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Chemical Papers 62 (4) 440–444 (2008)
DOI: 10.2478/s11696-008-0042-y

ORIGINAL PAPER

Extraction and analysis of ellagic acid from novel complex sources

[a]Antonio F. Aguilera-Carbo, [b]Christopher Augur, [a]Lilia A. Prado-Barragan, [c]Cristóbal N. Aguilar*, [a]Ernesto Favela-Torres

[a]*Department of Biotechnology, Universidad Autónoma Metropolitana, Iztapalapa, 09340, DF, México*

[b]*IRD-Unité Biotrans, IMEP Case 441, Faculté des Sciences de Saint Jérôme, Université Paul Cézanne, Av. Escadrille Normandie-Niemen, 13397 Marseille cedex 20, France*

[c]*Food Research Department, School of Chemistry, Universidad Autónoma de Coahuila, 25280 Saltillo, Coahuila, México*

Received 6 November 2007; Revised 4 February 2008; Accepted 5 February 2008

Ellagic acid (EA) was quantified by reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with photodiode array detection (DAD) in five fine-powdered plants collected from the semiarid region of Mexico. Samples analysed included *Jatropha dioica* branches (Dragon's blood), *Euphorbia antisyphyllitica* branches (Candelilla), *Turnera diffusa* Willd leaves (Damiana), *Flourensia cernua* leaves (hojasén) and *Punica granatum* husk (pomegranate) at two maturity stages ("turning" or intermediate and maturated fruit, considered as positive controls). The results demonstrated high EA concentrations in all tested samples which are novel sources of this natural antioxidant. The method developed for the EA analysis is fast and it showed an excellent linearity range, repeatability, intra- and inter-day precision and accuracy with respect to the methods reported for the EA analysis.
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Keywords: ellagic acid, semiarid plants, HPLC analysis

Introduction

Natural compounds derived from plants are part of our daily diet. In recent years, there has been an increasing attention in healthy way of life along with the search for new natural products. Among them are specific antioxidants, such as ellagitannins and ellagic acid, now in high demand (Clifford & Scalbert, 2000; Manach et al., 2004). Ellagitannins (ET's), one of the major groups of hydrolysable tannins, are esters of hexahydroxydiphenic acid (HHDP) and polyols, usually glucose or quinic acid. When exposed to acids or strong bases, ester bonds are hydrolysed and the hexahydroxydiphenic acid spontaneously rearranges into the water-insoluble dilactone, ellagic acid (EA) (2,3,7,8-tetrahydroxychromeno[5,4,3-*cde*]chromene-5,10-dione, according to IUPAC). EA is a highly thermodynamically stable molecule with four rings representing the lipophilic domain, four phenolic (hydroxyl) and two lactone groups (which can act as hydrogen bond donor or acceptor) representing the hydrophilic part (Barch et al., 1996; Bala et al., 2006). Presently, the main sources of EA and ellagitannins are oak trees (*Quercus robur*, *Q. alba*), walnut (*Junglans nigra*) and chestnut (*Castanea sativa*) trees (Bianco et al., 1998). Berry fruits are the other sources of ellagitannins and ellagic acid (Table 1), including strawberry (*Fragaria ananassa*), raspberry (*Rubus idaeus*), blackberry (*Rubus occidentalis*), as well as some seeds and nuts (*Bertholletia excelsa* Humb), pecan (*Carya illinoinensis*) (Clifford & Scalbert, 2000), and fruit, e.g. pomegranate (*Punica granatum* L.) (Atta-Ur-Rahman et al., 2001; Machado et al., 2002; Seeram et al., 2005; Koponen et al., 2007). EA has been associated with health improvement through its anti-atherosclerotic action (Aviram et al.,

*Corresponding author, e-mail: cag13761@mail.uadec.mx

Table 1. Main sources of naturally occurring ellagic acid

Plant source	Ellagic acid content/(mg g^{-1})
Quersus robus and *alba*	19.0–63.0[a]
Junglans nigra	28.0[a]
Castanea sativa	89.0[a]
Fragaria ananassa	0.4–0.6[b]
Rubus idaeus	1.2–1.5[b]
Rubus occidentalis	1.5–2.0[b]
Bertholletia excelsa Humb	0.59[b]
Carya illinoinensis	0.33[b]
Punica granatum	0.78[c]

a) Stoner and Gupta, 2001; *b*) Akiyama et al., 2001; *c*) El-Toumy and Rauwald, 2003.

2000). In addition, EA has been shown to have potent anti-carcinogenic properties resulting in a reduction of human colon, prostate, cervical, tongue, breast, oesophagus and skin cancer (melanoma) (Sharma et al., 1997; Stoner & Gupta, 2001; Varadkar et al., 2001; Asamy et al., 2003; Singh et al., 2003; Carrawey et al., 2004; Huetz et al., 2005). EA treatments also resulted in cell-cycle arrest and apoptosis (natural cell death) along with the inhibition of tumour formation (Seeram et al., 2005). In some cases, EA reduced cancer by ellagitannins and ellagic acid even after symptom development. Moreover, studies have suggested that ellagic acid prevented the development of cells infected by the human papilloma virus (HPV) (Stoner & Gupta, 2001). In addition, furosin, a structurally characterised ellagitannin, was also shown to be effective in osteoclast differentiation and prevention of bone diseases (Park et al., 2004).

In addition to the observed effects on human health, EAs also exert antibacterial (Atta-Ur-Rahman et al., 2001; Machado et al., 2002; Akiyama et al., 2001) and antiparasitic activities (Elkhateeb et al., 2005). Increasing requirements on ETs in food supplement and pharmaceutical industries result in their higher demand and the need for simple isolation and characterisation methods of such molecules. In the processes of tannin, ellagitannins and EA extraction, different solvents such as water, methanol, ethanol, acetone, hexane, ethyl acetate, butanol, etc., are used in different percentage (Atta-Ur-Rahman et al., 2001; Koponen et al., 2007; Lei, 2002). Many techniques of EA purification, with different purification steps, have been developed. Specific separation columns such as the Dianion LP-20 or Sephadex LH-20 (Seeram et al., 2005; Machado et al., 2002; Asamy et al., 2003), Sep-Pak tC18 and the cartridges Bond Elut PSA (Amakura et al., 2000) allowing the separation of compounds, are commonly utilised. Nevertheless, expensive materials and equipment are required. HPLC systems are commonly used for detection and quantification. Several methods based on the reverse phase C18 column technology, with solvents like methanol, ethanol, acetonitrile, mixed with acidified water (acetic or phosphoric acid) are reported. The major difficulty remains in the retention time as the use of solvents with high polarity results in long retention times. In addition, when solvent gradients are used, retention times increase from 30 min to 60 min. Another point is the validation using statistical analysis of acceptance criteria for biotech products (Wang et al., 2007). In the present work, a fast method for ellagic acid extraction, in combination with a HPLC scheme which reduces the time of analysis altogether, is presented. Additionally, the presence of EA was determined in five different plants presenting an alternative natural source of ellagitannins and ellagic acid.

Experimental

The analysis method was validated according to the guidelines of the international conference on harmonisation of technical requirements for registration of pharmaceuticals for human use (ICH) and recommendations of Wang et al. (2007).

Ellagic acid (high purity) was purchased from ICN Biomedicals Inc, USA. Solvents and mobile phases used were of HPLC grade and were obtained from Sigma-Aldrich, USA.

For the calibration curve (reference), a standard solution of different concentrations (0 μg mL^{-1}, 5 μg mL^{-1}, 10 μg mL^{-1}, 20 μg mL^{-1}, 30 μg mL^{-1}, 40 μg mL^{-1}, and 50 μg mL^{-1}) was prepared by dissolving EA in methanol. In the validation method, Czapek-Dox modified medium was used as described below (Treviño-Cueto et al., 2007) and dilutions of this solution were made for the linear calibration curve. The calibration curve was generated using six concentrations (in tetraplicates) of the EA standard solution, inter-day (different days) and intra-day (same day). The accuracy of the method was determined by recovery of known amounts of the EA standard in hexaplicates (Bala et al., 2006).

The samples for EA determination were prepared as follows: 1350 μg of EA were added to 7 mL of Czapek-Dox minimal medium and 20 mL of citrate buffer (pH 5.0, concentration 50 mM), EA concentration 50 μg mL^{-1}, mixed in an immersion blender (RIVAL immersion blender, model IB901 MX) and then immersed in an ultrasonic bath at 40 kHz (Bransonic, Model 2510R-MTH, Branson Corp., CT, USA) in a 50 mL conical tube for 30 min. Later, aliquots were taken to prepare final EA concentration of 0 μg mL^{-1}, 10 μg mL^{-1}, 20 μg mL^{-1}, 30 μg mL^{-1}, 40 μg mL^{-1}, and 50 μg mL^{-1}, and filled to 1.5 mL with the Czapek-Dox minimal medium.

Ellagic acid extraction: An aliquot was placed in a 2 mL conical tube and centrifuged at 36000 min^{-1} for 20 min. The supernatant was decanted and the precipitate was re-suspended in 1.5 mL of ethanol. The sample was ultrasonicated again for 30 min, filtered

through a 0.45 μm nylon membrane and injected into the HPLC system.

Plant samples for EA determination were prepared as follows: *Jatropha dioica* branches (Sangre de drago, Dragon's blood), *Euphorbia antisyphyllitica* branches (Candelilla), *Turnera diffusa* Willd leaves (Damiana), *Flourensia cernua* leaves (hojasén) and *Punica granatum* husk (pomegranate) turning and red stages were used as the plant sources of EA. All plant materials were collected from semi-desert areas near Saltillo, Coahuila, Mexico, except the *Punica* fruits which were collected from Sabinas, Coahuila, Mexico. Sixty milligrams of each powdered and dried plant sample were transferred to tubes with screw caps containing 4.95 mL of methanol and 50 μL of 6 M HCl (Lei, 2002). The tubes were closed and the content was incubated for 168 h at 90 ℃. After cooling, this material was filtered through a Whatman No. 41 in a vacuum system. An aliquot of 1.5 mL of the filtered fraction was centrifuged at 36000 min^{-1} for 20 min. The supernatant was decanted and the precipitate was re-suspended in ethanol (1.5 mL). The sample was ultrasonicated for 30 min, the material was filtered through a 0.45 μm nylon membrane and injected into the HPLC system.

Chromatography: HPLC analysis was carried out using a Varian Pro-Star 330 system using a modification of the Amakura et al. (2000) method. A Varian Pro-Star 330 photodiode array (PDA) detector with detection at 254 nm was used. Fractionation of the injected material was carried out on an Optisil ODS column (5 μm, 250 × 4.6 mm) at 25 ℃. An isocratic mobile phase consisting of acetonitrile (solvent A) and water : phosphoric acid (pH 2.5, solvent B), in the ratio of 30 : 70 was applied at the flow rate of 0.7 mL min^{-1}. Sample injection volume was 10 μL, retention time was 5.1 min.

Method validation: Linearity was evaluated using linear regression analysis (Bala et al., 2006). Precision was determined by repeatability (intra-day). Repeatability was assessed analysing six sample solutions of 40 μg mL^{-1} under the same operating conditions. Accuracy was determined by the recovery of known amounts of the EA standard solution on the Czapek-Dox medium in hexaplicate.

Table 2. HPLC method validation parameters of EA calibration curve (reference)

Parameters	HPLC
Linearity range/(μg mL^{-1})	5–50
Linearity	0.9947 ± 0.0025(0.25)^a
Repeatability/%	100.2 ± 1.15^b

a) Values are the mean ± SD (RSD) of $n = 4$ calibration curves; *b*) six replicates were analysed for each sample evaluated on six consecutive days.

Table 3. Estimated intra- and inter-day precision and accuracy of EA standard curve

	Intra-day		Inter-day^a	
Concentration μg mL^{-1}	Accuracy %	RSD^b %	Accuracy %	RSD^b %
10	99.3	0.55	98.6	0.54
30	100.4	1.66	100.3	2.50
50	99.8	1.90	99.5	1.34

a) Four replicates were analysed for each sample evaluated on four consecutive days; *b*) mean RSD value of $n = 4$ calibration curves.

Table 4. Estimated inter-day accuracy and precision of samples with EA in Czapek-Dox medium

	Inter-day^a	
Concentration μg mL^{-1}	Accuracy %	RSD^b %
10	105.05	0.52
20	108.08	8.84
30	109.19	1.47
40	102.11	0.645

a) two replicates were analysed for each sample evaluated on four consecutive days; *b*) mean RSD value of $n = 6$ sample treatments.

Results and discussion

The calibration curve for EA was constructed in the concentration range of 5 μg mL^{-1} to 50 μg mL^{-1} showing excellent linearity with a correlation coefficient of 0.9947 indicating a compliance with the Beer-Lamberts law. The relative standard deviation (RSD) was used to validate the determination repeatability (Table 2). Precision and accuracy were estimated intra- and inter-day for the same calibration curve. The accuracy was found to be in the range of 99.3–100.4 % and 98.6–100.3 %, respectively, in agreement with the true and observed values (Table 3). In order to recreate a complex medium, EA solutions of increasing concentrations were added to the Czapek-Dox medium and samples were prepared for HPLC analysis. Accuracy was in the range between 102–109 % (Table 4), and the correlation coefficient obtained as the mean value of slope values average was 0.9914 ± 0.00338, showing a good linearity. Ellagic acid was extracted and quantified from novel plant sources. The data are presented in Table 5.

The data in Table 1 demonstrate that all samples are either excellent or good sources of EA. The EA content has been previously reported in samples of *Punica granatum* husk and the results obtained in this study are in agreement with the literature (Seeram et

Table 5. Quantification of ellagic acid present in different plant sources

Plant	Ellagic acid/(mg g^{-1})^{a,b}
Punica ganatum turning	33.79 ± 7.43
Punica granatum red	12.80 ± 5.83
Euphorbia antisyphyllitica	2.18 ± 0.39
Flourensia cernua	1.59 ± 0.96
Tumera diffusa Willd	0.87 ± 0.59
Jatropha dioica	0.81 ± 0.43

a) Values are the mean of $n = 3$ experiments; *b*) dry base.

al., 2005; Machado et al., 2002; El-Toumy & Rauwald, 2002, 2003). However, for all other samples tested, this is the first study reporting their EA content and demonstrating the high potential of these sources of EA. More importantly, these novel plant sources represent an alternative for the extraction of ellagitannins and EA in order to prepare products for functional foods or neutraceuticals. These results will help in the development of new bioprocesses enhancing the release and recovery of EA.

Quantification of EAs has been previously reported in the literature (see Introduction). However, all these methods are tedious, and are not reproducible when complex organic materials are analysed. Moreover, most samples analysed contain a mix of phenols that could interfere with the EA detection. The presented methodology is simple and reproducible and results in fast estimation of the EA content in diverse plant materials.

Many techniques for the purification of EA have been developed, including the use of chromatography resins such as Dianion LP-20 or Sephadex LH-20 (Seeram et al., 2005; Machado et al., 2002; Asamy et al 2003), Sep-Pak tC18 or Bond Elut PSA cartridges (Elkhateeb et al., 2005). The method reported in this study is easy, fast and cheap as compared to these methods. This method takes advantage of the insolubility of EA in water, facilitating its precipitation and recovery. The pre-treatment of the sample achieved in the present study results in the removal of compounds that could interfere with the analysis.

Acknowledgements. This study was part of a research project financed by the Mexican program Fondo Sectorial SEP-CONACYT-51360. A. Aguilera-Carbo thanks the CONACYT for the fellowship assigned and also the Universidad Autónoma Agraria Antonio Narro for integral support.

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