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QUANTIFICATION OF ELLAGIC AND GALLIC ACID FROM LEAVES OF WILD FRUIT SPECIES BY UHPLC CHROMATOGRAPHY

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ABSTRACT

A simple, rapid and accurate chromatographic method was developed for the quantification of ellagic and gallic acid using UHPLC chromatography. The separation of the compounds was carried out using a Hypersil Gold column (150x4.6) by gradient elution with a mobile phase consisting of 0.5% formic acid aqueous solution and methanol, with a detection of the compounds at 278 nm. The obtained results confirm the presence of phenolic compounds in the leaves of some wild fruit species, respectively gallic acid varied between 5.12 (Malus sylvestris) and 82.16 (Rubus fruticosus) mg/100g DW, and ellagic acid had values between 4.28 (Crataegus monogyna) and 936.34 (Fragaria viridis) mg/100g DW. The used protocol proved to be useful for the accurate and rapid identification and quantification of the two phenolic acids.

INTRODUCTION

Phenolic compounds are recognized for their beneficial properties as bioactive compounds on the human body, and their role and importance have long been researched and investigated. From the category of phenolic compounds, the class of phenolic acids, the subclass of hydroxybenzoic acids, also belonging are gallic ($C_7H_6O_5$) and ellagic ($C_{14}H_6O_8$) acids. Gallic acid (MW 170.1195) and its dimeric derivative known as ellagic acid (MW 302.1926) can exist in free form, or bound as gallotannins and ellagitannins (Soong & Barlow 2006; Shakeri et al. 2018). Both are phenolic compounds with antioxidant activity, recognized for their curative and therapeutic properties against numerous ailments (Shakeri et al. 2018; Badhani et al. 2015). According to Hurtado-Nuñez et al. (2022) gallic and ellagic acids are present in foods, herbs, teas and dietary supplements. Liquid chromatography, using the HPLC technique (and more recently UHPLC), is among the most common methods used to determine phenolic compounds. Singh et al. (2014) mention sample preparation, concentration and nature of extraction solvent, extraction time and temperature as determining factors in the extraction of phenolic compounds of interest from plant samples. Although numerous studies have demonstrated the presence of gallic and ellagic acids predominantly in fruits and vegetables,

researchers have also evaluated other plant parts of some species of interest, such as leaves of *Eucalyptus globulus* (Liu et al. 2016), *Rubus* sp. (Chou et al. 2009), *Fragaria* sp. (Buricova et al. 2011), *Elaeagnus angustifolia* (Sun et al. 2021), *Camellia sinensis* var. *assamica* (Chen et al. 2015), *Terminalia Arjuna* (Singh et al. 2016), *Eugenia punicifolia* (Silva et al. 2023). New potential sources of ellagic acid and gallic acid can be further investigated, and this work aimed, in addition to establishing a fast and accurate method for the identification of compounds through the UHPLC chromatography, the quantification of these chemical compounds from leaves of some wild fruit species.

MATERIAL AND METHODS

Biological material

The plant material was harvested from a forest ecosystem, Bratovoeşti forest (44°05'N 23°54'E), located in the South-West part of Romania, Oltenia region, Dolj county. Leaves were collected during the BBCH 65 phenological stage, from several individuals within the species *Crataegus monogyna* (L.) Jacq., *Crataegus pentagyna* (L.) Waldst. et Kit., *Malus sylvestris* (L.) Mill., *Prunus spinosa* (L.), *Rosa canina* (L.), *Rubus caesius* (L.), *Rubus fruticosus* (L.) and *Fragaria viridis* (L.) Weston.

Sample preparation

The plant material was transported to the laboratory and dried in a dehydrator (Biovita DEH600D) at a temperature of 45°C to a constant weight. After grinding to a fine powder, the samples were stored in a refrigerator at a temperature of 4°C (±2°C). Sample preparation was performed according to the method described by Soong and Barlow (2006) with some modifications as follows: 1 g of dry sample was weighed with a Radwag AS 220 RD high-precision analytical balance in centrifuge tubes, over which 25 mL MeOH/H_2O (v/v) solution was added. The samples were vortexed for 3 min and ultrasonicated (Fungilab ultrasonic bath) for 60 min at a temperature maintained at 70°C, followed by centrifugation (Eppendorf Centrifuge 5430 R) for 15 min at 6000 rpm. The supernatant was filtered with Whatman filter paper no. 4 followed by 0.45 µm syringe microfilter in the vial.

UHPLC analysis

The analysis of phenolic compounds (ellagic and gallic acid) was performed on an ultra-high performance liquid chromatograph (Ultimate 3000 XRS Liquid Chromatograph, Thermo Scientific) combined with a Dionex Ultimate 3000 XRS autosampler, an XRS pump and a UV-VIS RS Diode. All substances were purchased from Sigma Aldrich. The mobile phase consisted of 0.5% formic acid aqueous solution (B) and MeOH (C). Samples were eluted with the following gradient: 85% B and 15% C from min 0-12; 50% B and 50% C from min 15–27; 85% B and 15% C from min 30–35 to restore initial conditions. The flow rate was 0.3 mL/min and the injection volume was 5 µL. The column used was Hypersil Gold (150x4.6) at a temperature maintained at 25˚C. The detection of phenolic compounds was performed by UV absorption at λ=278 nm. Each compound was identified based on its retention time and by comparison with standards under the same conditions.

Statistical analysis

The data obtained were interpreted using the Thermo Scientific Chromeleon Chromatography Data System (CDS) software and processed in the IBM SPSS

Statistics 26 statistical program. The results represent the mean and standard deviation of 3 consecutive determinations and are expressed in mg/100g DW.

RESULTS AND DISCUSSIONS

A UHPLC coupled to an RS Diode UV-VIS detector was used for the separation, identification and quantification of the two compounds of interest, ellagic and gallic acid from the leaves of some wild fruit species. Figure 1 shows the chromatogram of green strawberry leaves, highlighting compounds, absorbance and retention times. The obtained results can be found in table 1. Following the statistical processing of the obtained data (one-way ANOVA), significant differences were found between the analysed samples ($p < 0.05$), highlighted by the results of the Duncan's multiple range test. The highest amount of gallic acid was identified in wild blackberry (*Rubus fruticosus*) leaves, and the highest amounts of ellagic acid in green strawberry (*Fragaria viridis*) and blackthorn (*Prunus spinosa*) leaves. The obtained results are consistent with those mentioned by other researchers regarding the leaves of some species of *Fragaria* sp., *Rubus* sp., *Prunus spinosa*, as being rich in ellagic acid (Raudonis et al. 2013; Gudej & Tomczyk 2004; Muthukumaran et al. 2017; Stoenescu et al. 2022; Temiz & Okumuş 2022; Oszmiański et al. 2015).

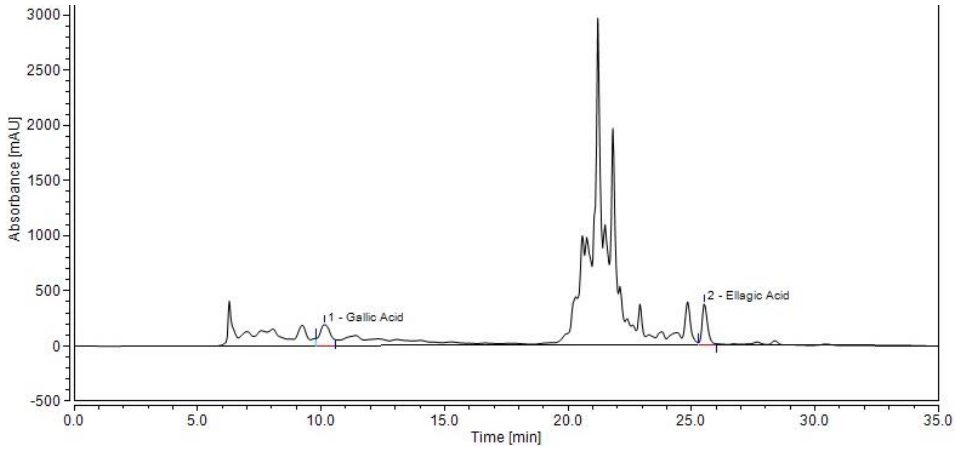

Figure 1. UHPLC chromatogram of green strawberry (*Fragaria viridis*)

The order of the presence of gallic acid (Table 1) in the performed analysis was: *Rubus fruticosus* < *Fragaria viridis* < *Rosa canina* < *Rubus caesius* < *Crataegus monogyna* < *Prunus spinosa* < *Crataegus pentagyna* < *Malus sylvestris*. Regarding the distribution of ellagic acid in the leaves of the analysed species, it was identified in the following order: *Fragaria viridis* < *Prunus spinosa* < *Malus sylvestris* < *Rubus caesius* < *Rubus fruticosus* < *Rosa canina* < *Crategus pentagyna* < *Crataegus monogyna*. The obtained results depend on genotype, location, phenological stage of the leaves, drying temperature, extraction procedure, and chromatographic conditions. Oszmiański et al. (2015) mention in their study regarding the antioxidant potential of some *Rubus* sp., ellagitannins as being the largest group of phenolic compounds found in the analysed leaves (51.59–255.01 mg/g DW). Many researchers mention the role and importance of wild fruit species leaves as a good source of antioxidants among other properties. European crab apple leaves are used

as decoction or teas in the treatment of various dermatological illnesses; blackberry leaves can be used for the treatment of diarrhoea, respiratory and genital diseases (Šavikin et al. 2013), for treating constipation; green strawberry leaves are used in treating gout, rheumatism, liver failure, whereas the aqueous leaf extract can be a direct vasodilator (Mudnic et al. 2009); dog rose leaves can be used as infusions for the treatment of kidney diseases, biliary colic, eczema (Kültür 2007), improving blood pressure (Güler et al. 2015); hawthorn leaves are well known for their treatment in cardiovascular diseases, regulating blood pressure; blackthorn leaves are used as laxative (Negrean et al. 2023) and together with flowers alleviates oxidative stress in diabetic condition (Temiz et al. 2021).

Table 1

Gallic and ellagic acid content (mg/100g DW) in the leaves of the studied species

Species	Gallic acid	Ellagic acid
Malus sylvestris (L.) Mill.	5.12±0.94^{d}	687.15±13.06^{c}
Rosa canina L.	34.89±0.23^{c}	150.74±4.02^{e}
Rubus caesius L.	29.49±0.12^{c}	377.56±0.57^{d}
Rubus fruticosus L.	82.16±14.60^{a}	356.89±30.73^{d}
Fragaria viridis (L.) Weston	44.88±0.08^{b}	936.34±3.97^{a}
Prunus spinosa L.	7.48±0.06^{d}	878.94±17.97^{b}
Crataegus monogyna (L.) Jacq.	11.88±1.36^{d}	4.28±1.07^{g}
Crataegus pentagyna L. (L.) Waldst. et Kit.	7.33±1.05^{d}	69.20±1.93^{f}

Mean±Standard Deviation; Different letters indicate statistically significant differences (Duncan's multiple range test, $p < 0.05$)

Among the various techniques for the identification of polyphenols, HPLC and more recently UHPLC are among the most important and widespread methods for the separation and quantification of phenolic compounds (Weber & Passon 2019). A first decisive role in the extraction of the analytes of interest is the extraction solvent. Singh et al. (2014) mention as extraction solvents of ellagic acid water, ethanol, acetone, ether and their combinations, the best results being obtained by using ethanol+ether+water. On the other hand, a ratio of 50:50 methanol:dimethylformamide had the best results in terms of ellagic acid extraction in the study by Williams et al. (2016). Vekiari et al. (2008) mention methanol as a good extraction solvent for ellagic acid, while Soong and Barlow (2006) use ethanol (50%) in their study as a much better extraction method compared to methanol (50% in the same study). Also, in the case of gallic acid, Baite et al. (2016) mention 50% methanol as a very good extraction solvent for this compound compared to 50% ethanol or alkaline water (the amount of gallic acid being double in methanol compared to ethanol). Dhanani et al. (2015) use hydromethanolic extracts (3:7, water:methanol) in fruit and bark of *Terminalia* species as a method for extracting some phenolic compounds including gallic and ellagic acid. According to Silva et al. (2023) phenolic compounds have more affinity with organic solvents such as methanol, ethanol, and aqueous solutions with acetone.

Another important aspect in the correct identification of the phenols is the use of mobile phase additives that improve the separation and resolution of bioactive compounds on the column (Li et al. 2010). In this matter, the specialized literature provides sufficient information regarding the substances used in the mobile phase. The mobile phase in liquid chromatography is a liquid passed through a chromatographic column in which the components of the mixture are separated at

different rates by adsorption to the stationary phase. Among the used solvents (in different concentrations) most frequently for the identification of ellagic acid according to the specialized literature are: water with methanol (Gudej & Tomczyk 2004, Stoenescu et al. 2022, Donno et al. 2023, Soong & Barlow 2006, Huerga-González et al. 2015), water with acetonitrile (Häkkinen 2000, Assunção et al. 2017, Lee & Kim 2018, Medic et al. 2021), acetonitrile with ethanol (Ratnam et al. 2006), acetonitrile/methanol/water (Silva et al. 2023).

Ionizable compounds such as acids or bases cause significant changes in retention and selectivity factors with changes in pH (agilent.com). In most cases used as an acidifying agent are: formic acid (De Andrade Neves et al. 2018, Sun et al. 2014, Chen et al. 2019, Silva et al. 2023), acetic acid (Soong & Barlow 2006, Huerga-González et al. 2015, Papoutsi et al. 2008, Stoenescu et al. 2022), trichloroacetic acid (Assunção et al. 2017), phosphoric acid (Nowak 2006), potassium dihydrogen orthophosphate (Bala et al. 2006, Ratnam et al. 2006, Vekiari et al. 2008), orthophosphoric acid (Gudej & Tomczyk 2004).

Regarding the chromatographic used columns, C18 (ODs or reverse phase columns) have been noted in the literature as being among the most used for the determination of ellagic acid (Daniel et al. 1989, Nuncio-Jáuregui et al. 2015, Donno et al. 2023, Silva et al. 2023). These are HPLC columns that use a C18 substance as the stationary phase and are used in environmental sciences and chemical determinations to analyse individual parts of their mixtures. C18 means that the molecules contain 18 carbon atoms, so the other atoms in the molecule can vary, resulting in significantly different substances (labcompare.com).

CONCLUSIONS

The method used and described is simple, precise, and accurate, allowing the analysis of compounds of interest unambiguously based on retention times compared to standard solutions. The leaves of the analysed species confirm the presence of gallic acid and in a larger amount of ellagic acid, compounds with intense antioxidant activity. The data obtained can be used in the pharmaceutical, therapeutic industry in nutraceutical products or functional foods beneficial to the human body.

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