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Comparison of different climatic seasons of Brazil in the value of phenolic compounds, oleuropein, and antioxidant capacity in olive leaves of three cultivars

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ABSTRACT

This study evaluated the effects of different Olive leaves cultivars harvested in the different climatic seasons on the total polyphenolic content (TPC), oleuropein content and antioxidant capacity. Arbosana presented higher TPC in summer and Koroneiki and Arbequina in spring. Oleuropein content presented a similar profile. DPPH had its best values for Arbosana and Koroneiki in the spring, followed by Arbequina in the summer. FRAP had the highest values in summer for all cultivars, with Arbosana presenting the higher value. ORAC had the highest values in winter for all cultivars, with Koroneiki presenting the higher value. Autumn had the worst results considering all the analysis. DPPH was the method more indicated to predict the antioxidant capacity of oleuropein according to statistical correlation. The results highlighted that the different harvest periods for Brazilian production and the different cultivars strongly influenced TPC, oleuropein content and the antioxidant capacity of olive leaf extracts.

PALAVRAS-CHAVE: olive leaves extract; Arbosana; Koroneiki; Arbequina; climatic conditions

INTRODUCTION

The olive trees (*Olea europaea L.*) are one of the oldest known plants cultivated worldwide. Native from Mediterranean countries, olive trees have been cultivated in Brazil, mainly in the south and southeast region, due to favorable climatic conditions for their development (Cavalheiro et al. 2015). The olive trees have better-growing conditions in temperate regions where annual temperature differences are high (Talhaoui et al. 2015; Özcan and Matthäus 2017). In these regions, the middle temperatures in the warm season are around 24°C (Weather and Climate 2022). The annual temperature differences in Brazil also has a high variation. However, the middle temperatures in the warm season are higher when compared with the European, around 30°C (Weather and Climate 2022). Despite this, the southeast region of Brazil presents areas with a microclimate that can provide necessary hours of cold for the flowering of olive trees (Teramoto et al. 2010). However, the effects of high temperatures on developing the compounds present in the Brazilian olive leaves have not yet been reported in the literature.

The main products of olive trees are olive oil and olive fruit, but their leaves, considered a waste of the olive industry, have been widely studied because of their compounds and health benefits (Espeso et al. 2021). The leaves grow in large quantities due to the higher vegetative power of the olive tree in tropical and soil conditions. For this reason, pruning and mechanical harvesting generate large amounts of waste that are renewable sources of value-added products with great healthy potential (Contreras et al. 2020). Just like olive oil and olive fruits have many important properties for human health, olive leaves have been used historically as a treatment for fevers and diseases such as stomach and intestinal diseases, malaria, and urinary tract infections (Özcan and Matthäus 2017). Recent studies also have shown that olive leaves have potential effects on glycemia and lipidemia (Acar-Tek and Ağagündüz 2020), anticancer effect (Boss et al. 2016), anti-inflammatory effect (Qabaha et al. 2018), antimicrobial and antioxidant activity (Borjan et al. 2020), besides their cosmetic activity (Rodrigues et al. 2015).

All these health effects are attributed to the phenolic compounds, a group of secondary metabolites with strong antioxidant activity that acts as a defense mechanism against microorganisms, external injuries, and UV radiation (Žugčić et al. 2019). These compounds accumulate mainly in fruits and leaves, especially during their growth and the first maturation phase (Ranalli et al. 2006). The most abundant phenolic compound found in olive leaves is oleuropein, a secoiridoid whose concentration is significantly higher in leaves than in fruits or oil (Žugčić et al. 2019; Otero et al. 2020). However, the presence and content of phenolic compounds in olive leaves depend on factors that can change plant characteristics (Ranalli et al. 2006; Pasković et al. 2020). These factors are named abiotic when environmental chemical and physical changes affect the plant characteristics (such as sampling time, climate conditions, soil apport, hydric deficiency, geographical zone, light exposition, others), and biotic when any living component or its metabolism affects the plant characteristics (such as fungi, bacteria, genotypes, biological cycle, others) (Talhaoui et al. 2015). Furthermore, many studies pointed out that phenolic compounds (mainly oleuropein) content in olive leaves can change according to cultivar and climatic season (Brahmi et al. 2013; Talhaoui et al. 2014; Ghasemi et al. 2018; Wang et al. 2019; Antunes et al. 2020). Thus, this study aimed to analyze the amount of phenolic compounds and antioxidant activity of olive leaves of three different cultivars according to the seasons of the

year in the southern hemisphere. The olive cultivar with the highest activity and the best leaves the drying and extraction condition were determined.

MATERIAL AND METHODS

MATERIALS

Ethanol (HPLC grade), methanol (HPLC grade), formic acid (HPLC grade), acetonitrile (HPLC grade), TPTZ (2,4,6 - Tris (2-pyridyl) - s - triazine); DPPH (2, 2 - diphenyl - 1 - picrylhydrazyl); TROLOX (6 - hydroxy - 2, 5, 7, 8 - tetramethylchromane - 2 - carboxylic acid); AAPH (2 ,2' - azobis (2-methylpropionamidine) dihydrochloride) and oleuropein were all purchased from Sigma-Aldrich (São Paulo, Brazil). Fluorescein sodium salt was purchased from Vetec Química Fina (São Paulo, Brazil). Folin-Ciocalteu phenol reagent was purchased from Dinâmica (Brazil). Ultrapure water (resistivity 18.2 MΩ cm−1 at 25 ◦C) was obtained with a Millipore OPak 2 (Millipore Corporation, Bedford, MA, USA). The other reagents used were of analytical grade.

OLIVE LEAVES

Three olive leaf cultivars (*Olea europea*, var. Arbosana, Koroneiki, and Arbequina) cultivated in Delfim Moreira city (Minas Gerais, Brazil) were analyzed. Approximately 1 kg of leaves of each cultivar were randomly collected in the last fortnight of the four climatic seasons corresponding to the southern hemisphere in 2016 (Summer - March 16, Autumn - June 02, Winter - September 20, and Spring - December 5). The olive leaves were promptly dried and stored at − 20 °C until used.

PRELIMINARY TESTS OF EXTRACTION AND DRYING

Preliminary tests with the Arbequina cultivar determined the best extraction and drying conditions. First, based on the study of Santos, Simão, Marques, Sackz and Corrêa (2016), extraction tests were performed with ethanol and methanol at different concentrations, namely: 20%, 40%, 50%, 60%, 70%, 80% and 100% (in ultrapure water, v/v). The best concentration of each solvent was verified by making extracts with fresh olive leaves of the Arbequina cultivar (0.5 g ground in liquid nitrogen according to section 2.4) and obtaining their absorbance values using the Total Polyphenolic Content - TPC. The best concentrations of solvents, 50% ethanol and 80% methanol, were used to make extracts to verify the best drying conditions of the Arbequina cultivar olive leaves. Then, the leaves of the Arbequina cultivar (300 g) were dried to natural, to the sun and an oven at 35 °C until constant weight. Then, they were transformed into fine powder by an electrical mill (Marconi, MA 630/1, Piracicaba, SP, Brazil). The material was passed through a sieve (20 mesh) to standardize the particle size. The best olive leaves drying method was verified by making extracts with the best concentrations of ethanol and methanol and obtaining their TPC. Extracts made with fresh leaves were used for comparison. Finally, leaves dried in an oven at 35 °C investigated the effects of solvent (ethanol 50% and methanol 80%), climatic seasons (summer, autumn, winter, and spring), and cultivars (Arbosana, Koroneiki, and Arbequina) on the TPC of the olive leaves using a full factorial experimental design (2 x 4 x 3).

Forty-eight assays were randomly obtained in duplicate. Then, the best solvent was identified to conduct the other analyses.

EXTRACTION

Dried olive leaves (0.2 g) were placed in a 50 mL Falcon tube with the addition of 0.36% disodium EDTA (ultrapure water, w/v) to prevent the degradation of phenolic compounds during extraction. The extraction solution was added to make up 10 mL. The tubes were shaken for one hour in an ultrasonic bath (UNIQUE, USC 1450, Brazil), followed by centrifugation (HETTICH, Routine 380R, Germany) at 9000 rpm for 10 min at 4 ºC. The supernatant was collected, and 10 mL of the extraction solution was added to the residue, which was again submitted to a second extraction. The procedure was repeated until the third extraction, remaining for 30 min in an ultrasound bath at 40 °C. The bath time was reduced to 15 min in the fourth and fifth extractions. Thus, the final volume of the combined extracts was 50 mL. The olive leaf extracts were stored at − 80 °C until used.

TOTAL POLYPHENOLIC CONTENT - TPC

The TPC measured by reducer capacity was determined by the Folin–Ciocalteu method adapted from Singleton, Orthofer and Lamuela-Raventós (1999). The extract or standard solution (300 µL) was added into a Falcon tube (15 mL) with 700 µL of ultrapure water, and 500 µL of Folin–Ciocalteu reagent. The tube was vortexed for 30 s. After 8 min in a dark box, 2.5 mL of 20% Na_2CO_3 solution was added, the volume was adjusted to 10 mL with ultrapure water, and the tube was vortexed for 30 s again. After 40 min in a dark box, the solution was added into a glass cuvette, and the absorbance was measured at 765 nm. A spectrophotometer UV/VIS Hitachi U2000 (Tokyo, Japan) was used for the analysis. By linear regression, results were expressed as Caffeic Acid Equivalent (mg CAE/g of dried extract) using an 8-points standard curve (0.0023 – 0.072 mg CA/mL). The measurements were taken in triplicate.

HPLC - DAD

Separation and identification of oleuropein in olive leaves extracts were monitored by High-Performance Liquid Chromatography (HPLC) using an Agilent apparatus (model 1260 Infinity Quaternary LC) equipped with a quaternary pump (1260 Quat Pump, G1311B), automatic injector (1260 ALS, G1329B) and diode array detector (DAD) coupled an integration system ChemStation®. Separation was carried out using a Zorbax C18 column (250 nm x 4.6 mm, 5 mm) preceded by a guard column with the same stationary phase at 35 ºC. Data acquisitions were performed with Agilent OpenLAB CDS software version A.01.04 (Agilent, Germany). Extracts and standard were injected (20 µL) directly into the HPLC column. A binary solvent system was used with solvent A (0.1% formic acid in ultra-pure water, v/v) and solvent B (0.1% formic acid and 40% acetonitrile in ultra-pure water, v/v) at a flow rate of 0.7 mL/min. The elution gradient adopted was: 0-15 min 0-20% of B; 15-70 min 20-70% of B; 70-85 min 70-100% of B; 85-110 min 100% B. Diode array detection occured under the following conditions: scan 200–600

nm, scan rate 1 Hz, and bandwidth 5 nm. Chromatograms were recorded at 280 nm, and acquired with a 10 Hz rate and 11 nm bandwidth (Silva et al. 2006).

Oleuropein was identified by comparison of retention times and absorption spectra and quantified with its standard. A 6-point standard curve with oleuropein (0.016 - 0.8 µg/mL) by linear regression quantified the content, expressed in mg of oleuropein/g of dried extract. The standard was prepared in 50% ethanol/water with 0.36% disodium EDTA (ultrapure water, w/v).

ANTIOXIDANT CAPACITY CHARACTERIZATION

DPPH radical scavenging activity

The DPPH was determined according to the methodology described by Brand-Williams, Cuvelier and Berset (1995) . The extracts (400 µL) were mixed with DPPH solution (3600 µL - 0.004% DPPH in ethanol absolute, w/v), and the solutions were left in the dark for 30 min at room temperature. The absorbance was measured at 517 nm using a spectrophotometer UV/VIS Hitachi U2000 (Tokyo, Japan). Trolox was used as a positive control and ethanol absolute was used as the control. Different concentrations of the extracts were evaluated (1.096, 0.822, 0.548, 0.274, 0.137 and 0.066 mg/mL). Equation 1 describes and the free radical scavenging activity (DPPH) (%).

$$\%DPPH = \frac{(Absorbance\ of\ control - absorbance\ of\ the\ sample)}{absorbance\ of\ control} \times 100 \qquad \text{(Equation 1)}$$

Results were expressed as IC50 values (mg/mL), referred to as the lower concentration of sample required to reach 50% of the antioxidant activity, and were calculated from the graph-plotted %DPPH against extract concentrations. The measurements were taken in triplicate.

Ferric reducing antioxidant power - FRAP

The FRAP was determined according to the methodology described by Benzie and Strain (1996) adapted to downscaling to optimize reagents and solvents. The reaction was prepared directly into transparent 96-well microplates pipetting 7.5 µL of extract, standard curve solutions or standard solution (ethanol), 22.5µl of ultrapure water, and 225µl of FRAP reagent (0.3M acetate buffer, with 10 mM TPTZ in 40 mM HCl and 33.3 mM $FeCl_3$ in equal parts). After 30 min at 37 °C in a dark oven, the microplates were allowed to cool to room temperature, and the absorbance was measured at 595 nm. A BioTek Synergy HT microplate reader (Winooski, USA) coupled with the Gen5™ 2.0 data software program was used for the colorimetric analysis. By linear regression, results were expressed as Trolox Equivalent (µmol TE/g of dried extract) using a 6-points standard curve (25 - 600 µMol Trolox). The measurements were taken in triplicate.

Oxygen radical absorbance capacity - ORAC

The ORAC was determined according to the methodology described by Ou et al. (2013), adapted to downscaling (to analyze on microplate) to optimize

reagents and solvents. The reaction was prepared directly into black 96-well microplates pipetting 22 µL of extract, standard curve solutions, or standard solution (75 mM sodium phosphate buffer - pH 7.4) and 150 µL of 13.57 µM sodium phosphate buffer in 75 mM sodium phosphate buffer - pH 7.4. After incubation at 37°C for 10 min in a dark oven, 25 µL of 0.15 M AAPH solution in 75 mM sodium phosphate buffer - pH 7.4 was pipetted. The absorbance was measured with fluorescent filters at 485 nm for excitation wavelength and 520 nm for emission wavelength in the kinetics of 81 min, observing the decay of fluorescence. A BioTek Synergy HT microplate reader (Winooski, USA) coupled with the Gen5™ 2.0 data software program was used for the fluorimetric analysis. By linear regression, results were expressed in Trolox Equivalent (µmol TE/g of dried extracts) using a 6-points standard curve (5 - 125 µM Trolox). The measurements were taken in triplicate.

EXPERIMENTAL DESIGN AND STATISTICAL ANALYSES

To verify the effects of solvent (ethanol and methanol), climatic seasons (summer, autumn, winter, and spring), and cultivars (Arbosana, Koroneiki, and Arbequina) on the total polyphenolic content of the olive leaves, the experiments were statistically designed and performed according to a full factorial experimental design (2 x 4 x 3) that was completely randomized and conducted in duplicate. An analysis of variance (one-way ANOVA) determined the differences between mean values using a general linear model in MINITAB 16® Statistical Software (Minitab Inc., State College, PA, USA). Multiple comparisons of mean values were performed by using Tukey's test with a 5% significance (p-value < 0.05 with 95% confidence level).

Analyses of variance compared the other data using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) software. Tukey's test of means examined differences in the mean values at a significance level of 5% (p-value < 0.05 with 95% confidence level). Pearson's correlation between the oleuropein content and the antioxidant capacity results of each method predicted the most suitable method to determine the antioxidant capacity of oleuropein.

RESULTS AND DISCUSSION

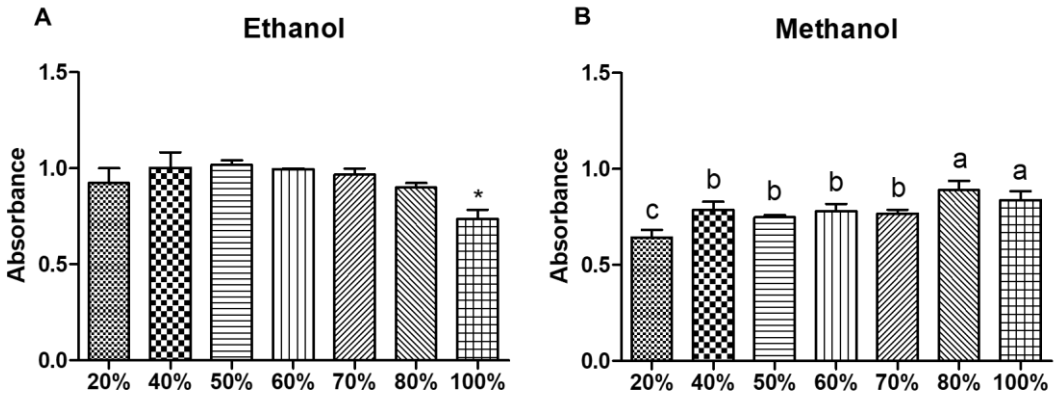
PRELIMINARY TESTS

Solvent

The solvent is one of the most important factors in obtaining a more effective and efficient extraction process. The mixture of solvents may be more efficient for extracting phenolic compounds than mono-solvent systems due to its ability to reach the polarity of diverse phenolic compound classes and improve their solubility (Azmir et al. 2013; Delgado-Povedano and Luque de Castro 2017). As a result, the highest absorbance of the ethanolic extracts occurred at a concentration of 50%. However, there was no significant difference between concentrations (except at 100%). For methanolic extracts, the highest absorbance occurred at a concentration of 80%. Likewise, there was no significant difference compared to the concentration of 100%. The concentrations were within a range well established in the literature as optimal for the extraction of the phenolic

compounds with both solvents in plant materials (Li et al. 2005; Dahmoune et al. 2014; Kaderides et al. 2019; Pinela et al. 2019; Vezza et al. 2019; Tarone et al. 2021; Zimare et al. 2021). Figure 1 show the absorbances of olive leaf extracts made with ethanol and methanol in different concentrations measured according to the TPC method.

Figure 1. Absorbances of the fresh olive leaf extracts of Arbequina cultivar made with ethanol (A) and methanol (B) in different concentrations measured according to the Total Polyphenolic Content method.



NOTE: Results are expressed as mean ± standard deviation. Different letters and * in the columns indicate a statistical difference ($p < 0.05$) between the samples.

Drying conditions

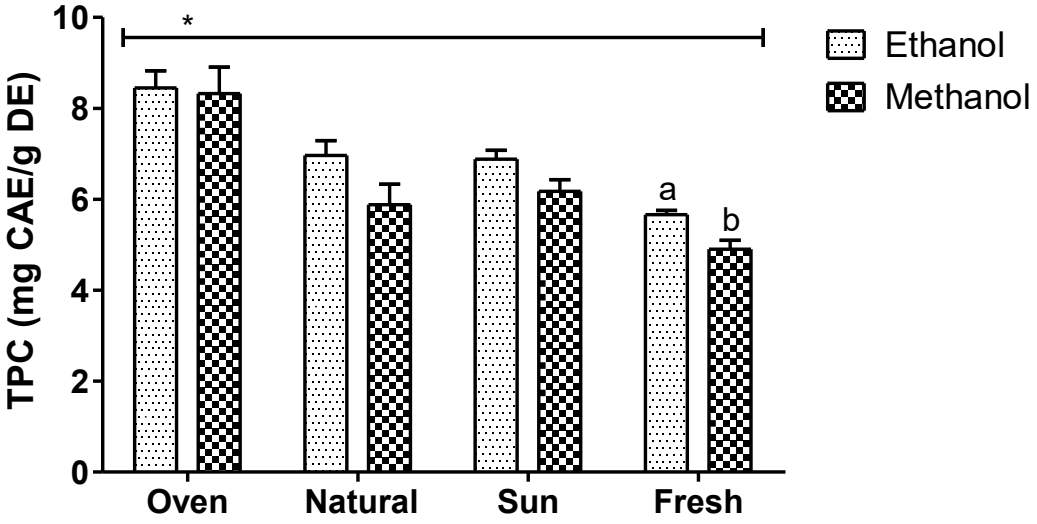
The extracts made with leaves dried in an oven presented the highest TPC. Ahmad-Qasem et al. (2014) found the highest TPC in extracts made with olive leaves dried in an oven when compared with the freeze-dryer method. Romani, Mulas and Heimler (2017) also found the highest TPC in extracts made with olive leaves dried in an oven compared to freeze-dryer and natural methods, but not when compared to fresh leaves due to the longtime of their drying processes. Only extracts made with fresh olive leaves presented a statistical difference ($P < 0.05$) between the solvents used. For this reason, it was decided to dry the olive leaves in an oven and investigate better the effect of solvents (ethanol and methanol) on TPC. Figure 2 exhibits the TPC of extracts of both solvents made with fresh and dried leaves.

Effect of solvent, climatic seasons, and cultivars on the TPC of the olive leaf extracts

The effects of solvent (ethanol and methanol), climatic seasons (summer, autumn, winter, and spring), and cultivars (Arbosana, Koroneiki, and Arbequina) on the olive leaf extracts were evaluated according to the TPC. The TPC was evaluated as a linear effect and as an interaction between solvent (X1), climatic seasons (X2), and cultivars (X3) function. The analysis of variance (ANOVA) indicated that the contributions of solvent, both linear and interaction with the other parameters, were not significant, as shown in Table 1S (Supplementary Material). The significance of the individual p- values, F-ratios, and R^2 are also presented in Table S1 (Supplementary Material). The model showed high values for R^2 (0.97), which indicates that the model could adequately represent the effects of solvent, climatic seasons, and cultivars on the olive leaf extract TPC. Figure 3 displays the linear effects of solvent, climatic

seasons, and cultivars on the TPC of the olive leaf extracts and the effects of interaction between solvent, climatic seasons and cultivars on the TPC of the olive leaf extracts.

Figure 2. TPC of ethanol and methanol extracts made with fresh and dried olive leaves natural, to the sun, and in an oven.



NOTE: Results are expressed as mean ± standard deviation. Different letters in the column and * in the groups indicate a statistical difference ($p < 0.05$) between the samples. Columns in the groups with no letters did not indicate a statistical difference ($p < 0.05$). CAE = caffeic acid equivalent. DE = dried extract.

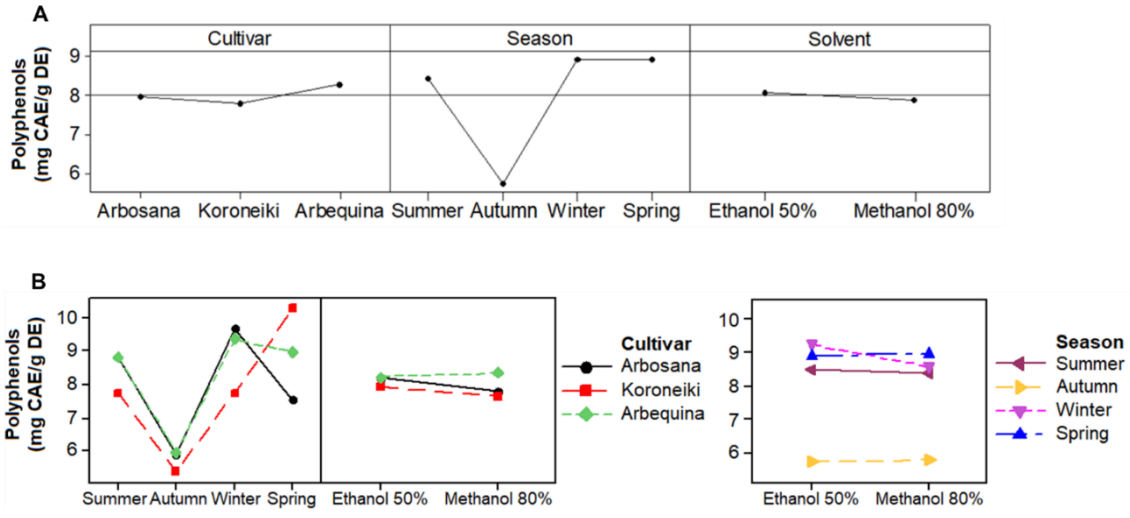


Figure 3. Main effects of solvent, climatic seasons, and cultivars (A) and interaction effects between solvent, climatic seasons, and cultivars (B) on the TPC of the olive leaf extracts.
NOTE: CAE = caffeic acid equivalent. DE = dried extract.

Through the seasons, Arbequina was the cultivar with higher TPC, and Koroneiki was the cultivar with the lowest TPC. Arbequina presented the highest

values of TPC in summer (9.03 mg CAE/g DE) and winter (10.03 mg CAE/g DE) and the second highest value in spring (8.90 mg CAE/g DE), with the values for these seasons being statistically equal. Arbosana presented similar values to Arbequina in summer and winter (8.64 and 9.32 mg CAE/g DE, respectively) but significantly lower in spring (7.72 mg CAE/g DE). Koroneiki presented lower values in summer and winter (7.84 and 8.40 mg CAE/g DE, respectively) than Arbequina and Arbosana but significantly higher in spring (10.12 mg CAE/g DE). Autumn had the worst result for all cultivars (5.97, 5.90, and 5.30 mg CAE/g DE, respectively), which presented the same behavior in summer, autumn, and winter and a significant variation in spring according to the cultivar, as displayed above (Figure 3). Cultivars and climatic seasons had a relevant role in the TPC of olive leaf extracts. The solvents did not present a significant statistical difference ($p < 0.05$). As the TPC results were (statistically) equal no matter the solvent, the other analyzes used 50% ethanol in the preparation of extracts. Ethanol was chosen because it is health safer than methanol (Azmir et al. 2013).

According to Talhaoui et al. (2015), the most important factors that influence phenolic compounds production of olive leaves are, for example, sampling time, climate conditions, biological cycles of the olive trees, and their genotypes/different cultivars. Lower values of TPC in the autumn season were found in literature compared with the other seasons in several cultivars, supporting the results reported in this study (Brahmi et al. 2013; Kabbash et al. 2019; Pasković et al. 2020; Sueishi and Nii 2020). The TPC in olive leaves is closely related to the biological cycle of the olive tree (Talhaoui et al. 2015). The polyphenols accumulate in the leaves during their growth and reach higher values in their terminal phase. During autumn there is a higher rate of degradation of phenolic compounds due to leaf senescence. Then, the duration of the leaves renovation period depends on the cultivar, which influences TCP (Ryan et al. 2003; Ranalli et al. 2006; Brahmi et al. 2013). Some cultivars can take the growth terminal phase of leaf later than others due to genetic factors (Ranalli et al. 2006), which can explain the high TPC variation in spring. Pasković et al. (2020) suggest that stress caused by the high air temperature variation can modulate the TPC. The authors found higher values of TPC in leaves harvested in March for some studied cultivars than in leaves harvested in January or October (northern hemisphere), which can explain the high TPC found in summer and winter. Several studies show that different cultivars can have a different production of phenolic compounds, promoting a TPC variation among them no mattering the harvest period (Ranalli et al. 2006; Nicolì et al. 2019; Lama-Muñoz et al. 2020; Lorini et al. 2021), which can explain the different TPC values between the cultivars studied in the same season.

OLEUROPEIN

The olive leaf extracts quantified the oleuropein, as displayed in Figure 4. Figures S1, S2, and S3 exhibits the chromatograms (Supplementary Material).

The oleuropein content demonstrated a similar profile to TPC for all cultivars, with a slight variation. It was expected since oleuropein is the main phenolic compound found in olive leaves (Žugčić et al. 2019). The Arbosana cultivar had the higher oleuropein content in summer (16.47 mg/g DE) followed by winter (12.91 mg/g DE). Koroneiki and Arbequina cultivars had the higher oleuropein content in spring (12.22 and 13.98 mg/g DE respectively), followed by summer (4.40 and

11.64 mg/g DE respectively), corroborating the TPC values. The autumn presented the lowest values of oleuropein content (0.92, 0.89, and 1.26 mg/g DE respectively), as in TPC results.

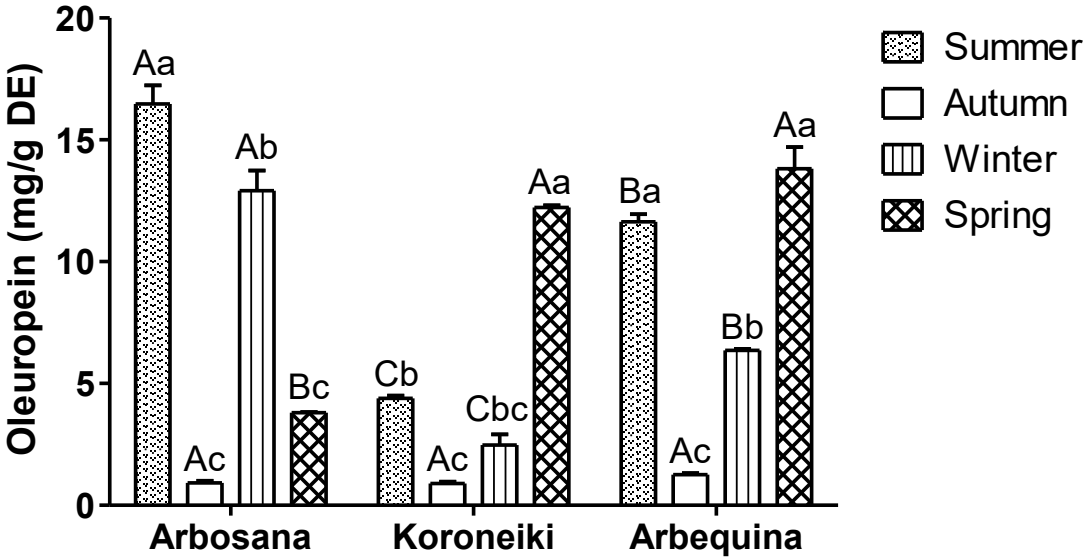


Figure 4. Oleuropein quantification of olive leaf extracts of three different cultivars harvested in the four different climatic seasons of the year.
NOTE: Results are expressed as mean ± standard deviation. Different letters in the columns indicate a statistical difference ($p < 0.05$) between the samples. Capital letters indicate statistical difference ($p < 0.05$) between the cultivars for the same climatic season, and small letters indicate statistical difference ($p < 0.05$) between the different climatic seasons in the same cultivar. DE = dried extract.

As discussed above, abiotic and biotic factors such as climate conditions, biological cycle, and genotypes/ different cultivars affect the phenolic compounds formation (Ranalli et al. 2006; Talhaoui et al. 2015). The TPC and oleuropein content varied depending on the biological cycle of the different cultivars and their sensibility to large temperature variations along the seasons. Arbequina and Koroneiki are cultivars more resistant to adverse weather conditions, being higher oleuropein content at the end of spring when the leaves are in the growth terminal phase or nearby this (Talhaoui et al. 2014; Kabbash et al. 2019). Already Arbosana cultivar is less resistant to adverse weather conditions than Arbequina and Koroneiki (Falcão 2019), being higher oleuropein content in summer and winter when the production of the phenolic compound is improved to protect against oxidative damage induced by adverse weather conditions (Talhaoui et al. 2015; Pasković et al. 2020). Kabbash et al. (2019) also found higher oleuropein content in spring than autumn in the Koroneiki cultivar. Studying other cultivars, Martínez-Navarro et al. (2021) observed higher oleuropein content in spring for the Manzanilla cultivar. The authors reported the higher oleuropein content in winter and summer for the Cornicabra cultivar, with autumn values the lowest for both cultivars, indicating the significant influence of the weather parameters in the different cultivars.

ANTIOXIDANT CAPACITY

The antioxidant capacity was measured in the olive leaf extracts by DPPH, FRAP, and ORAC, as exhibited in Figure 5.

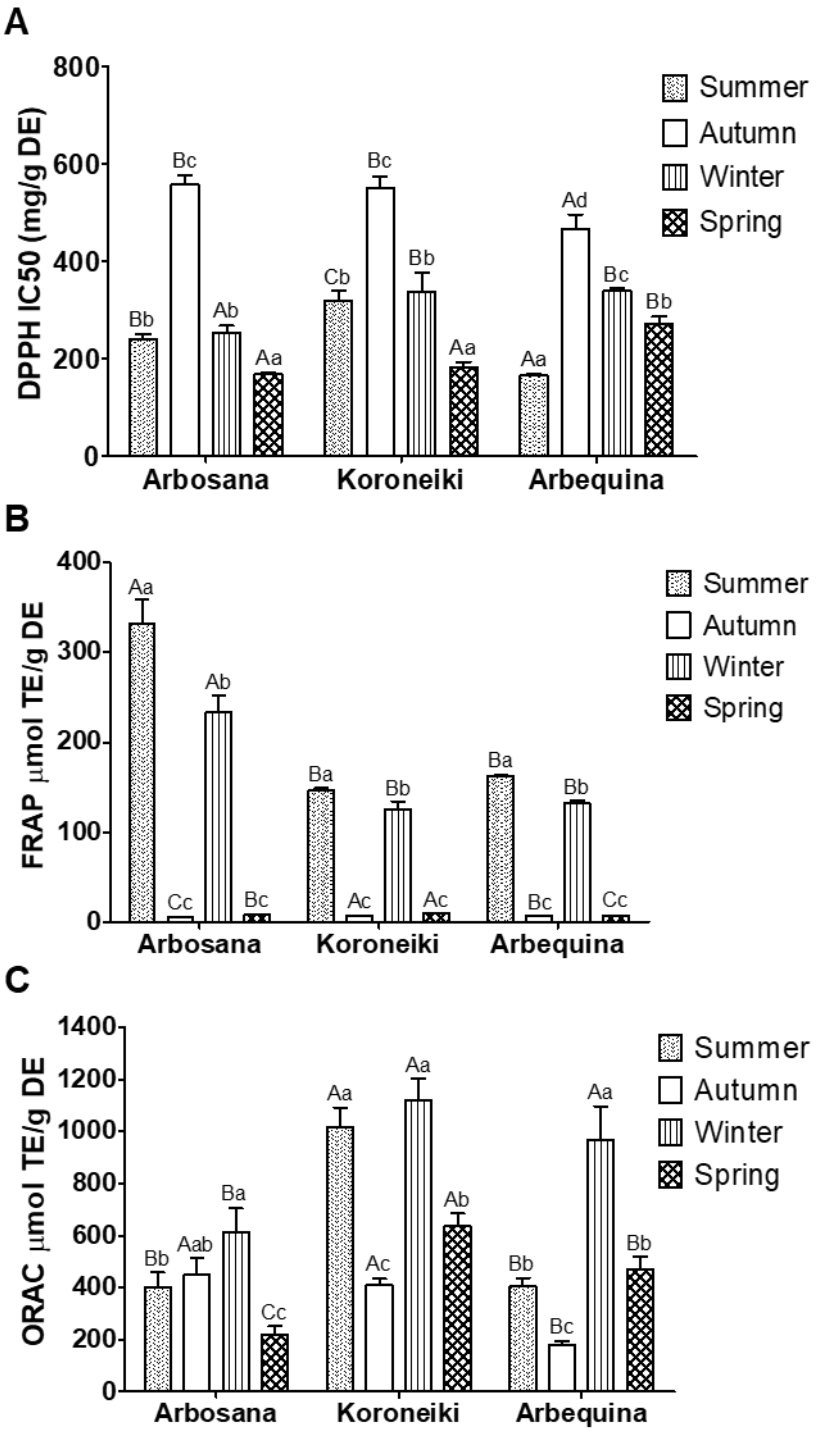


Figure 5. Antioxidants capacities DPPH (A), FRAP (B), and ORAC (C) of olive leaves extracts of three different cultivars harvested in the four different climatic seasons of the year. **NOTE:** Results were expressed as mean ± standard deviation. Different letters in the columns indicate a statistical difference ($p < 0.05$) between the samples. Capital letters indicate statistical difference ($p < 0.05$) between the cultivars for the same climatic

season, and small letters indicate statistical difference ($p < 0.05$) between the different climatic seasons in the same cultivar. TE = Trolox equivalent. DE = dried extract.

In DPPH analyses by the IC50 method is measured the concentration of the sample needed to reduce the radical DPPH from 50%. The smaller the amount used, the more antioxidant the sample. Arbosana had the best result for DPPH in spring (168.62 mg/g DE), contradicting the results of TPC and oleuropein, probably due to other phenolic compounds more reactive to DPPH present in this season. For Koroneiki and Arbequina, the best results were in spring (182.23 mg/g DE) and summer (164.84 mg/g DE), respectively, showing their high antioxidant activity came from oleuropein. For the FRAC analyses, all the cultivars had the best results in summer, followed by winter (331.39 and 233.27 µmol TE/g DE for Arbosana; 146.00 and 125.46 µmol TE/g DE for Koroneiki, and 162.43 and 131.75 µmol TE/g DE for Arbequina), with significant difference between Arbosana and the other two cultivars. The results for spring and autumn were the worst, without significant difference. These results are close to TPC, suggesting the formation of phenolic compounds more reactive to FRAP reagent to joust the stress caused by the higher temperature variation in these seasons. For ORAC analyses, all the cultivars had the best results in winter (614.42 µmol TE/g DE for Arbosana; 1117.14 µmol TE/g DE for Koroneiki, and 968.71 µmol TE/g DE for Arbequina), indicating that in this season were formed more compounds reactive to AAPH reagent. In this season, a significant difference between Arbosana and the other two cultivars was observed, showing this cultivar was less reactive to AAPH reagent. A Pearson correlation showed that DPPH ($R = - 0.71$) was the method more indicated to predict the antioxidant capacity of oleuropein compared to FRAP ($R = 0.56$) and ORAC ($R = - 0.07$).

Previous studies have reported that the antioxidant capacity of olive leaf extracts was closed related to their phenolic compound profile and content since these compounds can lower the levels of cellular reactive oxygen species (Brahmi et al. 2013; Antunes et al. 2020; Pasković et al. 2020). Furthermore, as the profile and content of phenolic compounds are affected by abiotic and biotic factors (Ranalli et al. 2006; Talhaoui et al. 2015) (as discussed in section 4.1), the antioxidant capacity also suffers the influence of these factors, but indirectly.

Antunes et al. (2020) found different antioxidant capacity values by FRAP, DPPH, and ABTS methods for the Arbosana, Koroneiki, and Arbequina cultivars harvested in the same period showing the cultivar had a significant effect on the antioxidant capacity of the olive leaves extracts. Lorini et al. (2021) reported the best DPPH and FRAP values for the Arbequina cultivar in summer, and Kabbash et al. (2019) also found the best DPPH values in spring than autumn in the Koroneiki cultivar, under the highest oleuropein content in this season. Finally, Sueishi & Nii (2020) measured the antioxidant capacity of the Nevadillo Blanco cultivar olive leaf extracts against five reactive oxygen species for one year. They observed different results among the reactive oxygen species, showing that the reactive oxygen species also influenced the antioxidant capacity of the olive leaf extracts due to their more or less interaction with the different profiles of phenolic compounds found in the different harvest periods. According to these authors, oleuropein was not an effective radical scavenger against the AAPH reagent, corroborating the result found for ORAC analyses.

The methods for measuring antioxidant capacity use different types of radicals that act at different sites, showing that the results depend on the type of compounds in the extracts and their interactions with the radicals. The antioxidant capacity presented a significant variation among the methods, showing the best results in the seasons of high TPC values. It indeed depended on the profile and content of phenolic compounds. The DPPH method is the most used in the literature to measure the antioxidant activity of olive leaf extract due to its reactivity with the oleuropein (Kabbash et al. 2019). The method indicated positive results related to oleuropein content.

CONCLUSION

Both the drying process of olive leaf and the solvent composition used to extract their phenolic compounds influenced the TPC of extracts, highlighting the importance of preliminary tests in choosing the parameters used in these methodologies. The TPC and oleuropein content varied depending on the biological cycle of the different cultivars and their sensibility to large temperature variations along the seasons. The antioxidant capacity presented a significant variation among the methods, showing the best results in the seasons of high TPC values. It indeed depended on the profile and content of phenolic compounds. DPPH was the method more indicated to predict the antioxidant capacity of oleuropein according to statistical correlation, demonstrating positive results related to oleuropein content.

Comparação de diferentes estações climáticas do Brasil no valor de compostos fenólicos, oleuropeína e capacidade antioxidante em folhas de oliveira de três cultivares

RESUMO

O presente estudo avaliou os efeitos de diferentes cultivares de folhas de oliveira colhidas nas diferentes estações climáticas no teor de polifenóis totais (TPC), teor de oleuropeína e capacidade antioxidante. A cultivar Arbosana apresentou maior TPC no verão e as cultivares Koroneiki e Arbequina na primavera, independente do solvente utilizado. O teor de oleuropeína apresentou um perfil semelhante. O DPPH teve seus melhores valores para Arbosana e Koroneiki na primavera, seguido por Arbequina no verão. O FRAP apresentou os maiores valores no verão para todas as cultivares, com Arbosana apresentando o maior valor. ORAC apresentou os maiores valores no inverno para todas as cultivares, com Koroneiki apresentando o maior valor. O outono teve os piores resultados. O DPPH foi o método mais indicado para predizer a capacidade antioxidante da oleuropeína segundo correlação estatística. Os resultados destacaram que os diferentes períodos de colheita para a produção brasileira e as diferentes cultivares influenciaram fortemente o TPC, o teor de oleuropeína e a capacidade antioxidante dos extratos de folhas de oliveira.

PALAVRAS-CHAVE: extrato de folhas de oliveira; Arbosana; Koroneiki; Arbequina; condições climáticas.

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Supplementary Material

Comparison of different climatic seasons of Brazil in the value of phenolic compounds, oleuropein, and antioxidant capacity in olive leaves of three cultivars

Table S1. Analysis of variance (ANOVA, α=0.05).

Source	TPC (mg GAE/L)	
	F-value	P-value
Linear effects		
X1 (cultivars)	7.88	0.002
X2 (seasons)	219.37	0.000
X3 (solvent)	2.73	0.112
Interaction effects		
X1*X2	33.88	0.000
X1*X3	2.21	0.133
X2*X3	2.58	0.078
X1*X2*X3	1.41	0.252
R^2	0.97	

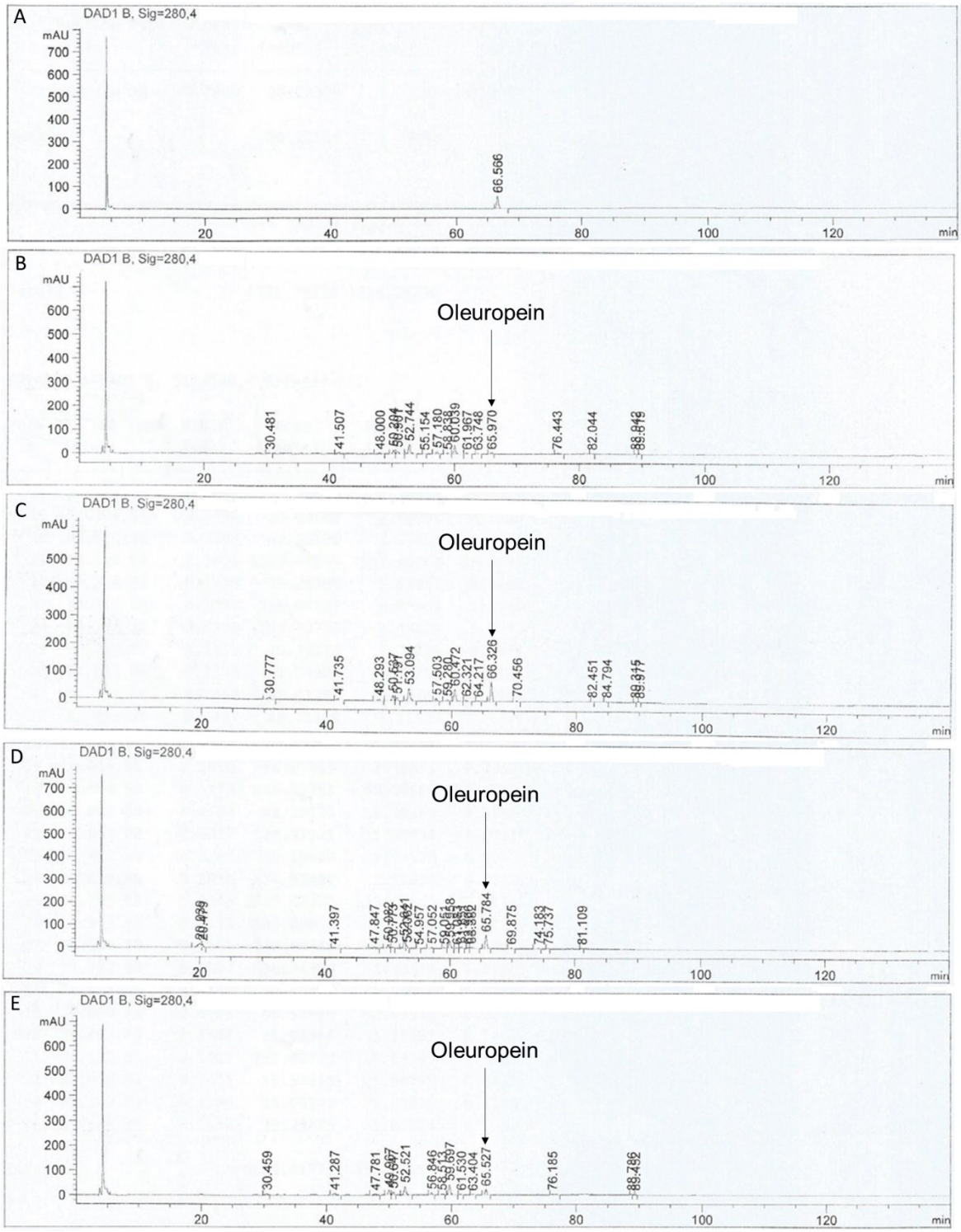

Figure S1. Oleuropein identification chromatograms of olive leaves extract of Arbosana cultivar harvested in the four different climatic seasons of the year. A – Oleuropein standard; B – Summer; C – Autumn; D – Winter; E – Spring.

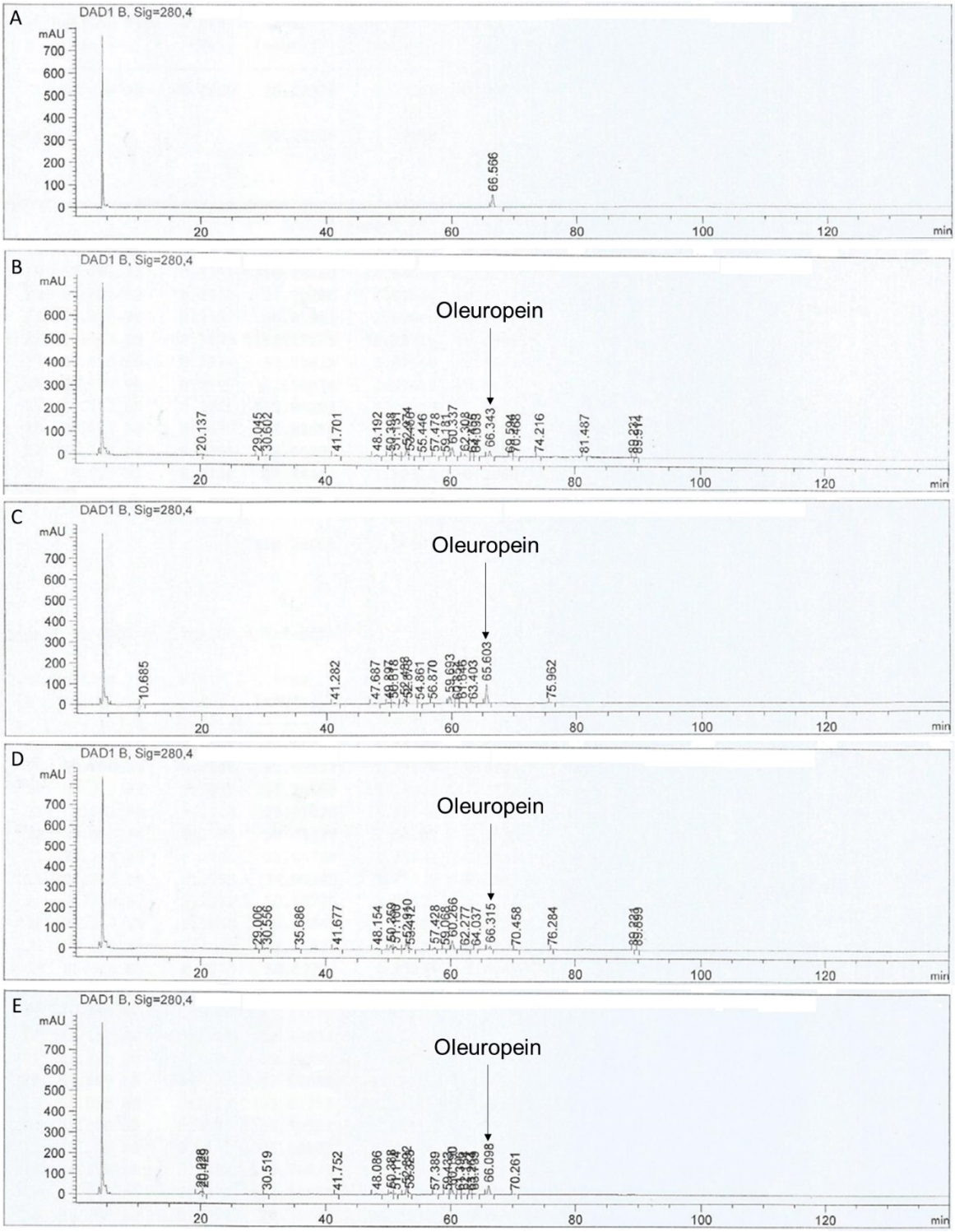

Figure S2. Oleuropein identification chromatograms of olive leaves extract of Koroneiki cultivar harvested in the four different climatic seasons of the year. A – Oleuropein standard; B – Summer; C – Autumn; D – Winter; E – Spring.

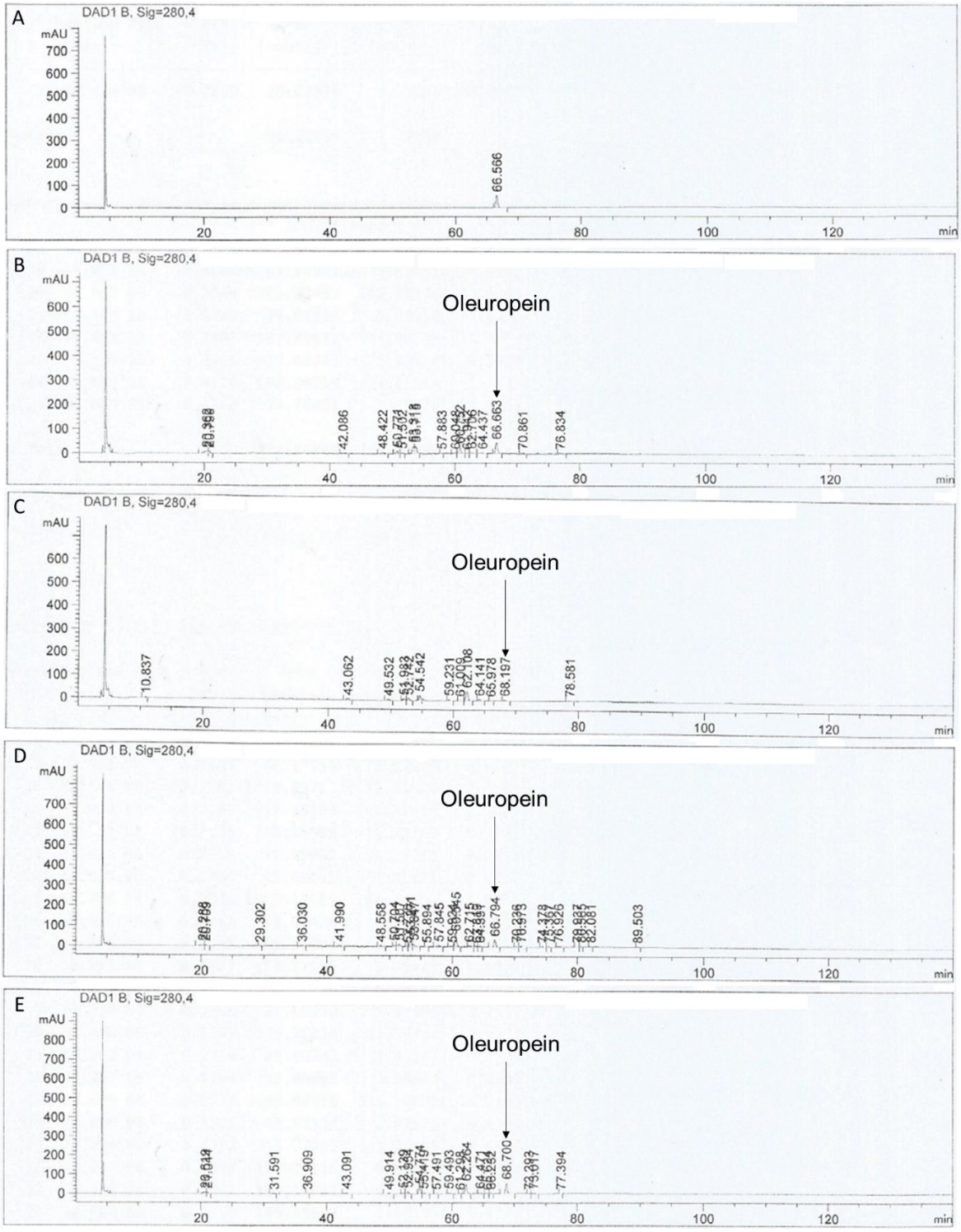

Figure S3. Oleuropein identification chromatograms of olive leaves extract of Arbequina cultivar harvested in the four different climatic seasons of the year. A – Oleuropein standard; B – Summer; C – Autumn; D – Winter; E – Spring.