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Hydrolyzable Tannins from Sweet Chestnut Fractions Obtained by a Sustainable and Eco-friendly Industrial Process

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Sweet Chestnut (*Castanea sativa* Mill.) wood extracts, rich in Hydrolyzable Tannins (HTs), are traditionally used in the tanning and textile industries, but recent studies suggest additional uses. The aim of this work is the HPLC-DAD-ESI-MS characterization of Sweet Chestnut aqueous extracts and fractions obtained through a membrane separation technology system without using other solvents, and the evaluation of their antioxidant and antiradical activities. Total tannins range between 2.7 and 138.4 mM; gallic acid ranges between 6% and 100%; castalagin and vescalagin range between 0% and 40%. Gallic Acid Equivalents, measured with the Folin-Ciocalteu test, range between 0.067 and 56.99g/100g extract weight; ORAC test results for the marketed fractions are 450.4 and 3050 μmol/g Trolox Equivalents/extract weight. EC_{50} values, measured with the DPPH test, range between 0.444 and 2.399 μM. These results suggest a new ecofriendly and economically sustainable method for obtaining chestnut fractions with differentiated, stable and reproducible chemical compositions. Such fractions can be marketed for innovative uses in several sectors.

Keywords: HPLC-DAD-ESI-MS, Sweet Chestnut, Hydrolysable tannins, DPPH test, Folin-Ciocalteu test, Membrane separation technology.

Tannins are polyphenolic secondary metabolites found abundantly in vascular plants, especially in the buds, stems, roots, seeds, bark and leaves, commonly with a dry plant material weight ranging between 5% and 10%. They are mainly involved in various mechanisms for protecting plants against pathogens, fungi, insects and herbivorous animals, despite the ability of several organisms to tolerate ingested tannins [1]. The presence of a high number of phenolic groups and their complex structure are what give tannins their main characteristics: they can interact both with polar groups of macromolecules of living organisms and with other substrates, such as metals. Tannins in general have the ability to prevent animal skin degradation transforming it into leather; they can act as mordants in dyeing processes, and link proteins and anthocyanins in solutions, so that they can be used to clarify wine and stabilize its organoleptic properties. But they also have specific biological properties that suggest more targeted uses as antioxidant, radical scavenging and antimicrobial agents [2]. Finally, according to more recent studies, they also have anti-inflammatory, antitumor, cholesterol-lowering, antiviral, and nematostatic properties [3-6].

Many recent studies on condensed tannins (CTs) and their biological properties have demonstrated that they are able to interact with biological systems by performing antioxidant, anti-allergy, anti-hypertensive, antiproliferative, and antimicrobial activities [7,8]. Accordingly, a few enriched plant extracts, in particular pine bark (Pycnogenol®) and grape seed extracts (Leucoselect™, Phytosome®), are marketed for their antioxidant properties. *Vitis vinifera* L. seed and leaf extracts are the main ingredients of several phytochemical remedies.

The so-called tannic acid, generally obtained from sweet chestnut (*Castanea sativa* Mill.) aqueous extract, is a typical product containing hydrolysable tannins (HTs). It consists of a not yet clearly identified mixture of different gallic acid esters of glucose. It is known for its ability to have beneficial effects on human health through the expression of some biological activities, related to its antimutagenic, anticancer and antioxidant properties. In addition, its ability to reduce serum cholesterol and triglycerides and suppress lipogenesis by insulin has been documented [9,10].

Despite the fact that HTs have numerous potentially exploitable properties, they are difficult to study due to their complex chemical structures and properties. Their low stability can jeopardize not only the analytical phase, but also their extraction from vegetal matrices and the standardization of the products. The use of modern high-performance chromatography tools (e.g. HPLC), associated with DAD-MS and DAD-MS-MS detectors, has recently allowed for a more detailed study of this interesting class of compounds. The complex composition of plant extracts rich in HTs also requires an in-depth study of fractionation techniques to isolate and stabilize noble fractions high in active molecules, and to identify the individual compounds responsible for the biological activity and new and innovative uses of such extracts and fractions.

In this work, fractions from sweet chestnut obtained via an environmentally sustainable process have been analyzed and characterized by chromatographic, spectrophotometric and spectrometric (HPLC-DAD-ESI-MS) methods in order to identify and quantify secondary tannic metabolites both as subclasses and single compounds. Hence, antioxidant and radical-scavenging activities have been evaluated for each characterized fraction using *in vitro* methods of Folin-Ciocalteu, stable radical DPPH• and ORAC, then compared with each other to identify the compounds or subclasses responsible for biological activities, with the aim of optimizing the production process and producing new standardized and stabilized fractions with higher concentrations of biologically active molecules, suitable for innovative uses in agronomy, cosmetics, foods and phytotherapy. Innovative and multifunctional uses can be considered for sweet chestnut fractions already marketed, and new and potential uses can be found for other fractions, not yet commercially available.

This work is a follow-up of previous studies by this research group [2], in which polyphenols, HTs in particular, from pomegranate (*Punica granatum* L.) peels and seeds, and myrtle (*Myrtus communis* L.) leaves were identified, characterized and compared to tannins from sweet chestnut raw extracts for their radical scavenging and antimicrobial activities. These new results have made it possible to evaluate additional and innovative uses not only for extracts from sweet chestnut and other plants rich in HTs, but also from plant materials currently considered production wastes or byproducts. The samples analyzed were ten process streams obtained from sweet chestnut wood, via a hot water extraction process followed by fractionation and concentration through a membrane technology system. This production process derives from the one of the MDF (Medium Density Fiberboard) panels that requires the removal of tannins from the wood before introducing the glue, so that panels can be modeled before hardening. Hence, the chestnut aqueous extract itself is a co-product. The plant, (the operating diagram of which is shown in the "Experimental" section), allows for refining the co-product which currently has its main use in the tanning and feed industry. Fractions 6 (liquid, concentrate from nanofiltration) and 10 (spray-dried obtained from fraction 6) are commercially available; the other fractions, not yet marketed, are reintroduced into the process, to be further refined or added to the extraction water. These fractions, with different compositions, could be optimized and marketed for specific uses as a result of this study. The use of only water as solvent makes ecologically and economically sustainable the described extraction and purification method, since the industrial processes are usually based on purification methods by extraction with organic solvents. Moreover, the use of a co-product for obtaining differentiated and refined marketable fractions eliminates many ecological and economic problems associated with the disposal of production waste.

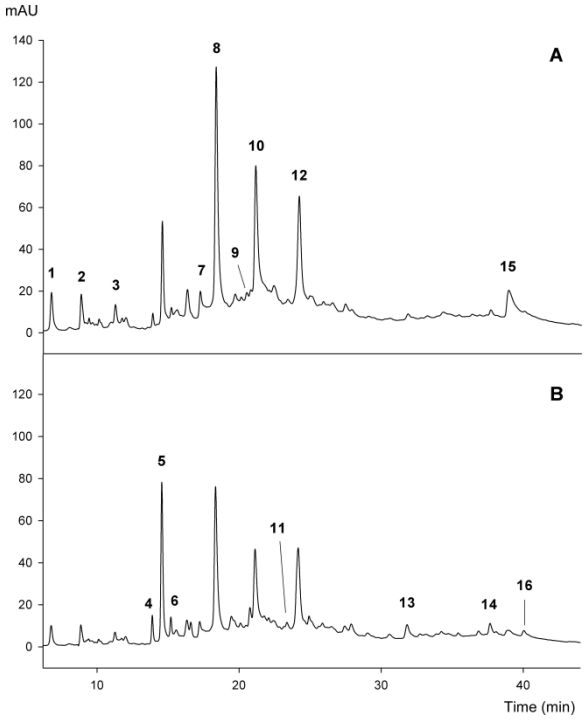

Figure 1: Chromatographic profile of the commercial liquid fraction of sweet chestnut (fraction 6), registered at 254 nm (A) and 280 nm (B). Peaks: 1. Vescalin; 2. Castalin; 3. Pedunculagin I; 4. Monogalloyl glucose I; 5. Gallic acid; 6. Monogalloyl glucose II; 7. Roburin D; 8. Vescalagin; 9. Dehydrated tergallagic-C-glucoside; 10. Castalagin; 11. Digalloyl glucose; 12. O-galloyl-castalagin isomer; 13. Trigalloyl glucose; 14. Tetragalloyl glucose; 15. Ellagic acid; 16. Pentagalloyl glucose.

Each fraction from the production process was characterized both by subclasses and single compounds through the HPLC-DAD-ESI-MS analysis. Figure 1 shows the chromatographic profile of the commercial liquid fraction of sweet chestnut (fraction 6), registered at 254 and 280 nm, with the list of the detected compounds.

The profile at 254 nm reports the presence of ellagic acid (wavelengths of maximum absorbance at 254 and 370 nm, as observed for standard solutions in aqueous and hydroalcoholic solvents) and its derivatives; at 280 nm, gallic acid (maximum absorbance at 272 nm for standard solutions in aqueous and hydroalcoholic solvents) and its derivatives are more evident. Single compounds were identified using both retention times and their spectroscopic and spectrometric data, according to previously reported data when available. Table 1 shows the identification of the single compounds found in the same fraction, numbered as in Figure 1. In the samples analyzed, only HTs are present which are based on different chemical structures. Their UV-Vis spectra and mass fragmentation patterns allow for their attribution to various molecular subclasses. UV-Vis spectra with an absolute maximum of absorbance at 254 nm or less have two typical profiles. In the first case, UV-Vis absorbance only has maxima at wavelengths lower than 240 nm and decreases to 0 mAU between 300 and 400 nm. This kind of UV-Vis spectra has a shoulder around 260-290 nm, more easily detected by observing them in combination with their first and second derivatives, the profiles of which deviate significantly as a result of slight variations in the spectrum [2]. These compounds are based on a core of glucose esterified with HHDP (hexahydroxydiphenoyl-) and/or NHTP (nonahydroxytriphenoyl-) groups [11].

Among the compounds listed in Table 1, numbers 1, 2, 3, 7, 8, 10 and 12 have this kind of UV-Vis spectra. Their ESI-MS fragmentation patterns confirm the structure described above, showing the presence of the ion at *m/z* 301 (HHDP- unit or deprotonated ellagic acid). The ion $[M - H - HHDP]^-$, deriving from the loss of a HHDP residue from the quasi-molecular ion, is also well evident in compound 3, identified as pedunculagin I (bis-HHDP-glucose), where this fragment, at *m/z* 481, is the most abundant. The identification of pedunculagin I was performed by comparing the spectral data (UV-Vis: λ_{max} = 222 nm, shoulder at 282 nm; ESI-MS: 783 $[M - H]^-$, 481; reported in Table 1) with those in literature: this compound has already been detected in other vegetable matrices such as fruits and leaves of strawberry (*Fragaria x ananassa*) [12,13] and walnut seeds (*Juglans regia* L.) [14], but to the author's knowledge, not yet in sweet chestnut wood. Compounds 8 and 10, vescalagin and castalagin, were identified by their UV-Vis spectra and ESI-MS fragmentation patterns. Both their UV-Vis spectra have an absorbance maximum at 224 nm and a shoulder at 276 nm. Their ESI-MS spectra show their main signal at *m/z* 933, $[M - H]^-$, and another signal at *m/z* 466, $[M - 2H]^{2-}$; the peak at *m/z* 301 is due to the ion $[\text{ellagic acid} - H]^-$. In this case we cannot see the ions at *m/z* 631, $[M - H - HHDP]^-$ that are detectable with electrospray ionization at a lower voltage (< 120 V). The spectrophotometric and spectrometric data of vescalagin and castalagin, epimers differing only for the stereochemistry of C1, are substantially the same, so we cannot distinguish between them just by their UV-Vis and ESI-MS spectra. However, they have different chromatographic retention times (Rt), so that we are able to identify each of them from previously reported data: chestnut extracts have previously [11] been analyzed by reverse phase HPLC-DAD with methods similar to ours, and vescalagin Rt was lower with respect to that of castalagin. This allows for identifying compound 8 (Rt=18.3 min) as vescalagin, and compound 10 (Rt=21.1 min) as castalagin.

Table 1: Single compounds qualitative analysis of the Sweet Chestnut liquid commercial fraction (fraction 6). R_t=retention time (min); λ_{max}=maximum absorbance wavelength (nm); sh = shoulder. *m/z* of the most abundant ESI-MS ion is underlined.

peak	Rt	Compound	λ_{max}	MW	negative ions *m/z*
1	6.8	Vescalin	230, 280 sh	632	631 $[M-H]^-$
2	8.9	Castalin	230, 280 sh	632	631 $[M-H]^-$
3	11.3	Pedunculagin I	222, 282 sh	784	783 $[M-H]^-$, 481
4	13.9	Monogalloyl glucose I	274	332	331 $[M-H]^-$, 271, 169
5	14.6	Gallic acid	272	170	169 $[M-H]^-$, 125
6	15.2	Monogalloyl glucose II	274	332	331 $[M-H]^-$, 271, 169
7	17.2	Roburin D	228, 286 sh	1850	933, 924, 915, 683, 301
8	18.3	Vescalagin	224, 276 sh	934	933 $[M-H]^-$, 466, 301
9	20.5	Dehydrated tergallagic-*C*-glucoside	250, 374	614	613 $[M-H]^-$, 301
10	21.1	Castalagin	224, 276 sh	934	933 $[M-H]^-$, 466, 301
11	23.4	Digalloyl glucose	274	484	483 $[M-H]^-$, 377, 271, 169
12	24.2	*O*-galloyl-castalagin isomer	220, 280 sh	1086	1085, 520, 542
13	31.8	Trigalloyl glucose	276	636	635 $[M-H]^-$, 465, 241, 169
14	37.7	Tetragalloyl glucose	276	788	787 $[M-H]^-$, 356, 169
15	38.9	Ellagic acid	254, 370	302	301 $[M-H]^-$
16	40.1	Pentagalloyl glucose	274	940	939 $[M-H]^-$, 469, 169

The same holds for compounds 1 and 2, castalin and vescalin, which are hydrolysis products of 8 and 10 respectively, lacking the HHDP residue. They are not always present in chestnut extracts, but were detected in all the fractions analyzed in this study.

The UV-Vis spectrum of compound 7 is consistent with a HT structure with a glucose core, esterified with HHDP or NHTP units. Its ESI-MS fragmentation pattern has been compared with that of roburin A and roburin D reported [11]: *m/z* 1849, 933, 924, 915, 616, 301 (roburin A) and *m/z* 933, 924, 915, 616, 301 (roburin D). The fragmentation pattern of compound 7 is very similar to those mentioned above, with the absence of the signals at *m/z* 1849 and 616. The UV-Vis spectra are also very similar, having absorbance maxima at 229, 280sh nm [11] and 228, 286sh nm (compound 7), so we can tentatively identify the compound 7 as roburin D. The other ESI-MS signals are due to the following ions: [vescalagin - H]$^-$ (*m/z* 933); [M - 2H]$^{2-}$ (*m/z* 924); [vescalagin - H_2O - H]$^-$ (*m/z* 915); [ellagic acid - H]$^-$ (*m/z* 301). The compound 12 has maximum UV-Vis absorbance at 220 nm with a shoulder at 280 nm, and an ESI-MS spectrum with fragments at *m/z* 1085 (the most abundant), 520 and 542. These data are consistent with a structure based on HHDP- or NHTP- glucose, possibly an isomer of *O*-galloyl-castalagin or *O*-galloyl-vescalagin. 1-*O*-galloyl-castalagin, recently tentatively identified in chestnut extracts [15], and the compound 12, have the same MS spectra, and UV-Vis profile ascribable to the same type of compound. They have also similar polarities, but their UV-Vis absorption maxima differ of 20 nm, making then plausible that these compounds are two isomers. Therefore, the compound 12 was tentatively identified as *O*-galloyl-castalagin isomer. Another kind of profile for UV-Vis spectra with absolute absorbance maxima at $\lambda \leq 254$ nm also shows a maximum relative absorbance around 370 nm. These compounds have a glucose core, esterified with at least one unit of ellagic acid, the lactonized form of hexahydroxydiphenic acid. In the analyzed fractions, two compounds were detected with this kind of UV-Vis spectra: 9 and 15. Compound 9 is tentatively identified as dehydrated tergallagic C1-glucopyranoside according to its spectrophotometric and spectrometric data, and previously reported data already observed in Acorns (*Quercus spp*.) [16] and cork from *Quercus suber* L. [17].

In actual fact, its UV-Vis spectrum shows an absolute absorbance maximum at 250 nm and a second one with a lower intensity at 374 nm. The quasi-molecular ion at *m/z* 613 and the presence of a fragment at *m/z* 301, [ellagic acid - H]$^-$, confirm the structure of this molecule. Such a compound could derive from tergallagic acid C1-glucoside (MW 631) via intramolecular esterification of the carboxyle group of one gallic acid unit and the oxydryl group linked to the C2 of cyclic glucose, with the loss of one molecule of water [16,17]. Finally, the UV-Vis spectra with maximum absorbance between 270 and 280 nm characterize gallic acid and organic polyhydroxylated cyclic compounds, such as D-glucopyranose or quinic acid, esterified with gallic acid units. The ESI-MS spectra of compounds 4 and 6, monogalloyl D-glucopyranose isomers, show an intense peak at *m/z* 271, [monogalloyl glucopyranose - H - 60]$^-$, due to the cross-ring fragmentation of the quasi-molecular ion [18,19]. The same fragmentation, in particular the presence of the peak at *m/z* 271, was also observed for compound 11 (digalloyl glucopyranose). Compound 16, identified as pentagalloyl glucopyranose, with a quasi-molecular ion at *m/z* 939, also shows the doubly-charged quasi-molecular ion (*m/z* 469). Moreover, we can note the absence of the typical spectral characteristics of the polygalloyl derivatives with depsidic links: a slight batochromic shift due to the increased conjugation inside the molecule, and the formation of a shoulder at about 300 nm, increasing with the number of the depside links, and clearly evident when observing the UV-Vis spectra in first and second derivatives [2,20]. Therefore we can conclude that the chromatographic profile of the commercial liquid fraction, registered at 280 nm, reports the presence of GTs with no depsidic links, in particular gallic acid (MW 170, 5), two isomers of monogalloyl glucopyranose (MW 332, 4 and 6), digalloyl glucopyranose (MW 484, 11), trigalloyl glucopyranose (MW 636, 13), tetragalloyl glucopyranose (MW 788, 14) and pentagalloyl glucopyranose (MW 940, 16).

Table 2 shows the quantitative analysis per single compound of the two commercial extracts. The results are expressed in mg/g and μmol/g and the Relative Standard Deviations (RSDs) are also reported. All RSDs are < 5% except for ellagic acid (8.7%), the chromatographic peak of which has a minor resolution. All fractions of the production process have been characterized by single compounds, but here we only show the results for these two fractions by way of example, as a final characterization according to tannic subclasses is more useful for our purpose.

The total tannins are 110.8 mg/g (151.1 μmol/g) in the liquid fraction and 224.8 mg/g (372.1 μmol/g) in the dry fraction, which means a weight of 11.1% and 22.5% respectively. The most abundant tannins in the commercial fractions are vescalagin and castalagin, with a predominance of the first one.

Table 2. Single compounds quantitative analysis of Sweet Chestnut commercial fractions: liquid (fraction 6) and dry (fraction 10). Peak numbers correspond to those in the previous Table 1; results are expressed in mg/g and μmol/g of sample. RSD % = Relative Standard Deviation (%).

peak	compound	content in commercial liquid fraction			content in commercial dry fraction		
		mg/g	μmol/g	RSD %	mg/g	μmol/g	RSD %
1	Vescalin	3.6	5.7	1.9	8.7	13.7	4.6
2	Castalin	3.2	5.1	0.6	8.8	14.0	4.9
3	Pedunculagin I	3.3	4.2	1.2	10.1	12.9	4.4
4	Monogalloyl glucose I	2.5	7.6	1.3	4.7	14.1	2.4
5	Gallic acid	1.5	9.0	1.9	13.6	79.8	2.1
6	Monogalloyl glucose II	2.7	8.1	4.7	5.0	15.2	4.3
7	Roburin D	4.0	4.4	1.2	9.6	10.4	3.4
8	Vescalagin	26.7	28.6	2.4	45.2	48.4	3.6
9	Dehydrated tergallic-*C*-glucoside	1.8	2.9	1.6	6.0	9.8	4.5
10	Castalagin	21.0	22.5	1.8	39.7	42.5	3.6
11	Digalloyl glucose	3.8	7.8	1.2	12.2	25.3	2.0
12	*O*-galloyl-castalagin isomer	20.8	19.2	1.9	32.0	29.5	2.2
13	Trigalloyl glucose	5.0	7.9	1.4	12.1	19.0	3.1
14	Tetragalloyl glucose	4.7	6.0	3.2	9.2	11.6	2.4
15	Ellagic acid	2.5	8.3	0.8	7.8	25.8	8.7
16	Pentagalloyl glucose	3.7	3.9	2.0	0.0	0.0	-
	total tannins	**110.8**	**151.1**	**1.0**	**224.8**	**372.1**	**2.5**

The weight percentage of vescalagin and castalagin on total tannins is 43.0% (liquid fraction) and 37.8% (spray-dried fraction). Vescalin and castalin are present in smaller amounts with respect to the total tannins: the percentage of vescalin and castalin is 6.1% in the liquid fraction and 7.8% in the spray-dried product, so we can easily hypothesize that the higher percentage of vescalin and castalin, where the corresponding percentage of vescalagin and castalagin is lower, is due to the partial hydrolysis of the latter. Ellagic acid is 2.3% in the liquid fraction and 3.5% in the dry one, and this increase reinforces the hypothesis of low stability of the high molecular weight ETs, and their partial hydrolysis during the production process. The amount of gallic acid is also lower in the liquid fraction: from 1.4% to 6.0% in the spray-dried, so we can infer that high molecular weight GTs are also partially hydrolyzed during the spray-drying process. In actual fact, only pentagalloyl glucose seems to be definitely unstable, since its content, 3.3% in the liquid fraction, drops to 0.0% in the dry fraction. The monogalloyl and tetragalloyl glucoside amounts also decrease slightly, from 4.7% to 4.3%, and from 4.3% to 4.1% respectively. The digalloyl and trigalloyl glucosides amounts rise from 3.4% to 5.4% and from 4.5% to 5.4% respectively.

Nevertheless, it is difficult to make assumptions about the stability of the single HTs, all present in the same sample, especially when we consider that the percentages of high molecular weight ETs, such as roburin D and dehydrated tergallagic C1 glucoside, are higher in the spray-dried than in the liquid fraction. Table 3 shows the characterization per tannin subclass made for every fraction of the production process. The total tannin content is expressed as mM for the liquid fractions and μmol/g for fraction 10 (spray-dried), and the compound percentages are calculated from their contents expressed in mM. The identification and quantification of the tannin subclasses present in each fraction give a more precise idea of which kinds of compounds are responsible for the biological activities. The compounds identified are almost the same for each fraction, while significant differences were found with regard to the quantitative aspects. The total tannin content varies between 2.7 mM and 138.4 mM for fractions 7 and 8 respectively (liquid fractions), and is 372.1 μmol/g for the spray-dried (fraction 10). Fraction 1 derives from the raw extract after only a coarse filtration, and contains almost the same percentages of gallic and ellagic derivatives, with a slight predominance of the first. The ET percentages are higher than those of the GTs in fractions 3, 4, 6 and 10 (nanofiltration concentrated fractions and spray-dried from fraction 6), whereas in the permeated ones (2 and 5), the GTs are predominant. Hence, the process of nanofiltration is quite effective in separating the GTs from the ETs. Fraction 5, already highly enriched with GTs (74.3%), gets filtrated via a reverse osmosis process to give a concentrate (fraction 8) with about the same qualitative composition and percentages as the tannic derivatives, but more highly concentrated (43.2 mM total tannins for fraction 5 and 138.4 mM for fraction 8). The osmosis permeate (fraction 7) only contains 2.7 mM of tannins, and 100% of gallic acid. This suggests that after optimization of this sequence of filtration and concentration steps, we could obtain a final fraction containing all the gallic acid present in the raw extract, purified and ready to be dried and marketed as a chemical. Up till now this has been reintroduced in the production process as extraction water. Conversely, the gallic acid content is very low (6.0%) in the commercial liquid fraction, consistent with the evidence that this is a concentrate from nanofiltration. The gallic acid amount is higher (62.2% on total tannins) in the corresponding nanofiltration permeate (fraction 5).

The vescalagin and castalagin content varies between 0.0% in fraction 7 and 40.5% in fraction 3, first concentrated via nanofiltration, confirming that large amounts of high molecular weight compounds are removed during the nanofiltration process. So we should consider optimization of the process capable of enhancing the differences found among all fractions. Such optimization could be based simply on variations of membrane molecular weight cut-off and filtration/concentration time, or on more complex types of intervention. Moreover, the dry commercial fraction was stable at the 6-month follow-up analytical control; the liquid fractions had some stability problems, in particular vescalagin and castalagin were partially hydrolyzed yielding vescalin and castalin. Further studies are necessary for obtaining new, stable and reproducible liquid fractions with a well-defined chemical composition and the possibility of uses, for example, in food or phytotherapy.

The antioxidant and antiradical activities were evaluated for each fraction with *in vitro* tests using the Folin-Ciocalteu reagent and stable radical DPPH• (1,1-diphenyl-2-picrylhydrazyl) respectively. An in-depth antioxidant activity estimation with the popular ORAC test was also performed on selected and already marketed fractions

Table 3. Quantitative analysis by subclasses (% calculated from their contents expressed in mM) for all the fractions from the productive process. A. % gallic acid on total tannins; B. % gallic acid on total GTs; C. % castalagin + vescalagin on total tannins; D. % castalagin + vescalagin on total ETs; E. % GTs on total tannins; F. % ETs on total tannins.

	A	B	C	D	E	F	TOT[a]
1	42.6	74.3	27.3	63.9	57.3	42.7	**27.2**
2	74.4	86.4	10.6	75.8	86.1	13.9	**15.7**
3	24.3	69.4	40.5	62.3	35.0	65.0	**82.0**
4	38.3	79.0	33.1	64.1	48.4	51.6	**75.3**
5	62.2	83.6	17.1	66.4	74.3	25.7	**43.2**
6	6.00	17.9	33.8	50.7	33.3	66.7	**121.0**
7	100.0	100.0	0.0	-	100.0	0.0	**2.68**
8	63.0	84.0	15.1	60.1	74.9	25.1	**138.4**
9	31.8	59.6	31.1	66.7	53.3	46.7	**63.6**
10	21.4	48.4	24.4	43.9	44.4	55.6	**372.1**

[a] Total tannins content expressed as mM for the liquid fractions and as µmol/g for the fraction 10 (spray-dried).

(6 and 10). The results of the Folin-Ciocalteu assay are expressed as total phenol content, measured as GAE (Gallic Acid Equivalents), which is considered to have an excellent correlation with the *in vitro* antioxidant activity, as previously confirmed by comparisons with other assays based on electron transfer reactions (e.g. FRAP, TEAC and ORAC) [21,22]. For the fractions under study, in view of the extraction method and the initial vegetal matrices, it can be assumed that there are no compounds, such as proteins containing tyrosine or other phenolic compounds, which interfere with the electron-transfer reaction between the samples under examination and the Folin-Ciocalteu reagent. The assay with DPPH• gives a measure of the antiradical activity of the samples, expressed as their EC_{50} (polyphenolic concentration inhibiting DPPH• activity to 50%). In the ORAC test, the antioxidant capacity is measured as Trolox Equivalents (TE) through the area under the curve (AUC) of the kinetic profiles of a target molecule (fluorescein) consumption, oxidized by free radicals produced by the free radical initiator AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride) in the presence of the sample. The AUC value obtained is compared with that obtained with Trolox in order to evaluate the antioxidant activity. The chemical mechanisms behind these three assays are different, and although the existence of a correlation among their results has previously been verified [11,22,23], it cannot be asserted that this is true in all cases, due to the complexity of the antioxidant processes of each sample and the variability of the factors that can affect them. Furthermore, chemical-based methods are useful for a first screening of a large number of samples due to the speed of execution, their low cost, and their ability to exclude products from the study with neither an antioxidant nor a radical scavenger activity. They may actually be crucial for demonstrating the effectiveness of a food or beverage preservative, but not completely predictive of its antioxidant capacity *in vivo*. The effectiveness of a compound or food in the human body is indeed conditioned by a number of other factors such as liposolubility, chemical stability and other chemical properties that determine bioavailability and metabolism, possible up-regulation of antioxidant and detoxifying enzymes, modulation of redox cell signaling, and gene expression [24]. Therefore, the measurement of the antioxidant and radical scavenging activity performed for the investigated samples should be considered an useful early evaluation that must be followed up, for the more active ones, by more in-depth *in vivo* or *ex vivo* experiments, while also taking into account previous studies on the bioavailability and metabolism of hydrolysable tannins. Table 4 shows the density values found for every chestnut liquid fraction, GAE, EC_{50} and TE values. The Folin-Ciocalteu results (GAE) are expressed both with respect to the sample weight, and the concentrations of tannic compounds, owing to the density differences existing among the samples. The GAE values, reported in both ways, should give an idea of the tannin concentrations in the first case, and of the tannin activities, based on their chemical structure, in the second. EC_{50}s are expressed as µM tannins in the sample by HPLC/DAD; TEs are expressed as µmol Trolox with respect to the sample weights. The GAE1 values are between 0.067 and 56.99 g/100g (for fractions 7 and 10 respectively); the GAE2 values are between 0.250 and 1.319 g/mmol (for fractions 7 and 6 respectively), and the EC_{50}s are between 0.444 and 2.399 µM (for fractions 3 and 7 respectively).

Table 4. Measured values of GAE, EC_{50} and TE. d: density (g/mL); GAE1: Gallic Acid Equivalents (g gallic acid/100g sample); GAE2: Gallic Acid Equivalents (g gallic acid/mmol total tannins by HPLC/DAD); EC_{50}: polyphenolic concentration inhibiting DPPH· activity to 50% (µM); TE: Trolox Equivalents (µmol/g sample).

	d	GAE1	GAE2	EC_{50}	µmolTE/g
1	1.01	2.382	0.636	0.695	
2	1.01	0.640	0.413	2.007	
3	1.06	9.148	1.182	0.444	
4	1.04	7.905	0.938	1.000	
5	1.03	2.370	0.565	1.429	
6	1.25	30.99	1.319	1.516	450.4
7	1.00	0.067	0.250	2.399	
8	1.07	7.367	0.616	1.510	
9	1.08	0.493	0.052	0.545	
10		56.99	1.210	0.586	3050

The TE values found for fractions 6 and 10 are 450.4 µmol/g and 3050 µmol/g respectively. Thus, a correlation can be assumed among the DPPH, ORAC and Folin-Ciocalteu results expressed as GAE1s, however further investigations are needed to achieve a statistically significant amount of data for each fraction obtained by the plant. Several works address the chemical characterization of chestnut wood for its uses in the wine industry, focusing mainly on the aromatic potential and organoleptic properties it can give to alcoholic beverages when used to produce barrels for ageing process. Recently, new studies have been presented on the uses of raw aqueous chestnut extracts in agriculture as water acidifiers, nitrogen stabilizers, and fertilizers with antimicrobial and nematostatic properties [25,26], or on possible uses of chestnut bark extracts as ingredients for food supplements [15]. Our study addresses for the first time the possibility of fractionating the raw aqueous extract to obtain new fractions with differentiated chemical compositions, for new, sustainable and innovative uses in a variety of fields. Further investigations will be carried out on the possibility of stabilizing the fractions in quali-quantitative point terms. The stability issue exists mainly for the liquid fractions, not for the spray-dried ones, the chemical composition of which appears to be stable with HPLC-DAD analyses after 6 months and up to one year. Moreover, the process repeatability will be taken into consideration, compatibly with the fact that the composition of a vegetable matrix in any case maintains a natural variability over time. A further added value is given by the ecological and economical sustainability of the process that uses waste wood as a raw material, and only water as solvent, partially recycled from the process itself as an osmosis permeate. This becomes important especially when if considering that most of the industrial processes for obtaining purified extracts currently involve the use of solvents and chemicals. The final product, sweet chestnut fractions with standardized and stabilized chemical compositions and specific biological properties, could be tested and approved for innovative uses in agronomy, cosmetics, feed, food and phytotherapy.

Experimental

General: Gallic acid and ellagic acid were of analytical grade, purchased from Extrasynthèse S.A. (Lyon, Nord-Genay, France).

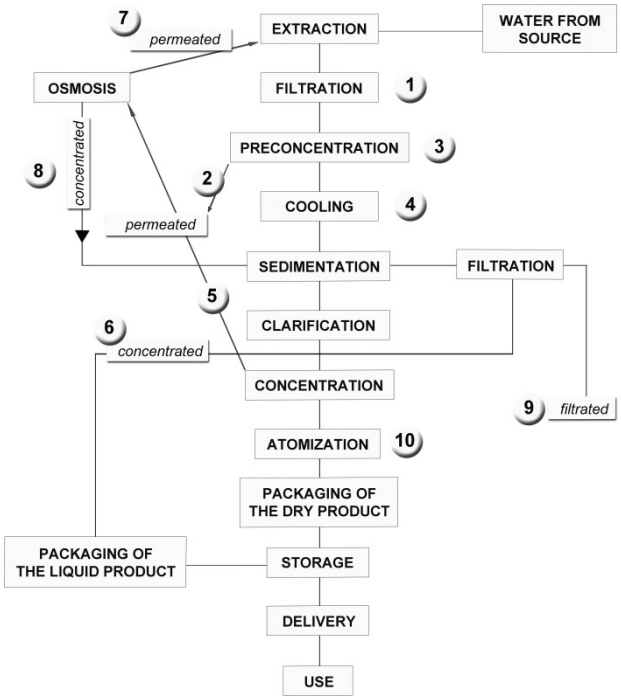

Figure 2: Operating diagram of the Gruppo Mauro Saviola extraction and fractionation plant: 1) filtered tannin broths; 2) permeate from nanofiltration step-1; 3) concentrate from nanofiltration step-1; 4) concentrate from nanofiltration step-2; 5) permeate from nanofiltration step-2; 6) concentrate from nanofiltration step-3; 7) osmosis permeate; 8) osmosis concentrate; 9) settled fraction from clarification step; 10) spray-dried obtained from fraction 6.

The DPPH• (1,1-diphenyl-2-picrylhydrazyl) radical (analytical grade) and the Folin-Ciocalteu reagent were purchased from Sigma (St Louis, MO, USA). All solvents (HPLC grade) and formic acid (ACS reagent) were purchased from Aldrich Company Inc. (Milwaukee, Wisconsin, USA).

Samples: The products under examination (courtesy Gruppo Mauro Saviola srl, Viadana, Mn, Italy) consist of ten process streams of the tannin extraction unit operating in Radicofani (Si), Tuscany, obtained using the plant whose diagram is shown in Figure 2: 1) filtered tannin broths; 2) permeate from nanofiltration step-1; 3) concentrate from nanofiltration step-1; 4) concentrate from nanofiltration step-1 (after cooling); 5) permeate from nanofiltration step-2; 6) concentrate from nanofiltration step-2; 7) osmosis permeate; 8) osmosis concentrate; 9) settled fraction from clarification step; and 10) spray-dried material obtained from fraction 6.

HPLC-DAD-ESI-MS Analysis: The HPLC-DAD-ESI-MS analyses were conducted using an HP-1100 liquid chromatograph equipped with a DAD detector and an HP 1100 MSD API-electrospray (Agilent Technologies) operating in negative ionization mode. A Luna, C18 250×4.60 mm, 5µm column (Phenomenex), operating at 26 °C was used. The eluents were H_2O (adjusted to pH 3.2 with HCOOH) and CH_3CN. A four-step linear solvent gradient starting from 100% H_2O (A) up to 100% CH_3CN (B) was performed with a flow rate of 0.8 mL/min over a 55-min. period, as previously described [2]. Mass spectrometer operating conditions were: gas temperature 350 °C at a flow rate of 10.0 L/min, nebulizer pressure 30 psi, quadrupole temperature 30 °C and capillary voltage 3500 V. The fragmentor was 120 eV. Identification of individual tannins was carried out using their retention times, and both spectroscopic and spectrometric data. Quantification of the single compounds was directly performed by HPLC-DAD using a five-point regression curve built with the available standards. Curves with an r^2>0.9998 were considered. Calibration was performed at the wavelength of the maximum UV-Vis absorbance, by applying the correction of molecular weight. In particular, galloyl-glucosides and gallic acid amounts were calculated at 280 nm using gallic acid as a reference. Ellagic tannins and ellagic acid were calibrated at 254 nm using ellagic acid as a reference. The determinations of the polyphenol contents were carried out in triplicate; the results are given as means and the standard error was <5%.

Antiradical Activity: The antiradical activity was evaluated using the stable radical DPPH• test, according to the reported procedure [27] with slight modifications.

In detail, the extracts were opportunely diluted and added, in a 1:1 amount, to an ethanolic solution of DPPH• (0.025 mg/mL). Measurements were carried out at 517 nm with a DAD 8453 spectrophotometer (Agilent Technologies) at time 0 and every 2 min. for the following 20 min. Antiradical activity (AR%) was calculated through the relationship: [$AR\% = 100 \times (A_0-A_{20})/A_0$], where A_0 and A_{20} were the absorbance of DPPH•, respectively at time 0 and after 20 min from the addition of the diluted extract. The EC_{50} of the extracts was determined through the use of five-point linearized curves AR% vs. ln(concentration in polyphenols), built determining the AR% for five different dilutions of each sample, and then calculating the molar concentration in polyphenols of the solution that inhibits the DPPH• activity by 50%.

Antioxidant activity with the Folin-Ciocalteu test: This was measured through the absorbance at 725 nm of a solution of the sample and the Folin-Ciocalteu reagent, after adding 20% Na_2CO_3 and incubating for 40 min., using a calibration curve built by measuring the absorbance of five reaction solutions containing gallic acid at different concentrations. The phenol content of each sample is expressed as GAEs (Gallic Acid Equivalents), both as [g/(100g of sample)] and as [g/(mmol of total tannins in the sample)], because of the variability of the fraction density.

Antioxidant activity with Oxygen radical absorbance capacity (ORAC) assay: The original method of Cao *et al*. [28] was used with a few modifications [29]. The final reaction mixture for the assay (2 mL) was prepared as follows: 1650µL 0.05 mM fluorescein sodium salt in 0.075 M sodium phosphate buffer, pH 7.0, 200 mL diluted sample, or 50 mM Trolox. The control was 0.075 M Na phosphate buffer, pH 7.0. Fluorescence was read every 5 min. at 37°C using an LS-5 spectrofluorometer (Perkin-Elmer, Norwalk, CT, USA) at 485 nm excitation, and 520 nm emission. When stability was reached, the reaction was initiated with 150 ml 5.55mM AAPH and fluorescence was read up to a value of zero. The ORAC value was calculated according to the formula: ORAC (µmolTroloxequivalents/g) = [(As-Ab)/(At-Ab)]kah where As is the area under the curve (AUC) of fluorescein in the sample, calculated with the ORIGIN 2.8 integrating program (Microcal Software), At is the AUC of the Trolox, Ab is the AUC of the control, k is the dilution factor, a is the concentration of the Trolox in mmol/L, and h is the ratio between the liters of extract and the grams of vegetable or oil used for the extraction.

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