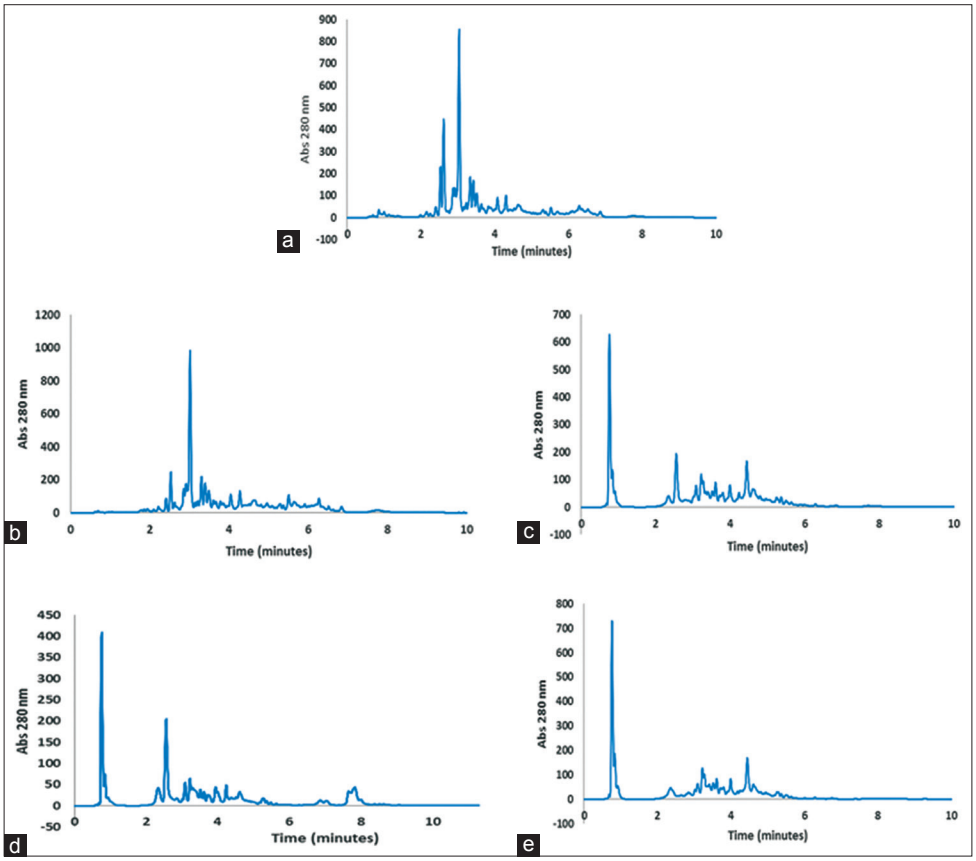

Fig. 4: Reversed-phase high-performance liquid chromatography of *Macadamia* protein concentrate obtained at different pHs. (a) *Macadamia* protein concentrate at pH 2.0 (b) *Macadamia* protein concentrate at pH 3.0 (c) *Macadamia* protein concentrate at pH 4.0 (d) *Macadamia* protein concentrate at pH 5.0 (d) *Macadamia* protein concentrate at pH 6.0. Column Zorbax EC C18 (Agilent Poroshell 120, 4.6 × 50 mm × 2.7 µm of particle size)

Table 1: Content of *Macadamia* protein concentrates obtained at different pHs and content of protein using the Dumas method

Sample	pH 2.0	pH 3.0	pH 4.0	pH 5.0	pH 6.0
% Concentrate	34.37±0.05[a]	34.41±0.19[a]	34.55±0.41[a]	33.41±0.06[b]	36.57±0.17[c]
% protein Dumas	20.923[a]	42.324[b]	51.594[c]	52.962[d]	51.564[c]

Different letters show statistical difference between the groups (p<0.05) ANOVA and Tukey's test

Table 2: Content of polyphenols *Macadamia*

Sample	pH 2.0	pH 3.0	pH 4.0	pH 5.0	pH 6.0
% Polyphenols	256.200±0.56[a]	180.256±0.56[b]	218.230±0.00[c]	367.340±0.00[d]	289.150±0.00[e]

Different letters show statistical difference between the groups (p<0.05) ANOVA and Tukey's test

as 70±80% of the total seed protein [19,20]. Using the SDS-PAGE electrophoresis method with a reductor agent, it was possible to observe globulins and albumins. Albumins present low molecular weight with 11 kDa approximately.

Nuts contain high amounts of vegetable proteins and fat-soluble bioactives as unsaturated fatty acids, phytosterols, phospholipids, phytostanols, essential oils, sphingolipids, tocopherols, tocotrienols, terpenoids, and squalene. Nut seeds are rich in a variety of other nutrients and provide dietary fiber, vitamins (such as folic acid, niacin, vitamin B6, and vitamin E), minerals (such as calcium, magnesium, and potassium), and many other phytochemicals (such as phenolic acids, flavonoids, lignin, hydrolysable tannins, proanthocyanidins, carotenoids, alkaloids, coumestan, and phytates). A healthy diet supplemented with one daily serving of nuts prevents cardiovascular events and possibly the development of other chronic diseases, including Type II diabetes, cancer, high blood pressure, and neurodegenerative diseases. Tree nuts and their co-products (skin or testa, hard shell, green leafy cover, hull, and leaf, among others) are rich sources of phytochemicals that possess multifunction alproperties such as antioxidant activities, anticarcinogenic, antimutagenic effects as well as antiproliferative potential [1,21]. In *Macadamia* nuts, it has been identified catechol, pyrogallol, and 3,4,5-trihydroxy phenolic compounds and its antioxidant activity in refined oil from *Macadamia* has been evaluated [22].

The Folin-Ciocalteu's reagent assay is the common method used to determine the total phenolic content (TPC) of nuts. TPCs of nuts, expressed as mg of GAE/100 g of sample, were reported in Phenol-Explorer database [23,24] with a range between 47 and 3673. Chestnut contained the highest TPC (1580-3673 mg GAE/100 g), followed by pecan (1284-2016), walnut (1558-1625), pistachio (867-657), hazelnut (291-835), peanut (0.1-420), almond (47-418), Brazil nut (112-310), cashew (137-274), *Macadamia* (46-156), and pine nut (32-68). The TPCs of nuts range from 32 to 1650 mg GAE/100 g of sample, with pecan, walnut, and pistachio having the highest values [25,26]. In the study reported by Kornsteiner *et al*. (2006), TPC (expressed as mg of GAE/100 g fresh weight) of nuts decreased in the order of walnut (1625) >pecan (1284) >pistachio (867) >peanut (420) >hazelnut (291) >almond (239) >Brazil nut (112) >*Macadamia* (46) >pine nut (32). In other studies, it was reported 86 mg GAE/100 g f sample from skin waste *M. tetraphylla* obtained with aqueous extraction [27]. In this study, the supernatant at pH 5.0 present 367,340 mg GAE/100 g protein of sample. Due to the above facts previously mentioned, *Macadamia* protein concentrate can be used as functional ingredients with bioactivity.

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