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Valorization of pumpkin seed hulls, cucurbitin extraction strategies and their comparative life cycle assessment

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ABSTRACT

In most cases, final agricultural products such as vegetables, fruits, and cereals are associated with the industrial generation of byproducts that are usually unexploited to express all their potential value or, in the worst but common case, directly disposed of as wastes. Even after industrial processing, plant biomasses still represent an invaluable source of unique and human-useful compounds. Within the circular economy, the valorization of industrial wasted biomasses is considered an opportunity to provide new higher-value products with a concomitant solution to waste accumulation issues.

In this framework, pointing to circularity, the seed hulls industrial residuals discarded as waste deriving from *Cucurbita pepo* L. (pumpkin) are hereby investigated to be re-entered in a circular valorization chain. After a full characterization of the biomass residual, the focus was set on cucurbitin, a biologically active non-proteic amino acid found only in the *Cucurbita* species. The present research investigates different water-based strategies for cucurbitin extraction and enrichment.

LCA comparative analysis has been performed to evaluate the environmental impacts of the extractions and assess the most sustainable strategy to yield cucurbitin.

1. Introduction

The agricultural sector generates mostly lignocellulosic wastes like straw (dry stalks of crops), molasses, spent grains, bagasse, husk (*e.g.*, wheat, rice, and maize), hulls (*e.g.*, groundnut, walnut, coconut, pumpkin seeds) and other plant waste every year around the world (Ajayi and Lateef, 2023; Elegbede et al., 2021; Ravindran and Jaiswal, 2016). Typically, all these agricultural residues are either burned or rotten in the fields, sometimes causing severe air, soil, and water contamination or waste along the food chain (Omran and Baek, 2022). Among industrial wasted biomasses, *Cucurbita pepo* L. (pumpkin) residuals represent an interesting source of high-value-added compounds and one of the most fascinating cases in which part of the full potential value deriving from the plant biomass is still not fully exploited. Pumpkin pulp is a commonly used aliment all over the world (Adelerin et al., 2022) while the seeds are employed as food or exploited for oil extraction (useful for the treatment of benign prostatic hyperplasia (Massironi et al., 2022). In both cases, a pretreatment for seed hulls removal is necessary.

Pumpkin seeds are known to contain phenolic compounds, amino acids, proteins, and polysaccharides (Andjelkovic et al., 2010). Many of these compounds are particularly interesting because of their antioxidant properties and positive effects on human health (Andjelkovic et al., 2010). Pumpkin seed hulls result less investigated in the literature

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(Dotto and Chacha, 2020), even if it is well established that skins and hulls as outer seed layers, usually contain much higher concentrations of bioactive compounds than inner layers as they represent the first defense line for a plant from the environment (Peričin et al., 2009).

Among them, cucurbitin (chemical structure depicted Fig. 1), a non-proteic amino acid known to be present exclusively in pumpkin species, is used as an antiallergen to prepare cosmetic and pharmaceutic products (Rabasso and Fadel, 2008) and as an anthelmintic for veterinary treatment (Hammond et al., 1997). The first studies on cucurbitin extraction and identification date back to the 60s, when the amino acid was isolated only from the *Cucurbita maxima*, *pepo,* and *moschata* species (Dunnill and Fowden, 1965). Cucurbitin was cited as one of the most active candidates, among others, while testing the antiparasitic properties of pumpkin seeds in the feed sector (Alhawiti et al., 2019; Grzybek et al., 2016). However, despite its potential applications, methodologies for cucurbitin isolation and synthesis have been poorly reported with most recent studies stuck on the first years of the 90s (Mamoun et al., 1995; Schenkel et al., 1992).

In our research, the seed hulls, byproducts of the industrial process that removes the hulls for the naked seeds before their commercialization was explored as a potential source of added-value compounds. A full characterization of the biomass was performed in order to understand the potential valorization of pumpkin seed hulls to achieve a circular process approach intending a zero waste strategy. Moreover, seed hulls were investigated as source of cucurbitin. The present research aims to develop and compare different water-based extraction strategies to valorize further these industrial biomass residuals targeting cucurbitin.

Finally, the evaluation of the most sustainable methodology to obtain the amino acid has been investigated through a comparative Life Cycle Assessment (LCA) by analyzing the environmental performances of applied extraction processes. In our studies, the LCA was performed to evaluate and quantify the environmental impacts of the developed extractions and to assess the most sustainable strategy to yield cucurbitin, identified and extracted for the first time from pumpkin seed hulls.

2. Materials and methods

2.1. Chemicals

Ethanol (97%), hydrochloric acid (37%), ammonia (35–37%), isopropanol, glacial acetic acid, ninhydrin, potassium phosphate monobasic, Dowex® 50WX8-100 ion-exchange resin, HPLC-grade water and HPLC-grade acetonitrile were purchased from Sigma-Aldrich Chemicals (Milan, Italy). Cucurbitin chloride standard was purchased from Phytolab (Vestenbergsgreuth, Germany). Milli-Q water was obtained from a purification system (Millipore, Billerica, MA, USA).

2.2. Biomasses

Cucurbita pepo L. seed hulls (SH), byproducts of the pumpkin seeds, were kindly donated by Indena S.p.A., located in the southeast part of Milan (Lombardy, Italy). The biomass was subjected to a pulverization step by means of a knife blender (Pulverisette 11-Fristch) for 20 s at 5000 RPM.

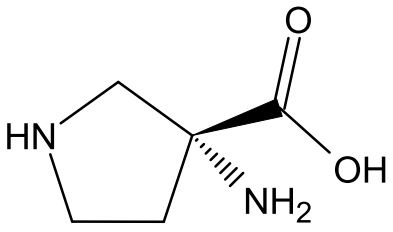

Fig. 1. Cucurbitin chemical structure.

2.3. Biomass characterization

The chemical composition of seed hulls (SH) biomass was evaluated in terms of the moisture, ash, lipid, soluble protein, carbohydrate, and phenolic compounds content, according to the described methods:

Moisture content: was determined gravimetrically and the ratio with its initial mass was calculated after 48 h drying in freeze-dryer (FreeZone6, Labconco Co.).

Ash content was determined by the calcination of biomass following the standard method of AOAC (1990).

Total lipid content was evaluated using the method described by Bligh (Bligh and Dyer, 1959). Briefly, the biomass was extracted with a mixture of chloroform, methanol and water (1:2:0.8) and homogenized. Subsequently, the solvent proportion was changed to 2:2:1.8. After phase separation, the apolar phase was collected, and the polar phase was extracted once more. The apolar phases were combined, and the solvent evaporated to dryness. Total lipid content was quantified gravimetrically in relation to the initial mass.

Soluble protein content was measured according to Bradford (1976) using a Bio-Rad protein assay kit.

Monosaccharides composition was elucidated by sulfuric acid hydrolysis according to conditions proposed by Seaman (Saeman et al., 1954). Two-step methanolysis and trifluoracetic acid (TFA) hydrolysis was applied to investigate hemicellulose and uronic acid content in the biomasses, following the method of Willför (Willför et al., 2009). The determinations were performed in high-performance anion-exchange chromatography coupled to pulsed amperometric detection (HPAEC--PAD) using a Dionex ICS-6000 system (Thermo Fischer, USA). A column CarboPacTM PA20 (3 × 150 mm) was used for the separation. The gradient method used Milli-Q water, 200 mM NaOH, and 100 mM NaOH +100 mM sodium acetate, as eluents A, B, and C, respectively at a flow rate of 0.4 mL/min. The quantification was performed against the calibration curve built with neutral sugars and uronic acids (fucose, arabinose, rhamnose, glucose, xylose, mannose, galacturonic acid, and glucuronic acid) standards. Cellulose content was determined by the difference between glucose content from sulfuric acid and methanolysis hydrolysis.

Total starch was enzymatically through α-amylase treatment and gravimetrically determined using the Total starch kit (Megazyme, Ireland).

Total free and bonded phenolic compounds (TPC): Free phenolic compounds were extracted according to the methodology described by Pérez-Jimenez (Pérez-Jiménez et al., 2008). Bonded phenolics were extracted using 2M NaOH saponification at 60 °C, overnight, followed by a partition with ethyl acetate 1:1. Total phenolic compounds were quantified by their interaction with Folin-Ciocalteu's reagent using an adaptation of the method proposed by Cicco (Cicco et al., 2009). In details, 15 μL of each biomass extract and 15 μL of Folin-Ciocalteu were added to the microplate wells. After 2 min, 200 μL sodium carbonate (5%) was added. The microplate was stored at 37 °C for 30 min and read at 760 nm in a ClarioStar (BMG Labtech, Germany) microplate reader. Gallic acid was used as the standard, and the results were expressed in mg GAE g^{-1} of dry sample.

Phenolic acids profiling: The phenolic acids profile was determined by high-performance liquid chromatography (HPLC) using a Waters HPLC-DAD system constituted by a separation module (Waters 2695, USA) coupled to a photodiode array detector (Waters 2996, USA). A ZORBAX StableBond C 18 column (Agilent Technologies, USA) was used for separation. A gradient method was performed with 2% acetic acid (v/v) and methanol as eluents according to the method described by Szydłowska-Czerniak (Szydłowska-Czerniak et al., 2011), with a sample injection volume of 50 μL. Calibration was performed using gallic acid, ferulic acid, ρ-coumaric acid, t-cinnamic acid, sinapic acid, and caffeic acid with a concentration range of 0.005–0.1 g/L of each standard. The individual components were quantified based on the retention times of the standards at 325 nm. Biomass extracts obtained

through saponification with sodium hydroxide and ethyl acetate partition were used.

2.4. Cucurbitin extraction

To understand the effectiveness of different extraction processes targeting cucurbitin, SH biomass was subjected to two different extraction strategies:

- **Water extraction**: exactly 100 g of SH biomass were introduced into a 1 L round flask and mixed with 750 mL of water. The mixture was magnetically stirred at 300 RPM at 60 °C for 6 h with a 500 mL silicon oil bath. Then, a stainless steel sieve (0.075 mesh) was used to separate the exhausted solid residuals from the water solution. The filtered solution was transferred into plastic tubes of 50 mL and underwent centrifugation (Rotofix 32 A-Hettich Zentrifugen) for 30 min at 6000 RPM to separate the fine solid particles still present in the liquid. Then, to reduce the volume of the liquid, the solvent was evaporated by rotary evaporation (60 mbar, 40 °C, 400 RPM) for 4 h.
- **Ethanol/water extraction (1:1)**: exactly 100 g of SH powder were introduced into a 1 L round flask and mixed with 500 mL of water/ethanol mixture (1:1). The mixture was magnetically stirred at 300 RPM at 60 °C for 6 h with the aid of a 500 mL silicon oil bath. Then, a stainless steel sieve (0.075 mesh) was used to separate the exhausted solid residuals from the water solution. The filtered solution was transferred into plastic tubes of 50 mL and underwent centrifugation for 30 min at 6000 RPM, in order to separate the fine solid particles still present in the liquid. Then, to reduce the volume of the liquid, ethanol was evaporated by rotary evaporation (60 mbar, 40 °C, 400 RPM) for 4 h.

The resulting water extract and water/ethanol extract derived from seed hulls will be labeled SH_{H2O} and $SH_{EtOH/H2O}$, respectively.

2.5. Cucurbitin enrichment

- **Polysaccharides and proteins separation**: proteins and polysaccharides were separated from amino acids and sugars by ethanol precipitation. A measured volume of ethanol was added to solutions deriving from SH water extraction to reach the ratio 1:1 = ethanol: water. The step of protein and polysaccharides precipitation was skipped for extracts deriving from ethanol/water extraction due to their absence in the obtained extract. The as-obtained mixtures were introduced into the refrigerator and kept at 4 °C for 2 h to promote proteins and polysaccharides precipitation. The suspensions were transferred into plastic tubes of 50 mL and underwent centrifugation for 20 min at 6000 RPM, to separate the solid from the liquid solution. The supernatants were transferred into 500 mL round flasks, and ethanol was evaporated by rotary evaporator for 15 min at 60 mbar and 40 °C. Then, obtained solutions derived from the precipitation procedure above and extracts derived directly after ethanol/water extraction were transferred into two 200 mL crystallizers and left at −20 °C for 12 h. Frozen extracts were freeze-dried (Edwards Pirani 1001) for 48 h.
- **Sugars separation**: to enrich the extracts in amino acids and separate them from sugars, the cation exchange resin DOWEX 50W-8X was used. The resin was activated with an aqueous solution of 1M HCl (3:1 v/w, solution/resin) for 30 min and then washed with milli-Q water until the pH was neutral. The resin beads were then introduced into a glass column (L = 10 cm, d = 1.5 cm) and put in contact with an aqueous solution of the extract deriving from the previous lyophilization (extract/resin ratio of 1:4 w/w). After 2 h, the eluate (containing free sugars) was collected. The absence of amino acids was confirmed by the ninhydrin assay. Consequently, a 2% ammonia aqueous solution was slowly added to the column until the pH of the eluate turned alkaline to desorb and collect amino acids. The eluate was collected in 10 mL glass tubes. The presence of amino acids in each fraction was confirmed through the ninhydrin assay. Elution was performed until the ninhydrin assay was negative, indicating the absence of amino acids. Collected aliquots were transferred into crystallizers and after 12 h at −20 °C, the frozen extract solutions were freeze-dried for 48 h.

Then, a second cycle of ion exchange resin was performed to fractionate amino acids based on their pk_a, to enrich the extracts in cucurbitin. The resin was activated with an aqueous solution of 1M HCl (3:1 v/w, solution/resin) for 30 min and then washed with milli-Q water until the pH was neutral. The resin beads were then introduced into a glass column (L = 10 cm, d = 1.5 cm) and put in contact with an aqueous solution of the extract deriving from the previous lyophilization (extract/resin ratio of 1:4 w/w). Adding aliquots of 3 mL, a gradient of aqueous ammonia solution was performed, increasing the ammonia concentration with the following steps: 0.5, 0.8, 1.0, 1.5, and 2.0 %. Each fraction was tested with the ninhydrin assay, and the pH was measured in the eluate all over the elution gradient.

2.6. Amino acids identification

Amino acids profile was evaluated by ion-exchange chromatography (IEC) (Hogenboom et al., 2017). Amino acids content was determined on 4 mg of the extract dissolved in 20 mL of trisodium citrate buffer (0.2 N; pH 2.2). The solution was then filtered on 0.22 μm cellulose acetate filter (Merck Life Science) prior to injection. An amino acid analyser Biochrom 30+ (Erreci, Milan, Italy) was used and the amino acid quantification was carried out with a multipoint calibration curve. Injection volume was 100 μL. Analysis were carried out in duplicate.

2.7. Cucurbitin identification and quantification

Thin Layer Chromatography: the presence of amino acids (in particular cucurbitin) in samples was detected by means of Thin Layer Chromatography (TLC). Sample aliquots were spotted on silica gel (Type 60 F254, Merck) 5 × 10 cm thin layer chromatography plates using a mixture of isopropanol, water, and acetic acid (4:2:1) as mobile phase. After elution, ninhydrin reagent (0.2% in ethanol) was sprayed on the plate. After heating, the presence of amino acids was detected by violet or orange visible spots.

High-Performance Liquid Chromatography: cucurbitin quantification was performed by means of High-Performance Liquid Chromatography (HPLC, Waters e2695, Milford, MA, USA) equipped with a Kinetex® Hilic column (100 Å, 2.6 μm, 4.6 mm × 100 mm) and a PDA (photodiode array, Waters 2996, Milford, MA, USA) detector. The mobile phase was a mixture of acetonitrile (85%) and potassium phosphate monobasic buffer aqueous solution (pH = 2.8; 12.5 mM) (15%) at a flow rate of 1 mL/min in isocratic mode. The injection volume was 5 μL. The total run time for each analysis was 20 min, and the wavelength was set at 210 nm. Data were processed with Empower 3 workstations. Before sample injections, 5 dilutions of a cucurbitin chloride standard water solution (10 mg/mL) were prepared in the range of 0.007–10.0 mg/mL. Standard solutions were filtered (0.2 μm nylon filters) and injected three times in the HPLC system. The limit of detection (LOD) and limit of quantification (LOQ) were calculated as 3- and 10-fold the signal-to-noise ratio.

2.8. Life cycle assessment

Life cycle assessment (LCA) calculations were performed using the SimaPro 8.5.1.17 software (PRE, Product Ecology Consultant) to analyze the life cycles of the experimentally obtained extracts. Ecoinvent v3.1 and ELCD v3.1 libraries were adopted to account for the secondary data. All inputs and outputs were referred to the extracts containing 1 kg of cucurbitin, chosen as the functional unit.

The generated inventory was assessed using the International Reference Life Cycle Data System (ILCD) and the single issue method Intergovernmental Panel on Climate Change (IPCC) 2013 with a time frame of 20 years.

In the execution of the calculations we used Ecoinvent 3 allocation, cut-off by classification – Unit library.

In performing the LCA analysis, the following approximations have been made:

- *Cucurbita pepo* L. seed hulls were considered virgin material.
- The energy consumption was estimated considering the tension, the resistance, and the intensity of the current of the instruments used during the experimental processes. All the instruments used a medium/low voltage (220V for the Italian Network). From the instrument power, measuring the amount of time of application has been estimated the energy consumption in kWh. This process could have caused some overestimation.
- It was preferred to select inputs defined as average for the Italian geographical location. When not possible, preference was given to those defined for the European geographical location over the Global geographical location.

3. Results

3.1. Biomass characterization

The valorization process is highly influenced by biomass chemical composition. For this reason, pumpkin seed hulls (SH) biomass was elucidated, and the results are described in Table 1.

SH biomass presents a relatively low moisture content (3.45%), which is interesting in the context of biomass storage for further valorization processing, thereby presenting the potential for a long storage period. The moisture content for SH is in accordance with literature results for *Cucurbita pepo* L. SH (Srbinoska et al., 2012).

Ash content is related to inorganic fractions where the mineral content is included. Ash content in SH represents less than 1% of the total biomass. The results are in accordance with the difference reported in the literature for hulled pumpkin seeds and kernels for *C. maxima* (Alfawaz, 2004). Srbinoska and co-workers reported an ash content in SH of 3.2% and 1.1% for *C. pepo* and *C. maxima*, respectively (Srbinoska et al., 2012).

Lipid content for SH represents 0.35% of the biomass in the present study. Srbinoska reported lipid extraction yield values between 4.44 and 6.55% for *C. pepo* SH and between 3.17 and 4.24% for *C. maxima* SH, which ranged in the function of solvent polarity index used in oil extraction and in function of cucurbita species (Srbinoska et al., 2012).

Table 1
Pumpkin seeds hulls chemical composition.

Compounds	Composition (mg/g)
Moisture content	34.5 ± 2.70
Ash	8.8 ± 0.10
Lipid	3.5 ± 0.5
Soluble protein	5.49 ± 0.62
Carbohydrate[b]	284.29 ± 17.67
Starch[c]	n.d.
Cellulose[d]	214.53 ± 10.59
Other polysaccharides[d]	47.47 ± 12.96
Phenolic compounds	3.07 ± 0.66
Phenolic acids	0.02 ± 0.001

[a] Result expressed in wet basis.
[e]Hemicelluloses from methanolysis.
[b]Total carbohydrate content determined as the sum of neutral sugars from sulfuric hydrolysis and uronic acids from methanolysis.
[c]Determined by enzymatic total starch kit.
[d]Determined by the difference in glucose content between sulfuric hydrolysis and methanolysis.

Protein content in pumpkin SH was investigated in terms of soluble proteins. Pumpkin SH presented a protein content of 0.55 % of total biomass. The low soluble protein content is in good accordance with the difference of protein observed in the whole seed and kernel in a study on hulled and hull-less genotypes of *C. pepo* seeds. Values of 3.48 and 3.31 g.100 g^{-1}, respectively, were reported, indicating that SH does not contain considerable amounts of soluble proteins (Charaya et al., 2023).

Total carbohydrate content was estimated as 28.43% of total biomass, from which 21.45% was represented by cellulose and 4.75% by hemicellulose presence. No starch was detected in the pumpkin SH. The total carbohydrate content is in line with previously reported values (Devi et al., 2018; Gohari Ardabili et al., 2011).

A close look into the SH monosaccharides composition (Fig. 2a) demonstrated that glucose is the major monosaccharide present in the biomass, which is attributed to the high content of cellulose, followed by xylose, galactose, and arabinose. Xylose presence in SH biomass might be associated with the presence of hemicelluloses as xyloglucan and xylan, which is in good agreement with the observations reported by Košťálová and co-workers (Košťálová et al., 2009). Total phenolic compounds (Table 1) were investigated as free and bonded fractions from SH (Fig. 2b). Despite the bonded fraction presenting values slightly higher than the free fraction of phenolic compounds, it was not statistically different ($p < 0.05$). This result suggests that free and bonded contributed equally to the global phenolic compounds in pumpkin SH biomass.

Among phenolic compounds present in the biomass, total phenolic acids count as 0.02 mg g^{-1} DW, representing approximately 65.15% of total phenolic compounds present in the biomass. In a literature study it was reported that phenolic acids are mostly presented in pumpkin SH in the bound form along with other cell wall compounds, being represented as esterified (0.024 mg g^{-1} DW) and insoluble-bonded (0.06 mg g^{-1} DW) (Peričin et al., 2009). The reported result is higher than observed in the present study for SH, which might be attributed to the agricultural conditions either to *C. pepo* subspecies investigated. Moreover, caffeic and ferulic acid were the only phenolic acids identified in the SH, at the concentrations of 0.01 ± 0.0017 and 0.01 ± 0.0019 mg g^{-1} DW, respectively.

3.2. Cucurbitin extraction strategy

Two extraction strategies were developed and compared in the present work with the aim of targeting cucurbitin. In the first strategy, water was selected and employed as the sole extraction solvent adopting the same condition of Dunnill and co-workers with some modifications (Dunnill and Fowden, 1965). The use of water has well-known benefits as a green extraction solvent because water is not only inexpensive and environmentally benign, but it is also non-flammable and nontoxic, providing opportunities for clean processing and pollution prevention (Pingret et al., 2013). In the case of the biomass of the present work, the water extract was expected to contain amino acids (the target of the work) and sugars, but also proteins and polysaccharides.

On the other hand, an ethanol/water (1:1) solvent commonly used for amino acids extraction but never applied for cucurbitin extraction was used for comparative purposes. Using a "bio-solvent" like ethanol should be considered a strategy anyway included in the green extractions. Ethanol can be produced from renewable resources by fermentation of sugar-containing biomasses or lignocellulosic materials, substituting the petrochemically fabricated solvents and avoiding fossil resource use and fossil fuel CO_2 emissions to the environment (Capello et al., 2007). The main reason behind the use of ethanol-water mixture as a comparative extraction strategy was to avoid the presence of polysaccharides and proteins in the extracts, in this way bypassing the protein and polysaccharides precipitation step since the extracts were expected to contain amino acids and sugars only. Moreover, ethanol is already regulated by the FDA as a food ingredient (*i.e.,* additive), and it is found as an active ingredient in oral, parenteral, and topical

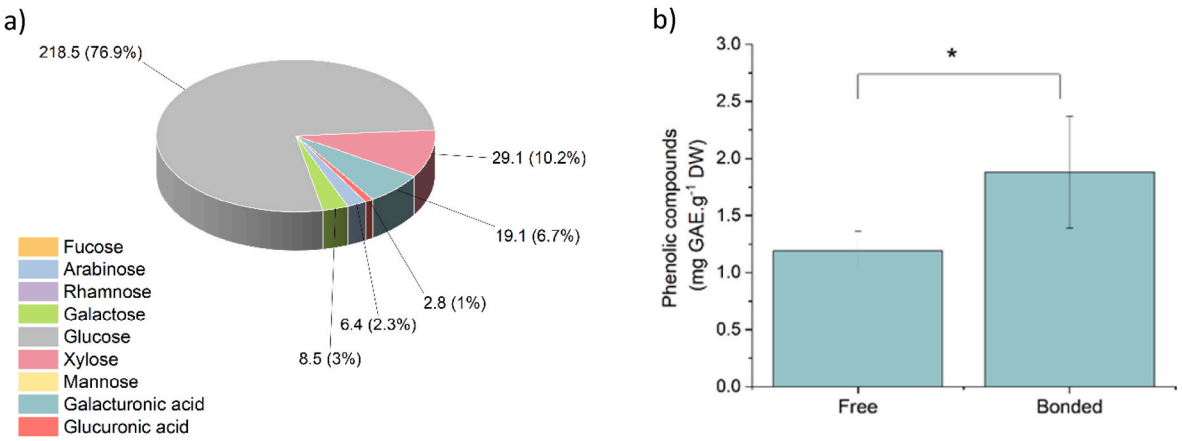

Fig. 2. Results from monosaccharides and phenolics composition. a) Monosaccharides composition of pumpkin seed hulls, represented as the sum of neutral sugars and uronic acids. b) Total phenolic compounds of pumpkin seed hulls expressed in terms of free and bonded compounds. *Indicates results not statistically different at ($p < 0.05$) by variance analysis (ANOVA).

(including inhalational) prescription and nonprescription drug products, in this way posing fewer issues in terms of residuals in the extracts (Parker, 1982).

Relevant results from extractions are listed in Table 2. Differences in extraction yields can be associated with the adopted extraction technique. As expected, water extraction provided higher yields compared to ethanol/water. This is related to the hindrance in the extraction of proteins when using a mixture containing ethanol together with water. In this case, the extraction is more selective (because proteins and polysaccharides are not extracted), and hence yields are lower.

After each extraction procedure, the presence of cucurbitin in the obtained extracts was preliminarily confirmed by TLC analysis. In fact, by means of ninhydrin assay, cucurbitin is revealed by an orange/violet spot. Cucurbitin can bond one or two ninhydrin molecules involving the amine of the aminopyrrolidine group or the amine sidechain group (Fig. S1, Supporting Information), resulting in a double color result.

3.3. Cucurbitin enrichment

3.3.1. Polysaccharides and proteins precipitation

Obtained extracts were subjected to further purification steps to separate proteins and polysaccharides by precipitation, in this way increasing the cucurbitin concentration in each sample (Yoshikawa et al., 2012; Zhang et al., 2018). Such procedure is mild and easily scalable, in this way avoiding any potential degradation of target molecules (Zhang et al., 2018). The amount of proteins and polysaccharides in the water extract was equal to 21.9%, whilst the remaining 78.1% of the extract weight was attributed, by difference, to the presence of amino acids and sugars, corresponding to 3.8% of the weight of the starting SH biomass. The amount of proteins was anyway low (around 1% of the SH biomass weight), as predictable considering that seed hulls are mainly composed of vegetable fibers (Dotto and Chacha, 2020; Glew et al., 2006). Results are reported Table S1.

As expected, the presence of proteins and polysaccharides was not detected in the extracts involving ethanol as a co-solvent.

3.3.2. Sugars separation

To further increase the concentration of cucurbitin in the extracts, Cation Exchange Chromatography (DOWEX 50W-8X) was employed. Two cycles were performed, with a resin regeneration step in between, the first to separate amino acids from free sugars and the second to further enrich the cucurbitin by amino acids fractionation based on their charge at different pH.

In the first cycle, free sugars were easily separated from the amino acids since sugars do not bear any net charge, while amino acids (including cucurbitin) are positively charged at neutral pH (pKa of strongest basic group of to the aminopyrrolidine group: 10.68; pKa) (Dunnill and Fowden, 1965; Mihranian and Abou-Chaar, 1968).

The highest amino acids extraction yield is related to SH_{H2O} (0.78%), while $SH_{EtOH/H2O}$ presented a slightly lower amount of amino acids (0.62%) after the first purification cycle. All results are, however in the same order of magnitude in terms of amino acids %, confirming that all the extraction procedures were effective in the amino acids extraction. As a consequence, the extract after proteins and polysaccharides removal was composed of amino acids in percentages varying from 17.9% to 23.6%.

After resin regeneration and amino acids absorption, the second cycle involved the application of a pH gradient in order to fractionate amino acids on the bases of their net charge at different pH to further increase the cucurbitin content in specific fractions, among the ones that are expected to contain only basic amino acids. Indeed, cucurbitin basicity arises due to the presence of the aminopyrrolidine group within its structure. When the aqueous ammonia elution gradient was performed, six main fractions were isolated. The presence of cucurbitin was monitored through TLC analysis and observed in the final two fractions containing the basic amino acids. No particular differences in terms of cucurbitin yields have been observed among obtained extracts after the second DOWEX cycle, characterized by 0.14–0.15% yields, corresponding to the % of basic amino acids (containing cucurbitin) by weight of the starting biomass.

Table 2
Results from extractions in water and ethanol/water solvents.

Sample	Starting Biomass	Extract weight	Extraction yield
SH_{Water}	100 g	4.81 ± 0.2 g	4.8 ± 0.2 %
$SH_{Ethanol/Water}$	100 g	4.17 ± 0.3 g	4.2 ± 0.3 %

3.4. Extracts characterization

3.4.1. Amino acids analysis

IEC analysis made it possible to determine the content of 21 amino acids in the extracts. The free amino acids (FAA) profile of $SH_{Ethanol/Water}$ extract, the sample obtained after the free sugars separation, is shown in Fig. 3 and amino acids quantification is reported in Table S2. IEC analysis was able to evidence the presence of cucurbitin, identified on the basis of the retention time of the cucurbitin commercial standard. The amount of cucurbit was about 77.31 μg/mg, in agreement with HPLC results. The extract was rich in alanine (ALA), gamma-aminobutyric acid (GABA), lysine (LYS) and arginine (ARG), whose

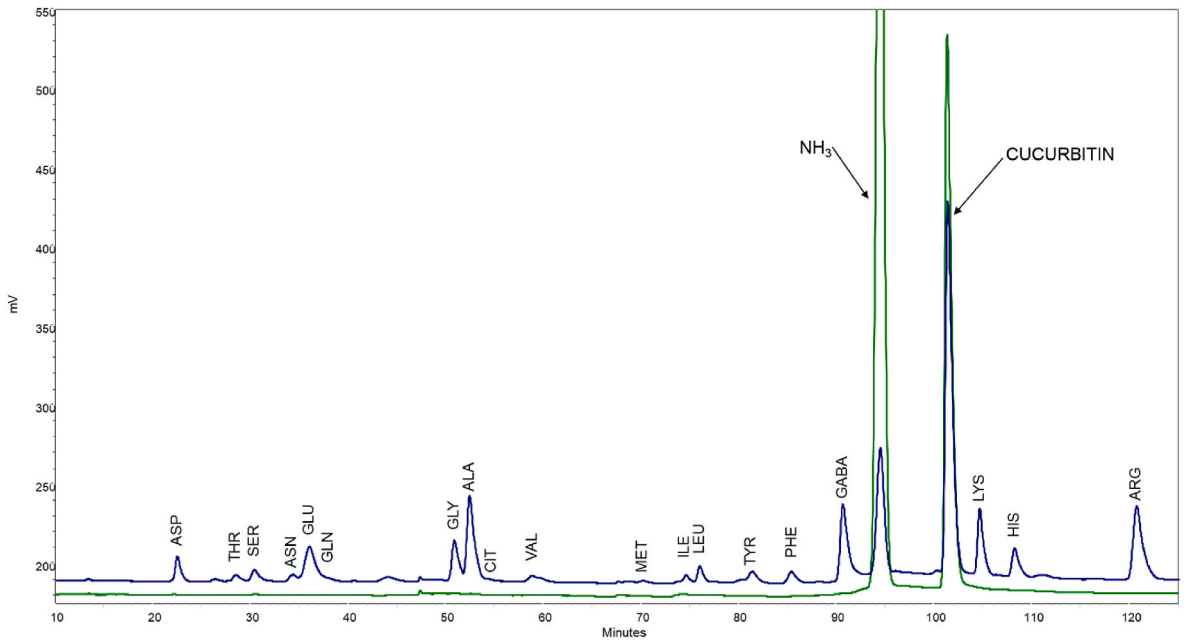

Fig. 3. IEC chromatogram at 570 nm of $SH_{Ethanol/Water}$ (blue line) and cucurbitin commercial standard (green line). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

contents approximately ranged between 25 and 78 μg/mg. These amino acids represent the 46% of the total FAA content. Citrulline (CIT), glutamine (GLN) and methionine (MET) showed the lowest values of 0.92 μg/mg, 1.59 μg/mg and 1.90 μg/mg, respectively. The extract showed high content of ammonia, due to the purification procedure by DOWEX resin.

3.4.2. HPLC cucurbitin quantification

In order to quantify the cucurbitin amount in each extract, preliminary injections with increasing concentrations of aqueous cucurbitin standard solutions were performed. Cucurbitin was eluted as sharp peaks at retention time of 11.5 min. In the operative concentration range the trend was with no saturation effects that could bend the linearity. The area under each peak was quantified by Empower 3 instrumental software and plotted versus the concentration. The best fit of experimental data in the plot "Peak area vs cucurbitin concentration (in mg/mL)" was then used for cucurbitin quantification in each sample (regression line reported in Figure S2).

From the correlation between the peak area and standard concentrations it was possible to obtain the equation for quantifying the concentration of cucurbitin in the samples, $y = 44547x - 5915.8$, in which, "y" is the peak area (expressed as units of absorbance) and "x" is the standard concentration (in mg/mL).

Limit of detection (LOD) and limit of quantification (LOQ) were calculated as 3- and 10- fold the signal-to-noise ratio, and they were found equal to 0.06 and 0.53 mg/mL, respectively.

The coefficient of determination (R^2) was found equal to 0.9995.

HPLC analysis of the extracts evidenced the presence of a main peak at the same retention time of the cucurbitin commercial standard.

In Table 3, cucurbitin quantification results are reported. Cucurbitin content in the biomass ranges from 0.039% (in case of water extraction) and 0.044% (in case of ethanol/water extraction).

To the best of the authors' knowledge, no results on cucurbitin identification and extraction from pumpkin seed hulls have been reported before.

Results of extract enrichments, displayed in Table 3, show that the concentration of cucurbitin increases significantly after the first purification step on DOWEX (from 0.81% to 5.0% for SH_{Water} and from 0.96% to 7.1% for $SH_{Ethanol/Water}$). After the second cycle on DOWEX, the concentrations of cucurbitin are always higher than 28%. Considering that the industrial targets for most cosmetic applications are usually extracts containing 1% of cucurbitin, results achieved in this work highlight the feasibility of the strategies hereby proposed for real-scale application (Renimel et al., 1992).

Table 3
Cucurbitin quantification.

Extraction procedure	Starting Biomass	Extracts	AA + S	AA	AA fraction containing Cucurbitin
SH_{Water}	110 g 0.04% cucurbitin	5.297 g 0.81% cucurbitin	4.139 g 1.04% cucurbitin	0.861 g 5.0% cucurbitin	0.154 g 28% cucurbitin
$SH_{Ethanol/Water}$	110 g 0.04% cucurbitin	4.586 g 0.96% cucurbitin	4.586 g 0.96% cucurbitin	0.687 g 7.1% cucurbitin	0.161 g 30% cucurbitin

3.5. Life cycle assessment

All the data related to both extractions in water and the ethanol/water were collected during the experimental process. For both extractions, SH was considered as a virgin material to which any kind of environmental impact is attributed. The two experimental pathways were divided into five steps given as output analogies products. Complete data, related to the carbon footprint per kg of cucurbitin in the extracts with a margin of exclusion of 0.5% of both two extraction techniques, are reported in Table S3.

As displayed in Fig. 4, relevant results show that the extract involving the ethanol as a co-solvent is the most impacting one, having attributed 1.88E+06 kg CO_2 eq, while, on the other hand, the carbon footprint of the extract involving just water as solvent is equal to 1.19E+06, meaning that is the 37% lower of the first one.

The higher indirect energy consumption of $SH_{Ethanol/Water}$, displayed in Fig. 4, is to be attributed to the higher amount of ethanol applied per unit of cucurbitin. Indeed, the indirect energy consumption is associated with the inputs and the different processes necessary to obtain ethanol.

In the International Reference Life Cycle Data System (ILCD) Characterization (main results reported in Fig. 5, all data reported in Fig. S3)

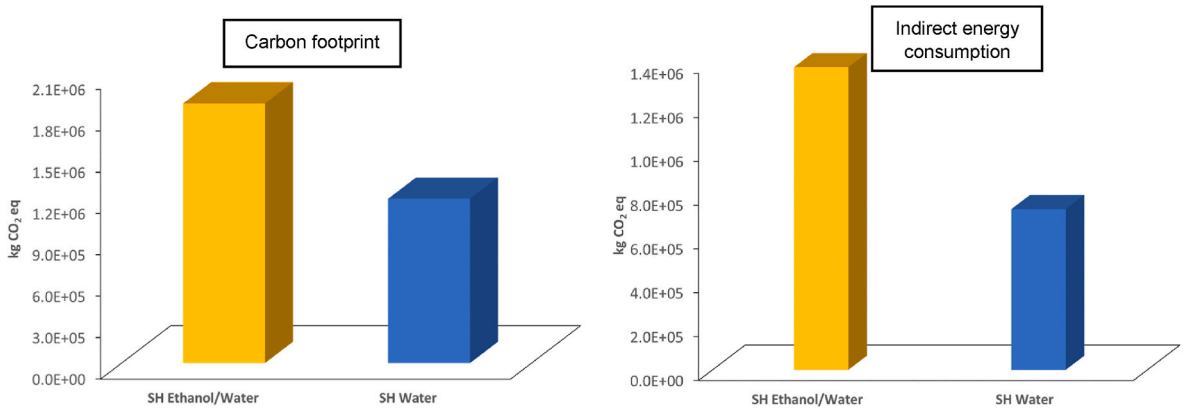

Fig. 4. Comparative LCA results, calculated per kg of cucurbitin in the extracts, for water and ethanol/water extractions. Left: Carbon footprint; Right: indirect energy consumption.

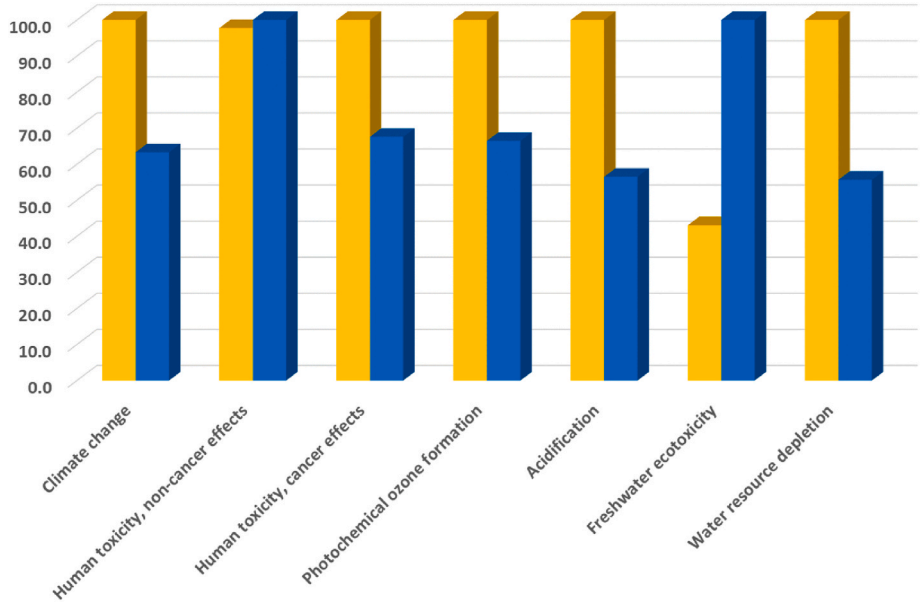

Fig. 5. Extract from ILCD results (complete results in Supporting Information).

each single impact category is expressed as normalized in respect of the category of the output having the higher impact.

Looking at results, it comes evident that for all the impact categories the $SH_{Ethanol/Water}$ output has a significantly higher impact than SH_{Water}, except for Freshwater Ecotoxicity, Human Toxicity non-cancer effects.

Analyzing in detail Human Toxicity cancer effect, Human Toxicity non-cancer effects and Freshwater Ecotoxicity we can highlight the following considerations:

- In **Human Toxicity cancer effect** both impact categories are reported in Comparative Toxic Unit for humans (CTUh), expressing the estimated increase in morbidity in the total human population per mass of a chemical emitted. Substances related with the 99.5% load in the Human Toxicity cancer effects, their impact and the compartment impacted by them are reported in Table S4. Data show that the substances most polluting are three and are all heavy metals. More precisely, Arsenic, Chromium and Nickel. Nickel and Chromium are related with the chemical industry, indeed are used in order to produce metallic pipes able to resist to corrosion and extreme temperatures (Sarzosa et al., 2017), while Arsenic is used in the production of ethanol, since it stimulates glycolic metabolism increasing the rate of fermentation (Takeshige and Ouchi, 1995). The impact on this category is higher for the $\mathbf{SH_{Ethanol/Water}}$ final output, due to the highest amount of ethanol necessary to perform the extraction.
- Substances related with the 99.5% load in the **Human Toxicity non-cancer effects**, their impact and the compartment impacted by them are reported in Table S5. The substance the most impacting is the Arsenic, then Barium, with Cadmium, Lead, Mercury, and Zinc. Zinc, Lead and Cadmium are applied in the chemical industry in order to produce pipes resistant to corrosion and extreme temperatures (Andreatta et al., 2017; Kakonyi and Ahmed, 2020; Wang et al., 2012). Barium is related to the oil and gas sector, indeed is used in the extraction processes for both oil and gas (Cappuyns, 2012), while Mercury is mainly attributed to the combustion of fossil fuels (Qian et al., 2009; Zhang et al., 2015) and Arsenic, as already explained, is involved in the production of ethanol.
- The most impacting substances for the **Freshwater ecotoxicity impact category** are finally reported in Table S6. In this case, the higher impact is related to the final extract obtained from the water extraction procedure (1.08E+07 CTUe for SH_{Water} against 4.65E+06 CTUe for $SH_{Ethanol/Water}$) accounting an impact more than double than the other extract. The impact is estimated as Comparative Toxic Unit for ecosystems (CTUe) expressing an estimate of the potentially

affected fraction of species (PAF) integrated over time and volume per unit mass of a chemical emitted (PAF m^3 d/kg), and the final output obtained from the calculation is the volume of water polluted in one year. In this case, the two substances related with the higher pollution of SH_{Water} are Antimony and Copper.

4. Conclusions

Pumpkin seed hulls biomass presented great potential for valorization, which includes a high content of carbohydrate compounds, represented by cellulose and xylose-derived hemicelluloses that could be used for cellulose fibers extraction for material applications or dietary fibers in the case of hemicellulose fraction. The presence of phenolic compounds in seed hulls is also attractive for valorization since the extraction in free form but also attached to the fibers can be of great interest for food and cosmetic applications.

When the target was set on cucurbitin, extractions using both water and ethanol/water (1:1) were confirmed to be both suitable and effective in the extraction of the amino acid, yielding extracts characterized by a content of cucurbitin in the range of 0.81–0.96 %, depending on the specific extraction. Further purifications were able to increase cucurbitin content up to 28–30%.

In perspective of potential applications, the enriched extract obtained after one only use of ion exchange resin was characterized by a cucurbitin content in the range of 5.0–7.3%, a result already competitive for its exploitation as bioactive agents in formulations, since it has been documented that extracts containing 1% of cucurbitin can already have an industrial application (Renimel et al., 1992). Avoiding a second step of further purification by ion exchange resin could reduce costs and time, thus reducing the related environmental footprint. However, higher cucurbitin concentration may enhance the final product bioactivities.

The LCA comparative analysis was performed to better clarify the sustainability of the selected extraction strategies. It demonstrates that the environmentally more sustainable extraction strategy is the one involving exclusively water as solvent. The same results are obtained from both the single-issue IPCC 2013 and ILCD Midpoint analysis methods. The difference in terms of environmental costs between the two extracts is mainly related to their energy consumption, both direct and indirect. Indeed, Ethanol/Water extract has a lower direct energy efficiency, meaning that providing the same quantity of cucurbitin requires more energy than the extraction in water. Moreover, the application of ethanol as co-solvent involves a higher ethanol demand per unit of cucurbitin (more than three times higher), instead of using ethanol just to make precipitate proteins and polysaccharides as in Water extract and being ethanol related with high energy demanding processes and highly harmful compounds, $SH_{EtOH/H2O}$ environmental impact is higher. In any case, this second aspect is the one weighting on the higher environmental impact of the Ethanol/Water strategy.

In conclusion, this study brings light on the potential benefits of utilizing pumpkin seeds byproducts as agricultural waste to extract added-value compounds and on the environmental implications of different extraction strategies. The investigation by means of life cycle assessment has highlighted the importance of comparing extraction practices in the development of cleaner production technologies. It was demonstrated that, by optimizing extraction processes and minimizing energy and resource consumption, the carbon footprint associated with cucurbitin production can be modulated. Furthermore, the findings of this research emphasize the significance of circular economy principles to foster the adoption of cleaner and more sustainable approaches in the valorization of agricultural byproducts, aligning with the global need for sustainable resource management and waste reduction.

In perspective, the biological activities of cucurbitin extracted from pumpkin seeds waste will be investigated, in this way coupling the use of a natural and plant-based origin product with the increasing demand for eco-friendly and health-conscious solutions in the pharmaceutical and feed industries.

CRediT authorship contribution statement

A. Massironi: Data curation, Formal analysis, Roles, Writing – original draft. **S. Biella:** Formal analysis. **P.F. de Moura Pereira:** Formal analysis, Roles, Writing – original draft. **F. Scibona:** Formal analysis. **L. Feni:** Data curation. **M. Sindaco:** Formal analysis. **D. Emide:** Formal analysis. **A. Jiménez-Quero:** Funding acquisition, Supervision, Writing – review & editing. **C.L.M. Bianchi:** Data curation. **L. Verotta:** Funding acquisition, Project administration, Supervision, Writing – review & editing. **S. Marzorati:** Data curation, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jclepro.2023.139267.

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