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Research Article

Compositional Study and Antioxidant Potential of Polyphenols Extracted from Corn By-Products, Using Ultrasound Extraction Method

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Abstract

The aim of this research was to determine the polyphenol composition and antioxidant activity of corn silk hairs. Two different methods were used to extract polyphenols: a (i) conventional extraction method with 70oC and 15 min; and an (ii) ultrasound extraction method with 40 kHz, for 15 minutes. A HPLC-DAD-UV/VIS system as was used to detect the polyphenols in the samples. The total antioxidant activity was measured by the spectrophotometric $ABTS^{\bullet+}$ free radical assay. The main polyphenols found were caffeic acid, chlorogenic acid, ferulic acid, pelargonidin and apigenin. The average content of each polyphenol detected as well as the total antioxidant activity were significantly dependent ($0.05<P<0.001$) of extraction method used and both were higher in samples extracted under ultrasounds. The total antioxidant activity was highly correlated with the presence of pelargonidin ($P<0.01$), ferulic acid ($P<0.01$) and apigenin ($P<0.01$). Based in our results, corn silk hairs can be used to extract polyphenols with enhanced biological activity and the ultrasounds shown to be very practical, feasible and accurate to maximize their polyphenol content. The knowledge regarding the composition of corn silk hairs can enable their use in pharmaceutical or industrial heath sector to produce coproducts with high biological added value.

Keywords: Corn wastes; Phytochemicals; Bioactivities; Enhanced value

Introduction

Corn (*Zea mays* L.) silk hairs also known as *Maydis stigma* is a popular corn byproduct which has been used for decades as medicinal herb in folk medicine of different countries all over the world even if the scientific evidences of their beneficial effects on human health are often contradictory. Corn silk hairs are usually considered as waste and they are discarded during industrial transformation of baby corn and super sweet corn products. They contain several nutritional compounds such as proteins, vitamins and minerals as well as phytochemical like phenolic acids, flavonoids and other polyphenols, which are claimed to be responsible for several beneficial effects on human health [1]. Corn silk hairs has been claimed to be a good herb to treat depression, hypertension, hyperglycemia, hepatitis, cystitis, gout, kidney stones, diabetes, nephritis and prostatitis [2-6]. Other uses include teas, powders and supplements to treat urinary related problems [7], which have been endorsed to the higher content in flavonoids [8]. Although the derived-products from corn silks such as pills, powders, teas or even other formulations, have been highly marketable all over the world, corn silk hairs are still considered as a waste and highly disregarded by corn industrial producers. As consequence, the opportunities to convert them into high added value coproducts are still limited. Thus, with this study we aim to reinforce the scientific knowledge about the beneficial effects of corn silk hairs trough the evaluation of their polyphenol composition and antioxidant capacity and how those properties linked each other and are affected with extraction method used. Also we want to evaluate if the ultrasonic treatment can be furthermore industrially developed for nutraceuticals and functional coproducts applications.

Material and Methods

Sampling

1 kg of corn silk hairs (*Zea mays* subsp. Mays L.) were collected, dried in a freeze-drier system (UltraDrySystems™, USA), milled into a fine powder, and stored in dark at 4°C until the extraction procedure.

Extraction procedure

Two different extractions methods, conventional and Ultrasound (UE) were performed in order to compare and establish a simple, practical and feasible extraction procedure. In the conventional extraction 100 mg of dried powered was placed in a 10 mL screw cap tubes and mixed with 10 mL of extraction solvent (70% methanol), followed by a vigorous agitation in a vortex (Genie 2, Fisher Scientific, UK). The tubes were then sealed and heated at 70°C (1083, GFL-Gesells chaft ffur Labortechnik mbH, Germany) during 15 minutes, and agitated every 5 minutes. The obtained extracts were then centrifuged (4000 rpm for 15 min (Kubota, Japan)), filtered consecutively through a Whatman No. 1 paper and syringe filters (0.2µm, 13mm Ø, Teknokroma, Spain) to a HPLC amber vials. The extracts were stored at -20°C prior to the LC analysis.

In the UE, the mixtures of 100 mg of dried powder with 10 mL of 70% methanol were placed in an ultrasonic bath device (34cm×10cm×31cm) with 40 kHz transducers annealed to a rectangular container (Bandelin Electronics, Berlin, Germany).The

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Table 1: Linear range, correlation coefficient (R^2), Limit of Detections (LOD) and Limit of Quantification (LOQ) of HPLC system used for polyphenols determination.

Analyte	Linear range (µg.mL^{-1})	Regression equations	R^2 (n=5)	LOD (µg.mL^{-1})	LOQ (µg.mL^{-1})
Chlorogenic acid	5.0-100	y = 21742x + 11157	0.9989	3.2	9.7
Caffeic acid	5.0-100	y = 52076x - 62599	0.9968	5.6	16.9
Ferulic acid	5.0-100	y = 31407x + 16117	0.9989	3.2	9.7
Apigenin	5.0-100	y = 38803x - 41352	0.9977	4.7	14.2
Pelargonodin	5.0-100	y = 168061x - 62214	0.9996	2.0	6.1

Table 2: Accuracy of HPLC system used for polyphenols determination.

Analyte	Prepared concentration (µg.mL^{-1})	Measured concentration (µg.mL^{-1})	Accuracy (%)
Chlorogenic acid	100.0	100.86	100.9
	50.0	49.38	98.8
	25.0	25.64	102.5
	10.0	10.70	107.0
	5.0	4.98	99.6
Caffeic acid	100.0	101.76	101.8
	50.0	46.31	92.6
	25.0	24.62	98.5
	10.0	11.28	112.8
	5.0	6.03	120.7
Ferulic acid	100.0	100.86	100.9
	50.0	49.01	98.0
	25.0	25.64	102.5
	10.0	10.70	107.0
	5.0	4.98	99.6
Apigenin	100.0	101.47	101.5
	50.0	46.90	93.8
	25.0	24.73	98.9
	10.0	11.03	110.3
	5.0	5.87	117.4
Pelargonodin	100.0	100.12	100.1
	50.0	50.34	100.7
	25.0	23.59	94.4
	10.0	10.39	103.9
	5.0	5.56	111.2

tubes were then sealed and irradiated for the pre-set extraction time (15 minutes). Then, the extracts were centrifuged (4000rpm for 15 min.) and the supernatants filtered, first in a Whatman No.1 paper Spartan filters. Finally, the extracts were filtered (0.2µm, 13mm Ø, Teknokroma, Spain) to amber flasks until HPLC analysis.

Extract purification and HCL hydrolysis

To remove impurities and undesirable non-phenolic compounds from the samples, a purification trough resin Sephadex LH20 (Sigma-Aldrich, Tauferkichen, Germany) and an HCl (Sigma-Aldrich, Tauferkichen, Germany) hydrolysis were performed. The columns with Sephadex LH-20, were firstly washed with 10 mL of ultrapure water followed by activation with 30 mL of methanol 20% (methanol: water). Then, 10 mL of extract were applied in the columns, followed by washing with 10mL of methanol 20%. After, the compounds were eluted with 10mL methanol 60%. An aliquot of 300µL of each eluted sample was evaporated until complete dryness. Then the residues were ressuspended in same volume of HCl (2 M in methanol 50%) and heated at 80ºC during 120 minutes. After the extracts were filtered through a syringe filters to HPLC vials amber and stored at -20ºC, until further analysis in HPLC.

Compositional determination by HPLC-DAD-UV/Vis

The phytochemical composition was assessed by HPLC-DAD-UV/Vis system [9]. The eluent was constituted by water with 1% of Trifluoroacetic Acid (TFA) (solvent A) and acetonitrile with 1% TFA (solvent B). Elution was performed using a flow rate of 1 mL.min^{-1} of solvent and a gradient starting with 100% of solvent A. The injection volume of 10µL. Chromatograms were recorded at 280, 320, 370 and 520nm with a C18 column (250 x 46mm, 5µm). The phytochemicals were identified using peak retention time, UV spectra and UV max absorbance bands, compared with those found for external commercial standards. The external standards apigenin, cafeic acid, chlorogenic acid, ferulic acid AND pelargonidin were purchased from Extrasynthese (Cedex, France). All standards were freshly prepared in 70% methanol (methanol: water) at 1.0mg.mL^{-1}. The quantification was performed using the internal standard (naringin, Sigma-aldrich, Germany) method and the levels were expressed as µg.g^{-1} dry weight (dw). Methanol and acetonitrile were purchased from Panreac chemistry (Lisbon, Portugal) and Sigma-Aldrich (Taufkirchen, Germany), respectively. The aqueous solutions were prepared using ultra-pure water (Milli-Q, Millipore).

Total Antioxidant Activity (TAA)

The TAA was measure trough the *in vitro* assay of 2,2′-azinobis-3-ethylbenzothiazoline-6-sulphonate ($ABTS^{\bullet+}$) radical cation [10] in a 96-well microplate. ABTS was generated by mixing a 7mM of ABTS at pH 7.4 (5mM NaH_2PO_4, 5mM Na_2HPO_4 and 154mM NaCl) with 2.5mM potassium persulfate (final concentration) followed by storage in the dark at room temperature for 16 h before use. The mixture was diluted with ethanol to give an absorbance of 0.70 ± 0.02 units at 734 nm using spectrophotometer (U-2000, serial 121-0120, Hitachi Ltd., Tokyo, Japan). For each sample, diluted methanol solution of the sample (100µL) was allowed to react with fresh ABTS solution (900µL), and then the absorbance was measured 6 min after initial mixing. The TAA was expressed as follows: TAA (%) = [((solvent absorbance - sample absorbance) / solvent absorbance) ×100]. All determinations in were performed in triplicate.

Statistical analysis

The results were expressed as mean values ± Standard Deviation (SD) of three replications. A two-way statistical analysis (ANOVA) was performed and Pearson's correlation coefficients were calculated.

Table 3: Polyphenols identified in the current study and respective Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), by elution order.

Phenolic compound identified	Rt (min)	UV (nm)	UV-DAD/VIS bands (nm)	Class
Apigenin	21.66	370	235, 268, 342	Flavone
Pelargonidin	22.41	520	280, 318, 489	Anthocyanin
Chlorogenic acid	22.87	320	242, 300, 325	Hydroxycinnamic acid
Caffeic acid	23.76	320	239, 299, 324	Hydroxycinnamic acid
Ferulic acid	29.91	320	244, 296, 325	Hydroxycinnamic acid

Table 4: Average content of polyphenols in corn silk hairs by-products, expressed as $\mu g.g^{-1}$ dw[1].

	Conventional	Ultrasound	*p-Value*[2]
Chorogenic acid	37.3 ± 0.6	42.4 ± 0.7	*P<0.01*
Caffeic acid	12.0 ± 0.5	13.7 ± 0.5	*P<0.05*
Ferulic acid	42.3 ± 0.6	48.1 ± 0.6	*P<0.001*
Apigenin	6.9 ± 0.6	7.9 ± 0.6	*P<0.001*
Pelargonidin	2.1 ± 0.1	2.6 ± 0.1	*P<0.001*

[1]Values expressed as mean ± SD of three replicates.
[2]Probability test values obtained by ANOVA variance analysis.

The software SPSS v.17 (SPSS-IBM, Orchard Road-Armonk, New York, USA) was used to carry out these analyses.

Results and Discussion

Method validation and analytical quality assurance

The method was validated for quantitative measurements using calibration curves and the analytical parameters tested were selectivity, linearity, precision, accuracy and recovery, Limit of Detection (LOD) and Limit of Quantification (LOQ). Five calibration curves for chlorogenic acid, caffeic acid, ferulic acid, apigenin and pelargonidin with 5 points each were established in the concentration range of 5.0 to 100 to $\mu g.mL^{-1}$. The LODs and LOQs were evaluated from the slope and residual standard deviation of the respective standard curves. An accuracy by spiking recovery test was assayed in which we prepared and injected in HPLC an amount of each polyphenol, ranging from 50 to 100 $\mu g.mL^{-1}$, followed by the respective determination of the amount found. The reference spiked samples were treated and analyzed using the same procedure adopted for the plant samples. Recovery rate was calculated by comparing the amount of each polyphenol detected in the spiked sample with the amount of each standard added. Instrumental precision was determined by replicate analysis of standard compounds (n=5). SPSS for windows version 17.0 were used to calculate all statistical parameters (means, standard deviations, coefficient of variation, minimum and maximum, correlation coefficient), and a t-test was used for determination of significant differences between the mean values. The method performance data are shown in Tables 1 and 2.

According our results, the extraction and quantification method demonstrated a good linearity, with R^2>0.99 Table 1, and recovered of polyphenols varied between 92.6 and 120.7% Table 2. The method showed good selectivity, since all polyphenols detected were well separated from other compounds with good resolution.

Polyphenols and antioxidant activity

The polyphenol profile and respective content of corn silk hairs are presented in Tables 3 and 4, and in the Figure 1 are illustrated three examples of typical chromatograms obtained at different wave length. In the Figure 2 is presented the variation in TAA. The main polyphenols found were ferulic acid with an average of 45.2 $\mu g.g^{-1}$ dw, followed by chlorogenic acid (39.9 $\mu g.g^{-1}$ dw), caffeic acid (12.9 $\mu g.g^{-1}$ dw), apigenin (7.4 $\mu g.g^{-1}$ dw) and pelargonidin (2.4 $\mu g.g^{-1}$ dw). The ferulic acid represented 42% of total polyphenols identified by HPLC. In average, the polyphenol content varied significantly (0.05<P<0.001) with extraction method Table 2 and were higher when UE settings was used. The higher content of polyphenol in samples from UE is in agreement with previous findings [11,12], in which it was observed a significantly (P<0.05) increment of polyphenols content from hemp, flax and canola seed cakes under ultrasonic treatments. Similar observation can be extending to the TAA the results. The extracts produced under UE had more AA (1.72 times higher) than extracts produced under conventional method. This higher antioxidant activity for UE extracts can be endorsed to the higher polyphenol content found in these types of samples. In fact, the Pearson´s correlation coefficients showed a high positive variation of TAA with the variation of pelargonidin (r^2=0.998, P<0.01), ferulic acid (r^2=0.992, *P*<0.01), apigenin (r^2= 0.988, *P*<0.01), chlorogenic acid (r^2=0.9869, *P*<0.01) and caffeic acid (r^2= 00.886, *P*<0.05), confirm the previous findings by other authors [13] in which they found a strong correlation between the content of polyphenols and the levels of AA. Based in these results we can state that the UE provides higher content of polyphenols and thus higher level of AA. In fact, under UE the solvent diffusion associated with the acoustical cavitation effect [14] leads to a higher cell walls disrupting, leading to a higher polyphenol movements from cells towards the solvents, thus increasing their content in solutes and by consequence increase the TAA. Therefore, UE can be a good choice to maximize the amount of polyphenols extracted.

Although these differences in polyphenols content, it was possible to observe a similar polyphenol profile between the two methods of extraction, which means that extraction method doesn't be affect the polyphenol profile. The presence in both type of extracts of ferulic acid, chlorogenic acid, caffeic acid, and pelargonidin, made this by-product very interesting from bioactive point of view, since all of them have been associated with important bioactivities. The ferulic acid has been reported as having anti-inflammatory activity and anti-oxidative properties [15] due to the inhibition of xanthine oxidase and COX-2 enzymes, which provide pro-inflammatory activity [16]. Analogous observations were made about biological role of caffeic acid and chlorogenic acid. In an *in vitro* and *in vivo* study [17] it was found that chlorogenic and caffeic acids play a protective against intestinal injuries caused by Radical Oxygen Species (ROS). Similar conclusions were taken in relation to pelargonidin [18], a natural red pigment present in many fruits and vegetables. Pelargonidin was found very protective of cells preventing oxidative effects toward atherosclerosis through the attenuation of Human Aortic Smooth Muscle Cells (HASMC), as well as aortic sprouting via the direct inhibition of Focal Adhesion Kinase (FAK) activity [18]. Also Edirisinghe and collaborators [19] found that the richness of strawberry in pelargonidin and related isomers might be the

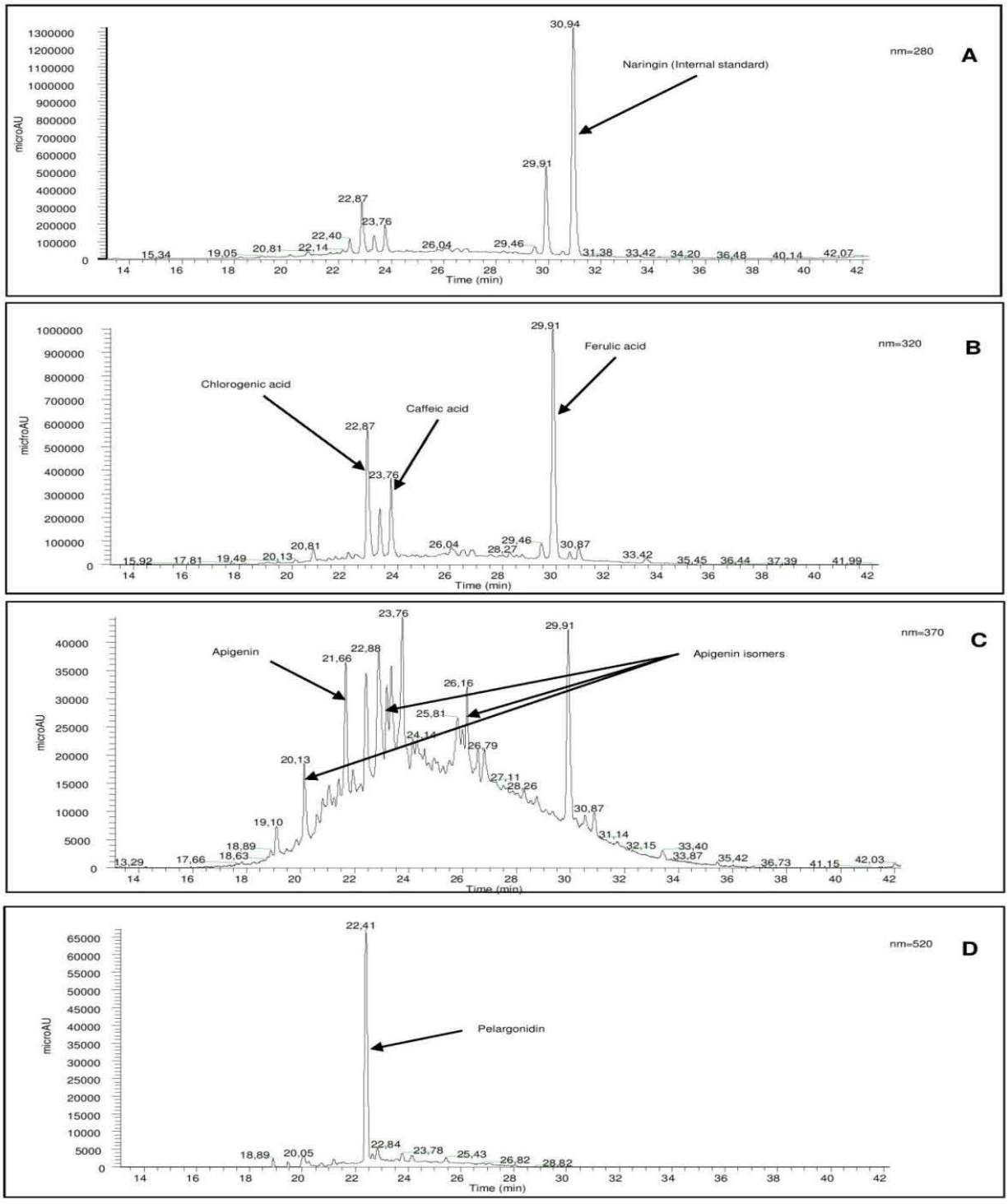

Figure 1: Chromatograms of corn silk hairs, recorded ant different wavelengths: 280nm (A), 320nm (B), 370nm (C) and 520nm (D).

primordial reason why these foods are pointed as protector against postprandial inflammation and insulin sensitivity. Another important polyphenol detected in the current study was the flavonoid apigenin. The presence of apigenin is very important, since this compound is widely associated with protective effect against Hsp90/Cdc37 complex, inhibiting the pancreatic cancer cell growth and migration [20], regulating the glucose and lipid metabolism, and ameliorate vascular dysfunction in type 2 diabetics [21]. Based in these findings, it seems that the richness of the above compounds in corn silk hairs supports the empirical evidences of their beneficial effects in human health preconized by traditional and folk medicine, such as wound healing capacity, anti-inflammatory and anti-glycaemic activities.

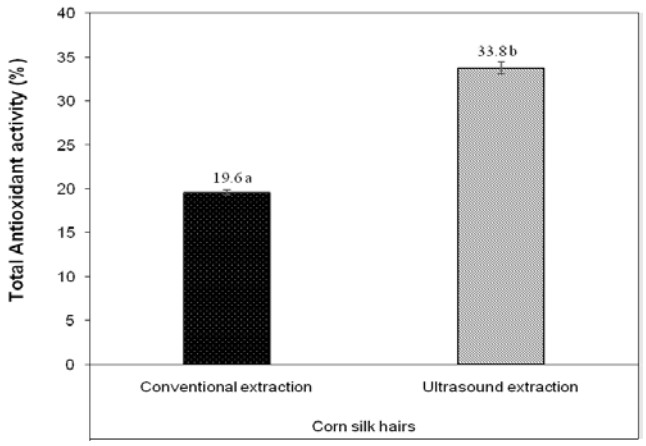

Figure 2: Total Antioxidant activity detected in corn silk hairs extracted under conventional and ultrasounds.

In fact, in several countries like Portugal, this type of by-product is widely used for decades as diuretic, with reduction of nephropathy via its anti-inflammatory activities, even if the main reasons for that were poorly understood.

To conclude, the method used to identify and quantify polyphenols in corn silk hairs is accurate and feasible and UE is a very useful method to maximizes the amount of polyphenols extracted. Our results reinforces the scientific evidences that corn silk hairs have high biological potential trough high content of polyphenols and their richness in pelargonidin, ferulic acid, apigenin, chlorogenic acid, and caffeic acid, turns this plant material highly valuable to be used as natural source of polyphenols and may contribute to its industrial applications for nutraceutical products development.

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