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Tannins from Different Parts of the Chestnut Trunk (*Castanea Sativa* Mill.): a Green and Effective Extraction Method and Their Profiling by High-Performance Liquid Chromatography-Diode Array Detector-Mass Spectrometry

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ABSTRACT: Chestnut wood is a rich source of hydrolyzable tannins, which include gallotannins and ellagitannins, such as castalin, vescalin, castalagin, and vescalagin. The study was aimed to improve knowledge on hydrolyzable tannins from chestnut wood, evaluating the extraction process, the curing time of wood, and the composition of the dry tannin extracts. Different extraction conditions (e.g., wood-chips/solvent ratio and temperature) were evaluated, and more than 50 ellagitannins and gallotannins were tentatively identified by HPLC-MS. The highest yields in tannins on dried matter were obtained for the samples with the higher curing time by the extraction carried out at 100 °C (maximum applied temperature). The extracts of different chestnut samples extracted at 100 °C showed a high content of total tannins, with values determined by HPLC-DAD ranging between 19.6 and 25.6% of the dry extract. The values of total tannin content determined using both the HPLC-DAD and the Folin-Ciocalteu methods, both expressed as gallic acid, were compared and correlated, and a multiplication factor of 3.25 was proposed. The use of this factor is a simple way to convert nonhomogeneous quantitative results on tannin concentration in chestnut wood present so far in the literature.

KEYWORDS: *vescalagin, castalagin, hydrolyzable tannins, Folin−Ciocalteu, mass spectrometry, seasoning time, quantitative determination*

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1. INTRODUCTION

Tannins are commonly found in different plant tissues such as leaves, roots, seeds, stems, and fruits, and among these, one of the richest sources is the pomegranate peel.[1] Tannins are also present in bark and wood as in the case of oak and chestnut wood.[2−5]

Sweet chestnut (*Castanea sativa* Mill) belongs to the family of *Fagaceae*, and it is one of the most common chestnut species in European countries, with the cultivation of chestnut for fruit production mainly concentrated in southern Europe, particularly in the Mediterranean area.[6] Hence, in the current context, it is interesting to take into account the presence of considerable amounts of tannins in chestnut wood and trunk for designing new, sustainable, and innovative ways of enhancing sweet chestnut cultivation and its exploitation as a source of active principles.[2,5,7] The industrial processing aimed to extract tannins from chestnut wood started in France, Italy, and ex-Yugoslavia.[8] In Europe, in 2016, the sweet chestnut covered more than 2.5 million ha, with approximately 90% of the cultivated areas concentrated in few countries such as France, Italy, Spain, Portugal, and Switzerland.[9]

Tannins in chestnut wood can vary depending on a variety of factors, including the species and the environmental conditions.[8] Chestnut wood extracts are a rich source of hydrolyzable tannins, which include gallotannins and ellagitannins such as castalin, vescalin, castalagin, and vescalagin.[10,11]

Traditionally, the extracts from chestnut wood are used in tanning and textile industries but also to clarify wine and stabilize its organoleptic properties.

Since 2018, in Italy, the regulation of the use of vegetable substances and preparations for food supplements includes the cortex of *Castanea sativa* Mill.[12] Tannins have long been overlooked because they are not bioavailable in the small intestine, but in these last years, it was demonstrated that they are degraded by the human microbiota producing small phenols that can re-enter the bloodstream as already observed for pomegranate ellagitannins.[5,13,14] Nowadays, the biological effects of phenol metabolites derived from microbiota fermentation is object of extensive studies, since several data suggest their role in mediating the benefits of polyphenols in human.[15] The chestnut extract and/or its fractions alone or in combination with other polyphenols have shown antioxidant, antidiabetic, antiproliferative, antibacterial, and antimycotic properties and a capacity of lowering nitrosamines and

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mycotoxins in raw food materials and food products.[16,17] Chestnut wood tannins at low concentrations can be effective as natural food additives, providing stability during processing and extending the shelf life of wine, meat, baked products, and gelatin.[18−20] Hydrolyzable tannins from chestnut and other vegetal species were proposed as potential substitutes of synthetic food preservatives due to their antioxidant and antimicrobial properties.[21,22] The availability of food grade commercial extracts of sweet chestnut wood makes them interesting candidates for this purpose.[7,16] In view of the recent increasing attention for environmental sustainability, they have also gained increasing importance as eco-friendly materials for food packaging based on biopolymers, as such, or functionalized with natural active ingredients. Sweet chestnut extracts rich in hydrolyzable tannins have proven their effectiveness in preventing microbial growth in food packaged with active films based on chitosan and chestnut extract.[22,23]

Several methods exist for extracting tannins from various vegetal materials and chestnut bark. The most used medium is the hot water and water at high pressure, but mixtures of organic solvents such as acetone, methanol, and ethanol added with different percentage to water were sometimes applied.[2,11,24−26] Compared with organic solvents, the use of water offers a simple and sustainable technique to efficiently recover tannins from chestnut wood. Recently, it has also been proposed to improve the extraction efficiency of chestnut wood by applying pressurized hot water at temperatures from 100 to 250 °C.[27] Indeed, the combination of high temperature and high pressure helps break down the cell walls and the release of tannins into the extraction medium. Two very recent studies investigated the efficiency of a subcritical water extraction to recover tannins from chestnut wood and peels, and exploited the possibility of a prefractionation of the phenolic compounds.[28,29]

However, as far as we know, and despite the widespread industrial use of chestnut wood and bark extracts, there is a knowledge gap regarding chestnut tannins that must be overcome. In particular, few data are related to the minor polyphenols present in the chestnut extract, the effects of the extraction temperature on tannin yield, the distribution of tannins in the different chestnut tissues, and the comparison between the quantification methods often applied to determine the total tannin content.

The aim of this study was to investigate hot water extraction procedures to recover tannins from different parts of the chestnut tree (chips from the basal trunk, bark, and inner part of the trunk) and from the whole trunk after short and long periods of wood seasoning. Furthermore, the tannin content was evaluated in a preliminary scale-up test using pressurized hot water in an autoclave and applying different extractive ratios (wood chips/water). Tannins were identified by HPLC-DAD-MS and the total content was determined on the final dry extracts. Finally, the results from the HPLC-DAD analysis were compared with those obtained with the Folin-Ciocalteu method to propose a suitable corrective factor to correlate the results obtained with the two different quantitative approaches.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents. Gallic acid of analytical grade was obtained from Extrasynthèse S.A. (Lyon, Nord-Genay, France). The Folin−Ciocalteu reagent was purchased from Merck (St. Louis, MO, USA). All solvents of both HPLC and HPLC-MS grade as well as formic acid and acetonitrile were purchased from Merck (St Louis, MO, USA).

2.2. Samples. The chestnut samples were purchased from an Italian factory located in the Lazio region over the years 2019−2021. The main characteristic and codes of the chestnut samples included in the study are reported in Table 1. These included noncured chestnut wood, chestnut cured for different times (9−48 months), whole trunk, and inner part and cortex considered separately. The curing time or seasoning time is related to the storage period of the whole trunk at room temperature in a covered area before the production of wood chips. For all the samples, the wood chips were produced few days before the extraction.

Table 1. List of the Chestnut Samples Included in the Study[a]

code	whole trunk
WT-T_0	whole trunk not seasoned (humidity 44.7%)
WT-T_9	whole trunk seasoned 9 months (humidity 11.7%)
WT-T_{18}	whole trunk seasoned 18 months
WUT-T_{24}	whole trunk obtained by cutting over 1 m from the ground old plants (18−20 years), seasoned 24 months
WT-T_{48}	whole trunk seasoned 48 months (humidity 14.3%)
code	**different part of the trunk**
UT-I-T_{24}	internal part of the upper trunk of old plants (18−20 years), seasoned 24 months
UT-C-T_{24}	external cortex of the upper trunk of old plants (18−20 years), seasoned 24 months
BT-T_{24}	basal whole trunk obtained only from the basal part of old plants (18−20 years), seasoned 24 months

[a]All samples were harvested from the same productive area, with the same size of the wood chips produced few days before the extraction. WT, whole trunk; T_x, time of seasoning; U, upper part of the trunk; I, inner part of the trunk; C, cortex; B, basal part of the trunk (approx. up to 1 meter from the ground).

2.3. Extraction Procedures at the Laboratory Scale. The extractions at laboratory scale were carried out applying two chestnut wood/water ratios: 1 g/10 mL and 1 g/15 mL; four different temperatures (i.e., 50, 70, 90, 100 °C) were tested working under magnetic stirring, with a first extraction step of either 30 or 60 min and further steps of 30 min each. To work with a representative sample, the extractions were carried out using 100 g of chestnut chips for each sample. For all samples, the average size of the chestnut shavings (grain size), evaluated by measuring 30 pieces of wood chips, varied from 15 × 20 to 20 × 20 mm, with an average thickness between 5 and 8 mm. During the extraction in hot water, all the chestnut wood chips adsorbed a water amount approximately corresponding to the starting weight of the sample. After the extraction, the samples were filtered with Whatman no. 1 paper to remove the wood chips. The obtained solutions were centrifuged at room temperature for 5 min at 13000 rpm to ensure the perfect solubility of the sample necessary for injection into the HPLC-DAD system. The % yield of each extract was calculated after freeze-drying the aqueous extracts and was expressed on dried chips (dry matter).

2.4. Scale-Up Procedure by the Autoclave. To test a possible scale-up of the extraction process, tests were carried out applying two extraction ratios: 7.4, and 4.9 dm^3/kg, using a total water volume of 112 dm^3. The extractor was an autoclave saturated with steam, the filling took place with the entry of water from below, and the applied pressure was 1.5−2 bar. The autoclave, produced by the lpvcaldaie factory (https://www.lpvcaldaie.it/prodotti/autoclave-orizzontale-700), was characterized by a basic set of control valves, including a continuous vent to ensure temperature uniformity. The model was 700 × 1000, customized according to our specific requests and suitable for the first scale-up. The sample of wood chips was put in a stainless-steel basket and then inserted into the extractive chamber. Hot water was added through several holes distributed in the chamber

Table 2. Phenolic Compounds in Aqueous Extracts of Chestnut Wood Tentatively Identified by MS Spectra in Negative Ionization Mode[a]

N°	Rt	compound	$[M-H]^-$	$[2M-H]^-$	$[M-2H]^{2-}$	fragment ions	references
1	3.1	caffeic acid hexoside	341			179	33
2	3.6	dipentosyl galloyl glucose derivative	763	1527		631, 499, 331	
3	4.1	vescalin	631	1263		481, 331, 301	10
4	4.4	vescalin isomer	631	1263		481, 331, 301	10
5	4.6	HHDP-glucose isom.1	481			331	10
6	4.8	monogalloyl glucose isom. 1	331	663		313, 271, 169	24
7	5.1	castalin	631	1263		481, 331, 301	10
8	5.6	castalin isomer	631	1263		481, 331, 301	10
9	5.7	galloyl dirhamnosyl glucose derivative	773	1547		481, 331, 301	
10	6.1	HHDP-glucose isom. 2	481			331,169	32,24
11	6.4	HHDP-glucose isom. 3	481			331,169	32,24
12	7.6	monogalloyl glucose isom. 2	331	663		331, 271	32,10
13	8.1	monogalloyl glucose isom. 3	331	663		331, 271	32,10
14	8.8	roburin E isom. 1	1065		532	924, 915, 301	34,11
15	9.4	roburin C	1981		990	1065, 915,301	34
16	9.6	gallic acid	169			125	
17	10.2	roburin A/D isom. 1	1849		924	924, 915, 616, 483, 331,169	34,11
18	11.1	roburin E isom. 2	1065		532	924, 915, 301	34,11
19	11.7	roburin A/D isom. 2	1849		924	1065, 924, 915, 616, 532	34,11
21	11.9	roburin E isom. 4	1065		532	924, 915, 532,484,466	32,10,11
22	12.7	roburin E isom. 5	1065			924, 915, 301	32,10,11
23	13.2	vescalagin	933	1867	466	301	32,10
24	14.7	vescavaloninic acid	1101		550	528	35
25	14.9	cascavaloninic acid	1101		550	528	35
26	16.2	digalloyl glucose isom.1	483	967		331,169	24
27	17.2	castalagin	933	1867	466	301	32,10,11
28	17.7	valoneic acid dilactone	469			425, 301	39,11
29	18.5	*β*-1-*O*-ethyl vescalagin isom 1	961		480	633, 331, 301, 169	32,34
30	18.9	*β*-1-*O*-ethyl vescalagin isom 2	961		480	633, 331, 301, 169	32,34
31	19.4	pedunculagin	951			783,633, 483, 331, 301	41
32	20.01	pedunculagin isom	951			783,633, 483, 331, 301	41
33	20.4	1-*O*-galloyl castalagin	1085			542,483, 301	10
34	21.1	digalloyl glucose isom. 2	483	967		331,169	24
35	22.3	digalloyl glucose isom. 3	483	967		331,169	24
36	22.4	valoneic acid	505			313	42
37	23.3	trigalloyl glucose isom. one kurigalin	635			317, 271, 169	10
38	23.7	di-HHDP-galloyl glucose casuarictin/potentillin	935			633,301	32
39	25.3	di-HHDP-galloyl glucose isom. 1	935			633,301	32
40	26.2	trigalloyl glucose isom. two kurigalin	635		317	317, 169	10
41	26.9	tellimagrandin I	785		392	301, 169	10
42	28.6	trigalloyl glucose isom. 3	635	1271		317, 169	10
43	30.5	trigalloyl glucose isom. 4	635		317	169	10
44	31.2	trigalloyl glucose isom. 5	635		317	271, 169	10
45	31.9	trigalloyl glucose isom. 6	635		317	169	10
46	32.5	tellimagrandin I isomer	785		392	301, 169	10
47	33.5	tetra-galloyl glucose isom. 1	787		393	317	10
48	35.9	tetra-galloyl glucose isom. 2	787		393	317	10
49	36.5	tetra-galloyl glucose isom. 3	787		393	317	10
50	36.7	trigalloyl-HHDP glucose	937				10
51	37.4	tetra-galloyl glucose isom. 4	787		393	317	10
52	37.8	tetra-galloyl glucose isom. 5	787		393	317	10
53	35.5	ellagic acid	301				
54	39.4	penta-galloyl glucose isom. 1	939		469	169	11
55	40.1	penta-galloyl glucose isom. 2	939		469	169	11
56	48.7	ellagic acid-deoxyhexose	447			301	
57	43.6	dehydrated ellagic acid dimer	585			301	39,40

[a]Isom − isomeric structure with the same mw.

until the fixed level was reached (in the rain of overheated water). The samples were extracted at 100 °C for a total extraction time of 90 min.

2.5. HPLC-DAD Analyses for Tannins. The extracts were analyzed by a HP 1260L liquid chromatography with a DAD detector (Agilent Technologies, Palo Alto, CA, USA); the column was a C18 Luna (250 × 4.6 mm, 5 μm; Phenomenex, Torrance, CA, USA), and the eluent was a binary mixture of acidic water (pH 3.2 by HCOOH) as the solvent A and CH_3CN as the solvent B. A multistep linear solvent gradient was applied as follows: 0.1−20 min, 5−15% B (v/v); 20−25 min, 15% B; 25−35 min, 15−25% B; 35−43 min, 25% B; 43−48 min, 25−100% B, maintaining this condition for 4 min and then returning at the starting conditions in 3 min. The total elution time is 55 min, the flow rate is 0.8 mL min^{-1}, and the oven temperature is 26 °C. The UV−vis spectra ranged from 200 to 500 nm, and the chromatograms were acquired at 254, 280, and 370 nm. The MS experiments were performed using the same chromatographic system and an Agilent 1260 Infinity MSD (G6125B) mass spectrometer applying a negative ionization mode with fragments from 150 to 250 V. The quantitative determination for all the phenolic compounds was done using the curve of gallic acid at 280 nm (linearity range 0.1−6.7 ug, R^2 1.000); data were expressed as mg/g dried matter (DM) or mg/g dried extract (DE).

2.6. Folin−Ciocalteu Method. The Folin−Ciocalteu method was applied as follows. The aqueous extract was diluted 1:20 v/v with water; 125 uL of the sample was added with 500 uL of water and 125 uL of the Folin−Ciocalteu reagent; and the sample remained in the dark for 6 min. Successively, 1.25 mL of a water solution at 20% Na_2CO_3 + 1 mL of distilled water were added to the sample, and the mixture was incubated for 85 min in the dark. The sample was then centrifuged (3000 rpm), and the solution was recovered to measure the absorbance at 725 nm according to a previous work.[30] Similarly, a blank sample was prepared, starting from distilled water. Calibration curves were built by measuring the absorbance of five solutions containing gallic acid at different concentrations treated with the same procedure applied to the sample. The phenol content of each sample was expressed as GAEs (gallic acid equivalents) as milligrams per gram of total tannins on dry extract (DE).

2.7. Statistical Analysis. Analyses were performed in triplicate, and the results were expressed as mean values ± the standard deviation. The Pearson coefficient was computed to test the possible correlations. Statistical significance was evaluated by the analysis of variance and *F*-test ($p \leq 0.05$) using Microsoft Excel statistical software. Fisher's LSD test was applied to compare the mean values using the software DSAASTAT v. 1.1.3.

3. RESULTS AND DISCUSSION

3.1. Identification of Tannins in the Aqueous Extracts. This part of the study was focused on defining the tannin profile of the water extracts of chestnut by performing HPLC-DAD-MS analyses in negative ionization mode. So far, most research on chestnut extracts has focused on the evaluation of the major compounds,[10,27,28,31] while minor polyphenols have received little attention. In this study, more than 50 ellagitannins and gallotannins were tentatively identified by combining data of MS fragmentation, UV−vis spectra, retention time, and earlier literature. Some authors recently reported a fractionation of total tannins extracted by supercritical water from chestnut wood and distinguished hydrolyzable and condensed tannins using different spectrophotometric methods.[28] Nevertheless, no further data were reported to describe the molecules belonging to these different classes.

Among the detected polyphenols, there are ellagitannins such as roburin, vescalin, castalin, vescalagin, and castalagin and gallotannins such as mono-, di-, and trigalloyl-β-D-glucose, trigalloyl-HHDP-glucose, and di-HHDP-galloyl-glucose (Table 2). Furthermore, several groups of isobaric compounds were highlighted, such as six isobars of trigalloyl glucose with $[M-H]^-$ at m/z 635, five isobars of tetragalloyl glucose with $[M-H]^-$ at m/z 787 and five isobars of roburin E with $[M-H]^-$ at m/z 1065. The identification of the major and minor polyphenols in chestnut wood extracts was possible thanks to the copresence, in most of the mass spectra, of three very diagnostic ions such as $[M-H]^-$, $[2M-H]^-$, and $[M-2H]^{2-}$. The doubly charged ions are common in MS negative ionization mode, particularly for polyphenolic molecules;[24,32] all these ion species were crucial to confirm the molecular weights of the compounds. Compound **1** $[M-H]^-$ at m/z 341 showed a loss of 162 Da and a fragment ion at m/z 179 indicating the structure of a caffeic acid hexoside, one of the most common hydroxycinnamic acids in oak wood.[33] The spectrum of the analyte **2**, with $[M-H]^-$at m/z 763, showed two fragment ions at m/z 631 and m/z at 499 attributable to the loss of one and two units of pentose, while the ion at m/z 331 was recognized as a monogalloyl glucose. Compounds **3**, **4**, **7**, and **8** are four isobaric molecules that produced $[M-H]^-$ at m/z 631 and showed the following fragments: two fragments at m/z 331 and 481, corresponding to monogalloyl glucose and HHDP-glucose, respectively, and one fragment at m/z 301, indicating the presence of an ellagic acid moiety in these molecules. According to their retention time in reverse phase,[10,24] these compounds were identified as vescalin (**3**), vescalin isomer (**4**), castalin (**7**), and castalin isomer (**8**). These compounds can be hypothesized as the precursors of the main tannins at higher molecular weight (mw), such as castalagin and vescalagin. Three isobaric molecules with $[M-H]^-$ at m/z 481 were tentatively identified as HHDP-glucose (**5**, **10**, and **11**) because of the two fragments at m/z 331 and m/z 169 corresponding to mono galloyl glucose and gallic acid, respectively, and the fragmentation pattern that was already observed by Comandini et al.[10] Compound **9** was tentatively identified as galloyl dirhamnosyl glucose derivative with $[M-H]^-$ at m/z 773 and fragment ions at m/z 481, 331, and 301, whereas compounds **6**, **12**, and **13**, tentatively identified as three monogalloyl glucose derivatives, were hypothesized as originated from a partial hydrolysis of precursors at higher molecular weight. Several roburins have been tentatively identified; five of them were roburin E and other isobaric forms with a $[M-H]^-$ ion at m/z 1065 (**14**, **18**, and **20**−**22**). Their mass spectra were in agreement with previous works[11,34] and showed a fragment ion at m/z 915 originated by the loss of a water molecule, and the ion at m/z 301 indicates the presence of the ellagic acid. Compound **15** was identified as roburin C, and this compound is glycosylated dimers of castalagin or vescalagin with a m/z of 1981, with a double charge at m/z 990 and diagnostic fragment ions at m/z 915 and m/z 301. Compounds **17** and **19** were identified as roburin A/D and its isomer by their mass spectra with the presence of the molecular ion at m/z 1849 and the double charged ion at m/z 924 and by diagnostic fragments ions at m/z 915 and m/z 301. The identification of the molecule by the fragmentation pattern was in agreement with previous data.[11,32,34] The two isobaric molecules **24** and **25** were identified as vescavaloninic or cascavaloninic acid with $[M-H]^-$ at m/z 1101; the molecular weight was confirmed by the presence of the corresponding double charged ion at m/z 550.[35] Another couple of isobaric compounds, **23** and **27**, showed the molecular ion at m/z 933, the double charged ion at m/z 466 and the fragment ion at m/z 301 and, according to previous works,[10,11,32] were identified as vescalagin and

castalagin, respectively. The three isomeric compounds **26**, **34** and **35** showed the [M-H]$^-$ ion at m/z 483, and diagnostic fragments at m/z 331 and m/z 169 attributable to monogalloyl glucose group and gallic acid, respectively, allowing identifying them as digalloyl glucose isomers. Another hydrolyzable tannin with m/z 469 was tentatively identified as valoneic acid dilactone (**28**). Another couple of isobaric molecules were compounds **29** and **30** with a molecular ion at m/z 961 and the same diagnostic fragment ion at m/z 331 and m/z 169 that allowed identifying them as two isomers of β-1-O-ethylvescalagin.[32,34] Compounds **31** and **32** were tentatively identified as pedunculagin and its isomer with m/z 951. Compounds **38** and **39** showed a [M−H]$^-$ at m/z 935 and a fragment at m/z 633 due to the loss of ellagic acid (m/z 301); they were tentatively identified as di-HHDP-galloyl glucose, also known as casuarictin or potentillin. Compounds **41** and **46**, with a [M-H]$^-$ ion at m/z 785 were tentatively identified as tellimagrandin I and tellimagrandin I isomer, according to a previous work.[36] Finally, according to their mass spectra and the previous literature,[37,38] several trigalloyl glucose (**37**, **40**, **42−45**), tetra-galloyl glucose (**47−49** and **51−52**), and penta-galloyl glucose derivatives (**54−55**) were tentatively identified. Compounds **56** and **57** were tentatively identified as ellagic acid derivatives such as ellagic acid deoxyhexose and dehydrated ellagic acid dimer, respectively.

Recently, Cravotto and co-workers,[29] evaluated the tannins in chestnut peels by using a microwave assisted subcritical water extraction to propose an industrial process to recover bioactive compounds from this byproduct. This tissue contains appreciable quantities of simple phenols such as catechin, epigallocatechin, gallocatechin, and ellagic acid, but the authors did not mention the presence of tannins typical of chestnut wood such as roburin, vescalin, castalin, vescalagin, and castalagin.

3.2. Preliminary Extraction Tests and Tannin Content by HPLC-DAD. The first laboratory-scale step was to collect information about the quantity of tannins extracted from chestnut wood in relation to the temperature applied during the extraction process. Hot water was used at 50, 70, and 90 °C working on different aliquots of the same chestnut sample (WT-T_9), and applying 30 or 60 min as extraction times. Meanwhile, each sample was treated by applying two extractive ratios of 1/10 and 1/15 w/v; a final step of extraction of 30 min was applied to all the samples. The applied conditions and the total amount of extracted tannins are summarized in Table 3, which showed that, at the lowest temperatures (i.e., 50, 70 °C), the recovery of total tannins is strongly reduced and that an important increase was observed passing from 70 to 90 °C. Furthermore, the longer extraction time for the first step (60 min) and the use of higher amount of water for the extraction (from 1/10 g/mL to 1 g/15 mL) did not increase the extracted tannins. This latter result indicated that an excess of solvent does not require efficient recovery of the tannins from the chestnut sample. This data are especially interesting when considering that during a future scalability of the extraction process reduced volumes of water will be required. To confirm the previous result, the second step of extraction (30 min) gave a recovery similar to that of the first step only for the lower temperature (50 °C), but was less relevant for the extraction done at 90 °C. In this latter case, after the first extraction the residual tannins in chestnut wood were approximately lower than 21% of the total tannins, it is presumable that some of these residual tannins could be recovered only after a simple washing of the extracted wood.

Table 3. Total Tannins Content Obtained By Extraction of Chestnut Wood Samples at the Lab Scale at Different Conditions of Time and Temperature and Applying Different Drug/Solvent Ratios[a]

		total tannins (mg/g DM)		
		first extraction step		second extraction step
temp.	extractive ratio (g/mL)	30 min	60 min	30 min
50 °C	1/10	4.8 ± 0.1	4.44 ± 0.12	3.5 ± 0.32
	1/15	4.03 ± 0.14	4.15 ± 0.86	3.25 ± 0.18
70 °C	1/10	5.97 ± 0.9	5.85 ± 0.36	2.45 ± 0.42
	1/15	5.18 ± 0.52	5.33 ± 0.17	2.51 ± 0.66
90 °C	1/10	10.65 ± 0.74	11.09 ± 0.55	1.85 ± 0.5
	1/15	9.18 ± 0.26	10.15 ± 0.32	2.14 ± 0.5

[a]Data are expressed as mean ± standard deviation of triplicates in mg/g on chestnut chips (DM).

These preliminary tests at different extraction temperatures were also aimed at comparing the phenolic profiles of the obtained extracts. As shown in Figure S1, the chromatographic profiles at 280 of the extract at 50 °C were very similar to that obtained at higher temperature (i.e., 90 °C), confirming the thermal stability of the chestnut tannins in these extraction conditions.

For maximizing the extracted tannins, in agreement with the data in Table 3, the following tests on the other chestnut samples were carried out only with water at 90 or 100 °C, with an extractive ratio of 1/10 w/v and an extraction time of 30 min for each step. In a previous study,[30] the authors assessed that an extraction with hot-water at 100 °C longer than 120 min did not increase the yield in total tannins.

This step of the study also focused on evaluating the effect of the different trunk seasoning times on the extracted tannins, working with three samples: WT-T_0 (no seasoning) and WT-T_9 and WT-T_{48} seasoned for 9 and 48 months, respectively. Moreover, to be exhaustive in the recovery of the tannins, three successive extraction steps of 30 min each were applied to these chestnut samples working at 90 and 100 °C. The results of this comparison are reported as the amount of total tannins in chestnut chips (Figure 1A), yields expressed as dried extract (DE) on chestnut chips (Figure 1B), total tannins per liter of water extract (Figure 1C), and total tannins on DE (Figure 1D).

Looking at the data in Figure 1, it is interesting to note the same trend for each sample: the highest values were for the first extraction step at the highest temperature both for the yields of dry extract (Figure 1B) and in terms of total extracted tannins (Figure 1A,C). Another important result was that the total extracted tannins increased according to the months of seasoning for all of the extractive steps. The statistical study applied to the same chestnut samples extracted at 90 or 100 °C indicated significant difference only for the WT-T_9 sample for the total tannins on dry chestnut wood (Figure 1A). On the opposite, evaluating the yields in DE were evaluated, all samples showed significant differences with higher values obtained for the higher temperature (Figure 1B). No significant differences were observed for the total tannin content in the liquid samples (Figure 1C). In agreement with the previous results, only one sample showed significant

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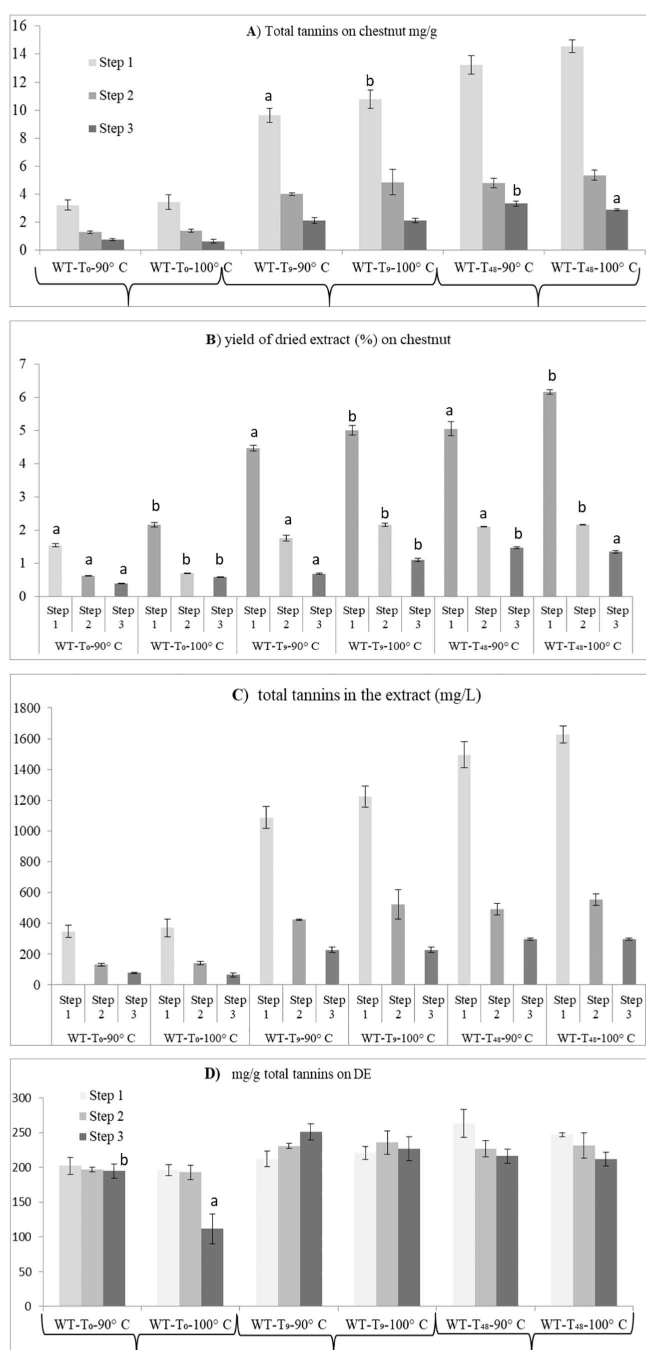

Figure 1. Comparison of the tannin content in three samples of chestnut wood at different seasoning times. The ratio wood chips/water was 1:10 (g/mL) for all the samples; the values are expressed as a mean of triplicates ± standard deviation. (A) Data on dried chestnut; (B) percentage of the dried extract on dried chestnut; (C) total tannins per liter of extract; (D) total tannins on dried extract (DE). WT, whole trunk; T_0, no seasoning; T_9, 9 months of seasoning; T_{48}, 48 months of seasoning. Different letters indicated statistically significant differences between samples extracted at 90 and 100 °C.

differences in mg/g of total tannins on DE (Figure 1D), but this was linked exclusively to the third extraction step.

Meneguzzo et al.[37] proposed 100 °C as the maximum extraction temperature for a cavitation process to guarantee an accelerated tannin release. These results, even if obtained under experimental conditions different from those applied in our work, confirmed that 100 °C is a good choice as an extraction temperature. A recent review[38] focused on the chestnut production chain discussed the extraction of tannins from different parts of the chestnut such as shells and leaves, but no data on wood extraction were reported. To the best of our knowledge, no data are available on the effect of using water at temperatures above 100 °C on extracted tannins.

Noteworthy, concerning the composition of the dried extracts (Figure 1D), the differences among the samples are strongly reduced, and the total amount of tannins on each sample shows very similar values, slightly higher for the two longer seasoned samples, namely, WT-T_9 and WT-T_{48}. The tannin content in these latter samples was close to 20−25% on DE, and only little variations were observed among the first, second, and third extraction steps (Figure 1D). It can be stated that the yield in tannin reached with only one extractive step is high and very similar independently from the different seasoning times of the wood. Overall, the results in Figure 1 made it possible to state that (i) it is crucial to carry out the extraction process at temperatures greater than 90 °C to maximize the yield in dry extract; (ii) the seasoning time of the trunk affects the concentration of tannins in the chestnut wood; and (iii) a single extraction is suitable for recovering about 65−70% of the total tannins.

This study did not evaluate temperatures above 100 °C because the differences in tannin recovery were slight or not significant ranging from 90 to 100 °C but also because we wanted to investigate experimental conditions that allowed maximum energy savings.

3.3. Scale-Up Test for Extraction in the Autoclave. In view of a future scale-up of the process, the last part of the study on the extraction conditions focused on evaluating the possibility of reducing the water volume and extraction time. For this purpose, an autoclave working with water at 100 °C under a slight pressure above the atmospheric (approximately 1.5 bar) value was used as the extractor. In accordance with the extractor capacity, two extractive ratios of 7.4 and 4.9 dm^3/kg of chestnut chips were tested and, to reduce the total time of the process, only a single extraction was carried out. Concerning the cortex, it was possible to apply only an extractive ratio of 7.4 due to the higher volume of the raw material and the need for more water to thoroughly wet the material. Because 30 min was approximately required to reach the final temperature, the total time of the process was fixed at 90 min. Whole chestnut wood samples with different seasoning times were extracted in these experimental conditions. For also exploring the possibility of obtaining extracts with different phenolic profiles, the cortex (UT-C-T_{24}), the inner part of the trunk (UT-I-T_{24}), and the basal part of the trunk (BT-T_{24}) were also analyzed. The last sample was included because, industrially, the basal part of the trunk after the cutting is then left on the field and not used for the extraction of the tannins.

According to the chromatographic profiles at 280 nm (Figure S2), several differences were observed among the samples. The phenolic profiles of the whole seasoned trunk and the inner part of the trunk were very similar, while the basal trunk of older plants showed an increased amount of castalagin. Gallic and ellagic acids prevailed in the cortex with a very low content of castalagin and vescalagin. From the literature, it was possible to identify another plant, *Anogeissus leiocarpus* (Combretaceae family) characterized by the presence of hydrolyzable tannins structurally similar to those found in chestnut wood. On the contrary of what was observed for chestnuts, this plant concentrates in the bark approximately

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70% of the total tannins of the entire trunk.,[32] with a lower amount in the inner part of the trunk.

The data in Table 4 provide quantitative information to evaluate the extraction results. It reports the % yield expressed

Table 4. Extractions Carried Out at 100 ± 2 °C Applying Two Different Extractive Ratios of Water/Chestnut Wood[a]

sample	extractive ratio (L/kg)	solid content (g/L)	total tannins (g/L)	% yield DE/DM	tot tannins (g/kg DM)	tot tannins (g/kg DE)
WT-T_{18}	7.4	7.25	1.60	5.8	12.7	219.7
WT-T_{18}	4.9	7.33	2.10	4.6	13.1	259.8
WUT-T_{24}	4.9	8	1.77	4.2	9.24	221.5
BT-T_{24}		10.5	1.41	5.5	7.34	140.2
UT-I-T_{24}		10.8	2.16	5.6	11.3	202.3
UT-C-T_{24}	7.4	5	0.43	2.6	3.6	108.9

[a]DM, dry matter; DE, dry extract.

as DE/DM, to provide information on the efficiency of the extraction process, indicating the amount of dry extract that is possible to recover from the chestnut chips. Regarding the composition of the extracts, the evaluation of their quality was related to their tannin content expressed as milligrams per gram of DE, as well as evaluated in the water solution and expressed as g/L.

Concerning the results of the two different extractive ratios applied to the sample WT-T_{18}, it can be assessed that applying a reduction of the water used for the extraction (ratio 4.9) the solid content in the samples did not change (Table 4). At the same time, with respect to the extractive ratio of 7.4, the yield DE/DM was lower, but the concentration of total tannins increased (2.1 g/L), the amount extracted from the chestnut sample was similar (13.1 g/kg DM), and the final dry extract was richer in total tannins (259.8 g/kg DE). These findings suggested that it is possible to reduce the water consumption during the extraction without reducing the extracted amount of tannins or the quality of the final extract.

Regarding the distribution of tannins in the different parts of the trunk, the whole trunk (WUT-T_{24}) had the highest solid content (10.5 g/L) and 1.77 g/L of total tannins. The same solid content was observed in UT-I-T_{24}, but the total tannin concentration was higher (2.16 g/L) than the concentration on DM (11.3 g/kg). These latter data confirmed that the ellagitannins of chestnut are more concentrated in the inner part and the lowest tannin content was in the cortex (UT-C-T24).

By determining some ratios between a single phenol and total tannins, differences were found between the whole trunk and the different parts of the chestnut, all aged for 24 months (Figure S3).

As the last test, the amount of nonextracted tannins applied to an extraction with hot water at the laboratory scale of the residual wheat chestnut chips (extractive ratio 1:10 g/mL, 30 min). It was assessed that after only one extractive step in the autoclave, the recovery of tannins was approximately 75% of the total content in chestnut wood. In the meantime, a parallel test was carried out: the water recovered after a washing of a few minutes of the chestnut chips recovered after the extraction was analyzed to determine the tannin concentration. It has been estimated that this simple operation allows the recovery of about 50% of the nonextracted tannins measured by the previous test.

In summary, the data in Table 4 demonstrate that the tannin content varies significantly depending on the different parts of chestnut trees and that the concentration in the basal trunk (BT-T24) is significantly lower than that of the whole trunk (WUT-T_{24}), while the highest concentration of tannins is in the inner part (UT-I-T_{24}). It can be assessed that the cortex alone of chestnut tree is not of interest as source of tannins. The one step extraction associated with a successive washing with a low volume of water at room temperature can increase the recovery of the tannins from chestnut wood.

3.4. Tannin Determination by the Folin−Ciocalteu Assay. It is important to have the possibility to compare the results obtained by HPLC-DAD determination, with the evaluation done by the Folin−Ciocalteu spectrophotometric method because both these procedures are widely applied to evaluate the tannin content in the extracts from chestnut

Table 5. Comparison of the Tannin Amount Expressed As Gallic Acid and Calculated With Two Analytical Procedures (HPLC-DAD At 280 Nm and Folin-Ciocalteu Methods) for Different Aqueous Extracts of Chestnut Wood Produced At Laboratory Scale and Using an Autoclave As Extractor[a]

		HPLC/DAD		Folin−Ciocalteu		
extract	extractive ratio(L/kg)	total tannins (g/L ext.)	total tannins (g/kg DE)	total tannins (g/L ext)	total tannins (g/kg DE)	folin/HPLCg/kgDE
WT-T_0	10	0.37	196.0	1.6	685	3.5
WT-T_9		1.23	220.7	4.4	758	3.4
WT-T_{48}		1.63	246.8	5.5	799	3.2
WTa-T_{18}	7.4	1.60	219.7	5.6	728	3.3
WTb-T_{18}	4.9	2.05	259.8	7.2	785	3.0
BT-T_{24}		1.41	140.2	5.8	552	3.9
WUT-T_{24}		1.77	221.5	5.3	663	3.0
UT-I-T_{24}		2.16	202.3	6.7	620	3.1
UT-C-T_{24}		0.43	108.9	2.1	382	3.5

[a]All the samples were obtained working with water at 100 °C and applying different volume/weight ratios (from 4.9 to 10 L/kg).

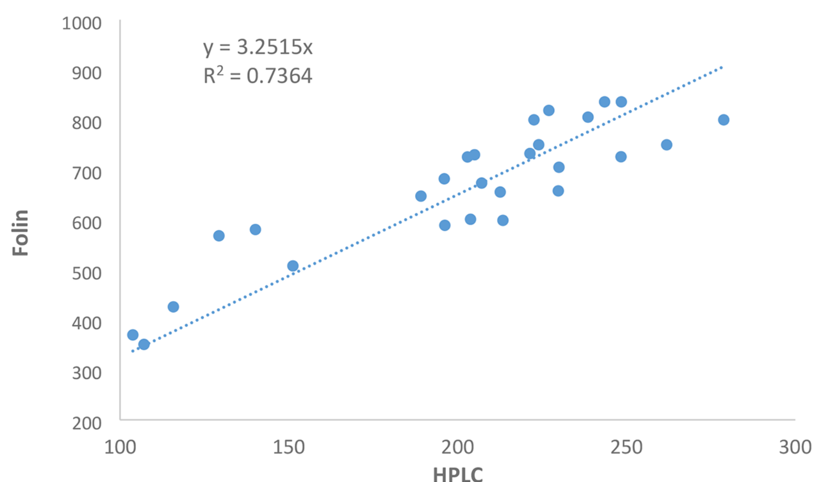

Figure 2. Linear fit of the quantitative data reported in Table 5 for the total tannin content in the dry extracts (DE). All data are expressed in mg/g of gallic acid used as external standard in both methods.

wood.[24] In this study, the findings were expressed using the same external standard (gallic acid) for both the methods, and the tannin content in the selected extracts is compared in Table 5. Overall, the spectrophotometric method gives much higher values with respect to those obtained by the chromatographic analyses by HPLC-DAD (that evaluating individually each compound can also provide specific information on the chemical changes of the extract over time). Since the Folin/HPLC ratios were similar for all the aqueous extracts obtained in different ways and from different parts of the trunk, a general multiplication factor can be proposed (ranging from a minimum of 3.0 to a maximum of 3.5) to pass from the total tannins by HPLC-DAD to those by the Folin−Ciocalteu method and vice versa. According to the data in Table 5 for the dry extracts (DE), the linear fit of the total tannin content expressed for both methods as mg/g of gallic acid allowed calculating this factor as the slope of the correlation line with a value of 3.25 (Figure 2).

Comandini et al.[10] reported the total tannin content in four commercial chestnut bark extracts determined only with the Folin−Ciocalteu method using gallic acid as an external standard with values between 239 and 560 mg/g of dry extract. By applying the conversion factor, the corresponding value determined by HPLC-DAD at 280 nm should be close to 74 and 172 mg/g DE. Recently,[28] results obtained applying a supercritical water extraction showed a highest total tannin content of approximately 84% determined by the Folin−Ciocalteu method. Applying the corrective factor shown in Figure 2, the corresponding concentration evaluated by the chromatographic method reached approximately 25.8%. These latter values, obtained applying the optimized procedure (100 °C and 60 min of extraction time), are in agreement with our results shown in Figure 1D.

Overall, the results of this study indicated that the extraction temperature and extractive ratio significantly affected the tannin content of the final extracts. Higher temperatures were decisive for a more complete extraction of the tannin, and the highest content of total tannins was obtained from chestnut woods, characterized by a longer seasoning time. A one-step extraction allowed recovery of approximately 80% of total tannins in chestnut wood. Different tannic contents in the inner or basal part of the trunk as well as in the bark were pointed out.

An important point to note for producers of chestnut tannin extracts is that dry extracts obtained from different whole trunk samples had similar percentages of total tannins, indicating a low variability in the composition of the final extract, which can be marketed.

Finally, a corrective factor for transforming data derived from chromatographic analysis into a spectrophotometric value and vice versa was defined, which can be useful for comparing values derived by two widely applied analytical methods to define the tannin content in chestnut extracts.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsfoodscitech.3c00272.

(Figure S1) Chromatographic profile of the chestnut wood WT-T9, with the extraction ratio of 1g/10 mL, at 50 and 90 °C; (Figure S2) chromatographic profiles at 280 nm of chestnut wood extracts (WUT-T_{24}, UT-C-T24, UT-I-T_{24}, BT-T_{24}); (**1**) vescalin; (**2**) castalin; (**3**) gallic acid; (**4**) vescalagin; (**5**) castalagin; (**6**) ellagic acid; and (Figure S3) different ratios between a single phenol/total tannin, for whole trunk (WT), internal part (UT-I), cortex (UT-C), and basal whole trunk (BT) (PDF)

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Notes

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■ LIST OF ABBREVIATIONS

HPLC-DAD-MS:high performance liquid chromatography-diode array detector-mass spectrometry; **DM**:dry matter; **DE**:dry extract; **GAE**:gallic acid equivalent; **HHDP**:hexahydroxydiphenoyl

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