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Analytical Methods

Extraction and determination of ellagic acid content in chestnut bark and fruit

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Abstract

Chestnuts are an important economic resource in the chestnut growing regions, not only for the fruit, but also for the wood. The content of ellagic acid (EA), a naturally occurring inhibitor of carcinogenesis, was determined in chestnut fruits and bark. EA was extracted with methanol and free ellagic acid was determined by HPLC with UV detection, both in the crude extract and after hydrolysis. The concentration of EA was generally increased after hydrolysis due to the presence of ellagitannins in the crude extract. The concentration varied between 0.71 and 21.6 mg g^{-1} (d.w.) in un-hydrolyzed samples, and between 2.83 and 18.4 mg g^{-1} (d.w.) in hydrolyzed samples. In chestnut fruits, traces of EA were present in the seed, with higher concentrations in the pellicle and pericarp. However, all fruit tissues had lower concentrations of EA than had the bark. The concentration of EA in the hydrolyzed samples showed a non-linear correlation with the concentration in the unhydrolyzed extracts.

Keywords: Ellagic acid; Chestnut fruit; Chestnut bark; HPLC

1. Introduction

Polyphenols are widely distributed in the plant kingdom and are important components of common foods, including tea, red wine, fruits, beverages and various medicinal plants. The importance of polyphenols arises from their effects on sensory properties, including astringency and colour, and possible health effects that they may have. One of the polyphenols is ellagic acid (EA), which has the structure shown in Fig. 1. EA is a dimeric derivative of gallic acid, which mainly exists in higher plants, including fruits and nuts, combined with its precursor, hexahydroxydiphenic acid or bound in the form of ellagitannins (Amakura, Okada, Tsuji, & Tonogai, 2000). EA was studied in the 1960s, mainly for its effects on blood clotting, its hemostatic activity and its effects in whitening of the skin, but reports about effects of EA on carcinogenesis were published in the following decades. Interest in EA has increased during the past few years due to its possible antimutagenic, antiviral and anticarcinogenic effects, proved by several studies, especially in laboratory animals, while a few works are reported in humans (Akagi et al., 1995; Hakkinen, Karenlampi, Mykkanen, Heinonen, & Torronen, 2000; Rommel & Wrolstad, 1993a; Sigman, Helmes, Fay, Lundquist, & Perry, 1984). Some ellagitannins have also been shown to possess anti-tumor-promoting activity, antibacterial and antiviral properties and host-mediated antitumor effects (Okuda, 2005). EA has also shown antioxidant activity as an inhibitor of in vitro lipid peroxidation and, because of its combined actions, it is used in the food industry. Extracts from red raspberry leaves or seeds, pomegranates, or other sources are said to contain high levels of ellagic acid, and are available as dietary supplements in capsule, powder, or liquid forms. A recent profusion of pomegranate

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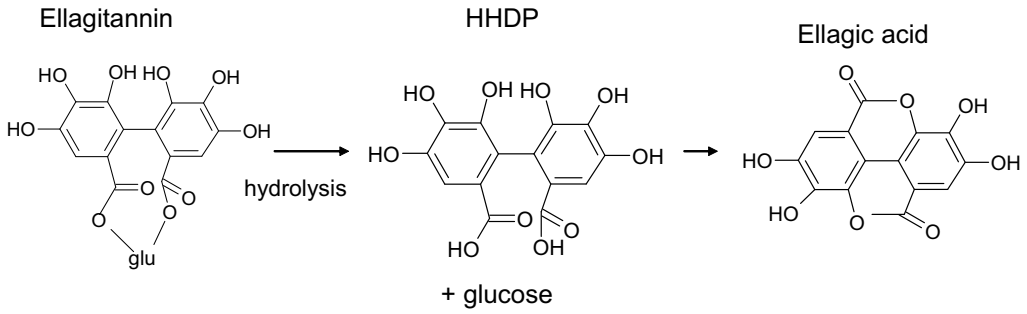

Fig. 1. Hydrolysis of ellagitannin to form ellagic acid through the production of hexahydroxydiphenic acid (HHDP) which is spontaneously lactonized to EA (Hakkinen et al., 2000).

nutraceutical products, "standardized to 40% ellagic acid" has appeared in the marketplace (Lansky, 2006).

Ellagic acid was reported to occur in 46 fruits, including raspberries, strawberries and cranberries, in significant quantities and also in nuts, walnuts, pecans, pomegranates and other plant foodstuffs (Amakura et al., 2000). Plants produce EA to protect themselves from microbial infection and pests. The barks of trees are rich in polyphenol components which help to protect the trees against predators and pathogens (Bianco, Handaji & Savolainen,1999; Lampire et al., 1998). In the case of chestnuts, EA is present in the bark, as reported by Mendes de Vasconcelos, Do, Bennett, Rosa, and Cardoso (2007) and in the wood too (Bianco, Handaji, & Savolainen, 1999) but there has not been any systematic study of the concentration of EA in different plant tissues, especially the parts of the fruit.

In order to assess the importance of chestnuts as dietary sources of EA and chestnut trees as potential sources of EA for use as food additives, it is important to determine the range of EA in various parts of the fruits and bark grown in different areas. The purpose of this study was to extract the EA and to determine its concentration in the tissues of fruit and bark of chestnut trees growing in five areas of Greece.

2. Materials and methods

2.1. Sampling

Mature fruits of the chestnut tree, *Castanea sativa*, were harvested from the ground in the main chestnut-producing areas of Greece. Fruits were collected from Elos, a region of Chania on Crete (Cr) and from Karpenisi (Ka), Parnon (Pa), Volos (Vo) and Kozani (Ko). The fruits were divided into three sections: the kernel that is eaten and the pericarp and pellicle that were manually removed. From the same areas, chestnut bark was also harvested from some 50 year-old trees. The external and internal parts of the chestnut barks were separated for analysis. All the samples were ground, lyophilized and then kept at −18 °C prior to analysis.

2.2. Extraction

The EA was extracted according to the method described by Bianco et al. (1999). An amount of 500 mg (in duplicate) of finally ground sample was suspended in 30 ml of methanol and the mixture was covered to prevent evaporation. The sample was stirred for 30 min at room temperature. Later, it was sonicated in a water bath for 20 min at room temperature. The extract was filtered through Whatman no. 1 filter paper (11 μm). The extracts were stored at −18 °C until analyzed before and after hydrolysis.

A small portion of filtrate was filtered again through a Millex Syringe 0.45 μm pore filter (Millipore Corporation Bedford, MA 01730, USA) prior to injection.

For hydrolysis, another portion of filtrate (20 ml) was evaporated to dryness using a rotary evaporator (30 °C water bath) (Heidolph instruments, Gmbh & Co. KG Schwabach, Germany). The residue was redissolved in 2 ml of methanol +2 ml of trifluoroacetic acid and the mixture was refluxed for 2 h at 85 ± 5 °C. The mixture was evaporated to dryness under N_2 and the residue was dissolved in 5 ml of methanol, and filtered through a 0.45 mm pore filter prior to injection (Hakkinen, Karenlampi, Mykkanen, Heinonen, & Torronen, 2000). Hydrolysis with HCl (final concentration 2.5 M) for 1 h at 90 °C was also effective in hydrolysing the ellagitannins.

2.3. HPLC analysis

The polyphenols fraction was isolated by methanolic extraction and ellagic acid was determined in the extract and in hydrolysed extracts by HPLC with UV detection. The detector was operated at 254 nm wavelength which corresponded to the experimentally found absorption maximum of the ellagic acid standard (Fig. 4).

An aliquot from the extract was diluted with an equal amount of water. The extracts were filtered through a 0.22 μm syringe filter prior to HPLC analysis. Ellagic acid was separated using a Hypersil ODS 5 μm column (250 × 4.6 mm) (MZ Analysentechnik, GmbH Mainz Germany). The solvent flow rate was 1 ml/min and the mobile

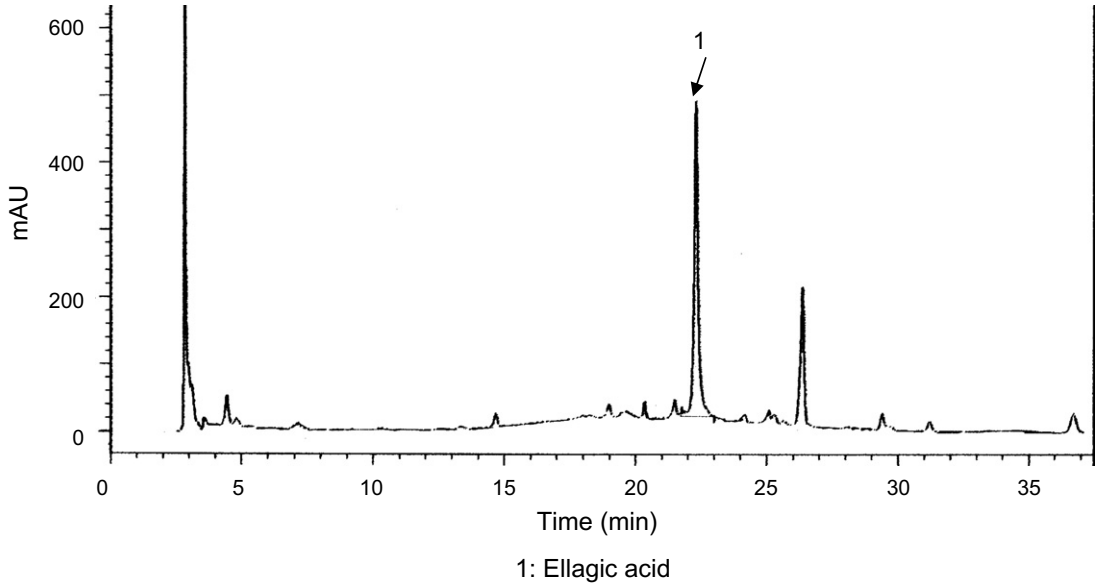

Fig. 2. A typical HPLC chromatogram of chestnut bark extract after hydrolysis.

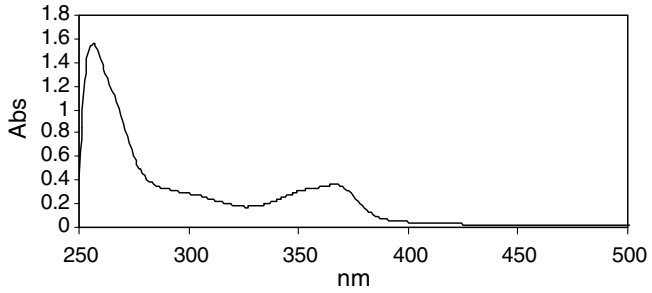

Fig. 3. Absorption spectrum of the HPLC peak from the EA standard. Spectra were sampled at the start of the peak, peak maximum and the end of the peak.

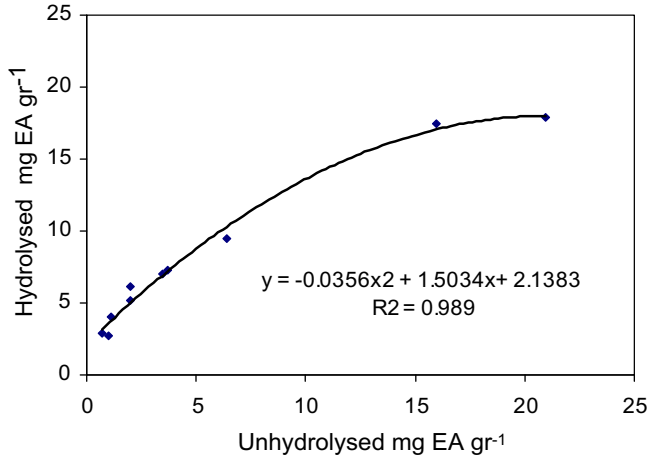

Fig. 4. Correlation between hydrolyzed and unhydrolyzed chestnut bark samples.

phase was a mixture of potassium dihydrogen phosphate solution (pH 2.5) and acetonitrile with the solvent programme as follows:

Time (min)	Acetonitrile%
0.00	7.0
8.00	7.0
25.00	32.00
30.00	35.00

3. Results and discussion

3.1. General

The EA in plants is present mainly in the form of ellagitannins and is bound to glucose. Acid hydrolysis transforms glucosylated and esterified EA into their aglycones, and liberates the parent compound EA as summarised in Fig. 1 (Daniel, Krupnick, Heur, Blinzler, Nims & Stoner,

Table 1
Ellagic acid content (mg g^{-1} in (d.w.)) in unhydrolyzed and hydrolyzed ground bark chestnuts ±SD of duplicate assays

Origin	Ellagic acid (mg g^{-1}) of raw chestnut barks	Ellagic acid (mg g^{-1}) of hydrolyzed chestnut barks
Vo_i	1.17 ± 0.03	4.18 ± 0.13
Vo_e	6.47 ± 0.13	9.6 ± 0.42
Cr_i	2.03 ± 0.04	6.2 ± 0.79
Cr_e	16.1 ± 0.07	17.6 ± 1.07
Pa_i	2.11 ± 0.06	5.43 ± 0.1
Pa_e	3.56 ± 0.07	7.26 ± 0.35
Ka_i	0.71 ± 0.03	3.02 ± 0.09
Ka_e	1.01 ± 0.02	2.83 ± 0.27
Ko_i	4.32 ± 0.01	8.5 ± 0.06
Ko_e	21.6 ± 0.39	18.41 ± 1.4

Vo_i: Volos internal, Vo_e: Volos external, Cr_i: Crete internal, Cr_e: Crete external, Pa_i: Parnon internal, Pa_e: Parnon external, Ka_i: Karpenisi internal, Ka_e: Karpenisi external, Ko_i: Kozani internal, Ko_e: Kozani external.

Table 2
The content of ellagic acid in chestnut fruit tissues (fruit, pericarp and pellicle) unhydrolysed and hydrolyzed (mg g^{-1} in (d.w.)) ±SD of duplicate assays

	Unhydrolysed			Hydrolysed		
	Seed (kernel) (mg EA g^{-1})	Pericarp (mg EA g^{-1})	Pelicle (mg EA g^{-1})	Seed (kernel) (mg EA g^{-1})	Pericarp (mg EA g^{-1})	Pellicle (mg EA g^{-1})
Ko	Trace	0.04 ± 0.004	0.03 ± 0.006	Trace	1.08 ± 0.01	0.57 ± 0.07
Ka	Trace	0.06 ± 0.002	0.04 ± 0.007	Trace	0.74 ± 0.03	0.77 ± 0.04
Cr	Trace	0.19 ± 0.02	0.091 ± 0.01	Trace	5.98 ± 0.68	0.64 ± 0.01
Par	Trace	0.11 ± 0.01	0.03 ± 0.005	Trace	1.76 ± 0.28	0.54 ± 0.05
V	0.05 ± 0.01	0.09 ± 0.008	0.04 ± 0.00	0.05 ± 0.007	1.34 ± 0.07	0.79 ± 0.06

Vo: Volos chestnuts, Cr: Crete chestnuts, Pa: Parnon chestnuts, Ka: **K**arpenisi Chestnuts, Ko: Kozani chestnuts.

1989). EA is a major product, as shown by a typical HPLC chromatogram of the hydrolysed product (Fig. 2) with detection at 254 nm, as justified by the UV spectrum of EA (Fig. 3).

3.2. Ellagic acid content in chestnut barks

The EA content in ground chestnut bark varied from 0.71 to 21.6 mg g^{-1} (d.w.) in unhydrolysed samples and from 2.83 to 18.4 mg g^{-1} (d.w.) in hydrolyzed samples (Table 1). For most samples of bark, the content of EA was markedly increased in hydrolyzed barks in comparison with unhydrolyzed. This is in line with the literature reports of hydrolysis in berries and walnuts (Daniel et al., 1989). The concentration of EA in the outer part of the bark was found to be significantly higher than that in the inner bark samples, in both hydrolyzed and unhydrolyzed samples, but in all cases it was much less than the amounts reported for chestnut wood samples (Bianco et al., 1999).

The concentrations of EA in raw and hydrolyzed chestnut bark samples showed a high degree of correlation (Fig. 4). The data fitted well to a quadratic polynomial equation. The curvilinear nature of the relationship suggests that some saturation of one or more enzymes involved in ellagitannin biosynthesis may occur at high ellagic acid concentrations.

The samples from different areas of Greece were significantly different in the EA and ellagitannin contents of bark samples. Samples from Kozani contained the most EA in the external part of bark in both hydrolyzed and unhydrolyzed samples, followed by samples from Crete.

3.3. Ellagic acid in chestnut fruit tissues

Table 2 shows concentrations of EA in different chestnut fruit tissues. EA occurred mainly in the pericarp and pellicle of the fruit and mainly in the form of ellagitannins. For most of the samples, the highest concentration of EA occurred in the pericarp, followed by the peel, with traces present in the seed. When considering pericarp and pellicles in the five areas, the most abundant EA was found in the pericarp of nuts. The presence of EA in pericarp and pellicle was elucidated for the first time. Concerning the chestnut kernels, the highest content of EA was found in the pericarp of Cretan chestnuts. The EA concentration varied from 0.04 to 0.19 mg g^{-1} in pericarp and from 0.03 to 0.091 mg g^{-1} in pellicle in the unhydrolysed and from 0.74 to 5.98 mg g^{-1} and from 0.54 to 0.79 mg g^{-1} for pericarp and pellicle, correspondingly, in the hydrolysed samples. The chestnut pulp contained traces of EA. The ripeness of chestnuts is reported to influence the concentration of EA present (Rommel & Wrolstad, 1993b), so further investigation of the contents of EA is needed at different stages of chestnut maturity and before they fall to the ground.

There are different reports about the concentration of EA in nuts. Some researchers report that the EA content measured in Brazilian nuts is below the HPLC detection limit (Daniel et al., 1989). However, in Finland, berries and nuts (not chestnuts) are used mainly as sources of EA in the total diet (Hakkinen et al., 2000). Portuguese researchers have reported that significant quantities of free ellagic acid could be extracted from chestnut kernels of several varieties (Mendes de Vasconcelos & Do, 2007). The current study has shown that Greek chestnuts harvested from the ground are not a significant source of EA with the fruit pulp containing traces of EA.

In conclusion**,** the results of the current study show that the bark of chestnut trees contains significant concentrations of EA. So, chestnut bark is potentially a useful source of EA for application in the food or pharmaceutical industry and in scientific research.

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References

Akagi, K., Hirose, M., Hoshiya, T., Mizoguchi, Y., Ito, N., & Shirai, T. (1995). Modulating effects of ellagic acid, vanillin and quercetin in a rat medium term multi-organ carcinogenesis model. *Cancer letters, 94*, 113–121.

Amakura, Y., Okada, M., Tsuji, S., & Tonogai, Y. (2000). High performance liquid chromatographic determination with photodiode array detection of ellagic acid in fresh and processed fruits. *Journal of Chromatography, 896*, 87–93.

Bianco, M. A., Handaji, A., & Savolainen, G. (1999). Quantitative analysis of ellagic acid in hardwood samples. *The Science of the Total Environment, 222*(1–2), 123–126.

Daniel, E. M., Krupnick, A., Heur, Y.-H., Blinzler, J. A., Nims, R. W., & Stoner, G. D. (1989). Extraction, stability and quantification of ellagic acid in various fruits and nuts. *Journal of Food Composition and Analysis, 2*, 338–349.

Hakkinen, S. H., Karenlampi, S. O., Mykkanen, H. M., Heinonen, I. M., & Torronen, A. R. (2000). Ellagic acid content in berries: Influence of domestic processing and storage. *European Food Research Technology, 212*, 75–80.

Lansky, E. P. (2006). Beware of pomegranates bearing 40% ellagic acid. Short communication. *Journal of Medicinal Food, 9*(1), 119–122.

Lampire, O. , Mila, I., Raminosoa, V., Herve du Phenoat, C., Faucheur, N., Laprevote, O., et al. (1998). Polyphenols isolated from the bark of *Castanea sativa* Mill. Chemical structures and autoassociation in honour of Pr G.H. Neil Towers 75th birthday. *Phytochemistry, 49*, 623–631.

Mendes de Vasconcelos, M., & Do, C. B. (2007). Primary and secondary metabolite composition of kernels from three cultivars of Portuguese chestnut (*Castanea sativa* Mill) at different stages of industrial transformation. *Journal of Agricultural and Food Chemistry, 55*, 3508–3516.

Mendes de Vasconcelos, M., Do, C. B., Bennet, R. N., Rosa, E. A. S., & Cardoso, J. V. F. (2007). Primary and secondary metabolite composition of kernels from three cultivars of Portuguese chestnut (Castanea sativa Mill) at different stages of industrial transformation. *Journal of Agricultural and Food Chemistry, 55*, 3508–3516.

Okuda, T. (2005). Systematics and health effects of chemically distinct tannins in medicinal plants. *Phytochemistry, 66*, 2012–2031.

Rommel, A., & Wrolstad, R. (1993a). Influence of acid and base hydrolysis on the phenolic composition of red raspberry juice. *Journal of Agricultural and Food Chemistry, 41*, 1237–1241.

Rommel, A., & Wrolstad, R. (1993b). Ellagic acid content of red raspberry juice as influenced by cultivar, processing and environmental factors. *Journal of Agricultural and Food Chemistry, 41*, 1951–1960.

Sigman, C. C., Helmes, C. T., Fay, J. R., Lundquist, P. L., & Perry, L. R. (1984). A study of chemicals in the wood and associated industries for the selection of candidates for carcinogen bioassay, 1. Naturally-occuring wood chemicals. *Journal of Environmental Science and Health, A19*(5), 533–577.