Biorefinery approach applied to the valorization of purple corn cob

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▪ ABSTRACT

The increased demand for textile products and the use of synthetic dyes have contributed to making dye wastewater one of the main causes of severe pollution problems. The use of natural dyes is therefore attracting big interest among the dyeing industries. Besides this, natural pigments need to be low-cost, with implications for the extraction methods used. Recovering pigments from waste materials and proposing a biorefinery approach can be a solution, reducing total costs and increasing total revenue, within the green economy.

This work reports a biorefinery approach to recover anthocyanins by set up a quick and cheap extraction method, starting from dried purple corn cob, to obtain pigments used to dye natural fibres. The residues of the cobs were extracted, recovering anthocyanins for nutraceutical purposes. The exhausted residue was proposed as animal bedding, closing the loop with zero waste produced, i.e. residual animal bedding is collected with food waste producing compost and/or biogas, and fertilizers. Water extraction allowed the recovery of 36.3% of anthocyanins, mainly composed of cyanidin derivatives (glycosylated and mainly monoacylated). The use of the first extract to dye fabrics gave good results in terms of colour strength and fastness. The subsequent extraction with ethanol allowed the recovery of additional 33.2% of residual anthocyanins. The anthocyanin-rich extract exhibited very good anti-inflammatory activity with high nutraceutical potential. Residual exhausted ground-up cobs are recommended to be used as animal bedding since fiber content and water retention ability were very similar to that of homologous commercial product. Moreover, the residual anthocyanins (183 ± 15 mg 100 g^{-1}) recalcitrant to extraction, conferred interesting properties to the proposed animal bedding.

KEYWORDS

Purple corn cob; Circular economy; Green chemistry; Anthocyanins extract; Natural dyeing; Bioactive compounds; Anti-inflammatory activity; Animal bedding.

Introduction

Textile industries, throughout the globe, produce and use approximately 1.3 million Mg of dyes, pigments, and dye precursors that cost around $23 billion each year.[1] Thus, textile industries generate toxic and hazardous chemical wastes which cause a threat to human health and the environment. For these reasons, the use of natural dyes is attracting more and more interest among the dyeing industries.[2] Since antiquity, natural dyes have been used to help to adorn human beings; for instance, in 2,000 BC the Chinese and the Sumerians in Iraq, already utilized indigo in painting and dyeing clothes.[1]

Natural pigment used as dyeing are generally extracted from vegetable substances, fungal species and animals. Among the plant pigments, anthocyanin are the most important pigments of the vascular plants and they are considered to be interesting for their use as natural water-soluble colourants[3] although natural fibres require dyeing in conjunction with metallic mordant such as Fe, Al, Cu, Pb, and Sn.[4,5] In addition anthocyanins are known to be nontoxic, and to act as antioxidant, bioactive components in nutraceutical,[6] antifungal, antibacterial[7] and UV-protective.[8]

The isolation of anthocyanin pigments from plants is typically done using solvent extraction processes. Anthocyanins are polar molecules and consequently more soluble in polar solvents: acetone is the most commonly used solvent, but it is also considered more toxic and hazardous,

needing to be handled with more precautions than ethanol, that, for example, is more environmentally friendly. The best extraction yield is obtained with this solvent, which is however expensive. Recently, in addition to conventional solvent-based anthocyanin extraction methods, other methods including supercritical CO_2,[9] ultrasound and microwave-assisted extraction[10] have been proposed.

Water is an essential medium for dyeing natural fabrics with natural polar pigments such as anthocyanins.[11] Nevertheless, using water greatly reduces extraction yields, leaving large amounts of anthocyanins in plant tissues, that in theory could be recovered for other purposes by a cascade approach, *i.e.*, biorefineries, aimed at limiting the waste produced and increasing the numbers of products.

In recent years, in Italy, several farmers have started the production of pigmented maize because different epidemiological and preclinical studies have demonstrated that regular consumption of anthocyanin-rich foods is associated to a reduced risk of chronic diseases, such as cardiovascular diseases, cancer, obesity and diabetes.[12-15] The most effective anthocyanins appeared to be both cyanidin-3-glucoside (CyG) and pelargonidin-3-glucoside (PlG).[14] Several varieties of coloured maize rich in different pigments have been studied and developed by classical breeding[16-21] that could be used as cheap sources of dyes and natural mordants, after their extraction by hydro-alcoholic solution from corn residues, *i.e.,* cobs and corn stover, that need to be valorised.

Earlier investigations indicated that in purple corn, pigments were located at especially high levels in the inedible husk and cob tissue, and it was also found that corn varieties and the extraction methods employed can affect anthocyanins content.

On average, the seeds that represented 82% of dry matter of the ear contains about 55% of total anthocyanins (ranging from 0.2 to 115 mg 100 g^{-1})[22-26] being the remaining fraction of anthocyanins, *i.e.* 45%, in the cob.

Non-food biomass such as corn cob is recalcitrant to biodegradation due to the low water content and owing to the lignocellulosic nature of the materials, organized as the rigid and compact structure of the cell walls.[27] These features make the purple corn cob an excellent raw material rich in anthocyanins, which remains stable over time, and these molecules are available to be extracted at any time.

This work aimed to recover anthocyanins from purple dried corn cobs by proposing a quick and cheap extraction method to obtain anthocyanin pigments to be used to dye different natural fibres (cotton and wool). In addition, waste streams generated were successively recovered by a cascade approach to produce nutraceutical products and pigmented litter for pets.

Experimental section

Corn crop and plant material. A new variety of pigmented F1 hybrid corn (*Zea mays* L.) carrying B (Booster 1) and Pl (Purple Plant) regulatory genes, was used as the source of plant material in this study.[20] Plants were grown in the experimental field (2018 crop year) of the University of Milan located in Landriano (Italy) (N 45°180', E 9°150').

The ears, harvested and dried, were shelled (by electric sheller); kernels were collected separately and preserved. Corn cobs obtained were ground by grinder (electric chipper, Home and Garden 0600853600, 2200 W, Bosch, Robert Bosch GmbH), and reduced to particle sizes of 1-3 mm. The dried and milled corn cobs had a moisture content of about 4% fresh weight.

Anthocyanins extraction and quantification on raw material.

Conventional extraction. Anthocyanins from corn cob were extracted by using two conventional solvent types: *i*. ethanol 50% acidified with 0.01% HCL 6 M and *ii*. acetone solution 70%.[28] Solvents were mixed with the ground purple cobs (solid-liquid ratio of 1:50 w/v) and stirred at room temperature. After 12 h stirring at room temperature, the mixture was centrifuged at 3,000 rpm for 10 min, and the resulting clear supernatant was filtered and analyzed for its anthocyanins content.

Total anthocyanins content (TAC). Anthocyanins quantification was spectrophotometrically determined using the pH-differential method.[29] To the aliquots, properly diluted, of extract were added 3 ml of 0.025 M of potassium chloride buffer (pH 1.0) and 0.4 M of sodium acetate buffer

(pH 4.5); after ~ 20 min, absorbance at 520 and 700 nm was measured. TAC was calculated as CyG equivalent per 100 g sample.

Biorefinery process.

The raw material was subjected to an anthocyanin's extraction process by two sequential extractions, each one having a different purpose, illustrated in the scheme in Figure1.

Step 1: water soluble anthocyanins extraction for direct dyeing. Extraction was performed by adopting a solid-liquid ratio of 1:10 (w/v), which allowed us to extract an anthocyanins concentration of almost 300 mg L^{-1}, that was indicated as an adequate concentration for dyeing fabric with anthocyanins.[30] To determine the effects of temperature on anthocyanins recovery some experimental extraction tests were performed at different temperatures,[31] *i.e.*, 25, 50, 70, 85 & 100 °C (Figure S1).

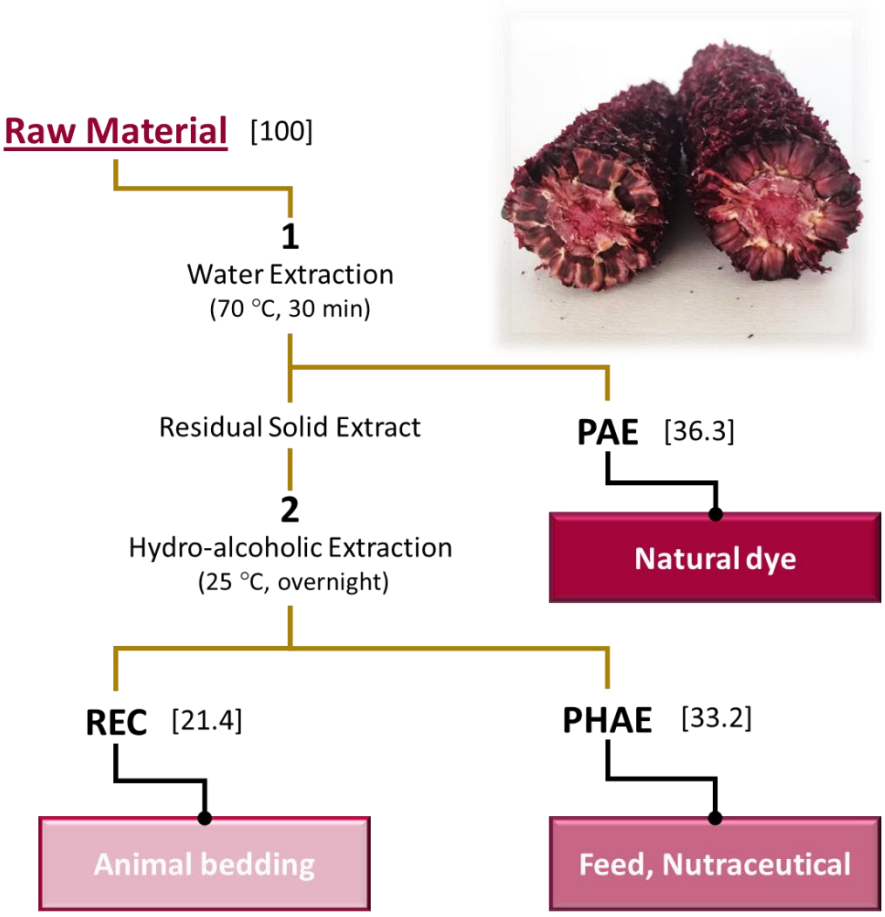


Figure 1. Framework of the biorefinery approach in valorizing purple corn cob. In square brackets, the relative anthocyanins yield expressed in percent of starting content [100] in raw material. Biorefinery products: pigmented aqueous extract (PAE); pigmented hydro alcoholic extract (PHAE); residual exhausted cob (REC).

In the light of these assessments, the first extraction method was done using 100 g of ground corn cob which were suspended in 1 L of water at 70 °C and stirred for 30 min. The suspension was filtered and the pigmented aqueous phase clarified by centrifugation at 3,000 rpm for 10 min; clear supernatant was filtered and analyzed for TAC. The first corn cob extract, named

pigmented aqueous extract (PAE), was characterized and employed for fabric dyeing tests. The corn cob solid residue was collected separately and subjected to a second extraction.

Step 2: hydroalcoholic anthocyanins extraction for nutraceutical use. Water based solvent is quite effective in the extraction of bioactive molecules from purple corn cob but the remaining solid residues evidently contained other anthocyanins (persistent reddish color still present) that can be recovered by using stronger solvents and/or a second extraction. Therefore, following the first extraction, the resulting cake was treated with hydroalcoholic solvent, *i.e.*, 50% ethanol slightly acidified with 0.01% HCl 6 M[28], at room temperature, overnight and under stirring, for a further anthocyanin extraction. Doing so, the solution pH was below 2, enhancing anthocyanins solubility and stability and total anthocyanins yield, without hydrolyzing their original form.[28]

The suspension was filtered and clarified by filtration with filter paper. The pigmented hydro alcoholic extract (PHAE) was successively characterized in both quantitative and qualitative terms and evaluated for nutraceutical properties, based on antioxidant and anti-inflammatory capacities. The lignocellulosic solid phase was washed for 30 min with distilled water (1:5 w/v), to eliminate the residual acid, collected by filtration and dried at 60 °C overnight.

Recovery of depleted cobs.

The dried residual exhausted cob (REC in Figure 1), retained after the anthocyanin extraction process, was subsequently characterized and analyzed to determine its suitability to be used as animal bedding

Quantification and characterization of phytochemicals in biorefinery products.

Total anthocyanins content (TAC). Anthocyanins quantification was spectrophotometrically determined such previously reported for raw material[29].

Anthocyanin compounds. Anthocyanins identification was performed by a chromatographic analytical approach on both of the different liquid extracts (PAE and PHAE) and the final solid residue (REC).

HPLC-DAD anthocyanins characterization was conducted with LC-20AD Series Shimadzu Prominence liquid chromatograph (Kyoto, Japan). The instrument was equipped with SPD-M20A diode-array detector. The chromatographic separation of the pigments was carried out by Luna® Omega C18 column (Torrance, USA) 3 µm, 3.0 × 150 mm. The mobile phase was composed by aqueous solution of 1% (v/v) formic acid (solvent A) and 100% acetonitrile (solvent B); the gradient program was: 10% B for 1 min, 15% B at 10 min, 20% B at 20 min, 45% B at 40 min, then returning to 10% B. Samples were properly diluted in solvent A and analysis was conducted under the following chromatographic conditions: flow rate of 0.4 mL min^{-1}, injection volume of 10 µL and column temperature of 30 ºC. Anthocyanins were detected at 520 nm and 280 nm by using a diode-array detector set up for an acquisition in the range 200-600 nm and an acquisition rate of 1.25 scans s^{-1} (peak width 0.2 min). LabSolution software version 5.90 (Shimadzu Corporation, Kyoto, Japan) was used both to control the HPLC system and for data processing.

Anthocyanins recognition was confirmed by liquid chromatography coupled with mass spectrometry (LC/MS). Detection was made by the LC/MS system 6130 Series from Agilent Technologies (Agilent Technologies, CA, USA) equipped with an Electro Spray Ionization (ESI) source and liquid chromatography sprayer and operated in the positive-ion mode. Analyses were performed by using both the same chromatographic separation conditions and column above described; injection volume was of 2 μL. Mass spectra were obtained under positive ion conditions using total ion scan (SCAN) from m/z 100 to 1000 and selected ion monitoring (SIM) modes. The MS settings were optimized for CyG signal-to-noise ratio. The capillary voltage was of +5.0 kV, the nebulizer pressure of 1.7 bar, the drying gas flow of 9 L min^{-1} and the drying temperature 350 °C. Compounds were detected as positive ions ($[M–H]^+$) at different channels: m/z 449 CyG, m/z 535 (cyanidin-3-malonyl glucoside, CyMG), m/z 433 (pelargonidin-3-glucoside, PlG), m/z 463 (peonidin-3-glucoside, PnG). For data processing ChemStation software Rev.B.04.03 (Agilent Technologies, CA, USA) was used.

Total Polyphenols (TP). The determination of the TP was carried out directly on diluted aliquots of the PAE and PHAE extracts. For the quantification of total polyphenol contents of REC, 0.5 g of dried material was weighed, mixed with 20 mL of 70% (v/v) aqueous methanol and shaken at room temperature for 6 hours. TP was determined by the Folin-Ciocalteu assay[32]; briefly, to the extracts (PAE, PHAE and REC extracts) properly diluted were added 1 mL of Folin-Ciocalteau reagent. The mixture was neutralized with the addition of 2 mL of 20% (v/v) aqueous Na_2CO_3 solution and left at room temperature for 15 min until the characteristic blue colour developed. Absorbance at 725 nm was measured with UV-Vis spectrophotometer (UV-Vis Cary 60, Agilent Technologies, USA) against a blank. The total phenolic content was

determined by means of a calibration curve prepared with gallic acid and expressed as mg of gallic acid equivalents (GAE) per 100 g of corn cob.

Antioxidant Capacity (AC). Antioxidant capacity was directly carried out on aliquots of the PAE and PHAE extracts as above reported for polyphenol quantification. REC antioxidant capacity was assessed starting from 5 g of REC extracted with 100 mL of 70% acetone for 6 h at room temperature. The radical scavenging activity was measured according to the ABTS [2,29-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] method described by Pellegrini et al.[33] Briefly, the $ABTS^{\bullet+}$ radical solution was generated by reaction of a 7 mM solution of ABTS in water with 2.45 mM of $K_2O_8S_2$ (in darkness at room temperature for 16 h). The blue-green $ABTS^{+}$ solution was diluted with 50% ethanol until it reached an absorbance of 0.700 ± 0.020 at 734 nm, measured with a UV-Vis spectrophotometer (UV-Vis Cary 60, Agilent Technologies, USA). Trolox was used as the antioxidant standard. Extracts were diluted appropriately with 50% (v/v) ethanol. The reaction was carried out using 100 µL extracts, or Trolox standard solution, plus 3,900 µL of radical solution. The decrease of absorbance was monitored at 734 nm after 15 min. The results were expressed as mmol Trolox equivalents (TE) antioxidant capacity 100 g^{-1} corn cob.

Anti-inflammatory properties. To test the anti-inflammatory properties of PHAE extract, the cells from human intestinal epithelial (Caco-2 cells) (ECACC 86010202, Public Health England) were used according to the method described in our previous work.[32] Prior to the assay, PHAE was dried in a rotatory vapor to obtain the residual powder, subsequently dissolved in Dulbecco's Modified Eagle's Medium (DMEM). In order to evaluate the anti-inflammatory property of PHAE extract, the Caco-2 cells were simultaneously incubated with IL-1β (20 ng mL^{-1}) and anthocyanins-rich extract for 2 hours. Cells incubated only with IL-1β were

considered as the control whereas the intact cells (considered as blank) were used to measure the cells' viability. Effects of the anthocyanin molecules on inflammatory cytokines were measured through the fold changes in IL-8 cytokine gene expression quantified by Real Time-PCR.

A concentration of total anthocyanin content of 25 μg mL^{-1} was considered for the tested samples. CyG standard was used as the positive control. For each sample the treatment was performed in three biological replicates.

Dyeing process, colour strength and fastness.

PAE was employed as a natural dyestuff for dyeing cotton (240 g m^{-2}) and wool (190 g m^{-2}) fabrics.

Cotton fabric pretreatment. Before dying cotton, the fabric was put through a pretreatment developed by Wang et al.,[30] by soaking with succinic acid and sodium hypophosphite (NaH_2PO_2) at the same concentration (20 g L^{-1}). The impregnated fabric was dried for a short time (4 min in total) at 90 °C, then 180 °C.

Cotton and wool mordanting and dyeing. Dyeing was performed as indicated previously in dye-tests[30], with minor revisions, adopting a fabric to liquor ratio of 1:50 (w/v). Alum (*i.e.,* $AlK(SO_4)_2 \cdot 12\ H_2O$, cream of $KO_2CCH(OH)CH(OH)CO_2H$)), or tin salt ($SnCl_2$) were used as mordants and dissolved directly in the dye bath (10% weight of fabric) to obtain PAE+Alum and PAE+Tin treatments, respectively. The procedure was conducted at 60 °C for 1 hour. The dyed fabrics were then rinsed with cold water and finally dried at room temperature overnight.

Colour analysis. Dyed fabrics colour data were evaluated by CIELAB parameters detected with a tri-stimulus colorimeter (Croma Meter Cr 400 Konica Minolta, Nieuwegein, Netherlands) instrument (D65 illuminant standard, 10° standard observer). In CIELAB colour space, colours are designated in coordinates, L* coincides to brightness (100 = white, 0 = black), a* to the red-green coordinate (positive sign = red, negative sign = green), and b* to the yellow-blue coordinate (positive sign = yellow, negative sign = blue); C* is the quantitative parameter of the chroma of the colour, and h^* is the quantitative parameter of the hue of the colour. Colour strength (K/S) for dyed samples was determined according to the Kubelka-Munk equation.[30]

Light fastness. Light fastness of dyed cotton and wool fabrics was measured according to an internal method derived from ISO standard method 105-B02 (2014). The samples were exposed with Solarbox 1500 (Erichsen GmbH & Co., Hemer, DE), a tabletop large test chamber equipped with 1500-Watt Xenon lamp of correlated colour temperature 5500 °K to 6500 °K, and irradiance control. The light of the Xenon lamp was filtered with UV 310 + IR filters, light exposure time 48 h. The light fastness test was assessed against to the standard Blue-scale reference and the values assigned starting from 1 (poor) to 8 (excellent).

Washing fastness. Washing fastness of cotton and wool dyed fabrics was evaluated according to standard method ISO 105-C02 (1989) with some modifications. Fabrics were washed at 50 °C for 45 min using water solution (1:50 w/v) of neutral detergent (Green Oasis brand, ICEFOR, Italy) 10 g L^{-1}. Fabrics were subjected to three laundering cycles with interposed rinsing and drying. After tests, the colour changes were related to the standard grey-scale (marks 1–5; 1 = poor, 5 = excellent).

Characterization of Residual Exhausted Cob.

The exhausted corn cob material was characterized for its water holding capacity (WHC) and fibre detergent analysis in order to assess its ability to act as animal bedding in comparison to commercial corn cob products litter (CCL) (Lettosan Croci, Va, Italy)

The WHC was characterized following EN methodology (EN 13041, 2012). The neutral detergent fibre (NDF), cellulose and lignin contents in the corn cob dry matter were estimated using the fibre detergent method, determined according to protocols developed by Ankom Technology (ANKOM Technology Corporation, Fairpoint, NY), based on the work of Goering and Van Soest.[34]

For pH measurement, both raw material and REC (20 g) were mixed with 40 mL of deionized water for 30 min stirring, filtered and used for direct pH measurement.

To test halochromic properties REC was exposed to acidic and alkaline buffers. Two buffer solutions, at pH 5.0 (0.4 M of sodium acetate buffer) and pH 8.0 (0.01 M TRIS-HCL buffer), were prepared and used to wet dry REC, then after 10 min it was photographed under natural light.

Informatic tools. Statistical analyses were conducted using the software IBM SPSS 25, (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp). In particular, one-way ANOVA analyses were carried out with a significance threshold set at 0.05. Multiple comparisons were performed with the Duncan and Gabriel methods.

▪ Results and Discussion

Anthocyanins content in feedstock.

The massive extraction of anthocyanins from dry raw material was conducted to detect the total amount of pigments (TAC) present in the corn cob. Both solvents used, *i.e*., 50% ethanol and 70% acetone, gave similar anthocyanins content, *i.e.,* 854 ± 24 and 931 ± 44 mg CyG eq. 100 g^{-1}, respectively (Table 1).

Table 1. Phytochemicals contents and antioxidant activity for corn cob and derived extracts

	TAC[a] (mg CyG eq. 100g^{-1})	TP[b] (mg GAE 100 g^{-1})	Antioxidant Activity (mmol TE 100 g^{-1})
Conventional extraction			
EtOH extract	854 ± 24a	1,674 ± 127a	5.35 ± 0.47a
Acetone extract	931 ± 44a	1,212 ± 98b	4.76 ± 0.52b
Biorefinery products[c]			
PAE	310 ± 18b	708±14c	3.36 ± 0.17c
PHAE	284 ± 17b	419±79d	2.16 ± 0.25d
REC	183 ± 15c	441±21d	1.97 ± 0.10d
CCL[d]	-	303±34e	0.70 ± 0.04e

[a]TAC: Total Anthocyanins Content; [b]Total polyphenols; [c]products obtained during sequential extraction process such as described in Figure 1. [d]CCL: commercial corn cob litter for pets (CCL), followed by the same letter, within each column, are not significantly different (Tukey test, $p < 0.05$).

These values fell into the range reported in the literature, *i.e.,* 202– 4,600 mg 100 g^{-1} corn cob[35-37] in which variability depended on both corn variety[9,10] and pedoclimatic conditions. Light (photoperiod length) and temperature, have been reported to affect anthocyanins content when varieties adapted to tropical areas are cropped in a temperate climate.[8]

Biorefinery first step: PAE for dyeing.

The first product of the biorefinery process, PAE, yielded 310 mg CyG equivalent 100 g^{-1} corn cob (Table 1) that represented about one third of TAC (36%) reached by conventional extraction with 50% ethanol. The increase of temperature positively affected the recovery of anthocyanins by using water solvent and the optimum extraction temperature was reached at 70 °C (Figure S1). Exceeding this temperature gave no significant increase in anthocyanins yield. Moreover, extraction time was established at 30 min, as no differences occurred when tests were prolonged to 60 min, as already observed.[31]

In PAE, from a qualitative point of view, it emerged that glycosylated anthocyanins were represented mainly by three aglycones: cyanidin, present as CyMG and CyG, pelargonidin as PlG and PlMG and peonidin, as PnG and PnMG (Table 2, chromatogram in Figure S2). In addition, minor anthocyanins, constituting less than 10% of TAC, were found but not clearly identified. These compounds, probably, can be ascribed to hypothetical dimalonyl- or ethyl-malonyl-derivatives.[38] Chromatogram in Figure S2 showed that there were no free anthocyanidins in water extract, indicating that the mild extraction conditions adopted did not

affect anthocyanins integrity. In fact, free aglycones are normally obtained by basic and acid hydrolysis.

The anthocyanin profile of the PAE (Figure S2) matched that found in the literature for similar plant material.[39,40] CyG and CyMG were the two major anthocyanins since they accounted for about 60% of the total anthocyanins present (Table 2), and, among them, CyMG was two-fold CyG. In corn, anthocyanins acylation occurs prevalently through the linkage with malonic acid, which may be present as mono, di-, or tri-malonyl, glycosylated.

Table 2. Distribution of anthocyanins and relative composition in biorefinery products

anthocyanins	PAE	PHAE	REC
		(% TAC)	
CyG	22.2	31.4	25.4
PlG	4.6	6.7	5.6
PnG	7.2	9.3	7.9
CyMG	40.7	30.3	34.1
PlMG	6.8	6.8	6.1
PnMG	9.0	6.8	7.6
Others	9.7	8.7	13.3

The presence of -COOH group(s) and their dissociation forms promote the protonated form of the flavylium cation of the anthocyanins (Figure S3) which protects them from colour degradation, deriving from the increase in pH. Lower pH values promote anthocyanins stability, solubility in water and brilliant red colour.[28] Effectively, different anthocyanin forms exist in equilibrium, and the increase of pH favors the deprotonation or hydration of the flavylium cation, towards molecular forms that lose stability and become colourless (carbinol pseudobase and chalcone) with respect to the coloured flavylium cation (red) and the quinoidal base (blue).[41] These different pigment chemical forms coexist in PAE that showed a pH which was not extremely acidic (pH of corn cob extract of about 4.2). However, the prevalence in the extract of the acylated anthocyanins (malonyl and dimalonyl derivates) (66% of TAC) (Table 2), can favor the maintenance of colour stability. Despite pH conditions, anthocyanin stability is also affected by heat, and the pigment is easily converted into the colourless chalcone form during prolonged heating.[42]

Water extraction from the cob also allowed the solubilisation of other phenolic compounds. In PAE, TP content was of 708 ±14 mg GAE 100 g^{-1} corn cob (Table 1) that represented the major moiety (45%) of the total sequentially extracted polyphenols, *i.e.*, (PAE + PHAE + EC) = 1,568 mg GAE 100 g^{-1} (Table 1), with those for PHAE and REC of 419 ± 79 mg GAE 100 g^{-1} and 441 ± 21 mg GAE 100 g^{-1} corn cob, respectively. Phenolic compounds found in PAE might have important roles in dyeing, for instance by contribution to maintain anthocyanin colour stability (copigmentation phenomena involving polyphenols)[43] or, together with anthocyanins extracted, by implementing UV radiation absorption[44] and promoting a protective function in the dyed fabric against solar radiation damage. Moreover, polyphenols, which include the anthocyanin family, can act as antioxidants or free radical scavengers, thus preventing oxidative stress. In this

regard, many authors have reported that also anthocyanins may act with protective effect on biological oxidative damage.[45,46] Generally, the pigmented kernel exhibits higher antioxidant activity than non-pigmented corn.[25,47] Antioxidant activity was associated with the presence of bioactive compounds inclusive of anthocyanins and soluble phenols. The use of different solvents in the extraction stream induced slight differences in antioxidant activity (Table 1). Nevertheless, PAE resulted in better antioxidant activity in comparison with the PHAE, because of the presence in the extract of more water-soluble phenols rather than the anthocyanins concentration.

Dyeing properties. PAE, once obtained, was directly employed for eco-friendly natural dyeing on cotton and wool fabrics (Figure 1). PAE dye concentration corresponded to 350 mg L^{-1} anthocyanins and tincture tests were conducted using only PAE or by adding mordanting agents (PAE+Alum, PAE+Tin) to implement and improve the fixing of colour to natural fibres. To assess the quality of the dye, the colour strength (K/S), the changes in the original colour profile (a*, b*, C*, h*) and the light and wash fastness of fabric dyed were explored (Table 3). Generally, protein fibres of animal origin have more affinity to natural dyes in comparison with cellulosic plant ones. In fact, dyed wool always showed a better colouring behaviour than cotton, which shows lower values of K/S and light-wash fastness. However, the choice to include cotton in dyeing trials with PAE is mainly due to the modification to which the fabric was subjected during a pre-treatment with succinic acid, as suggested by Wang et al.[30], which enhanced the affinity of the cellulose substrate to dye, especially in the presence of a metal-salt based mordant. Particularly, the presence of tin in the dye bath improved the colour depth of the fabrics, measured by K/S value. The increase was of almost two-fold, for both cotton and wool with

respect to other dyeing tests, *i.e.*, in wool, PAE+Tin treatment exhibited the highest K/S value (4.29) while PAE+Alum slightly affected K/S in comparison with mordant-free dyeing.

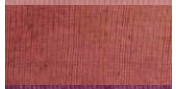
The addition of the metal salt in the dye bath caused a bathochromic shift in a visible range of red anthocyanins towards purplish red extract as already well described in dyeing with natural pigments.[30,48] This chemical mechanism is known as metal copigmentation mediated by the complexation of anthocyanins with metals (Cu, Al, Sn, Fe), with the result that it increased anthocyanins' colour stability to temperature, light, oxygen and pH.[49] Among aglycones present in PAE, cyanidin, which has at least two free hydroxyl group in the B-ring (see anthocyanins molecule in Figure S3), are capable of metal chelation. In dyed fabrics the maximum absorption (λ_{max}) in the visible range (shown in Table 3) shifted from 518 nm of PAE to 528 nm for PAE+Alum (λ_{max} = 10 nm) and to 549 nm for PAE+Tin (λ_{max} = 31 nm). Moreover, PAE+Tin induced, in addition to colour stability, an enhanced colourant hyperchromic effect, as sustained by increases in absorbance value (data not shown).

The use of Tin mordant, besides enhancing colour strength such as suggested by the K/S parameters, (Table 3), i.e. K/S number increase with colouring strength, conferred on fabric dyes a shade difference. In addition, the use of this mordant affected the colour brightness parameter L* (L* of 100 = white, L* of 0 = black). In particular, for cotton the presence of Tin determined a darkening of the colour, *i.e.* L* value decreased from 56.69 for PAE and 59.8 for PAE + Alum to 40.9 for PAE + Tin. On the contrary, for wool the L* parameters indicated a slightly brightness increase, i.e. from 27.31 for PAE and 25.63 for PAE + Alum to 32.34 for PAE + Tin (Table 3). Interesting was also data relative to the colour hue, such as well resumed by h* parameter, that increased a lot in presence of Tin mordant, *i.e.* 337.10 for cotton and 335.93 for wool. The formation of metal-anthocyanin complexes was suggested changing colours from

reddish-purple to violet (Table 3), such as indicated by the modification of b* parameters (it indicates the yellow-blue coordinate: positive sign = yellow and negative sign = blue), that assumed negative value, being the parameters a* that corresponded to the red-green coordinate (positive sign = red and negative sign = green) unmodified, independently from the nature of the fabric. Otherwise, PAE+Alum dyed fabrics displayed the ability to keep the original colour characteristics of PAE mordant-free and in fact b* parameters did not change for both cotton and wool (Table 3).

No less important than colour characteristics are the colour fastness. The rating results of light and washing fastness are included in Table 3 and show that the addition of mordant was necessary to improve colour durability of the fabric dyed with PAE. Colour fastness to light showed that, once again, in wool PAE+Tin the rating increased from poor (1) to fair (3) in Blue-scale grade, and this was an acceptable result, considering the susceptibility of the anthocyanins to the (sun)-light and the importance of their co-pigmentation as above described. Both cotton and wool dyed with anthocyanins-based PAE were gently washed with neutral anionic soap and after three cycles of laundering a good wash stability was shown for cotton (3 and 4 value for Alum and Tin, respectively) and better for wool fabrics (4-5 value).

Table 3. Dyed fabrics, colorimetric and fastness properties

		λ_{max}[d] (nm)	CIELAB space L*	a*	b*	C*	h*	K/S	Fastness Light	Wash
Cotton										
Undyed			94.01	-0.07	2.94	3.02	103.26			
PAE[a]		281, 518	56.69	25.61	3.15	25.80	27.02	1.61	1	1
PAE+Alum[b]		283, 528	59.80	23.48	3.34	23.71	28.10	1.98	1	3
PAE+Tin[c]		278, 549	40.90	23.47	-9.91	25.47	337.10	3.54	3	4
Wool										
Undyed			86.58	-1.31	15.10	15.16	94.94			
PAE[a]		278, 519	27.31	22.42	8.20	23.88	20.09	1.98	1	2
PAE+Alum[b]		278, 529	25.63	22.92	8.63	24.45	20.64	2.10	2	4
PAE+Tin[c]		278, 547	32.34	14.48	-6.47	15.86	335.93	4.29	3	4-5

[a]Pigmented aqueous extract obtained during sequential extraction process such as described in Figure 1. Mordant added: [b] Alum, potassium aluminium sulphate, and [c] Tin salt, $SnCl_2$. [d]Maximum absorbance (nm) in the UV-Visible range.

In plants, anthocyanins may possibly limit photo-oxidative injury of the photosynthetic tissues of leaves and fruits by acting as a screen from high-energy light excesses, attenuating the incident radiation in visible and UV parts of the spectrum and scavenging reactive oxygen species. Anthocyanins preferably absorb in the visible green and ultraviolet (UV) regions.[49] However, they exhibit a lower absorbance in the UV region in comparison with other colourless flavonoids and phenolic compounds.[44] Outside the biological system, and according to previous studies, characteristic absorption spectra of specific anthocyanins extracted from purple corn cob (PAE), showed two regions in the UV-Vis (Figure 2).

The first region, peaking around 280 nm (UV-B), is due to the presence of aromatic rings and is typical of all phenolic family compounds; the second long-wave band was situated in the 450-550 nm range (blue-green) and it was due to the flavylium cation. The maximum absorbance in the visible range varied for different aglycones, and the aglycone of CyG, which was responsible for the reddish colouration of purple corn cobs, peaked at around 518 nm. The same absorption spectrum was described for CyMG, which means that acylation did not substantially change the absorption characteristics of the pigment.

Natural dyed cloth can exert a role in the protection of human skin against the harmful effects of UV irradiation. Particularly in humans, it is widely accepted that there is an actual direct or indirect association between UV radiation and the development of skin cancer.[50]

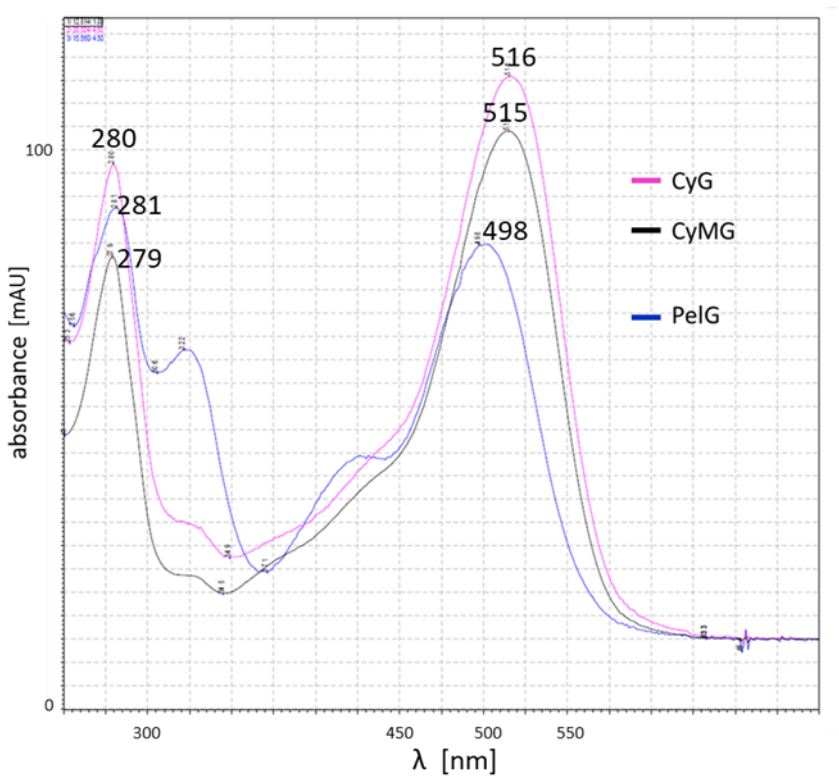


Figure 2. UV-VIS spectra with relative absorption maxima of some anthocyanins (CyG, CyMG, and PlG) founded in PAE and separated by HPLC-DAD.

As a covering factor, dyed fabrics can absorb an important moiety of UV radiation, greater than undyed ones. The protection efficiency, described by the Ultraviolet Protection Factor (UPF)[51], increased in those fabrics dyed with natural pigments.[52,53] In our study, anthocyanins metal co-pigmentation induced, in the PAE +Tin dyeing solution, a shifting of the radiation absorbance (nm) in the UV-visible range, i.e. λ_{max} shifted from 518 nm to 549 nm (Table 3), but it did not interfere with anthocyanins absorption in the UV range, i.e. λ_{max} remained quite stable, *i.e.* from 281 nm to 278 nm (Table 3 & Figure 2). Among numerous extracts of natural colourants, it has been demonstrated that fabrics coloured with red onion peel, rich in

anthocyanins' aglycones (cyanidin, delphinidin, pelargonidin, and peonidin), showed an excellent UPF value (50+), in the presence or in the absence of mordant.[54] PAE dyed fabrics could show the same important feature.

Biorefinery second step: PHAE for nutraceuticals

The extraction yield obtained showed that the anthocyanins water extraction was not an exhaustive extraction process (Table 1) and that about 64% of total anthocyanins (TAC) remained in the residual solids extracted (Figure 1). Therefore, aiming to recover as much as possible of the valuable biomolecules contained in the corn cob, a subsequent extraction with hydro-alcoholic solvent slightly acidified (0.01% HCl) was performed, obtaining a second extract, *i.e.*, PHAE, that accounted for 33% of the total corn cob anthocyanins content (Figure 1), equal to 284 mg CyG equivalent 100 g^{-1} (Table 1). PHAE showed a different anthocyanins pattern with respect to PAE (Table 2) and in particular, the CyG/CyMG ratio for PHAE (1.03) was higher than that estimated for PAE (0.54). It would seem that the acylated anthocyanidins forms (CyMG, PlMG and PnMG) were preferentially extracted by cold water during the first step, while the following hydro-alcoholic extraction acted preferentially on glycosylated but not acylated anthocyanins retrieval (CyG, PlG and PnG).

The antioxidant activity of extracts was investigated individually both for PAE and PHAE. However, considering the antioxidant activity per mg of anthocyanins, *i.e.,* antioxidant efficiency, it appeared that PHAE contained anthocyanins which exhibited a better antioxidant performance than PAE (Table 1). Comparing anthocyanins composition of both extracts (Table 2), there was no clear evidence that the acylation of anthocyanins can improve antioxidant

capacity. Nevertheless, it has been shown that the number of hydroxyl groups on the B-ring of the chemical structure of anthocyanidins could be responsible for their antioxidant activity (Chen et al., 2018). A linear relationship was observed in both extracts between antioxidants and total polyphenols that included the anthocyanins moiety ($r = 0.92$, $p < 0.05$; $n = 3$), but it would seem that the antioxidant efficiency of PAE was higher compared to PHAE.

This might be due to the fraction of non-pigmented polyphenols extracted preferentially during the first extraction in water. The second extraction with ethanol solution became necessary to obtain an additional 33% of yield since the water alone was not effective to obtain this result (data not shown). Furthermore, ethanol is a non-toxic solvent and water-alcohol solution reduces the time and cost to concentrate the biomolecules.

In PHAE the yield of total polyphenols was almost halved compared to the amount found in PAE while TAC was more or less the same. Thus, in PHAE the antioxidant activity might be mostly ascribable to the TAC.

The anti-inflammatory properties of purple corn anthocyanins have recently been reported by Zhang et al.[55] Purple corn anthocyanins have shown an effective down-regulation on the secretion levels of pro-inflammatory cytokines such as IL-6 and TNF-β in a wide range from - 2.5 to -60.1% and -9.7 to -64.8%, respectively.

PHAE was investigated for its anti- inflammatory effects (Figure 3) on the IL-8 cytokine production in the Caco-2 cells stimulated by IL-1β, potent pro-inflammatory cytokine. As shown, in response to cytokine IL-1β treatment, the expression of IL-8 gene was increased up to 63-fold, whereas in the presence of the anthocyanins-rich extract (IL-1β+PHAE), the gene expression was down-regulated 10-fold, similarly to the effect given by CyG (IL-1β+CyG(std)).

The PHAE and CyG can decrease the cytokine secretion significantly (p<0.05) in the stimulated Caco-2 cells.

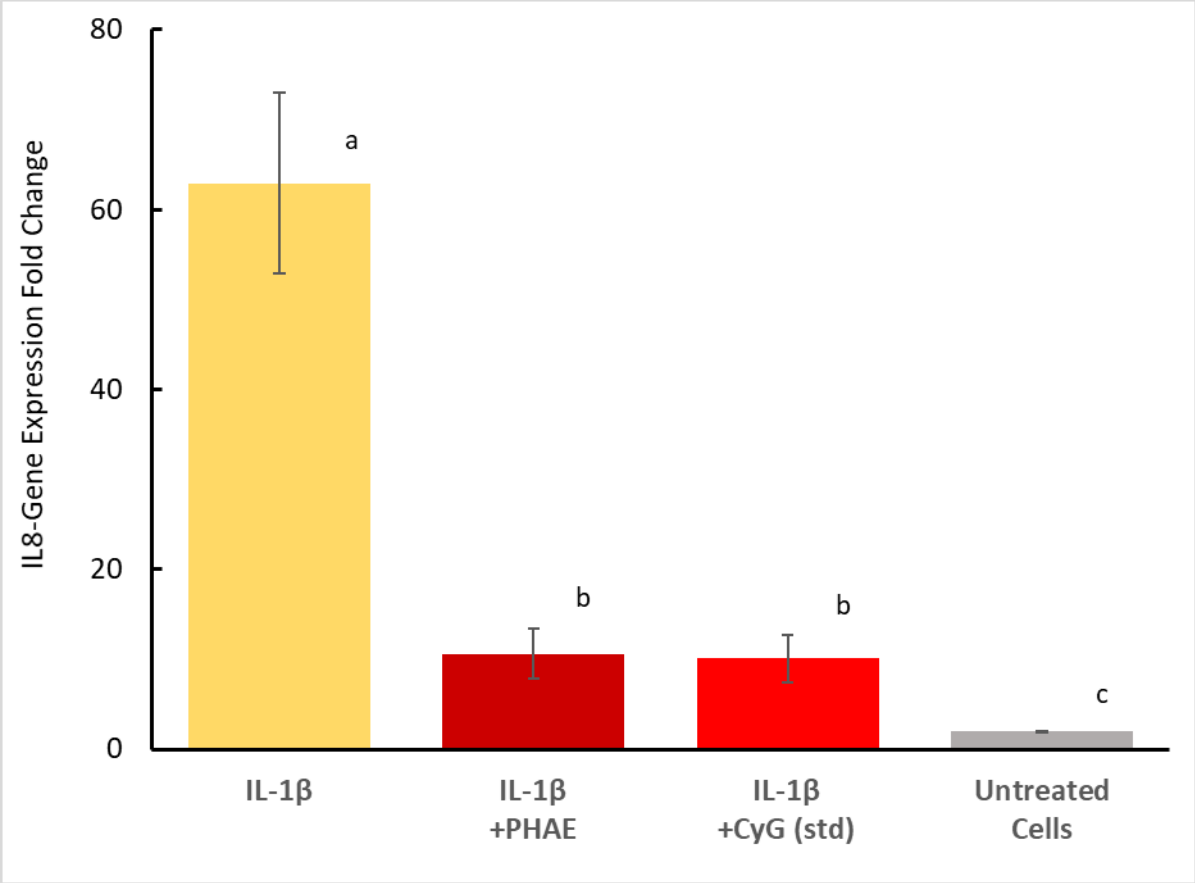


Figure 3. Immunosuppressive effects of PHAE and CyG pure standard (25 μg mL^{-1}) on IL-8 gene expression in the Caco-2 cells stimulated by pro-inflammatory cytokine IL-1β, expressed as the change folds. Untreated cells are not stimulated. Bars with different letters are significant different (Duncan's test, p<0.05).

The characterization of pigmented corn cob revealed that the PHAE anthocyanins-rich extract (Table 2) was represented by the anthocyanins in glycosyl and acyl bound forms. In fact, in the

hydro-alcoholic extract, anthocyanins CyG and CyMG were present at the same relative contents, which could suggest a lower efficiency for CyMG than CyG to suppress the inflammation within the Caco-2 cells, which propose that the glycoside form of cyanidin has a greater anti-inflammatory property than its malonyl form.[56] Zhang et al.[57] investigated the associations between various glycosylated anthocyanins and phenolic compounds in the corn and anti-inflammatory markers, and found that the CyG was more correlated to suppressing the inflammatory cytokines than CyMG. The same results were observed for the PlG and PlMG in the pigmented corn. All this implies that the presence of glycosyl-bound anthocyanins (but not the malonyl moiety) facilitate the regulation of inflammatory responses and the transcription of the modulatory signaling pathways in the cells. Anthocyanins from pigmented corn not only have suppressing activity on the inflammatory cytokine gene expression, but also have regulatory effects on the enzymes related to the inflammation. The strong anti-inflammatory properties of CyG at 20 μg mL^{-1} have been reported previously in the macrophages induced by lipopolysaccharides, in which it has inhibited nitric oxide (NO) production.[58] Two main anthocyanins in the pigmented corn, CyG and PlG, showed the highest inhibitory effect on inducible nitric oxide synthase (iNOS) expression (−28.9%), and the greatest potency in suppressing cyclooxygenase-2 (COX-2) expression (−51.8%).[55] However, it was noteworthy that other flavonoids rather than anthocyanins, usually found in the hydro-alcoholic extracts of pigmented corn cob, collaborated towards the overall anti-inflammatory properties. In this regard, quercetin, rutin and luteolin, have been reported for their active anti-inflammatory activity in the pigmented corn.[55]

The mechanism of the anti-inflammatory property of pigmented corn anthocyanins has been described through the inhibiting of the protein complex NF-κB which is an important family of transcription factors playing a pivotal role in the regulation of the immune system.[55]

These results illustrate how PHAE can supply nutraceutical and functional molecules that could be supplemented to improve the nutritional value of final products, *i.e.* food/feed.

REC utilization as animal bedding.

Residual exhausted cob (REC) was proposed to be used as animal bedding (*e.g.,* for cats, rodents, birds and reptiles), that after its use can be separately collected with organic food waste and addressed to anaerobic digestion and/or composting producing biogas and biofertilizers, thus closing the biomass cycle, with zero waste production.

Chemical and physical characteristics of REC were compared with those of a commercial corn cob litter (Table 4).

Table 4. Comparison between REC and homologous commercial cob litter

	pH	WHC[a]	DM[b]	Ash	NDF[c]	Cellulose	Lignin
				(g 100 g^{-1} DM)			
REC	4.2[d]	73.4	95.1	0.81	93.3	27.3	23.1
CCL	5.9	62.8	96.2	1.25	94.9	33.9	14.3

[a]WCH: water holding capacity. [b]DM: dry matter. [c]NDF neutral detergent fibre. [d]The data are mean values obtained from three independent experiments. Standard deviations were not more than 10% for each determination.

Chemical characteristic of residual exhausted cob (REC) and commercial animal bedding were very close. In particular dry matter and ashes content were quite identical (Table 4) as well as the presence of fibre, *i.e.* cellulose and hemicellulose. However, the water holding capacity (WHC), that represents an important parameter since the retention of liquid is greatly appreciated for animal bedding, was higher than that measure for commercial product, *i.e.* 73.4 *vs*. 62.9 g 100 g^{-1} DM. Moreover, commercial corn cob litter for pets (Table 1) shown the minor antioxidant activity due low total polyphenols (TP) concentration and a total absence of anthocyanins. Despite the extractions, REC still contains anthocyanins, *i.e.*, 183 ± 15 mg 100 g^{-1} corn cob, which could not be removed (see the purple colour in Figure 4, i.e. control), and their positive effect might be exploited. Cyanidin (in the form of CyG and CyMG) is the aglycone most representative in REC (Table 2).

A previous study[59] reported a strong antimicrobial activity (against *Salmonella enteritidis*, *Staphylococcus aureus*, and *Candida albicans*) exerted by extracts of a purple corn hybrid. Thus, thanks to the inhibition of bacterial activity, the presence of residual anthocyanins in REC may be useful, *e.g.*, to limit the release of unpleasant odours from the litter.

The colour change property of anthocyanins extract, because of pH variations, has been already exploited as potential natural pH indicators in film packaging applications.[60,61] Similarly, we have tested the halochromic properties of REC, soaking it with pH buffers at units 5 & 8, to simulate the limits of pH range of animal urine (Figure 4).

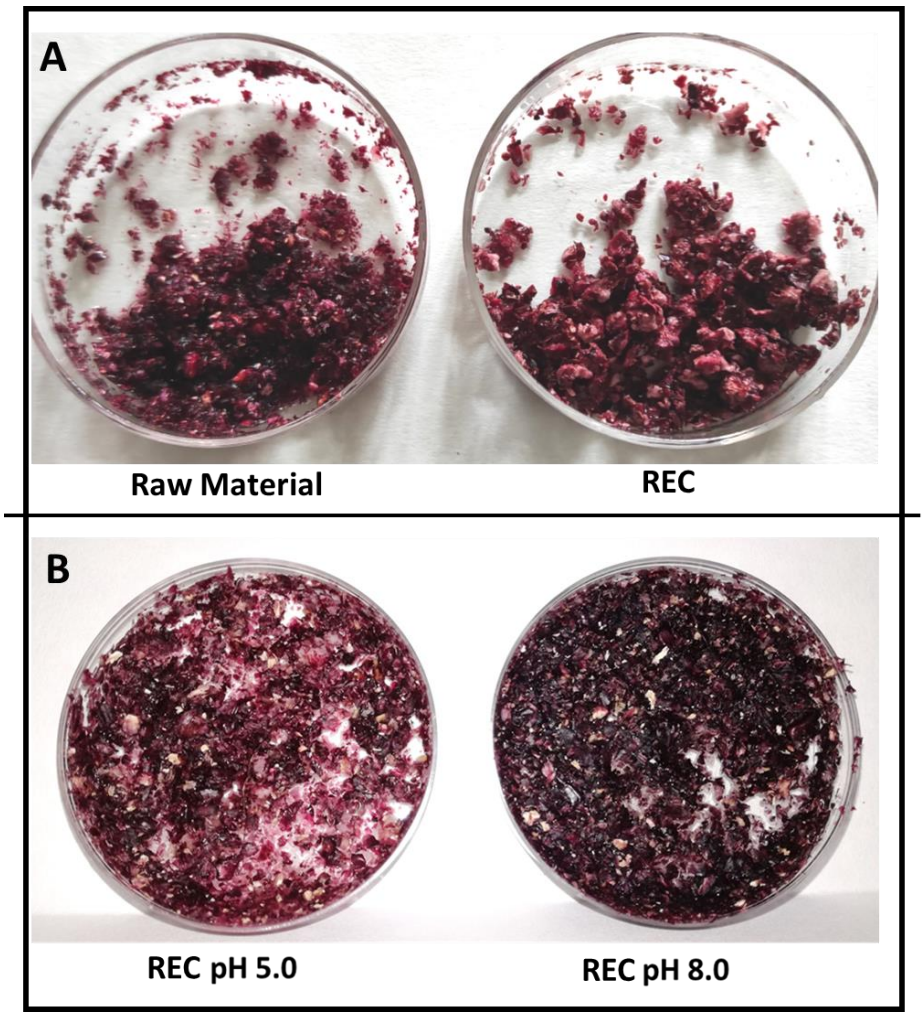


Figure 4. Halochromic property of purple corn cob: (A) raw material before biorefinery process (top left) and residual exhausted cob (REC) (top right) soaked with water (pH of 4.2); (B) REC soaked with different pH buffers: pH 5.0 (bottom left) and pH 8.0 (bottom right).

The result showed a perceptible REC colour change between the two treatments (pH 5.0 *vs* pH 8.0 in Figure 4B). The prevalence of the colourless chalcone form of anthocyanins pigment at these pH[41] associate to ligno-cellulosic matrix confer a brownish colour (background colour) which slightly mask the color change. This is evident in the test with distilled water (Figure 4A) in which corn cob before and after extraction did not show differences in pH (4.2) and therefore in colour. Nevertheless, at pH 8.0 the change in colour was more marked, since the quinone-

base form of anthocyanins became the main molecular form, shifting the REC colour towards green and blue shades. The use of the REC as a pH indicator could be useful to pet owners to evaluate when to replace the litter box or even can give, in some cases, indications on the state of the animal's health.

▪ Conclusion

Purple corn cob represents a sustainable source of high added-value products recoverable by a biorefinery approach, according to the concept that the bioeconomy must be restorative and regenerative. The proposed biorefinery concept was analyzed in detailed aspects of the extraction and the potential for subsequent valorization of the products, and useful data were obtained.

Water extraction gave an anthocyanins-rich extract (PAE) fraction usable as a natural dye, showing, also, UV protection of the dyed clothes. Consumers can take advantage of textile products of excellent aesthetic appearance and also for their sustainability since they are dyed with natural pigments, with good durability and acceptable stability. The PHAE extract subsequently extracted demonstrated a good anti-inflammatory property and it can be used in food/feed or beverages to enhance health benefits.

Subsequently the purple lignocellulosic solid residue (REC) can be considered as a feasible animal bedding for pets, which may also include a pH indication capacity, and which can be compostable once discharged, nulling the waste produced.

Further studies needed in order to provide more data with regards biorefinery process of purple corn cob, *i.e.* mass balance, energy efficiency including cost analysis.

Associated Content

SI Supporting Information

Preliminary experiments on water extraction anthocyanins (based on temperature and sample-to-solvent ratio); HPLC chromatograms of corn cob extracts; chemical structure of anthocyanins.

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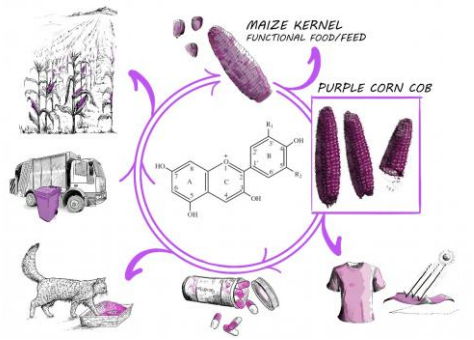

Synopsis

Cascade extraction approach allows makes purple corn cob recovery versatile and useful