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Separation and Determination of Biophenols in Olive Oil Samples Based on the Official Method of the International Olive Council and Commission Regulation (EU) No. 432/2012

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Abstract: The purpose of this study was to evaluate the key quality characteristics of olive oil with a main focus on the biophenolic content, its beneficial effects on health and the contribution of various quality characteristics to its health claims, as well as its influence from various parameters. Samples from different traditional oil-producing regions of Greece were initially examined for the most important quality parameters, such as the percentage of free acidity, the number of peroxides and the spectrophotometric investigation in the ultraviolet. The samples were then tested for their biophenolic content, expressed in milligrams of tyrosol, and its derivatives per 20 g of olive oil using the analytical technique high-performance liquid chromatography (HPLC). Then, the biophenol contents of the analyzed samples were compared based on the presented intensities in terms of the variety and the area of cultivation, the altitude, the type of cultivation, the extraction system in the mill and the time interval from olive harvest to oiling. Finally, according to the results, the content of biophenols in olive oil and its health claims were found to be influenced by the various parameters with which they were compared.

Keywords: olive oil quality; health claim; tyrosol; hydroxytyrosol; oleuropein; ligstroside aglycon; phenolic compounds; HPLC/DAD; European Commission Regulation 432/2012

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1. Introduction

Olive oil is obtained from the fruits of the European olive (*Olea Europea*) exclusively by means of mechanical and natural methods or treatments, without the addition of chemical improvers or other treatment, and at appropriate temperatures that do not cause oil deterioration [1,2]. Because of this, it can be consumed immediately, like all natural juices, but differs from other vegetable oils because it is used in its natural form, having a unique pleasant taste, excellent aroma and great biological and nutritional value. As a key ingredient in the Mediterranean diet, it is now one of the most popular food products and has a steadily growing international demand, equivalent to functional foods [3].

The components of olive oil are divided into saponifiable (triglycerides and free fatty acids), covering 98.0–99.0%, and unsaponifiable (hydrocarbons, aliphatic and aromatic alcohols, phenols, sterols, tocopherols, fat-soluble vitamins, volatile organic compounds, dyes, triterpenic acids, etc.), which while occupying a small percentage, play an important nutritional and biological role [4]. It is now well known for olive oil that the nutritional benefits that protect health have a scientifically proven basis. A large number of scientific

data show that the consumption of olive oil improves cardiovascular function through anti-inflammatory action in a wide range of pathological conditions [5]. As a result, a number of compounds have been isolated and characterized in recent years [6]. Its high nutritional value, compared to other vegetable oils, is based on the high levels of monounsaturated fatty acids it contains, as well as the presence of some secondary components, such as phenolic compounds, which are responsible for many of the health-promoting properties and the unique organoleptic characteristics of olive oil [7].

The polar phenolic fraction of olive oil, also known as bioactive phenols, is in fact a complex mixture of compounds with various chemical structures. Phenols include compounds that contain at least one benzene ring with at least one hydroxyl substituent. Phenolic compounds are divided into seven categories: phenolic acids, simple phenols, flavonoids, secoiridoids, lignans, hydroxy-isochromans and pseudophenols. As polar compounds, they are mainly water soluble, slightly fat soluble and have a strong antioxidant activity [8]. The phenolic components present in olive oil are different from those of olives. Those contained in olives are mainly glycosides, such as oleuropein, verbascoside, luteolin and rutin [4]. In contrast, the phenolic fraction of olive oil consists of a heterogeneous mixture of compounds with the most abundant being the simple phenols hydroxytyrosol and tyrosol (3,4-DHPEA and p-HPEA); the aglycones of oleuropein and ligstroside; the aldehydic forms of oleuropein aglycon and ligstroside aglycon (3,4-DHPEA-EA and p-HPEA-EA), which belong to the secoiridoids; the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol (oleacein, 3,4-DHPEA-EDA); the dialdehydic form of decarboxymethyl elenolic acid linked to tyrosol (oleocanthal, p-HPEA-EDA); lignans (1-acetoxypinoresinol and pinoresinol); acetylated hydroxytyrosol; flavonoids (luteolin and apigenin); phenolic acids (p-coumaric acid and vanillic acid) and other secondary substances [9]. Phenolic compounds, such as antioxidants in olive oil and especially hydroxytyrosol and its derivatives, have received special attention in recent years because they are important parameters in terms of the quality, stability, shelf life, organoleptic characteristics and health benefits of olive oil [7]. Thus, they increase the shelf life of olive oil by slowing down oxidation and affect organoleptic characteristics such as color, bitterness, astringency, and aroma [10–13].

In general, biophenols have several beneficial properties. These include: antioxidant action, anti-inflammatory action, antiatherosclerotic action, anticancer action, antimicrobial action, antiviral action, skin protection and antiaging action [14]. Studies show that consuming olive oil rich in biophenols reduces the risk of developing neurodegenerative diseases, cardiovascular disease, certain cancers and HIV-1 infection [15–18]. The systematic and continuous intake of the hydrophilic phenols of olive oil has a long-term positive effect on the incidence of various types of cancer and chronic diseases such as cardiovascular disease and type II diabetes [19]. Thus, in 2011, the Panel on Nutrition, Novel Foods and Food Allergens (NDA) Panel of the European Food Safety Authority (EFSA) concluded that there is evidence for a cause-and-effect relationship between the consumption of bioactive phenolic compounds in olive oil and the protection of low-density lipoprotein (LDL), i.e., cholesterol particles, from oxidative damage [20]. As a result, the European Commission introduced the use of a health claim, which can only be used for olive oils containing at least 5 mg of hydroxytyrosol and its derivatives per 20 g of olive oil, and noted that positive results are ensured by a daily intake of 20 g of olive oil [20].

The chemical composition of olive oil varies and depends on many different parameters. For this reason, its content of phenolic components is affected by the variety of the olive, the cultivation area, the applied cultivation techniques, the environmental-climatic conditions, the degree of ripeness of the fruit, the method of collecting the fruit, and the type of processing applied during production [21–23]. The greatest influence seems to be exerted by the ripening stage of the olive fruit during the harvest but also by the cultivated olive variety due to the sequence of specific chemo-types [23,24]. Thus, the qualitative and quantitative compositions of phenolic components are greatly influenced by many variables related to production processes, the maturation stage and storage conditions [25].

Finally, the total content of bioactive phenols in the extra virgin olive oil shows a wide range between 60 and 1.200 mg/kg, depending on the factors mentioned above [26,27].

The purpose of this study is to evaluate the key quality characteristics of olive oil with a main focus on the total bioactive phenolic content and the existence of a health claim. In addition, through this research, we want to focus on the contribution of the biophenolic content to the various quality characteristics of olive oil, its quantitative influence from various parameters, and also the beneficial effects on health through the consumption of olive oil with a health claim.

2. Materials and Methods

2.1. Olive Oil Samples

The 90 samples analyzed during this research were mostly extra virgin (EVOO) and virgin (VOO) olive oils from different Greek regions, which came from olive trees of different varieties. After production, which took place between November 2019 and January 2020, the samples were collected and stored in dark hermetically sealed bottles at room temperature until the analyses were completed, as exposure to oxygen and light leads to oxidation of the oil and therefore affects the results. The analysis of the samples took place shortly after their production and collection. After the initial evaluation, in terms of their basic quality characteristics, the biophenols were determined according to the official method of the International Olive Council, using the analytical HPLC technique, in order to find existing health claims.

The parameters that were known for the origin of each olive oil sample were the following:

- Olive variety (Koroneiki, Manaki, and Thasitiki);
- Cultivation area (prefectures of Aitoloakarnania, Zakynthos, Argolida, Dodecanese, Kavala and Heraklion);
- Olive fruit extraction system in the mill (two-phase system; three-phase system);
- Type of cultivation (organic; conventional);
- Altitude location of cultivation (ountainous; semi-mountainous; lowland);
- The time interval between harvest and oiling in days (0, 1, 2, 3, and 4).

2.2. Chemical Analysis of Olive Oil

The quality control of the olive oil samples was conducted through the use of known chemical parameters, which are the free fatty acids, the peroxide value and the specific absorption constants in the UV (K_{232}, K_{270} and ΔK), according to the official methods of the International Olive Council, with the codes COI/T.20/Doc/N°34, N°35 and N°19, respectively [28].

The method used in the present study to determine the bioactive phenols of olive oil is based on the direct extraction of small polar phenolic compounds of olive oil using methanol/water (80/20, *V/V*) as solvent and the subsequent separation and quantification by HPLC with the aid of a UV diode array detector (DAD) at 280 nm and using syringic acid as an internal standard. The content fraction of natural and oxidized oleuropein-ligstroside derivatives, lignans, flavonoids, tyrosol, hydroxytyrosol and phenolic acids is expressed as mg of hydroxytyrosol and its derivatives per 20 g of olive oil. This determination was made in accordance with the official method of the International Olive Council, with the code COI/T.20/Doc/N°29 [28]. All reagents and standards used in the experimental procedure were of high purity (HPLC grade). In addition, the main parts of the used HPLC (SHIMADZU) were a Shimadzu DGU-20A5R Degasser, a Shimadzu LC-20AD Prominence HPLC Pump and a Shimadzu SPD-M20A HPLC Photodiode Array Detector (PDA). Finally, the HPLC was equipped with a C18 reverse phase column (Thermo SCIENTIFIC, ODS HYPERSIL, Dim. 4.6 mm × 25 cm, 5 μm).

3. Results

According to the results of the initial quality control of the samples from the assays for the determination of free fatty acids, the peroxide value and the specific absorption

constants in the UV (K_{232}, K_{270} and ΔK), the samples were divided into the qualitative categories EVOO (n = 59), VOO (n = 29) and LOO (lampante olive oil) (n = 2) according to Annex 1 of the Commission Regulation (EEC) No. 2568/91. The results for free fatty acids played an important role in the separation of the samples in these three categories, since the results of the peroxide value (p.v.) and the specific absorption constants in the UV (K_{232}, K_{270}, and ΔK) did not show significant differences and were in accordance with those of extra virgin olive oils, i.e., p.v. $\leq$ 20 mEq O2/kg and ($K_{232} \leq 2.50$, $K_{270} \leq 0.22$, and $\Delta K \leq 0.01$).

The results of the analyses submitted for the 90 olive oil samples and mainly the found concentrations of biophenols were compared with the various parameters that were known for each olive oil sample, which were mentioned in the previous section. This took place to determine whether the concentration of the bioactive phenolic components of olive oil is affected by these parameters.

In addition, as mentioned above, according to European Regulation 432/2012, an olive oil can be marked with "Health Claim" if it has a high content of biophenols, with at least 250 mg of hydroxytyrosol and its derivatives per 1 kg of olive oil. Thus, the samples found to have a concentration of biophenols greater than or equal to 5 mg of hydroxytyrosol and its derivatives per 20 g of olive oil were determined to be positive in the health claim, while those that were less than 5 were determined to be negative in the health claim.

3.1. Comparison of Concentrations of Biophenolic Components of the Samples with the Various Parameters

According to the results, 60 of the 90 olive oil samples analyzed may have a health claim on their label, having more than 250 ppm of biophenols. Hence, we have a success rate of 66.7% in the health claim, which is very positive for the olive oils of Greece. It is very important to emphasize that out of the 60 samples with health claims, 37 were of the Koroneiki variety, 21 of the Manaki variety and only 2 of the Thasitiki variety. This can be seen in Figure 1 below, where Thasitiki has the lowest biophenol values, with the majority (16 out of 18 samples) being lower than 5 mg biophenols/20 g olive oil. On the contrary, the Koroneiki and Manaki varieties are close to the results with a high success rate, since Koroneiki has 37 samples with health claims out of 46 in total (80.4%) and Manaki 21 out of 26 samples (80.8%). Between these two varieties, Koroneiki has on average higher values and three samples that show very high values, two between 750 and 1000 ppm and one close to 1250 ppm.

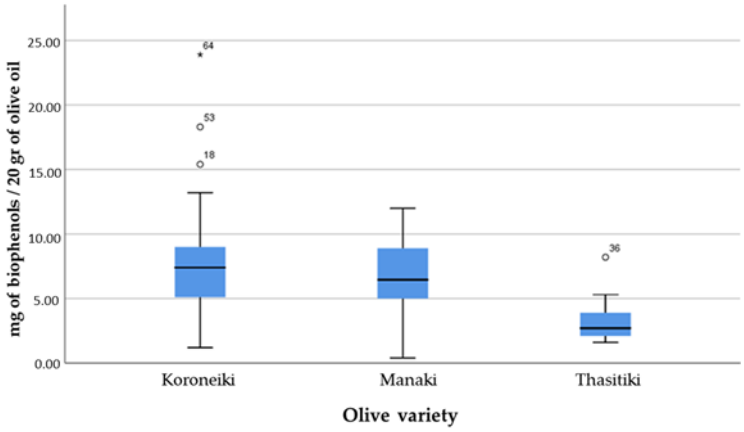


Figure 1. Comparison of biophenol concentrations with the olive variety.

Regarding the cultivation area, it is shown in Figure 2 that the prefectures of Kavala (Thasitiki) and Heraklion (Koroneiki) have the lowest concentrations of biophenols (<250 ppm). They are followed by Argolida (Manaki) and the Dodecanese (Koroneiki) with very good

values (higher than 250 ppm), Aitoloakarnania (Koroneiki) with slightly higher prices than the previous two and finally Zakynthos, where all three samples had very high concentrations.

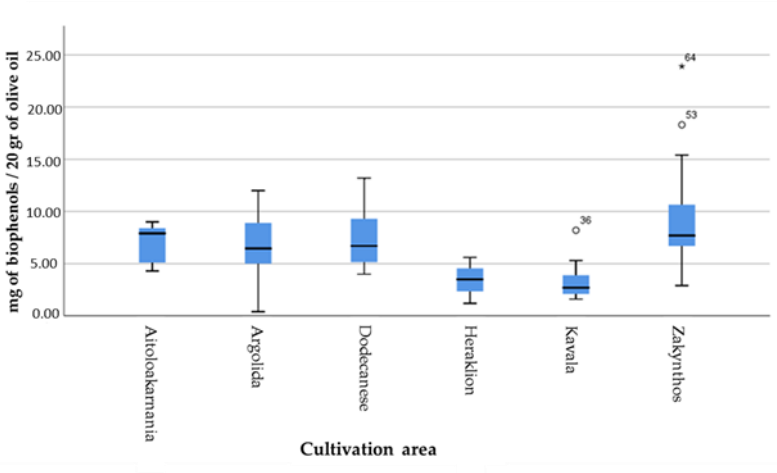


Figure 2. Comparison of biophenol concentrations with the area of olive cultivation.

Of the four prefectures with the Koroneiki variety, the success rate in the health claim in each prefecture, i.e., samples with a biophenol concentration greater than or equal to 250 ppm, is as follows:

- Heraklion 33.3%;
- Aitoloakarnania 77.8%;
- Dodecanese 80.0%;
- Zakynthos 89.5%.

From Figure 3, we conclude that the three-phase mills have a slightly higher success rate in the health claim, but the two-phase mills generally have higher values, with the three samples exceeding 750 ppm.

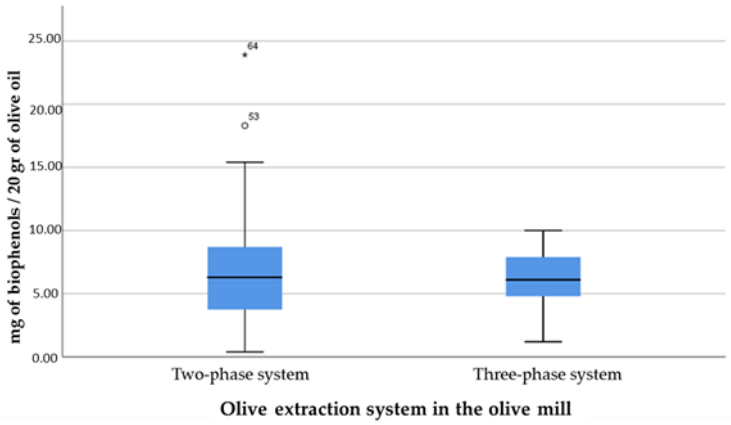


Figure 3. Comparison of biophenol concentrations with the extraction system in the mill.

In the above two figures (Figures 4 and 5), where the amount of bioactive phenols is compared with the type of cultivation and with its altitude, we see that organic cultivations also have a higher success rate in terms of health claim and higher concentration values on average. Comparing lowland and semi-mountainous locations, the lowland region is superior due to it having the largest percentage of existing health claims, with higher values of concentrations. In Figure 5, the sample with the highest concentration is presented, close to 1250 ppm, where the olive cultivation is located in a mountainous location. This finding is important for further study in future surveys, but it is not statistically acceptable because it is only one sample and it may be a random result.

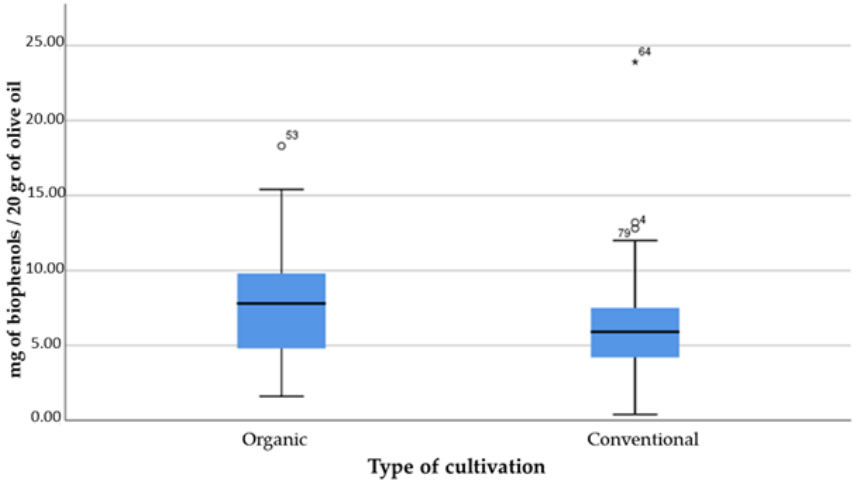


Figure 4. Comparison of biophenol concentrations by the type of cultivation.

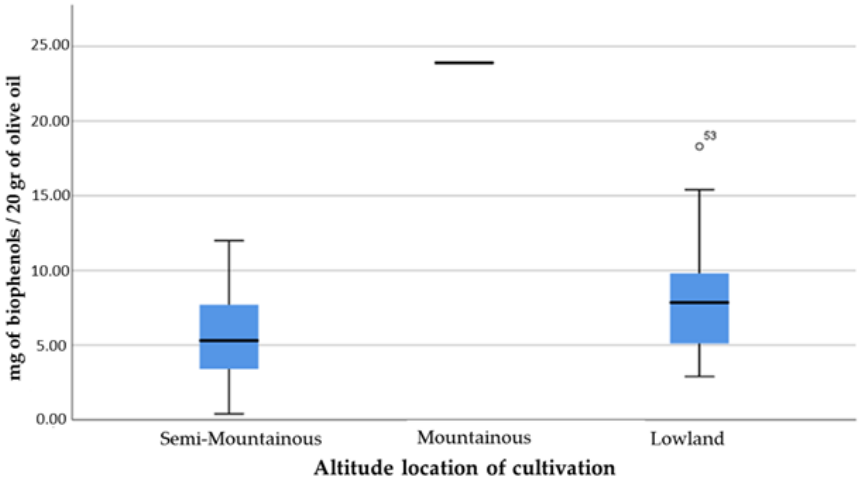


Figure 5. Comparison of biophenol concentrations by the altitude location of cultivation.

Finally, from Figure 6, we see that the time period from the harvest of the fruit to its oiling is important, since as this period increases, the concentrations of bioactive substances and the percentage of samples with a health claim decrease. Therefore, over 0, 1 and 2 days, we saw a decrease in concentrations as the days went by and a negative effect on the health claim at 3 and 4 days, as all samples, except one in 3 days, are below 250 ppm. It is very important to emphasize that the three samples with the very high concentrations were found at 0 and 1 days.

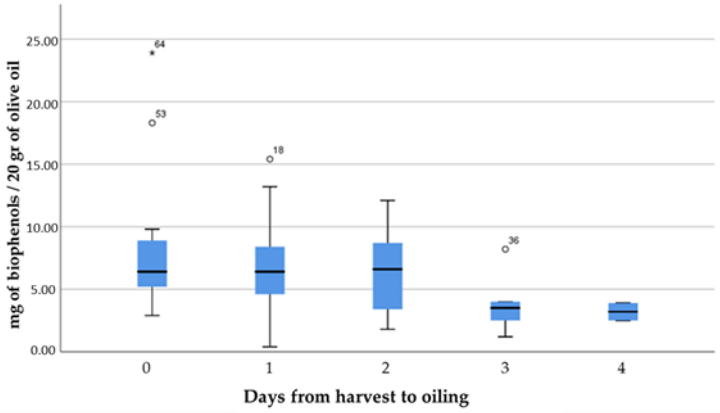


Figure 6. Comparison of biophenol concentrations with the time interval between harvest and oiling in days.

3.2. Comparison of Some Quality Characteristics of Olive Oil with the Results for the Health Claim

To examine any existing differences in the quality characteristics of the olive oil samples that showed a positive result in the health claim with those that were negative, we compared them with the results of the health claim.

Of the 59 EVOOs, 45 were positive in the health claim (76.3%). Of the 29 VOOs, 15 had a health claim (51.7%). Of the 2 LOO, none had a health claim (0%). The qualitative characteristic that contributed to the separation of the samples into the three categories and appeared to show differences in the olive oils that had a health claim and in those that did not was the % free acidity.

Figure 7 shows two diagrams, where on the right the % free acidity is compared with the presence or absence of a health claim in the samples, while on the left the peroxide value is compared with the same factor. What is observed is that the peroxide value does not show significant differences. On the contrary, the % free acidity shows significant differences, with the positive samples in the health claim being at lower values and the majority of them having % free acidity <0.8%. The olive oils that did not show health claims had quite high values of % free acidity and they were not classified as extra virgin olive oils. Finally, it is important to mention that several samples with low values of % free acidity (<0.8%) did not show a health claim since their content of phenolic compounds was slightly less than 5 mg per 20 g of oil.

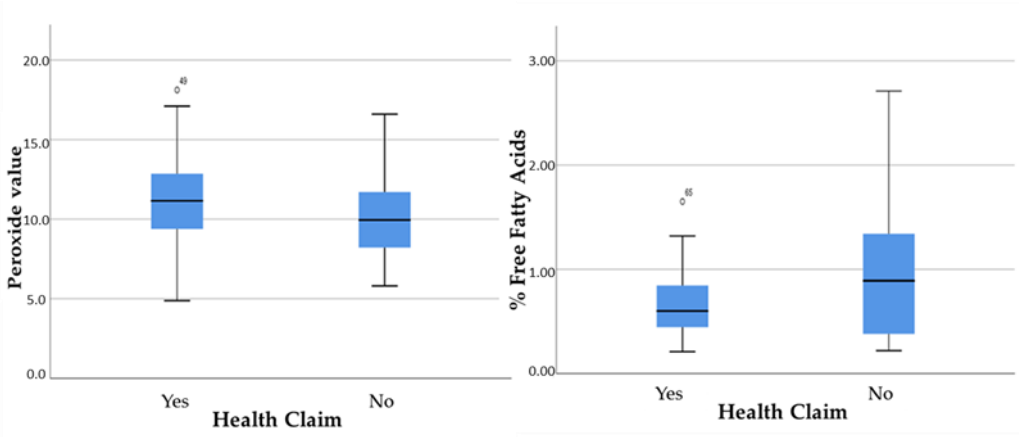


Figure 7. Comparison of the % free acidity (**right**) and the peroxide value (**left**) with the results for the health claim.

4. Discussion

4.1. Summary of Results

Taking into account all the information of the previous sections, the beneficial role of the bioactive phenolic components of olive oil in health is now scientifically accepted. In addition, the existence of scientifically substantiated opinions of the European Food Safety Authority, which concerns the possibility of labeling health claims on the olive oil label, is now well established. This directive is based on the correlation between the intake of extra virgin olive oil and the prevention and control of a variety of pathological conditions and diseases such as cardiovascular disease, cancer and neurodegenerative diseases.

According to the results of this study in addition to the findings of other studies, it seems that the effect of variety, area, altitude of cultivation, method of extraction, cultivation technique and direct oiling of olives and other factors is very important and largely determines the quality characteristics and the content of biophenols of the olive oil produced.

The total content of biophenols in extra virgin olive oil varies widely, depending on both the factors already mentioned and the analytical technique used. Table 1 presents references to recent research, which mainly examine the content of biophenols in olive oils based on health claim legislation, with various variations on the analytical techniques applied.

Table 1. References to similar studies on biophenols and health claim.

N	Sample	*n*	Country	Method	Purpose	Compound	Statistical Analysis	Ref.
1	EVOO	12	Italy Spain	FC HPLC	Comparison of the effectiveness of analysis methods for the determination of olive oil biophenols based on EU Regulation 432/2012.	Biophenols	*t*-test	[29]
2	EVOO	108	Italy	HPLC-DAD	Comparison of hydroxytyrosol and tyrosol levels based on EFSA health claim, from variations in acid hydrolysis.	Biophenols	ANOVA *F*-test	[30]
3	EVOO OO	25	Italy	(HPLC-DAD-ESI/MS) HPLC-DAD FC	Development of a method for the determination of polyphenols and comparison of its results with those of the IOC and FC method.	Biophenols	ANOVA	[31]
4	EVOO	284	Italy	HPLC-DAD FC	Comparison of biophenolic contents in PDO and non-PDO olive oils based on the regulation on the EU health claim 432/2012.	Biophenols	*t*-test	[32]

Therefore, in order to ensure high-quality olive oil with high concentrations of biophenols and with a health claim, strict controls are recommended at all stages of the olive production process. It is important to mention that during the various stages of oiling, large amounts of waste are generated, which are associated with the loss of the polyphenolic components of olives and olive oil.

In conclusion, according to all the results of the analytical methods applied, for the total 90 samples of olive oil examined, the following findings are summarized:

- In terms of variety, Koroneiki showed the lowest levels of free fatty acids and the highest concentrations of biophenolic components, presenting a plethora of extra virgin olive oils with the health claim. This was followed by the Manaki variety with quite good results, while the Thasitiki had high levels of free fatty acids and 88.9% failure in the health claim; however, it had the lowest peroxide value;

- In Argolida, we only had samples from Manaki and in Kavala from Thasitiki. Thus, from the other areas with the Koroneiki variety, Zakynthos showed the best results and the highest percentage in the health claim, followed by Aitoloakarnania;
- None of the quality characteristics were significantly affected by the way the olive oil was produced in the mill and the extraction system. However, the two-phase system had slightly higher concentrations of phenolic components and three very high values. It should be noted that in a two-phase system, all the samples of the Thasitiki variety significantly lowered the average value;
- Based on the cultivation technique, the olives from organic olive cultivations had lower values of % free acidity, higher concentrations of phenolic compounds and a higher percentage of olive oils with health claims compared to conventional cultivations. The same applies to the lowland region in relation to the semi-mountainous location of the cultivations;
- A very important factor for the quality of olive oils, as the results showed, is the interval between the harvest of the olive fruit and its oiling. The shorter this period, the better the quality of the oil. After the intervals of 0 and 1 day, the lowest values of % free acidity and the highest concentrations of bioactive phenolic components appear, while at intervals of 3 and 4 days these characteristics change dramatically in the opposite;
- Finally, a general observation is that the number of peroxides did not appear to be affected by the changing factors, except for the variety. In contrast, the content of bioactive phenolic compounds and the values of % free acidity are equally affected by all parameters.

4.2. Future Suggestions and Prospects

Regarding the experimental framework and based on the experience gained from the present study, there are some specific proposals that may be included as basic prerequisites when preparing a new experimental design in this field.

Initially, it is recommended to focus on the Koroneiki and Manaki varieties. On the one hand, they are predominantly cultivated varieties in Greece and on the other hand, as in previous studies, their higher content of phenolic components was confirmed, as well as their lower values of free acidity, which indicates that these varieties produce extra virgin olive oils of the highest quality. Of course, it would be very important to investigate other varieties that are widely grown in Greece in a representative portion of samples so that the results are more accurate and statistically acceptable.

In addition, to what extent each factor affects each of the quality characteristics of olive oil and especially the phenolic content depends on many parameters. Therefore, research must be conducted where each time only the variable in question changes, while the other factors (variety, area, altitude, cultivation technique, etc.) remained constant. However, there must be a significant number of samples for each variable in order to produce accurate results.

Regarding the selected methods of analysis and their experimental conditions, it is proposed to introduce them in most cases as they showed very positive responses with quite high repeatability.

Additionally, the change in the results of the total phenolic components and consequently the validity of the health claim in the olive oils could be investigated, in a reasonable period of time (6 months; 12 months), in different storage conditions (light, temperature, and humidity) and processing (cooking).

Finally, it would be interesting to observe the change in all the quality characteristics of olive oil over time in various storage and/or processing conditions, and between olive oils with high phenolic content, i.e., with a health claim and without.

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