

Identification and quantification of phenolic compounds in fresh and processed table olives of cv. 'Kalamata'

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Abstract

Mediterranean diet is almost synonymous to the healthy lifestyle and diet nowadays. Some of the major components of the diet are the products of the olive tree, fruits and olive oil, which are classified as medical foods, due to their nutraceutical benefits and their protective properties against cancer, cardiovascular diseases, age-related diseases, neurodegenerative disorders and other diseases. The key contributors to these properties are the phenolic compounds such as hydroxytyrosol, tyrosol and oleuropein. Table olives are being processed with several methods in order to reduce the bitterness of the olive fruit and the impact of the processing on phenolic compounds has not been studied extensively. In the present study, changes in the concentration of the most important phenolic compounds were quantified in fresh, Greek-style and Spanish-style processed olive fruits of cv. 'Kalamata', using two different analytical methods for identification and quantification: high-performance liquid chromatography diode array detector (HPLC-DAD) and ultrahigh-performance liquid chromatography tandem mass spectrometry (LC-(ESI)-MS/MS). The phenolic compounds that were identified and quantified were hydroxytyrosol, tyrosol, verbascoside, rutin, oleuropein and luteolin. Both processing methods used altered the phenolic compounds concentration in 'Kalamata' olive fruits compared to untreated fruits. In both analytical methods, a statistically significant increase in verbascoside and hydroxytyrosol concentration and a statistically significant decrease in rutin concentration was observed in both, Greek-style and Spanish-style, processed olive fruits.

Keywords: HPLC-DAD; LC-(ESI)-MS/MS; Mediterranean diet; phenolic compounds; processing; table olives

Introduction

Plants are an inexhaustible source of bioactive compounds that in recent years have gained enormous interest in their therapeutic use. The scientific community has focused on the search for compounds from plant extracts to prevent and control a large number of diseases. Indicatively, it is reported that 50% of drugs aiming at fighting cancer come directly or indirectly from plant products (Kingston, 2011). Among the thousands of

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plant products, the products of the olive tree, extra virgin olive oil (EVOO) and olive fruits, historically play a particularly important social and economic role in the Mediterranean countries. The main area of cultivation is the Mediterranean region, where 90% of olive oil worldwide is consumed and 80% of the market share of exports is controlled (Bartolini and Petruccelli, 2002; Vasto *et al.*, 2014). Olive tree products are a significant source of healthy fatty acids as part of the Mediterranean diet and considered as rich as in beneficial compounds 'superfoods' (Vasto *et al.*, 2014; Gerber and Hoffman, 2015). The abundance of phenolic compounds in EVOO, such as polyphenols (Barbaro *et al.*, 2014; Rigacci and Stefani, 2016), have been studied excessively over the years for their anti-microbial, antioxidant and anti-inflammatory action (Cicerale *et al.*, 2012). Research has shown that the consumption of olive products is linked with the reduction of risk of several diseases, such as cardiovascular diseases (Esti *et al.*, 1998), neurodegenerative diseases and diseases associated with aging (Khalatbary, 2013; Salis *et al.*, 2020) and diabetes (Salas-Salvadó *et al.*, 2014).

Of the bioactive compounds, phenolic compounds have gained interest in the scientific community in recent years, mainly due to their antioxidant and anti-inflammatory properties. Phenolic compounds are derived from the metabolism of primary products, such as carbohydrates, fats and amino acids, and are called secondary products due to their biosynthesis. The most important phenolic groups present in the olive fruit are phenolic acids, phenolic alcohols, flavonoids and secoiridoids (Macheix *et al.*, 1990; Ryan and Robards, 1998; Soler-Rivas *et al.*, 2000). The most important phenolic alcohols in the olive fruit are tyrosol and hydroxytyrosol (Macheix *et al.*, 1990; Mazza and Miniati, 1993; Romero *et al.*, 2002). The most frequently mentioned and important flavonoids in the olive fruit are luteolin and rutin (Esti *et al.*, 1998; Ryan and Robards, 1998; Romani *et al.*, 1999). The most prevalent secoiridoid found in the olive fruit is oleuropein, while verbascoside is the predominant phenolic acid. Hydroxytyrosol has been shown to have a wide range of biological effects, including anti-cancer, cardioprotective, anti-microbial, neuroprotective and other effects (Visioli *et al.*, 2002; Parkinson and Cicerale, 2016; Tomé-Carneiro *et al.*, 2016; Valenzuela *et al.*, 2017; Robles-Almazan *et al.*, 2018). Oleuropein has a strong antioxidant activity, which is widely recognized and along with its anti-inflammatory properties, is considered to be the basis of anti-cancer, neuroprotective, cardioprotective and other actions (Visioli *et al.*, 2002; Hassen *et al.*, 2015; Basdeki *et al.*, 2020). Tyrosol appears to be an effective antioxidant and has beneficial effects against hyper intention, atherosclerosis, heart failure, obesity and other actions (Di Benedetto *et al.*, 2007; Chang *et al.*, 2019; Salis *et al.*, 2020). Rutin has demonstrated antioxidant, cardioprotective, anti-cancer, neuroprotective and other pharmacological properties (Janbaz *et al.*, 2002; Nassiri-Asl *et al.*, 2010; Richetti *et al.*, 2011; Javed *et al.*, 2012; Valenzuela *et al.*, 2017). A growing body of research shows that lutein has antioxidant, anti-cancer, anti-inflammatory and neuroprotective properties (Lin *et al.*, 2008; Qiao *et al.*, 2012; Theoharides *et al.*, 2015). Finally, verbascoside has demonstrated anti-inflammatory properties and it has been suggested that it could have anti-cancer properties (Lee *et al.*, 2006; Korkina, 2007; Lenoir *et al.*, 2011; Akdemir *et al.*, 2011).

The most important factors affecting the biosynthesis of phenolic compounds are genotype, tissue, growth stage, biotic factors, microclimate and cultivation techniques (Karakaya and Nehir, 1999). The processing method of the final products has also a significant effect on the phenolic compound concentration. Olive fruit processing methods affect the taste and can significantly alter the health properties of the olive fruit, mostly because different processing methods can lead to different capacity of hydrolysis products (Marsilio *et al.*, 2005; Zoidou *et al.*, 2010; Charoenprasert and Mitchell, 2012). The main methods of processing olive fruits which are designed to reduce oleuropein concentration, but consequently affect the other phenolic compounds, leading to a different phenolic compound profile, are: a) the Greek-style processing method: ripe olives are treated with brine, b) the Spanish-style processing method: unripe olives are treated with sodium hydroxide, fermented and pasteurized and c) the Californian-type processing method: unripe olives are treated with sodium hydroxide and air oxidation (Karkoula *et al.*, 2012). The greatest volume of studies is based on the phenolic compound profile of olive oil, while the phenolic compound composition of the fruit is different, it changes after treatments and remains insufficiently explored (Boskou *et al.*, 2005).

In the past, several strategies of analysis and quantitation of phenolic compounds have been used for the products of the olive tree, but most of them have focused on olive oil (Bianco *et al.*, 2003; Servili *et al.*, 2004; Bendini *et al.*, 2007; Carrasco-Pancorbo *et al.*, 2007; Fu *et al.*, 2009). Techniques based on HPLC have been widely used for the identification of hydrophilic phenolic compounds in olive oil and olive fruits (Mateos *et al.*, 2004; Siliani *et al.*, 2006; Kanakis *et al.*, 2013; Mitsopoulos *et al.*, 2016; Romero *et al.*, 2017). The employment of UV methods, such as DAD, has seen a great use the past years for the quantitation of phenolics such as oleuropein, hydroxytyrosol and tyrosol (Boskou *et al.*, 2005), phenolic acids and flavonoids (Artajo *et al.*, 2007). It seems though that methods based on UV have a disadvantage because of their lack of sensitivity needed to measure the full range of hydrolysis products of oleuropein that appear during the processing of the table olives (Kumral *et al.*, 2013). It is also mentioned that they are characterized by long time of separation and in most cases higher detection limits are required than MS methods to identify certain phenolics of the olive products (Segura-Carretero *et al.*, 2010; Johnson *et al.*, 2018). As a result, different methods have developed for the analysis of chemical compounds, such as mass spectrometry methods, which are more sensitive in detecting limits of quantification, than UV methods (Kanakis *et al.*, 2013; Melliou *et al.*, 2015; Johnson *et al.*, 2018). MS methods seem to be more sensitive in the evaluation of hydrolysis products of oleuropein and they generally have been adopted for their higher sensitivity, selectivity, higher mass accuracy and the simultaneous quantitation in the analysis of phenolic compounds (Jerma Klen *et al.*, 2015; Melliou *et al.*, 2015; Johnson *et al.*, 2018). MS methods have been focused mainly on studying olive oil (Bendini *et al.*, 2007; Dierkes *et al.*, 2012).

The aim of this research was to study the changes in the concentration of the most important phenolic compounds in the olive fruits cv 'Kalamata', one of the most important Greek table olive variety, that have been treated with two different, Greek-style and Spanish-style, processing methods. The study of the effect of the different processing methods on the phenolic compounds of the olive fruit was conducted by using two different methods of analysis and quantification, HPLC-(DAD) and LC-(ESI)-MS/MS.

Materials and Methods

Olive fruit samples

Three different styles of table olive fruits were included in the present study; fresh fruits, olives fruits processed in Greek-style method and olive fruits processed in Spanish-style method. All fruits were sampled from the same orchard in Agios Vlasios, region of Viotia, in November 2019 in the stage of full maturity and black color and from several trees in the orchard. For the processed table olive fruits, packages of 500 gr each were prepared, specifically for the study, and treated by a table olive production enterprise. Fruits processed with Greek-style method were placed in sodium chloride solution 10% with implementation of acetic acid. After this process, olives were exposed to oxygen conditions for 24 hours and finally were packed in fresh brine 8% and pH 4. Fruits processed with Spanish-style method were placed in sodium hydroxide solution 2%. After this process, fruits were extracted, washed and placed in sodium chloride solution. Salt concentration in the solution was 6% w/v. After the end of lactic fermentation, when the pH dropped to 4, the salt concentration increased to 8%. At their final stage of processing, the fruits were packed in fresh brine 8%. Three replicates were made for each category of unprocessed and processed olive fruits, each sample included six fruits.

Extraction method/Sample preparation

The pit from the olive fruits was removed before the extraction. After pit removal, the flesh of the olive fruits was cut in small pieces and analytically weighted. The samples were homogenized with IKA Ultra-Turax T25 Basic, with 25 mL n-hexane, twice. Then, the samples were centrifuged for 10 min at 2500xg (in Labofuge 400 Functionline centrifuge), and the n-hexane layer was discarded. The residual solid phase was then extracted for 30 min, in supersonic bath with 80% methanol for four times. Each time, the samples were centrifuged for

10 min at 2500 xg and the extracts were transferred to 100 mL flasks. The volume was adjusted with 80% methanol. Sample preparation method was the same for the two different methods of analysis that were used.

Identification and quantitation of phenolic compounds using the HPLC-(DAD) technique

Identification and quantitation of the concentration of the phenolic compounds in the flesh of the olive fruit was performed using the HPLC-(DAD) technique, with the HPLC Agilent Technologies series 1200. Separation of the sample compounds was performed with a Zorbax Eclipse XDB-C18 rapid analysis column, 1.8 mm, measuring 50 X 4.6 mm, maintained at 45 °C. The UV-Vis detector used was an Agilent Technologies series 1200 Diode-Array Detector (DAD). The sample was introduced into the analytical column via a Rheodyne model 7725i valve, with a circular volume of 20 mL. The injection volume of the sample in the analytical column was 5 mL.

Mobile phase consisted of 0.05% formic acid in deionized water (A) and methanol gradient grade (B) was delivered in gradient mode as follows: 0 min 25% B, 12 min 70% B, 18 min 100% B, 24 min 100% B, with re-equilibration time 6 min. Between analyses the column was rinsed with 2.2% v/v acetic acid solution. A 40-min cleaning program was used to clean the column at regular intervals as follows: 10 min – 100% acetic acid solution, 10 min -100% acetonitrile solution, 10 min – 100% acetic acid solution. The wavelength switching program was adjusted in the DAD detector, based on the UV spectra of the phenolic compounds detected in the sample. The identification of each compound was done after comparing the sample peaks with standard phenolic compounds. The eluting phenolic compounds were detected in the range of 280 to 340 nm. The quantitation of the above phenolic compounds identified in the samples was performed using commercial standards for these compounds, using a standard phenolic curve, for each of the compounds used. The assay was expressed as µg of phenolic compounds per gram of olive fruit fresh tissue. Quantitation of the identified phenolic compounds was performed according to the method of the external standard.

Identification and quantitation of phenolic compounds using the LC-(ESI)-MS/MS technique

The identification and quantitation of the individual phenolic compounds of the olive fruit tissue was performed using the reverse Twin Mass Spectrometry technique with electrospray ionization (LC-(ESI)-MS/MS). The analyses were performed with the Agilent Technologies series 1260 HPLC system Agilent Technologies 6460 Triple-Quad Mass Detector, with electrospray ion source. The chromatographic column used was reverse phase, which had as filler material silanol groups chemically linked to 18 carbon atom chains. More specifically, the separation of the sample compound was performed with a Zorbax Eclipse XDB-C18 rapid analysis column, measuring 50 X 4.6 mm, with a particle diameter of 1.8 mm. The rapid analysis column used was kept at 50 °C. The sample was inserted with a Hamilton HPLC syringe into the injector and the volume of the injected sample was 5 mL. The measurements were recorded with the program Agilent Technologies MassHunter Workstation Software – Data Acquisition (ver. B.03.01).

Mobile phase consisted of 0.05% formic acid in deionized water (A) and methanol gradient grade (B) was delivered in gradient mode as follows: 0 min 25% B, 12 min 70% B, 18 min 100% B, 24 min 100% B, with re-equilibration time 6 min. Between analyses the column was rinsed with 2.2% v/v acetic acid solution. A 40-min cleaning program was used to clean the column at regular intervals as follows: 10 min – 100% acetic acid solution, 10 min -100% acetonitrile solution, 10 min – 100% acetic acid solution. Detection of eluting compounds in the samples was performed through the system (ESI)-MS/MS, using the ion source parameters, as follows: nebulization gas (N₂) pressure: 50 psi, drying gas (N₂) flow: 10L/min and temperature: 350 °C, capillary voltage: 4 kV, negative polarity. Data were obtained in dynamic ion default monitoring (SRM) mode. The specific optimized parameters for the phenolic compounds were used to obtain the data: precursor ion, ion produced, fragmentation tendency, collision tendency. The tendency of each compound was made after comparing the peaks of the sample with the peaks of standard phenolic compounds. For all compounds, peak areas were identified using Agilent MassHunter Workstation Software – Qualitative Analysis (ver. B.06.01). Calibration curves were plotted, sample concentrations were calculated using Microsoft Excel Software and

expressed as μg of phenolic compounds per gram of olive fruit fresh tissue. The conditions for determination of phenolic compounds in the fruit of cv. 'Kalamata' using the LC-(ESI)-MS/MS method is presented in Table 1.

Table 1. LC-(ESI)-MS/MS conditions for determination of phenolic compounds in the fruit of cv. 'Kalamata'

Compound	Molecular weight (Mw)	Fragmentor voltage (vf)	Precursor ions (m/z)	Product ions (m/z)	Collision energy (vcol)
Hydroxytyrosol	154	130	153	123	11
Tyrosol	138	120	137	106	13
Verbascoside	624	116	623	161	33
Rutin	610	135	609	300	42
Oleuropein	540	180	539	275	19
Luteolin	286	135	285	133	25

Statistical analysis

The effect of the phenolic concentration factor on the phenolic compounds examined at the different stage of the fruit with or without treatment, and the treatment method was assessed by one-way analysis of variance (ANOVA). The comparisons of phenolic compounds for all samples, of all stages of the olive fruit were made based on the method of honest significant difference (HSD, Post-hoc Test) of the method Tukey-HSD. Statistical analyses were performed with JMP 15.0 software (SAS Institute, Cary, NC, USA) and XLSTAT 2020.3.1 software (Addinsoft).

Results and Discussion

The phenolic compounds that were identified and quantified, using both analytical methods, were hydroxytyrosol, tyrosol, verbascoside, rutin and luteolin (Figures 1, 2). Oleuropein was identified, in low quantities, when using the LC-(ESI)-MS/MS analytical method.

Both processing methods used, Greek-style and Spanish-style, altered the phenolic compound concentration in 'Kalamata' olive fruits compared to untreated fruits (Table 2). In both analytical methods, a statistically significant increase in verbascoside and hydroxytyrosol concentration and a statistically significant decrease in rutin concentration was observed in both, Greek-style and Spanish-style, processed olive fruits (Figures 1, 2 and Tables 2, 3). Tyrosol concentration significantly decreased in all processed olive fruits when using the HPLC-(DAD) technique while the decrease in tyrosol concentration was statistically significant only in Greek-style processed olive fruits when using the LC-(ESI)-MS/MS technique. Luteolin concentration was significantly increased in all processed olive fruits when using the LC-(ESI)-MS/MS technique while there was no statistically significant change in its concentration when using the HPLC-(DAD) technique. Oleuropein was identified, in low quantities, only when using the LC-(ESI)-MS/MS analytical method and a decrease was observed in processed fruits that was statistically significant in the Spanish-style processed fruits. Verbascoside was the predominate phenolic compound in all processed olive fruits, while rutin was the predominate compound in fresh olive fruits (Tables 2, 3). Verbascoside and hydroxytyrosol concentration was significantly higher in the Greek-style processed fruits when using the LC-(ESI)-MS/MS analytical method while no statistically significant difference was observed in the concentration of the other phenolic compounds between the Greek-style and Spanish-style processed olive fruits using both analytical methods.

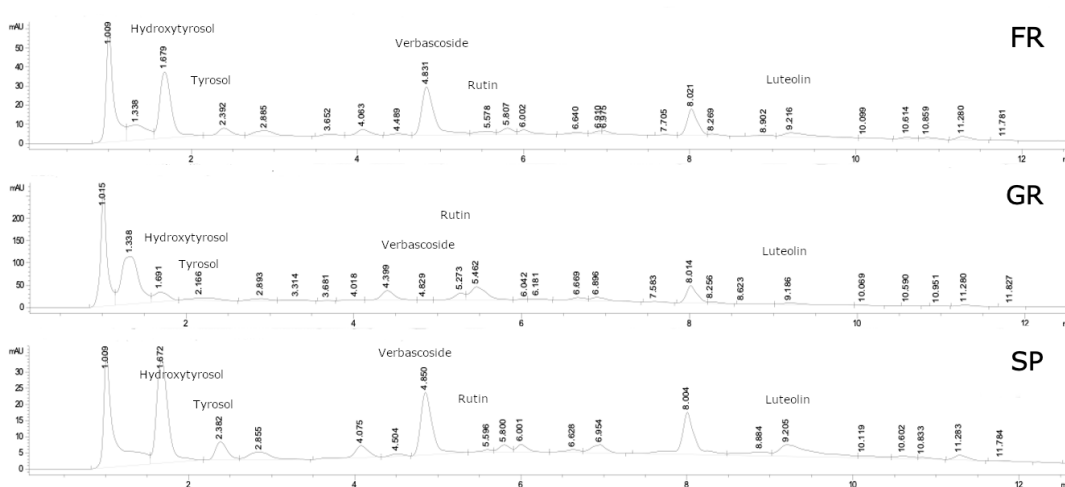


Figure 1. HPLC-(DAD) chromatogram recorded for the phenolic compounds extracted from Fresh (FR), Greek-style (GR) and Spanish-style (SP) olive fruits.

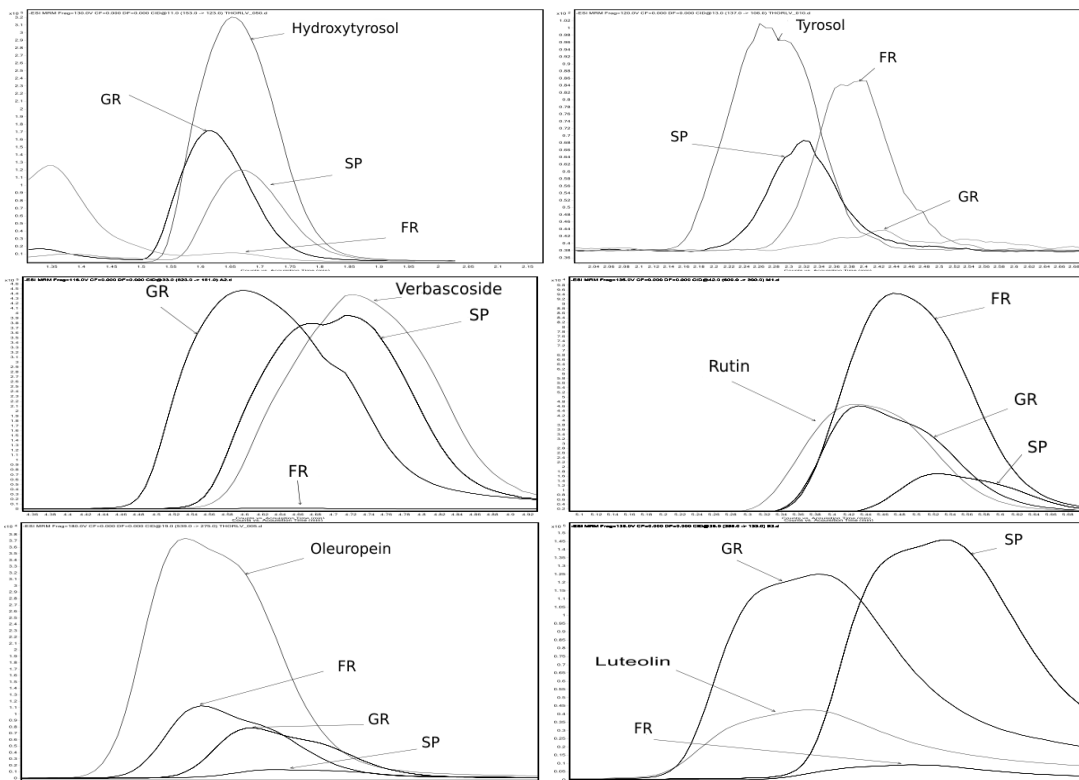


Figure 2. LC-(ESI)-MS/MS overlaid chromatogram of each phenolic compound extracted from Fresh (FR), Greek-style (GR) and Spanish-style (SP) olive fruits
Name = standard compound

Verbascoside is a major derivative of hydrocinnamic acid in olive pulp and its presence is related to the variety and the harvest time (Charoenprasert and Mitchell, 2012). In this study, verbascoside concentration significantly increased in all processed olive fruits, using both analytical methods. Its concentration ranged from 31.50 $\mu\text{g/g}$ fresh tissue (ft) in unprocessed fruit to 504.26 $\mu\text{g/g}$ ft in Greek-style processed fruit using the HPLC-(DAD) analytical method and from 28.33 $\mu\text{g/g}$ ft in unprocessed fruit to 332.03 $\mu\text{g/g}$ ft in Greek-style

processed fruit using the LC-(ESI)-MS/MS analytical method (Table 2). Verbascoside concentration was higher in the Greek-style than in the Spanish-style processed fruits, but the difference was statistically significant only when using the LC-(ESI)-MS/MS analytical method (Table 3). High verbascoside concentration in processed olive fruits was also observed by Melliou *et al.* (2015), Sahan *et al.* (2013) and Selli *et al.* (2018).

Table 2. Mean concentration of the phenolic compounds in tissue samples of fresh fruits (FR), Greek-style (GR) and Spanish-style (SP) treated olive fruits of cv. 'Kalamata', expressed in μg per g of fresh tissue

Compound ($\mu\text{g}/\text{gr}$)	HPLC-(DAD)			LC-(ESI)-MS/MS		
	FR	GR	SP	FR	GR	SP
Hydroxytyrosol	273.43 \pm 8.72	367.83 \pm 32.53	333.96 \pm 10.82	nq	90.66 \pm 1.94	63.10 \pm 2.62
Tyrosol	215.56 \pm 14.30	84.33 \pm 1.99	123.66 \pm 8.82	37.23 \pm 4.39	18.26 \pm 0.97	27.93 \pm 0.76
Verbascoside	31.50 \pm 11.76	504.26 \pm 39.49	453.66 \pm 54.06	28.33 \pm 6.65	332.03 \pm 6.85	283.80 \pm 6.92
Rutin	653.80 \pm 49.51	5.50 \pm 0.78	3.40 \pm 0.75	296.03 \pm 24.06	17.70 \pm 1.79	6.63 \pm 0.38
Oleuropein	nd	nd	nd	7.66 \pm 1.13	5.80 \pm 0.55	2.90 \pm 0.90
Luteolin	106.06 \pm 9.12	92.40 \pm 4.68	118.16 \pm 8.45	144.23 \pm 9.33	228.2 \pm 14.18	245.46 \pm 2.13

nd=non detected, nq=non quantifiable

Table 3. Comparison of samples of fresh (FR), Greek-style (GR) and Spanish-style (SP) samples

Compound	HPLC-(DAD)			LC-(ESI)-MS/MS		
	GR-FR	SP-FR	GR- SP	GR-FR	SP-FR	GR- SP
Hydroxytyrosol	*	*	ns	***	***	***
Tyrosol	***	**	ns	**	ns	ns
Verbascoside	***	**	ns	***	***	**
Rutin	***	***	ns	***	***	ns
Oleuropein	-	-	-	ns	*	ns
Luteolin	ns	ns	ns	**	**	ns

*Significant set at $\alpha < 0.05$. ns = not significant, significance level * < 0.05 , ** < 0.01 , *** < 0.001 . Tukey HSD Post-hoc Test, One-way Analysis of Variance ANOVA.

Hydroxytyrosol concentration in the present study ranged from 273.43 $\mu\text{g}/\text{g}$ ft in unprocessed fruit to 367.83 $\mu\text{g}/\text{g}$ ft in Greek-style processed fruit using the HPLC-(DAD) analytical method (Table 2). Using the LC-(ESI)-MS/MS analytical method, hydroxytyrosol was detected in minute, non-measurable quantities, in fresh fruits while its concentration increased in the processed fruits, 63.10 $\mu\text{g}/\text{g}$ ft and 90.66 $\mu\text{g}/\text{g}$ ft in Spanish-style and Greek-style respectively. There was a statistically significant increase of hydroxytyrosol concentration in processed fruits compared to the fresh fruits (Table 3). The concentration was higher in the Greek-style than in the Spanish-style processed olive fruits but the difference was statistically significant only when using the LC-(ESI)-MS/MS analytical method. Increase in hydroxytyrosol concentration in processed olive fruits was also reported by Marsilio *et al.* (2001) while higher hydroxytyrosol concentration was observed in Greek-style processed olive fruits (Marsilio *et al.*, 2005; Johnson *et al.*, 2018).

Luteolin concentration ranged from 92.40 $\mu\text{g}/\text{g}$ ft in Greek-style processed fruit to 118 $\mu\text{g}/\text{g}$ ft in Spanish-style processed fruit when using the HPLC-(DAD) analytical method and from 144.23 $\mu\text{g}/\text{g}$ ft in fresh fruits to 245.46 $\mu\text{g}/\text{g}$ ft in Spanish-style processed fruit when using the LC-(ESI)-MS/MS analytical method (Table 2). Its concentration was significantly increased in Greek-style and Spanish-style processed olive fruits when using the LC-(ESI)-MS/MS technique (Table 3) while there was no statistically significant difference in its concentration between the Greek-style and Spanish-style processed olive fruits. There was no significant difference in luteolin concentration between all fruits when using the HPLC-(DAD) analytical method. In a

similar study, increase in luteolin concentration in processed fruit was reported (Ambra *et al.*, 2017) while Melliou *et al.* (2015) reported that luteolin concentration was similar in fresh and processed fruits.

Rutin, according to the literature, is the major flavonoid in the olive fruit and this compound is always present, even when other compounds are absent (Esti *et al.*, 1998; Servili *et al.*, 2004). A statistically significant decrease in rutin concentration of processed fruits was observed using both analytical methods. Rutin concentration ranged from 3.40 $\mu\text{g/g}$ ft in Spanish-style processed fruits to 653.80 $\mu\text{g/g}$ ft in fresh fruit when using the HPLC-(DAD) analytical method and from 6.63 $\mu\text{g/g}$ ft in Spanish-style processed fruits to 296.03 $\mu\text{g/g}$ ft in fresh fruits when using the LC-(ESI)-MS/MS technique (Table 3). There was no statistically significant difference in rutin concentration between Greek-style and Spanish-style processed fruits using both analytical methods. Many studies report a decrease in rutin concentration in processed olive fruits (Brenes-Balbuena *et al.*, 1992; Marsilio *et al.*, 2005; Melliou *et al.*, 2015). Vinha *et al.* (2005) and Mitsopoulos *et al.* (2016) report high rutin concentration in fresh olive fruits.

Tyrosol concentration ranged from 84.33 $\mu\text{g/g}$ ft in Greek-style processed fruits to 215.58 $\mu\text{g/g}$ ft in fresh fruits when using the HPLC-(DAD) analytical method and from 18.26 $\mu\text{g/g}$ ft in Greek-style processed fruits to 37.23 $\mu\text{g/g}$ ft. in fresh fruits when using the LC-(ESI)-MS/MS technique (Table 3). Tyrosol concentration significantly decreased in all processed olive fruits when using the HPLC-(DAD) technique while the decrease in tyrosol concentration was statistically significant only in Greek-style processed olive fruits when using the LC-(ESI)-MS/MS technique. Brenes-Balbuena *et al.* (1992) also observed a decrease in tyrosol concentration in processed olive fruits.

Oleuropein is among the most important phenolic compounds found in the olive fruit. In our study, using the LC-(ESI)-MS/MS technique, oleuropein was detected in low concentration in all samples and a reduction in oleuropein concentration was observed in the processed fruits that was statistically significant in the Spanish style processed fruit (Table 3). Oleuropein concentration ranged from 2.90 $\mu\text{g/g}$ ft in the Spanish-style processed fruits to 7.66 $\mu\text{g/g}$ ft in fresh fruits. Decrease in oleuropein concentration in processed olive fruits was also reported from Ambra *et al.* (2017) and Johnson *et al.* (2018), while higher concentration of oleuropein in Greek-style than Spanish-style processed fruits was also reported (Brenes-Balbuena *et al.*, 1992; Marsilio *et al.*, 2001; Johnson *et al.*, 2018).

Comparing the two processing methods used it was observed that verbascoside and hydroxytyrosol concentration was significantly higher in the Greek-style processed fruits when using the LC-(ESI)-MS/MS analytical method (Tables 2, 3) while no statistically significant difference was observed in the concentration of the other phenolic compounds between the Greek-style and Spanish-style processed olive fruits using both analytical methods. Studies comparing the two processing methods have shown higher phenolic compound concentration in the Greek-style than Spanish-style processed olive fruits (Blekas *et al.*, 2002; Marsilio *et al.*, 2005; D'Antuono *et al.*, 2016; Johnson *et al.*, 2018).

Comparing the two analytical methods used, some differences were observed. Using the HPLC-(DAD) technique, oleuropein was not detected in the olive fruits (Table 3). UV techniques are characterized for long separation times and for higher detection and quantitation limits, than MS techniques, that do not support the identification of specific phenolic compounds in olive products (Segura-Carretero *et al.*, 2010). LC-MS/MS methods have higher sensitivity in the analysis of phenolic compounds (Segura-Carretero *et al.*, 2010; Jerman Klen *et al.*, 2015; Melliou *et al.*, 2015; Johnson *et al.*, 2018) and the LC-MS/MS method has reported good results with low quantification limits in the phenolic compounds analysis (Lopez-Fernandez *et al.*, 2020).

Conclusions

In the present study, changes in the concentration of the most important phenolic compounds were quantified in fresh, Greek-style and Spanish-style processed olive fruits of cv. 'Kalamata', using two different analytical methods for identification and quantitation: high-performance liquid chromatography diode array

detector (HPLC-DAD) and ultrahigh-performance liquid chromatography tandem mass spectrometry (LC-(ESI)-MS/MS). The phenolic compounds that were identified and quantified were hydroxytyrosol, tyrosol, verbascoside, rutin, oleuropein and luteolin.

In both analytical methods, a statistically significant increase in verbascoside and hydroxytyrosol concentration and a statistically significant decrease in rutin concentration was observed in both, Greek-style and Spanish-style, processed olive fruits. Tyrosol concentration significantly decreased in all processed olive fruits when using the HPLC-(DAD) technique while the decrease in tyrosol concentration was statistically significant only in Greek-style processed olive fruits when using the LC-(ESI)-MS/MS technique. Luteolin concentration was significantly increased in all processed olive fruits when using the LC-(ESI)-MS/MS technique while there was no statistically significant change in its concentration when using the HPLC-(DAD) technique. Oleuropein was identified, in low quantities, only when using the LC-(ESI)-MS/MS analytical method and a decrease was observed in processed fruits that was statistically significant in the Spanish-style processed fruits. Verbascoside was the predominate phenolic compound in all processed olive fruits, while rutin was the predominate compound in fresh olive fruits.

Verbascoside and hydroxytyrosol concentration was significantly higher in the Greek-style processed fruits when using the LC-(ESI)-MS/MS analytical method while no statistically significant difference was observed in the concentration of the other phenolic compounds between the Greek-style and Spanish-style processed olive fruits using both analytical methods.

Although there were differences among the phenolic compound quantities detected using the two analytical methods, similar trends were observed between untreated/treated olive fruits.

Overall, both processing methods used altered the phenolic compounds concentration in 'Kalamata' olive fruits compared to untreated fruits. The phenolic compounds are key contributors to the antioxidant properties of the olive fruit and these results can be useful for producing commercial products with beneficial health properties.

Authors' Contributions

Conceptualization: MH; Data curation: CS; Formal analysis: CS; Investigation: CS, MH and IEP; Methodology: MH and IEP; Project administration: MH; Resources: MH, IEP, and CS; Supervision: MH and IEP; Validation: MH, IEP, CS; Visualization: MH; Writing-original draft: CS; Writing-review and editing: CS, MH and IEP. All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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