Bioactive substances from hazelnut processing residues via Soxhlet extraction:
set up, mass balances and yield

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# 1.Introduction

Hazelnut (*Corylus avellana L*.) is one of the most popular tree nuts consumed for human food worldwide, ranking second in production after almond. Turkey, specifically the Black Sea region, is the world leading producer of hazelnut, contributing over 72% to the global production, although other important producing areas include Georgia, Spain and Italy (FAO, 2020). In Italy, the Campania region has been the leader in the field production of hazelnut in 2020, with an amount of about 480000 q and less than half in the province of Avellino (ISTAT, 2020). The hazelnut skin (*perisperm*), hard shell (*pericarp*), green leafy cover (*floral bracts*) and the hazelnut tree leaves represent the byproducts of the roasting, the cracking, the shelling/hulling, and the harvesting processes, respectively.

The present paper is in the framework of a R&D project aimed at valorization of the above non-edible parts. As the public opinion is aware and under the focus of current R&D activities, residues and wastes of biogenic origin are more and more considered as a valuable source of both bioactive substances and biofuels, whatever their original moisture content is (Adiletta et al., 2020; Casa et al., 2021).

It is well known that a diet rich in tree nuts adds benefits because of their mono- and polyunsaturated fatty acid content (Ros et al., 2006), their high level of dietary fiber (Salas-Salvadó et al., 2006) and the presence of several bioactive molecules in the kernel and skin ranging from tocopherols to arginine and to polyphenols (Andrés et al, 2002), which might exert positive cardiovascular effects such as low-density lipoprotein (LDL) protection from oxidation or enhanced endothelial function (Andrés et al, 2002). The antioxidant capacity of various nut byproducts has been widely investigated, and several works have acknowledged that nut byproducts are especially rich sources of natural phenolic compounds with potential bioactivity (Shahidi et al., 2007). Phenolic compounds are the primary bioactive components in plants. Consequently, the utilization of natural phenolic antioxidants instead of synthetic ones has recently raised considerable interest among food scientists, manufacturers and consumers. In particular, the skins from roasted hazelnut (Shahidi et al., 2007) and green leafy covers (Alasalvar et al., 2006) have been investigated to exploit the content of some phenolic acids.

The bioactive compounds of interest can be separated, in principle, from the source hazelnut matrix by means of a conventional liquid-solid extraction triggered by an organic solvent. Nowadays, a switch to “greener” solvents like limonene or even water would represent a convenient step along the way to more environmentally sustainable processes. In any case, the extraction process generates solid residues, in a wet or dry state, which are to be disposed of. They are suitable candidates for subsequent valorization steps to biofuels, e.g., based on mild thermal processing: biomass torrefaction is the most representative one (Brachi et al., 2016; Chen et al., 2021).

Therefore, the idea underlying this work is to extract bioactive compounds using a “green” solvent.

Among the above-mentioned hazelnut residues, green leafy covers (or leafy husks) have been privileged here because, based on a preliminary bibliographic analysis, they appear now of greater interest and exhibit greater knowledge gaps. Samples at a different “maturation” degree have been tested, i.e., in a green (wet) and a dry state.

# 2. Materials and Methods

Different samples of husks were directly collected from the trees in a hazelnut orchard at Pizzolano (Fisciano, SA). Dry brown ones (see Figure 1.A and Table 1) were taken in September 2020; green leafy husks were sampled on August 5, 2021. These latter, when not directly tested, have been frozen and stored at –18 °C.

|  |  |
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| Immagine che contiene testo  Descrizione generata automaticamente |  |

**Figure 1**. Pictures of the “as collected” hazelnut husks: A. Dry brown leafy husks sampled on September 8, 2020; B. Fresh green leafy husks sampled on August 5, 2021

Samples of dry cuticles separated from roasted hazelnuts were kindly provided by cooperating companies in the Campania region, precisely PRODAL Scarl and Grimaldi Srl. They (see Figure 2 and Table 2) were obtained after industrial roasting of hazelnuts harvested in 2020.



**Figure 2**. Picture of the roasted hazelnut cuticles

For the measurement of the moisture content, the KERN MLB analyzer was used, with a sample load of 2 g and a final T = 105 ° C. For all samples, the determinations of the moisture content have been conducted in triplicate.

A conventional Soxhlet extractor was adopted in the present study, which is equipped with a 250 W electric heater (by Falc), a 500 mL glass flask, a 43x123 mm cellulose thimble and a 300 mm long Graham condenser for solvent vapor condensation.

The selected solvent was the “greenest” one, i.e., water; actually, double-distilled water was used with a loaded volume of 300 mL. For comparison, pureethanol of analytical grade was used at the same load in a few tests.

The Soxhlet extraction tests were carried out on the “as collected” samples, without any grinding. In the case of frozen green leafy husks, they were gently thawed to room temperature and allowed to lose the dripping water. In the case of roasted cuticles, the PRODAL sample was taken as the first reference material and, for such a reason, was gently sieved to obtain a 2-4 mm size cut. It is worth saying that the cuticles have a skin-type shape and, as a consequence, the 2-4 mm size fraction yields particles that are very irregular and far away from the conventional sphere-like solids. The sample mass to be loaded into the cellulose thimble was varied according to the moisture content of the test sample (see Tables 1 and 2) to keep fixed the mass of dry solids in the extraction experiments. For the necessary verification, each test was at least duplicated, as it is possible to check from the color codes in the rows of the Tables 1 and 2.

**Table 1.** Summary of the Soxhlet extraction tests with “as collected” hazelnut husks

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Soxhlet test No. | Sample | Sample mass (g) | Sample moisture content (% wt.) | Extraction solvent | Solvent volume (mL) |
| 21 | Dry husks “as collected” 2020-09-08 | 8.0 | 9.60 | water | 300 |
| 29 | Dry husks “as collected” 2020-09-08 | 8.0 | 9.60 | water | 300 |
| 22 | Green husks “as collected” 2021-08-05 | 22.5 | 61.84 | water | 300 |
| 23 | Green husks “as collected” 2021-08-05 | 22.5 | 61.84 | water | 300 |
| 24 | Green husks “as collected” 2021-09-08 | 22.7 | 67.53 | water | 300 |
| 30 | Green husks “as collected” 2021-08-05 after thawing | 22.5 | 62.64 | water | 300 |
| 31 | Green husks “as collected” 2021-08-05 after thawing | 22.5 | 63.50 | water | 300 |
| 26 | Green husks “as collected” 2021-08-05 | 22.5 | 65.70 | ethanol | 300 |
| 27 | Green husks “as collected” 2021-08-05 | 16.5 | 54.49 | ethanol | 350 |
| 28 | Green husks “as collected” 2021-08-05 | 16.5 | 54.49 | ethanol | 500 |

The effectiveness of the extraction has always been confirmed by the change in color of the extracted solution (see Table 1 and 2). Then, each extracted solution was stored in a glass bottle away from light.

**Table 2.** Summary of the Soxhlet extraction tests with roasted hazelnut skins

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Soxhlet test No. | Sample | Sample mass (g) | Sample moisture content (% wt.) | Extraction solvent | Solvent volume (mL) |
| 25 | PRODAL 2-4 mm size cut | 14.3 | 7.25 | water | 300 |
| 1 | Grimaldi “as collected” | 20.0 | 9.19 | n-hexane | 300 |
| 4 | Grimaldi (residue after extraction with n-hexane) | 14.3 | = = = | ethanol | 400 |
| 2 | Grimaldi “as collected” | 20.0 | 9.19 | ethanol | 300 |

The UV spectrophotometric analysis has been preliminary used for a qualitative assessment of the actual presence of bioactive compounds in the Soxhlet extracted solutions. To this end, a laboratory UV spectrophotometer has been used and at least one extracted solution has been tested for each reference sample of hazelnut residue. In particular, the presence of polyphenols was expected in the 200-300 nm wavelength window.

All the Soxhlet extracted solutions have been subjected to a quantitative assessment of their content of polyphenols and tannins as representative families of bioactive compounds. The method of Singleton and Rossi (1965) using *Folin and Ciocalteu's phenol reagent* was followed for the quantitative determination of total polyphenols as g (gallic acid equivalent)/L (extracted solution). The method of Price et al. (1978) was adopted for the quantitative determination of tannins as g/L (extracted solution).

# 3. Results and discussion

First of all, the Soxhlet experimental procedure has been checked for the correct closure of the mass balances. The most critical case has been taken into consideration, corresponding to the test condition in which there is contemporary presence in extraction of both ethanol (EtOH) and water, the latter being introduced as “moisture” of the wet husks (WH).

The test sample of the green hazelnut husks was weighed and the moisture content of another sample taken from the same feedstock was measured. This allowed the calculation of the masses of the two macro-components, i.e., water and dry solids (DS).

After extraction, the wet spent husk was accurately separated from the wet thimble, both were weighed on an analytical scale, after which they were dried under forced air circulation in a lab hood and re-weighed to determine the mass of the dry husk. The difference between the initial weight (i.e., wet spent husk and wet thimble) and the final weight (i.e., dried spent husk and dried thimble) provided the mass of the liquid (i.e., ethanol and water) removed from solids by evaporation during drying.

The liquid extracted solution was also weighed, then its density was simply measured with the aid of a precisely graduated balloon. By using the mixing rule for density of miscible liquids, the masses of the two components, i.e., ethanol and water, were determined by calculation.

Three simple mass balance equations were written for dry solids, ethanol and water, respectively. They are not reported here for brevity. Their solution, which was quite straightforward, yielded the masses of total solids dissolved in the extracted solution, and of EtOH and H2O losses to atmosphere, these latter likely due to incomplete condensation in the Graham condenser.

The scheme of the material flows in the Soxhlet experimental procedure is shown in Fig. 3.



**Figure 3**. Schematic representation of the material flows in the lab Soxhlet apparatus: A – Biomass test sample; B – Reagent grade ethanol; C – Extracted solution; D – Loss due to incomplete condensation (Evaporation loss); E – Spent “wet” biomass solids; NOTE: The recirculating streams are dashed.

The closure of the mass balances was quite satisfactory. Small and reasonable values were obtained for the masses of total solids dissolved in the extracted solution and the H2O loss to atmosphere; only the EtOH loss to atmosphere appeared as a negligible mistake because of the negative sign (see the value –0.08 g for the “D” stream in Table 3), but its absolute value was by far smaller than any other value reported in Table 3.

**Table 3.** A check of the mass balances during the Soxhlet extraction tests with ethanol as solvent

|  |  |  |
| --- | --- | --- |
| Samples | Corresponding stream in Fig.3 | #26 |
|  |  | Green hazelnut husks (WH) |
|  |  | **INPUT, g** |
| H2O in WH | A | 14.78 |
| DS in WH | A | 7.72 |
| EtOH (anhydrous) | B | 236.70 |
| TOTAL |  | 259.20 |
|  |  | **OUTPUT, g** |
| EtOH evaporated from drying solids | E | 47.84 |
| H2O evaporated from drying solids | E | 2.66 |
| Husk (dry) | E | 7.00 |
| H2O in the extracted solution | C | 10.51 |
| EtOH in the extracted solution | C | 188.94 |
| EtOH loss due to incomplete condensation \* | D | -0.08 |
| H2O loss due to incomplete condensation \* | D | 1.62 |
| Dissolved solids in the extracted solution \* | C | 0.72 |
| TOTAL |  | 259.20 |
| \* Unknown variable obtained as the solution of mass balance equations |

All in all, the Soxhlet experimental procedure proved to be both reliable and accurate enough.

Representative results of the UV spectrophotometric analysis of Soxhlet liquid extracts are shown in Fig. 4. The profiles reported in Fig. 4 have been smoothed with respect the originally acquired data by introducing a “baseline averaging” for absorbance in its profile as a function of the wavelength.

|  |  |
| --- | --- |
| A | B |
| C | D |

**Figure 4**. Representative UV spectrophotometric profiles: A. test #30: extract of husks in water; B. test #26: extract of husks in ethanol; C. test #2: extract of roasted cuticle in ethanol (after 1:1 dilution); D. test #1: extract of roasted cuticle in n-hexane

All of the Soxhlet extracted solutions exhibit a peak near and across 270 nm, which can be easily attributed to polyphenols, although the peak is different in height and shape from sample to sample.

The extracted solutions using ethanol or n-hexane in the Soxhlet apparatus exhibit another peak approximately in the range 600–700 nm, which is however one order of magnitude smaller in absorbance. In the case of green husks (Figure 3.B), it is located at 680 nm and reveals the presence of chlorophyll, likely to be present at a low extent.

**Table 4.** Results of the Soxhlet extraction tests with “as collected” hazelnut husks

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Soxhlet test No. | Sample | Liquid-to-dry solids ratio(mL/g) | Polyphenols g (GAeq)/L | Tannins(g/L) | Theoretical yield in total Polyphenols (g/g DS) | Theoretical yield in Tannins (g/g DS) | Color of extract |
| 21 | Dry husks “as collected” 2020-09-08 | 41.48 | 0.26 | 0.16 | 0.0079 | 0.0049 | Brown |
| 29 | Dry husks “as collected” 2020-09-08 | 41.48 | 0.19 | 0.29 | 0.0055 | 0.0084 | Reddish yellow |
| 22 | Green husks “as collected” 2021-08-05 | 34.94 | 0.12 | 1.37 | 0.0024 | 0.0279 | Light brown |
| 23 | Green husks “as collected” 2021-08-05 | 34.94 | 0.21 | 0.72 | 0.0049 | 0.0168 | Light brown |
| 24 | Green husks “as collected” 2021-09-08 | 40.70 | 0.17 | 0.44  | 0.0048 | 0.013 | Brown |
| 30 | Green husks “as collected” 2021-08-05 after thawing | 35.69 | 0.18 | 5.68 | 0.0058 | 0.182 | Amber yellow  |
| 31 | Green husks “as collected” 2021-08-05 after thawing | 36.53 | 0.21 | 1.51 | 0.0058 | 0.0414 | Amber yellow  |
| 26 | Green husks “as collected” 2021-08-05 | 34.94 | 0.11 | 1.56 | 0.0032 | 0.0454 | Yellow |
| 27 | Green husks “as collected” 2021-08-05 | 46.61 | 0.11 | 4.51 | 0.0033 | 0.135 | Light green |
| 28 | Green husks “as collected” 2021-08-05 | 66.59 | 0.04 | 2.34 | 0.0019 | 0.112 | Light green |

**Table 5.** Results of the Soxhlet extraction tests with roasted hazelnut skins

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Soxhlet test No. | Sample | Liquid-to-dry solids ratio(mL/g) | Polyphenolsg (GAeq)/L | Tannins(g/L) | Theoretical yield in total Polyphenols (g/g DS) | Theoretical yield in Tannins (g/g DS) | Color of extract |
| 1 | Grimaldi “as collected” | 15.00 | ND | ND | = = = | = = = | Pale yellow |
| 4 | Grimaldi (residue after extraction with n-hexane) | 27.97 | 2.01 | 16.85 | 0.0337 | 0.283 | Brown |
| 2 | Grimaldi “as collected” | 16.52 | 2.34 | 7.10 | 0.0309 | 0.094 | Brown |
| 25 | PRODAL 2-4 mm size cut | 22.62 | 2.99 | 7.28 | 0.0620 | 0.151 | Dark brown |
| \* ND = Not Detected |

As concerns hazelnut husks, testing of a "*fresh feedstock*", i.e., the green leafy covers with a high moisture content in the order of 60–70% wt. (see samples in Table 1 and 4), was privileged in investigating the extraction potential of bioactive compounds. For brevity, the results available so far can be summarized as follows:

* the Soxhlet extraction turns out effective with both solvents tested on the “as collected” husks, even on the "*fresh*" ones with a water content as high as 65% wt. or more
* generally, the content of tannins in the extracted solution (g/L) exceeds that measured for polyphenols [g (GAeq)/L], sometimes by more than one order of magnitude (see tests #30 and #28), with both solvents tested
* water is more effective than ethanol in the Soxhlet extraction of polyphenols, with a minimum value of 0.12 g (GAeq)/L (see tests #22) that is in any case larger than the concentrations provided by ethanol
* the previous statement seems not applying to the Soxhlet extraction of tannins by ethanol (see tests #26-28) as compared to the corresponding water extraction tests (see tests #22-24)
* in any case, the measured concentration of tannins appears much more scattered within the same subset of tests (see the color code) in Table 4

The "theoretical" yield from the feedstock is calculated in Tables 4 and 5 by the relationship, respectively in total polyphenols and tannins:

 $Theoretical yield=\frac{volume of extracted solution ∙ measured concentration}{mass of dry solids in the sample}$ ()

under the assumption of “complete extraction” for the Soxhlet technique. The following comments apply:

* The theoretical yield in total polyphenols is three orders of magnitude lower than the mass of dry solids in the original (wet) feedstock. This finding provides a straight and sharp indication of the best attainable performance when a solvent extraction process is outlined for hazelnut husks taken as a biomass feedstock at a given processing capacity (e.g., in kg/h).
* The theoretical yield in tannins is more scattered, even within the same subset of tests (see the color code in Table 4), but it can reach a value that is just one order of magnitude lower than the mass of dry solids in the original (wet) feedstock (see tests #27 and #28 in Table 5), apparently when extraction is performed with ethanol.
* The water-based extraction seems to provide a slightly better performance than the ethanol-assisted Soxhlet: roughly, the theoretical yield in polyphenols is 5•10-3 g (total polyphenols)/g (dry solid) in the average when water is used as a solvent against 3•10-3 with ethanol.

As concerns the roasted hazelnut skins (see samples in Table 2 and 5), the early results are briefly commented here:

* It is confirmed that the compounds of interest here, i.e., the families of polyphenols and tannins, are not extracted by n-hexane as they have not been detected in the quantitative analytical tests on the extract (see test #1 in Table 5). In principle, a non-polar solvent like n-hexane can be used for defatting the roasted hazelnut cuticles and the defatted solid residue can undergo a subsequent extraction step devoted to polyphenols and tannins. This is what was done and is actually confirmed by the test #4 (see Table 5), which yielded a concentration of polyphenols in the extracted solution equal to 2.01 g (GAeq)/L and of tannins as high as 16.85 g/L. This latter result coming after the two-stage extraction is very promising but needs to be further confirmed because the one-stage extraction with ethanol seems to yield a concentration of tannins that is at least halved with the same sample of roasted hazelnut skins (see test #2 in Table 5) and with another one from a different source (see test #25 in Table 5), too.
* Generally, the concentration of total polyphenols [g (GAeq)/L] in the extracted solutions from the roasted hazelnut cuticles (see Table 5) appears larger than that obtained from the “as collected” hazelnut husks (see Table 4). Correspondingly, the theoretical yield in total polyphenols appears to be one order of magnitude higher, i.e., 5•10-2 g (total polyphenols)/g (dry solid) in the average when roasted hazelnut cuticles undergo Soxhlet extraction against 5•10-3 g (total polyphenols)/g (dry solid) in the above-mentioned case of “as collected” hazelnut husks. This comparison is to be confirmed, too.

# 4. Conclusions

* The Soxhlet technique shows that water, the greenest solvent overall, is suitable for extracting polyphenols from the green leafy covers of hazelnut, i.e., agricultural residues that have been poorly taken into consideration so far in the framework of waste valorization and appear now of great interest.
* Using a "*wet feedstock*",i.e., fresh leafy cover samples with a moisture content as high as 60-70% wt., does not impair the extraction process and favors its performance even in the case another polar solvent like ethanol is used instead of water.
* An additional advantage of the extraction technique investigated in this work is that the green leafy covers are processed “as collected”, i.e., without any other pretreatment like drying or size reduction; this represents a significant simplification and provides added value in a possible industrial process implementation in terms of green solvent extraction of bioactive compounds.
* Further work is required. Apart from an additional set of Soxhlet tests for extension of the investigation conditions and verification of the reported results, especially for the case of roasted hazelnut cuticles, a further exploitation is currently in progress by means of other suitable analytical methods like HPLC in order to identify in the Soxhlet extracts individual phenolic acids, i.e., gallic, caffeic and p-coumaric acid, having the role and the appeal of being bioactive compounds.
* Finally, the present work analysis is one of the planned steps along the route to a biorefinery implementation project aimed at a thorough valorization of hazelnut (and possibly other nuts) residues while yielding valuable products (bioactive compounds and solid biofuels) and pursuing circular economy goals.

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