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Total Antioxidant Capacity, Total Phenolic Content and In Vitro Predicted Bioavailability of Olive Oil Fortified with Herbs and Waste By-Products, towards Sustainable Development

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Abstract: Nowadays, the high demand for healthy foods and sustainable products, has led the food industry to explore innovative food technologies, such as fortification with bioactive compounds like antioxidants and polyphenols, that may be sourced from herbs or by-products. The aim of the present study was to explore the enrichment of refined olive oils with natural bioactive compounds such as the herbs rosemary (*salvia rosmarinus*), basil (*ocimum basil*), sage (*salvia officinalis*), lemon balm (*melissa oficinallis*), st. john's wort (*hypericum perforatum*), pink savory (*Thymbra satureja),* dittany (*origanum dictamnus*), and by-products such as pomace from olives, olive leaves (*olea europaea* tree), orange peel (*citrus sinensis)*, lemon peel (*citrus limon*), pomegranate peel (*punica granatum*) and mandarin peel (*citrus reticulate*). The fortification of the refined olive oils was performed with the use of different methodologies such as Conventional maceration (CM), Incubation shaking maceration (ISM), and Ultrasound assisted maceration (UAM). Their phenolic content and antioxidant capacity were measured with Folin–Ciocalteu assay and Ferric Reducing Antioxidant Power (FRAP) assay respectively. All methods demonstrated that different parameters such as time of maceration, temperature and sample concentration, play an important role in the fortification process of the refined olive oils. The predicyed bioavailability of the antioxidant and phenolic compounds in the fortified oils was determined with in vitro digestion and ranged from 4.84% to 53.11%. Furthermore, the refined olive oils fortified with pomace, basil, st. john's wort, and pomegranate peel presented the highest antioxidant and phenolic predicted bioavailability indices during the in vitro process compared to the control refined olive oil. Finally, fortification with natural herbs or by-products can be considered an innovative method for the improvement of the nutritional value of refined olive oils.

Keywords: refined olive oil fortification; herbs; plant by-products; conventional maceration (CM); incubation shaking maceration (ISM); ultrasound assisted maceration (UAM); in vitro predicted bioavailability

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1. Introduction

The concept of 'Mediterranean diet' was first studied by A. Keys and F. Grande as the traditional dietary pattern found in olive-growing areas of Crete, Greece and Southern Italy in the late 1950s and early 1960s [1]. General descriptions of Mediterranean diet (MedDiet) are similar amongst studies, emphasizing at key components like olive oil [2]. Olive oil (OO), a vegetable liquid fat obtained from olives (the fruit of *Olea europaea*), is MedDiet's principle source of fat, while it is characterized by a high content of monounsaturated fatty acids [3]. In its unsaponifiable fraction it contains a variety of bioactive compounds, such as antioxidants, associated with its organoleptic characteristics and several health benefits [4].

The European Regulation of 29/2012 classifies OO in three market categories: (i) extra virgin olive oil (EVOO) defined as the "superior category olive oil obtained directly from olives and solely by mechanical means"; (ii) virgin olive oil (VOO) which is "olive oil obtained directly from olives and solely by mechanical means"; and (iii) olive oil, composed of refined olive oils (ROO) and virgin olive oils and it is "oil comprising exclusively olive oils that have undergone refining and oils obtained directly from olives" [5]. Due to the different treatment methods of olive oils, quality characteristics vary between categories [6], while refined olive oil lacks optimal taste, aroma, and natural antioxidants, compared to extra virgin and virgin olive oil [7]

Most of the world's olive oil is produced in Mediterranean countries. However, over 20% of this production is of such poor quality, that has to become refined in order to be fit the regulatory assumptions for human consumption [8]. The primary reason that extensive amounts of olive oil undergo the refining process, is the low quality of the original olive fruit [9]. Nevertheless, the refining process eliminates antioxidant compounds that exist in higher-quality olive oils, and therefore it limits the antioxidant effect of the final product [10]. Moreover, the lack of antioxidant components, like phenolic compounds, has negative effects on the oxidative stability of the final product [11]. Therefore, a fortification of refined olive oil with antioxidants may present a research interest.

Food fortification is a method to improve the nutritional status of food products and, consequently, a way to manage micronutrient deficiencies in the general population [12]. Food fortification leads to functional food products, while their consumption is related to the promotion of human health [13]. Dietary surveys indicate that functional foods play a role in mitigating risks related to the lack of several important nutrients; however, the number of foods suitable for fortification is considerably limited by several factors, including technological properties, leading to unacceptable taste and appearance, cost, and consumer expectations [14]. In addition, consumer acceptance of functional foods is linked to the consumer's knowledge of the health effects of specific ingredients, while the role of healthiness in the food choice is continuously increasing [15].

Many natural antioxidants are derived from plant materials, such as fruits, vegetables, herbs and spices [16]. Fruits and vegetables are ranked in the options of health-conscious consumers and represent a prominent segment in the functional and nutritional food sector [17]. In recent years has been proposed the term "fruit and vegetable waste" (FVW) which is defined as an indigestible part of the produce that is thrown away at a certain point, for example, during handling, collection, processing, or shipping [18]. Recent studies report that waste peels generated through fruit and vegetable processing are to be recognized as specialized residues owing to their high levels of residual bioactive compounds like phenols, tannins and phytochemicals [19]. FVWs can therefore be used to extract and isolate potential bioactive compounds that can be used in the food industry to enrich conventional foods and develop innovative food products [20,21].

Other antioxidant sources may be herbal plant parts (roots, leaves, branches/stems, bark, and flowers), which are commonly rich in terpenes (carvacrol, citral, linalool, and geraniol) and phenolics (flavonoids and phenolic acids). These compounds can also be effective as food additives [22]. Herbal extracts, have been demonstrated to be excellent sources of natural antioxidant molecules, but with more limited ranges of applications due to their strong flavor characteristics [23]. Their application in the food industry is steadily increasing, and finding better ways of isolating and incorporating bioactive compounds from herbal extracts is part of ongoing research [24].

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The aim of the present study was to investigate the effect of different methods for the fortification of refined olive oils with herbs and by-products in order to increase their content in antioxidants and polyphenols. More specifically, rosemary, basil, sage, lemon balm, st. john's wort, pink savory, dittany, pomace, olive leaves, orange peel, lemon peel, pomegranate peel and mandarin peel were used for the fortification of the refined olive oils. The enhanced olive oil extracts were obtained by ultrasound-assisted maceration, incubator-assisted maceration and conventional maceration and evaluated for their total antioxidant activity and total phenolic content. The predicted bioavailability of their bioactive compounds was also determined after performing in vitro digestion process.

2. Materials and Methods

2.1. Chemicals and reagents

All chemicals were attained from Sigma-Aldrich (St. Louis, MO, USA) and Merck Chemicals (Darmstadt, Germany).

2.2. Sample collection and preparation

Herbs and plant by-products were collected from Lemnos Island, in the North Aegean, Greece, between November of 2021 and February of 2022. The samples included: rosemary (*salvia rosmarinus*), basil (*ocimum basilicum*), sage (*salvia officinalis*), lemon balm (*melissa oficinallis*), st. John's Wort (*hypericum perforatum*), pink savory (*thymbra satureja*), dittany (*origanum dictamnus*), pomace from olives, olive leaves (*olea europaea* tree), orange peel (*citrus sinensis*), lemon peel (*citrus limon*), pomegranate peel (*punica granatum*), mandarin peel (citrus reticulate). All collected samples were dried in a drying heating oven (Binder GmbH, Tuttlingen, Germany) at 60°C for 48h and stored in sealed bags at dark conditions till further analysis.

For the fortification of the refined olive oil, refined olive oil was purchased from a local certified producer from Lemnos, Greece (Sousalis, Lemnos, Greece). Extra virgin and virgin olive oil that was used for the phenolic content analysis was purchased also from the same producer from Lesvos, Greece (AES Stypsis, Lesvos, Greece).

Three different methods, as described in Table 1, were used for the fortification of the refined olive oil: conventional maceration (CM), incubator shaking maceration (ISM) and ultrasound-assisted maceration (UAM).

Table 1. Methodology used for the olive oil fortification.

Methods for Oil Aromatization	Temperature(°C)	Duration (min/hours)/days)	Food Mass of Herbs and By-Products (g)
Conventional maceration (CM)	37°C	15days/30days	2.5g/5g
Incubator shaking maceration (ISM)	37°C	1h/2h/3h	1g/2g/3g
Ultrasound assisted maceration (UAM)	30/40°C	30 min/60min	1.5g/3g

2.2.1. Conventional Maceration (CM)

Conventional maceration was performed according to the method reported by Caporaso et al. (2013), with some modifications regarding the quantity of the herbs and maceration time [26]. Samples were prepared in 250 mL Erlenmeyer flasks using either 2.5g or 5g of the dried sample in 30 g of refined olive oil. They were maintained at 15 and 30 days of maceration, in the dark, at room temperature (20 ± 2 °C). Then, the samples were filtered, and 2 g of enriched olive oil was extracted and analyzed the same day.

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2.2.2. Incubator Shaking Maceration (ISM)

The incubator shaking maceration process was performed according to Karoui et al., (2010) methodology with some modifications. These referred to the quantity of the herbs, the incubation time and the temperature of the method [27]. Dried samples of 1g, 2g, or 3g were used for the fortification of 30 g of refined olive oil in glass duran bottles. Each bottle was then placed in the incubator (SKI-4, P.R.C.), and the temperature was set at 37°C. Each bottle remained in the incubator for either 1 hour, 2 hours or 3 hours. Then, each of the above samples was filtered and analyzed in duplicate the same day.

2.2.3. Ultrasound Assisted Maceration (UAM)

Samples were prepared in 250 mL glass bottles using either 1.5g or 3g of the dried samples per 30 g of refined olive oil. Each bottle was then placed in an ultrasound water bath (Elmasonic P 70 H, Elma-Hans Schmidbauer GmbH & Co. Singen, Germany) for 30 or 60 minutes at 30°C or 40°C. Each extraction process was performed in duplicate. All of the above produced samples were then filtered and analyzed on the same day.

2.3. Sample Extract Analysis

2.3.1. Preparation of sample for antioxidant and phenolic analysis

The polar fraction of the sample extracts was prepared according to Soares et al. (2020) & Nakbi et al. (2010) with some modifications [26,29]. Briefly, 5mL of methanol/water (40:10 v/v) were added to 2g of oil sample and vortexed for 1min. The mixtures were then placed in the ultrasound water bath (Elma Elmasonic P 70H Type Elma-Hans Schmidbauer GmbH & Co., Singen, Germany) for 15 minutes at room temperature. The samples were centrifuged for 25 minutes at 4.000g (OHAUS model: FC5718R, Germany).

2.3.2. Total phenolic content by Folin-Ciocalteau assay

The total phenolic content of the above prepared samples was determined by the Folin-Ciocalteau method. This assay is calculating the reductive capacity of the Folin-Ciocalteau reagent. 100μL of Folin-Ciocalteau reagent and 20μL of the extracted oil were placed in 96-well plates and the absorbance was measured at 765nm with a spectrophotometer (SPARK, TECAN, Switzerland) [30]. A standard gallic acid (GAE) curve was used to describe the total phenolic content and the results were expressed in mg GAE per L of dried food sample and performed in triplicate. All chemicals were purchased from Sigma-Aldrich.

2.3.3. Total antioxidant activity by Ferric reducing antioxidant power assay

The total antioxidant capacity of olive extracts was measured by the ferric reducing antioxidant power (FRAP) assay [31–33]. The FRAP method is based on the alteration of the TPTZ-Fe+3 to TPTZ-Fe2+. 50μL of Fe2+, 20μL of TPTZ solution, and 20μL of sample extract were placed in 96-well plates. The absorbance is measured at 595nm with a spectrophotometer (SPARK, TECAN, Switzerland). The total antioxidant capacity was determined with the use of a standard FeSO4 curve, and the results were expressed in mmol of Fe2+ per L of sample extract in triplicate. All chemicals were purchased from Sigma-Aldrich.

2.4. In vitro digestion analysis

The in vitro gastrointestinal assay was used to stimulate the gastrointestinal digestion process and to estimate the predicted bioavailability of antioxidants and polyphenols. The methodology followed was as described by Kapsokefalou et al. (2006) with some modifications [33]. In more detail, 2mL from each extract was added into 6-well plates and mixed with 0.1mL of human pepsin. The samples were placed in a shaking incubator (Shaking Incubator SKI-4, P.R.C.) for 2 hours in 37°C. At the end of the incubation a dialysis membrane was added to each well and piperazine-N,N′-bis(2-

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ethanesulfonic acid) (PIPES) buffer reagent and the pH was adjusted to 6.3. Then the samples were allocated in the shaking incubator (1h, 37oC) and a mixture of pancreatin and bile salts (0.5mL) was added in each well and 2h of incubation was performed at 37oC. The supernatant (fraction above the dialysis membrane) was centrifuged at 5000g for 15 minutes at 4oC. The antioxidant capacity and total phenolic content were estimated by performing FRAP and Folin-Ciocalteau assays as described above.

2.5. Statistical Analysis

Statistical analysis was carried out using SPSS 21.0 software (SPSS Inc, Chicago, IL, USA). Shapiro-Wilk test was performed to check for normality in continuous variables with a $p<0.05$. The total antioxidant capacity and total phenolic content of the fortified olive oil samples are expressed as mean ± standard deviation (SD). A three-way factorial ANOVA was performed to investigate the differences between the different maceration conditions in the phenolic and antioxidant content of the enriched refined olive oils for each method. A least significant difference (LSD) test was completed to detect significant differences between the samples ($p<0.05$). Pearson's correlation test was conducted to investigate correlations of antioxidant capacity and polyphenols before and after in vitro digestion process.

3. Results

3.1. Refined Olive Oil

In the following table (Table 2), the total phenolic content and the total antioxidant capacity of three selected olive oils (virgin, extra virgin and refined) are presented.

Table 2. Total phenolic content and total antioxidant capacity of virgin, extra virgin and refined olive oil.

	Total Phenolic Content	Total Antioxidant Capacity
Olive Oil Type	**Folin-Ciocalteau (mg GAE/L)**	**Frap (mmol Fe2+/L)**
Refined Olive Oil (ROO)	10.83 ± 1.36a	0.20 ± 0.03a
Virgin Olive Oil (VOO)	15.34 ± 3.14b	0.47 ± 0.05b
Extra Virgin Olive Oil (EVOO)	20.47 ± 2.87c	0.50 ± 0.09c

*Data are presented as mean ±SD *Different letters in each column indicates statistically significant differences ($p<0,05$).*

Extra virgin olive oil presented the highest values of total antioxidant capacity, and total phenolic content followed by virgin olive oil, while refined olive oil appeared the lowest values (*$p<0.05$*).

3.2. Conventional Maceration (CM)

The total phenolic content and the total antioxidant capacity of the fortified refined olive oils using conventional maceration at 15 and 30-day periods in two different quantities (2.5 g and 5 g) are presented in Tables 3 and 4.

Table 3. Total phenolic content of fortified refined olive oils using conventional maceration (CM).

	Total Phenolic Content (mg GAE/L)					
Food Sample	**CM (15 Days)**		**CM (30 Days)**		**P_1**	**P_2**
	2.5 g	**5 g**	**2.5 g**	**5 g**		
			Herbs			
Rosemary	38.55 ± 8.91	35.91 ± 2.52	21.37 ± 9.98	29.84 ± 5.66	*	NS

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Basil	21.05 ± 6.54	17.88 ± 1.86	19.24 ± 3.92	22.49 ± 11.97	NS	NS
Sage	24.42 ± 6.76	35.15 ± 2.87	35.00 ± 9.07	47.34 ± 6.14	*	*
Lemon Balm	15.55 ± 1.39	19.80 ± 0.15	18.67 ± 2.19	22.92 ± 3.84	NS	NS
St. John's Wort	36.21 ± 3.33	43.53 ± 4.10	22.59 ± 7.84	36.63 ± 5.72	NS	*
Pink Savory	50.82 ± 2.97	55.06 ± 8.99	44.60 ± 11.39	49.97 ± 6.10	NS	NS
Dittany	22.96 ± 1.17	27.64 ± 1.52	34.52 ± 6.00	37.83 ± 8.44	*	NS
By-products						
Pomace	13.13 ± 3.84	16.82 ± 5.85	14.14 ± 2.74	14.84 ± 1.79	NS	NS
Olive Leaves	36.57 ± 5.18	34.81 ± 13.64	21.82 ± 8.33	26.15 ± 12.48	**	NS
Orange Peel	27.36 ± 10.08	40.07 ± 3.86	26.15 ±11.45	37.25 ± 25.93	NS	**
Lemon Peel	44.40 ± 6.55	35.39 ± 4.78	28.72 ± 10.66	16.50 ± 3.38	**	*
Pomegranate Peel	16.57 ± 1.03	15.49 ± 2.41	16.96 ± 1.27	18.16 ± 1.81	NS	NS
Mandarin Peel	34.77 ± 7.13	36.28 ± 2.46	17.97 ± 6.60	31.42 ± 27.24	NS	*

*Data are expressed as mean ± SD. P_1: statistical differences between samples prepared with conventional maceration (CM) for 15- and 30-day period. P_2: statistical differences between samples of different mass macerated for the same day period. Significance level *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, NS: non-significant ($p>0.05$)*

All fortified refined olive oils that resulted from the conventional maceration showed an increased phenolic content in comparison with the non-fortified refined olive oil which was used as a control, with the average values ranging from 13.13 ± 3.84 mg GAE/L to 55.06 ± 8.99 mg GAE/L. The highest phenolic content in all conditions for the CM was demonstrated by the fortified refined olive oil with pink savory; values ranging from 44.60 ± 11.39 mg GAE/L to 55.06 ± 8.99 mg GAE/L, with the highest value at 5 g concentration of herb for the 15th day period. The lowest content was observed in pomace, ranging from 13.13 ± 3.84 mg GAE/L to 16.82 ± 5.85 mg GAE/L with the lowest value at 2.5 g concentration for the 15-day period. The fortified refined olive oils that showed significant differences in the phenolic content compared to either the 15th day and/or the 30th day are the following: rosemary, sage, lemon peel, dittany, and olive leaves ($p<0.05$), compared to the rest of the fortified olive oils. Specifically, the refined olive oils enriched with waste by-products showed a decrease in the phenolic content except of pomegranate when analyzed on the 30th day (16.96 ± 1.27, and 18.16±1.81 mg GAE/L) instead of the 15th days (16.57±1.03, and 15.49 ±2.41 mg GAE/L).

Table 4. Total antioxidant capacity of fortified refined olive oils using Conventional Maceration (CM).

Food Sample	Total Antioxidant Capacity (mmol Fe2+/L)				P_1	P_2
	CM (15 Days)		CM (30 Days)			
	2.5 g	5 g	2.5 g	5 g		
Herbs						
Rosemary	0.39 ± 0.05	0.40 ± 0.02	0.36 ± 0.01	0.38 ± 0.03	NS	NS
Basil	0.31 ± 0.01	0.35 ± 0.01	0.38 ± 0.01	0.38 ± 0.01	*	NS
Sage	0.89 ± 0.30	1.54 ± 0.07	1.05 ± 0.25	1.63 ± 0.07	***	***
Lemon Balm	0.33 ± 0.02	0.40 ± 0.02	0.41 ± 0.01	0.54 ± 0.02	***	***
St. John's Wort	0.38 ± 0.02	0.39 ± 0.02	0.33 ± 0.05	0.34 ± 0.01	*	NS
Pink Savory	0.56 ± 0.02	0.74 ± 0.03	0.52 ± 0.05	0.68 ± 0.07	*	***
Dittany	0.39 ± 0.02	0.44 ± 0.03	0.43 ± 0.01	0.51 ± 0.05	**	**
By - products						
Pomace	0.28 ± 0.03	0.28 ± 0.03	0.28 ± 0.05	0.31 ± 0.06	NS	NS
Olive Leaves	0.31 ± 0.05	0.32 ± 0.05	0.32 ± 0.02	0.31 ± 0.04	NS	NS
Orange Peel	0.35 ± 0.01	0.37 ± 0.02	0.34 ± 0.03	0.33 ± 0.01	NS	NS

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Lemon Peel	0.35 ± 0.03	0.35 ± 0.02	0.33 ± 0.01	0.32 ± 0.02	NS	NS
Pomegranate Peel	0.28 ± 0.01	0.28 ± 0.01	0.33 ± 0.04	0.35 ± 0.01	**	NS
Mandarin Peel	0.35 ± 0.02	0.34 ± 0.03	0.33 ± 0.24	0.29 ± 0.01	*	NS

*Data are expressed as mean ±SD. P_1: statistical differences between samples prepared with conventional maceration (CM) for 15 and 30 day periods. P_2 : statistical differences between samples of different mass macerated for the same day period. Significance level *** p < 0.001, ** p < 0.01, * p < 0.05, NS: non-significant (p>0.05).*

Table 4 summarizes the total antioxidant capacity values that resulted from the CM extraction and ranged from 0.28±0.01 mmol Fe2+/L to 1.63±0.07 mmol Fe2+/L. The highest total antioxidant values are consistently displayed by the refined olive oil fortified with sage; values ranging from 0.89±0.30 mmol Fe2+/L to 1.63±0.07 mmol Fe2+/L with the highest antioxidant capacity at 5 g concentration at the 30th day extraction period. Other samples with relatively high antioxidant values and significant differences between the day period of extraction are the following: Basil, sage, pomegranate peel, dittany, pink savory, and St. John's Wort ($p<0.05$). Alternatively, those fortified with pomace and olive leaves have relatively low total antioxidant content, with values generally below 0.35 ± 0.01 mmol Fe2+/L. The total antioxidant capacity of those fortified with rosemary, orange peel, lemon peel, olive leaves and pomace, showed non-significant changes ($p>0.05$) between the different conditions, while those enriched with lemon balm, dittany and sage, demonstrate a stronger correlation ($p<0.01$).

3.3. Incubation Shaking Maceration (ISM)

Different quantities (1, 2 and 3 g) of the 7 herbs and 6 plant by-products were used to fortify refined olive oils using an incubator shaking method for 3 different time durations (60, 120 and 180 minutes) in a temperature of 37°C. The total phenolic content of the enriched refined olive oils by incubation method is demonstrated in Table 5.

Table 5. Total phenolic content of fortified refined olive oils using Incubation Maceration.

Total Phenolic Content (mg GAE/L)												
Food Sample	**ISM (60 min)**			**ISM (120 min)**			**ISM (180 min)**			**P_1**	**P_2**	**P_3**
	1g	**2g**	**3g**	**1 g**	**2 g**	**3 g**	**1 g**	**2g**	**3g**			
Herbs												
Rosemary	13.61 ± 0.70	12.15 ± 1.01	13.30 ± 2.01	14.98 ± 1.39	15.29 ± 2.98	17.16 ± 2.19	12.81 ± 1.46	12.70 ± 0.60	14.58 ± 1.19	NS	NS	NS
Basil	15.54 ±1.48	12.83 ± 1.59	13.26 ± 1.74	15.64 ± 1.23	12.29 ± 0.63	16.36 ± 3.84	11.31 ± 0.78	13.13 ± 0.96	13.09 ± 1.30	NS	NS	NS
Sage	17.85 ± 3.07	21.81 ± 4.99	33.22 ± 17.41	17.25 ± 3.27	28.61± 4.00	32.51 ± 5.83	17.77 ± 3.32	25.22 ± 1.03	36.07 ± 4.35	NS	NS	NS
Lemon Balm	28.95 ± 7.10	27.59 ± 6.23	33.13 ± 4.44	27.65 ± 9.16	25.11± 2.79	27.70 ± 1.65	26.54 ± 7.10	28.98 ± 2.21	23.32 ± 1.21	NS	NS	NS
St. John's Wort	42.66 ± 12.85	34.28 ± 7.56	39.96 ± 3.79	31.91 ± 1.84	34.43± 1.57	27.92 ± 3.12	40.26 ± 12.85	36.55 ± 3.27	26.49 ± 1.46	**	NS	NS
Pink Savory	35.10 ± 25.07	23.64 ± 2.56	24.28 ± 2.98	19.70 ± 10.46	24.40± 1.92	27.30 ± 1.53	25.24 ± 18.74	31.46 ± 15.24	27.49 ± 2.03	NS	NS	NS
Dittany	26.75 ± 10.26	14.68 ± 2.01	23.71 ± 8.67	21.99 ± 15.34	23.08± 1.49	12.81 ± 0.98	39.49 ± 40.55	14.00 ± 7.90	13.08 ± 2.99	NS	NS	NS
By - Products												
Pomace	13.43 ± 1.83	11.67 ± 1.23	22.07 ± 18.79	18.01 ± 2.46	17.18± 2.08	16.54 ± 2.16	18.60 ± 4.53	16.94 ± 2.16	16.30 ± 0.89	NS	NS	NS
Olive Leaves	29.03 ± 4.46	14.71 ± 1.81	12.04 ± 1.14	17.37 ± 9.91	12.51± 1.96	13.31 ± 5.21	13.99 ± 2.85	20.12 ± 5.69	13.73 ± 1.81	NS	NS	NS
Orange Peel	16.35 ± 9.86	11.37 ± 1.01	10.68 ± 3.94	12.37 ± 1.05	17.01± 4.89	16.74 ± 5.44	13.58 ± 0.88	14.72 ± 1.49	13.72 ± 2.32	NS	NS	NS
Lemon Peel	9.94 ± 1.41	11.13 ± 0.41	13.18 ± 1.37	13.19 ± 1.80	11.59 ± 2.32	12.63 ± 0.75	13.27 ± 1.84	11.69 ± 1.08	10.77 ± 1.04	NS	NS	NS
Pomegranate Peel	9.99 ± 0.62	9.06 ± 1.14	13.20 ± 1.09	10.85 ± 0.95	10.84± 1.09	11.53 ± 1.33	13.09 ± 1.40	12.77 ± 0.64	15.59 ± 2.48	NS	NS	NS

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Mandarin Peel	22.35 ± 10.60	13.89 ± 2.47	23.74 ± 1.59	16.15 ± 1.01	13.05± 1.49	12.69 ± 1.37	23.51 ± 6.79	14.60 ± 3.03	16.17 ± 3.71	*	NS	NS

*Data are expressed as mean ± SD. P_1: statistical differences between samples prepared with 60 min versus 120 min of incubation maceration. P_2: statistical differences between samples prepared with 120 min versus 180 min of incubation maceration. P_3: statistical differences between samples prepared with 60 min versus 180 min of incubation maceration. Significance level *** p < 0.001, ** p < 0.01, * p < 0.05, NS: non-significant (p>0.05).*

From the 13 samples, the highest phenolic content was observed in the refined olive oil enriched with St. John's Wort ranging from 26.49±1.46 mg GAE/L to 42.66±12.85 mg GAE/L, while the lowest was demonstrated by the pomegranate peel and lemon peel enriched olive oils with values ranging from 9.06±1.14 mg GAE/L to 15.59±2.48 mg GAE/L and 9.94±1.41 mg GAE/L to 13.19±1.8 mg GAE/L, respectively.

The sample concentration did not significantly affect the total phenolic content for the following fortified olive oils: rosemary, Basil, orange peel, lemon peel, lemon balm, pomegranate, pink savory and pomace (p> 0.05). The total phenolic content of the refined olive oil fortified with sage showed a significant difference at 3 g (p < 0.01). The refined olive oil fortified with dittany demonstrated a significant difference between 1 and 2 g of the sample (p<0.001) and 2 to 3 g of the sample during the oil extraction (p<0.001). The refined olive oils fortified with St. John's Wort and Mandarin Peel presented high values of antioxidant capacity between 1 and 2 g of sample (p<0.05) while the refined oil enriched with olive leaves showed a significant difference at the concentration of 3 g (p<0.05).

In Table 6 the total antioxidant capacity of the fortified refined olive oils is displayed. Among the studied samples, the sage-enriched refined olive oil displayed the highest total antioxidant capacity with significant differences at all time points and concentrations with values ranging from 0.51±0.06 mmol Fe2+/L to 1.28±0.20 mmol Fe2+/L (p<0.05). Specifically, the highest value was observed at the 180 min duration and 3 g sample concentration (1.28±0.20 mmol Fe2+/L). The lowest total antioxidant value was presented in the olive oil enriched with mandarin at the sample concentration of 1 g at the 60 min duration with a value of 0.19±0.02 mmol Fe2+/L.

Table 6. Total antioxidant capacity of fortified refined olive oils using Incubation maceration.

Total Antioxidant Capacity (mmol Fe2+/L)												
Food Sample	**ISM (60 min)**			**ISM (120 min)**			**ISM (180 min)**					
	1 g	**2 g**	**3 g**	**1 g**	**2 g**	**3 g**	**1 g**	**2 g**	**3 g**	**P_1**	**P_2**	**P_3**
Herbs												
Rosemary	0.30 ± 0.03	0.28 ± 0.02	0.36 ± 0.02	0.32 ± 0.04	0.34 ± 0.02	0.31 ± 0.03	0.30 ± 0.06	0.30 ± 0.03	0.31 ± 0.03	NS	NS	NS
Basil	0.34 ± 0.01	0.53 ± 0.18	0.32 ± 0.02	0.31 ± 0.02	0.34 ± 0.03	0.40 ± 0.08	0.23 ± 0.08	0.34 ± 0.04	0.33 ± 0.03	*	*	***
Sage	0.55 ± 0.02	0.64 ± 0.04	1.02 ± 0.15	0.52 ± 0.07	0.92 ± 0.10	0.65 ± 0.08	0.51 ± 0.06	0.91 ± 0.12	1.28 ± 0.20	NS	***	***
Lemon Balm	0.30 ± 0.02	0.31 ± 0.01	0.34 ± 0.03	0.33 ± 0.01	0.38 ± 0.02	0.36 ± 0.02	0.33 ± 0.01	0.35 ± 0.06	0.32 ± 0.03	NS	NS	NS
St. John's Wort	0.29 ± 0.02	0.33 ± 0.03	0.40 ± 0.01	0.38 ± 0.01	0.42 ± 0.04	0.43 ± 0.04	0.35 ± 0.03	0.41 ± 0.04	0.36 ± 0.04	*	NS	NS
Pink Savory	0.41 ± 0.05	0.34 ± 0.05	0.63 ± 0.18	0.31 ± 0.02	0.49 ± 0.07	0.46 ± 0.11	0.45 ± 0.06	0.39 ± 0.08	0.58 ± 0.06	NS	*	NS
Dittany	0.26 ± 0.08	0.27 ± 0.05	0.31 ± 0.03	0.29 ± 0.08	0.41 ± 0.06	0.49 ± 0.14	0.31 ± 0.06	0.47 ± 0.06	0.24 ± 0.05	***	*	*
By-products												

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Pomace	0.33 ± 0.05	0.30 ± 0.04	0.33 ± 0.01	0.35 ± 0.07	0.39 ± 0.01	0.43 ± 0.03	0.34 ± 0.03	0.32 ± 0.02	0.35 ± 0.02	**	*	NS
Olive Leaves	0.28 ± 0.08	0.24 ± 0.02	0.30 ± 0.02	0.36 ± 0.01	0.25 ± 0.01	0.25 ± 0.01	0.31 ± 0.03	0.36 ± 0.06	0.34 ± 0.04	NS	*	**
Orange Peel	0.23 ± 0.04	0.26 ± 0.03	0.31 ± 0.05	0.29 ± 0.01	0.29 ± 0.03	0.35 ± 0.02	0.28 ± 0.02	0.34 ± 0.05	0.30 ± 0.02	*	NS	NS
Lemon Peel	0.29 ± 0.01	0.27 ± 0.08	0.35 ± 0.06	0.34 ± 0.04	0.33 ± 0.03	0.33 ± 0.03	0.27 ± 0.03	0.28 ± 0.03	0.32 ± 0.04	NS	NS	NS
Pomegranate Peel	0.30 ± 0.02	0.28 ± 0.02	0.29 ± 0.02	0.33 ± 0.04	0.37 ± 0.07	0.34 ± 0.03	0.35 ± 0.04	0.31 ± 0.04	0.31 ± 0.02	*	NS	NS
Mandarin Peel	0.19 ± 0.02	0.26 ± 0.01	0.26 ± 0.02	0.30 ± 0.02	0.31 ± 0.03	0.35 ± 0.01	0.28 ± 0.02	0.25 ± 0.05	0.29 ± 0.02	***	*	NS

*Data are expressed as mean ±SD. P_1: significant differences between 60 min and 120 min of incubation maceration. P_2 :significant differences between 120 min and 180 min of incubation maceration. P_3: significant differences between 60 min and 180 min of incubation maceration. Significance level *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, NS: non-significant ($p>0.05$).*

In general, the refined olive oils enriched with mandarin peel, orange peel, and olive leaves, showed the lowest total antioxidant values amongst the different time durations and concentrations. The effect of the herb and by-product concentration on the total antioxidant capacity of the fortified refined olive oils was inconsistent across the tested samples. In more detail, the total antioxidant levels did not significantly differ compared to the g of the following samples: rosemary, basil, orange peel, lemon peel, pomegranate peel, dittany, and pink savory ($p>0.05$). So, the quantity of the above food samples did not show any significant differences between antioxidant capacity levels. On the other hand, the g of the following food samples: sage, olive leaves, lemon balm, St. John's Wort, and mandarin peel, showed a significant difference in their antioxidant capacity ($p<0.05$). So, the quantity of the above-mentioned samples increased the antioxidant content of refined oil in different periods. The different sample g used for the enrichment of the refined olive oils did not significantly affect the total antioxidant capacity of those fortified with rosemary, pomegranate, olive leaves, lemon balm, orange peel, lemon peel and mandarin peel ($p>0.05$). Furthermore, 1, 2, and 3 g of sage presented the highest antioxidant levels compared to other samples that were used for the olive oil enrichment ($p<0.001$). The refined olive oil enriched with Basil presented statistically significant differences between 1 and 2 g of sample ($p<0.001$), while pink savory showed a significant difference only at 3 g of sample concentration ($p<0.001$). Finally, St. John's Wort displayed the least statistical differences in its antioxidant capacity, between 1 and 2 and 2 to 3 g of sample used ($p<0.05$).

3.4. Ultrasound Assisted Maceration (UAM)

Different quantities (1.5 and 3 g) of 7 herbs and 6 plant by-products were used to enrich refined olive oils using an ultrasound-assisted method for 30 and 60 minutes in temperatures of 30°C and 40°C. The total phenolic contents of the enriched refined olive oils are displayed in Table 7.

Table 7. Total phenolic content of enriched fortified olive oils using Ultrasound Assisted Maceration.

Food Sample	Total Phenolic Content (mg GAE/L)										
	30° C		40° C		30° C		40° C		P_1	P_2	P_3
	UAM (30 min)		UAM (30 min)		UAM (60 min)		UAM (60 min)				
	1.5 g	3 g	1.5 g	3 g	1.5 g	3 g	1.5 g	3 g			
Herbs											
Rosemary	36.99 ± 35.50	20.51 ± 7.42	14.95 ± 4.21	19.67 ± 0.89	17.84 ± 1.12	21.54 ± 5.62	25.50 ± 4.88	15.00 ± 1.74	NS	NS	NS

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Basil	58.15 ± 39.34	23.19 ± 9.80	16.30 ± 2.98	18.16 ± 0.32	16.43 ± 1.35	24.01 ± 3.28	23.52 ± 3.13	19.08 ± 2.85	**	*	***
Sage	43.41 ± 5.69	30.36 ± 1.11	27.64 ± 3.58	47.90 ± 6.86	38.91 ± 6.34	51.16 ± 5.12	29.95 ± 9.35	35.75 ± 2.90	NS	*	NS
Lemon Balm	0.22 ± 0.04	12.21 ± 1.57	0.23 ± 0.06	0.14 ± 0.02	0.33 ± 0.08	0.22 ± 0.01	0.28 ± 0.06	0.21 ± 0.02	NS	NS	NS
St. Johns Wort	0.37 ± 0.17	12.57 ± 1.10	0.59 ± 0.50	0.16 ± 0.06	0.31 ± 0.09	0.17 ± 0.03	0.26 ± 0.04	0.14 ± 0.00	NS	NS	NS
Pink Savory	24.71 ± 3.17	26.15 ± 3.96	24.72 ± 6.27	33.90 ± 4.54	20.49 ± 3.15	37.50 ± 3.59	31.80 ± 6.16	38.49 ± 10.28	NS	**	NS
Dittany	15.37 ± 1.38	-	12.01 ± 4.02	-	12.65 ± 2.08	-	12.32 ± 1.77	-	NS	-	NS
By-products											
Pomace	13.49 ± 1.36	14.23 ± 0.75	16.97 ± 1.49	15.95 ± 3.48	15.71 ± 3.76	17.88 ± 2.36	16.56 ± 1.92	14.36 ± 1.39	NS	NS	NS
Olive Leaves	11.29 ± 4.09	17.25 ± 5.21	13.59 ± 2.26	11.09 ± 1.14	15.17 ± 1.27	12.56 ± 1.50	11.58 ± 1.85	11.27 ± 1.45	NS	NS	NS
Orange Peel	38.71 ± 10.17	36.28 ± 24.60	36.62 ± 5.24	64.35 ± 25.31	19.95 ± 3.95	21.64 ± 2.44	21.64 ± 2.93	42.67 ± 16.98	***	***	***
Lemon Peel	27.03 ± 11.22	32.52 ± 18.03	47.21 ± 19.65	41.46 ± 16.24	29.34 ± 11.51	28.47 ± 7.73	17.37 ± 1.08	20.63 ± 1.89	***	NS	NS
Pomegranate Peel	37.90 ± 13.25	19.14 ± 5.08	42.31 ± 4.76	43.23 ± 5.85	26.08 ± 4.01	29.59 ± 4.99	24.05 ± 3.36	28.57 ± 12.42	**	NS	*
Mandarin Peel	0.21 ± 0.02	9.76 ± 1.24	0.23 ± 0.03	0.11 ± 0.04	0.24 ± 0.05	0.11 ± 0.03	0.22 ± 0.04	0.12 ± 0.02	NS	NS	NS

*Data are expressed as mean ±SD. P_1: significant differences between ultrasound-assisted maceration time (30min and 60min). P_2: significant differences between sample g per food sample (1.5 g and 3 g). P_3: significant differences between ultrasound-assisted maceration temperatures (30°C and 40°C). Significance level *** p < 0.001, ** p < 0.01, * p < 0.05, NS: non-significant (p>0.05).*

The refined olive oil fortified with Basil showed the highest total phenolic content with a value of 58.15±39.34 mg GAE/L at the 30 min period, 1.5 g sample concentration and 30oC. On the other hand, mandarin peel displayed the lowest total phenolic content with a value of 0.11±0.04 mg GAE/L at two different conditions: 30 min, 3 g, 40oC, and 60 min, 3 g, 30oC. Furthermore, the refined olive oils enriched with rosemary, sage, orange peel, pink savory and pomegranate peel had relatively higher total phenolic content, while those fortified with dittany, pomace, olive leaves, and St. John's Wort presented lower phenolic values compared to the rest of the samples. Significant differences between the different parameters were observed about Basil and orange peel ($p<0.05$).

The total antioxidant capacity of the enriched refined olive oils is described in Table 8.

Table 8. Total Antioxidant capacity of fortified refined olive oils using ultrasound-assisted Maceration.

Food Sample	Total Antioxidant Capacity (mmol Fe^{2+}/L)								P_1	P_2	P_3
	30°C 30 min		40°C 30 min		30°C 60 min		40°C 60 min				
	1.5 g	3 g	1.5 g	3 g	1.5 g	3 g	1.5 g	3 g			
Herbs											
Rosemary	0.34 ± 0.05	0.31 ± 0.07	0.23 ± 0.08	0.53 ± 0.07	0.38 ± 0.05	0.32 ± 0.06	0.36 ± 0.02	0.42 ± 0.04	NS	NS	NS
Basil	0.42 ± 0.03	0.30 ± 0.04	0.27 ± 0.08	0.40 ± 0.07	0.39 ± 0.04	0.38 ± 0.07	0.36 ± 0.02	0.66 ± 0.17	**	*	NS
Sage	0.63 ± 0.10	0.68 ± 0.13	1.05 ± 0.13	1.52 ± 0.56	1.32 ± 0.13	1.56 ± 0.10	1.69 ± 0.07	1.42 ± 0.15	***	**	***
Lemon Balm	0.27 ± 0.03	0.33 ± 0.07	0.32 ± 0.05	0.32 ± 0.05	0.33 ± 0.09	0.52 ± 0.08	0.35 ± 0.06	0.46 ± 0.04	**	*	NS
St. John's Wort	0.32 ± 0.08	0.35 ± 0.05	0.29 ± 0.03	0.39 ± 0.11	0.26 ± 0.05	0.33 ± 0.01	0.23 ± 0.02	0.28 ± 0.02	NS	NS	NS
Pink Savory	0.45 ± 0.02	0.52 ± 0.04	0.51 ± 0.07	0.41 ± 0.05	0.41 ± 0.03	0.50 ± 0.03	0.59 ± 0.06	0.60 ± 0.03	NS	NS	NS
Dittany	0.44 ± 0.03	-	0.41 ± 0.02	-	0.37 ± 0.06	-	0.40 ± 0.02	-	NS	-	NS
By - products											
Pomace	0.29 ± 0.01	0.32 ± 0.01	0.32 ± 0.02	0.32 ± 0.02	0.30 ± 0.07	0.31 ± 0.02	0.34 ± 0.04	0.32 ± 0.01	NS	NS	NS
Olive Leaves	0.33 ± 0.03	0.22 ± 0.03	0.32 ± 0.05	0.28 ± 0.05	0.31 ± 0.04	0.31 ± 0.03	0.27 ± 0.04	0.29 ± 0.03	NS	NS	NS
Orange Peel	0.37 ± 0.08	0.51 ± 0.15	0.97 ± 0.16	1.24 ± 0.24	0.36 ± 0.03	0.33 ± 0.05	0.58 ± 0.04	0.56 ± 0.05	***	*	***
Lemon Peel	0.46 ± 0.08	0.56 ± 0.18	1.17 ± 0.07	1.03 ± 0.08	0.46 ± 0.02	0.61 ± 0.21	0.54 ± 0.03	0.44 ± 0.04	***	NS	***

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Pomegranate Peel	0.50 ± 0.11	0.53 ± 0.24	1.25 ± 0.09	1.37 ± 0.15	0.59 ± 0.09	0.43 ± 0.03	0.53 ± 0.03	0.57 ± 0.14	***	NS	***
Mandarin Peel	0.26 ± 0.04	0.26 ± 0.07	0.30 ± 0.12	0.27 ± 0.03	0.22 ± 0.03	0.23 ± 0.05	0.23 ± 0.04	0.21 ± 0.04	NS	NS	NS

*Data are expressed as mean ±SD. P_1: significant differences between ultrasound-assisted maceration time (30min and 60min) P_2: significant differences between sample g per food sample (1.5g and 3g). P_3: significant differences between ultrasound-assisted maceration temperatures (30°C and 40°C). Significance level *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, NS: non-significant ($p>0.05$).*

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The fortified refined olive oil fortified with sage demonstrated the highest total antioxidant capacity and significant differences at all conditions and time intervals with values ranging from 0.63±0.10 mmol Fe2+/L to 1.69 ±0.07 mmol Fe2+/L ($p<0.05$). The highest value was observed at the 1.5 g, 60-minute duration and 40°C. The refined olive oils fortified with orange peel also showed relatively higher values regarding their antioxidant content and significant differences between all conditions ($p<0.05$).

In comparison, those fortified with mandarin peel, olive leaves or lemon balm had relatively lower values under most of the conditions. Some fortified refined olive oils that demonstrated higher phenolic content, also showed high total antioxidant capacity, such as sage (from 0.63±0.10 to 1.69±0.07 mmol Fe2+/L) and pomegranate peel (from 0.43±0.03 to 1.37±0.15 mmol Fe2+/L. However, some refined olive oils such as those enriched with dittany and mandarin peel, even though they presented high phenolic content, presented relatively low antioxidant capacity.

It is important to be mentioned that dittany extraction could not be performed in 3 g of the plant in the refined oil because the herb absorbed the larger quantity of the oil. Therefore, some measurements for both antioxidant capacity and phenolic content could not be obtained, making this maceration method not applicable to this specific herb.

3.5. Evaluation of total antioxidant and phenolic content of fortified olive oil prior and after in vitro digestion process

In vitro digestion analysis was conducted for the fortified refined olive oil samples that presented among the highest amounts of phenolic content and total antioxidant capacity, as well as valuable sensory factors such as aroma, color, and texture during the extraction process.

The antioxidant capacity and total phenolic content of the different fortified olive oils with selected waste by-products and herbs, before and after in vitro digestion simulation, are presented in Table 9.

Table 9. Total antioxidant capacity and total phenolic content of selected fortified olive oils before and after in vitro digestion and their predicted bioavailability indices.

	Before Digestion		After Digestion					
Food Sample	**Total Antioxidant Capacity (mmol Fe2+/L)**	**Total Phenolic Content (mg mgGAE/L)**	**Total Antioxidant Capacity (mmol Fe2+/L)**	**Total Phenolic Content (mg mgGAE/L)**	**Total Antioxidant Capacity BAvI %**	**Total Phenolic Content BAvI %**	**P1**	**P2**
Plant By-products								
Orange peel	1.24±0,24d	64.35±25,31d	0.06±0,01a	13.28±10,84a	4.84	20.64	*	***
Pomegranate peel	1.25±0,09d	42.31±4,77c	0.08±0,02a	20.95±13,93a	6.40	49.52	*	**
Pomace	0.37±0,05bc	20.27±4,86b	0.11±0,10a	9.86±8,39a	29.73	48.64	*	*
Herbs								
Basilicum	0.42±0,03c	58.15±39,34d	0.09±0,03a	11.29±7,29a	21.43	19.42	*	***
St. John's Wort	0.32±0,08b	17.38±8,59b	0.07±0,01a	9.23±8,47a	21.88	53.11	***	NS

** Data are expressed as mean ±SD. * Different letters in the same group (antioxidant capacity or phenolic content) indicate significant differences ($p < 0.05$) between samples. * BAvI: Bioavailability Index. * P1: Sample correlations between before and after in vitro digestion for total antioxidant capacity. * P2: Correlations between samples before and after in vitro digestion for total phenolic content. Significance level *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ NS: non-significant ($p>0.05$).*

The average values of the total antioxidant capacity before digestion of the extracts varied from 0.32±0.08 to 1.25±0.09 mmol Fe2+/L. Olive oil that fortified with pomegranate peel showed the highest

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antioxidant capacity, followed by orange peel (1.24±0,24 mmolFe^{2+}/L) with a non-significant difference ($p>0.05$). The values for Basil, St. John's wort and pomace are considered lower with mean values of 0.42±0.03 mmolFe^{2+}/L, 0.32±0.08 mmolFe^{2+}/L and 0.37±0.05 mmolFe^{2+}/L, respectively. After the simulated in vitro digestion analysis, the values of total phenolics and total antioxidant capacity content decreased significantly ($p<0.05$). The total antioxidant capacity values range from 0.06 to 0.11 mmolFe^{2+}/L after digestion. Regarding the predicted bioavailability of total antioxidant capacity of the selected extracts, the refined olive oil enriched with pomace has the highest bioavailability (29.7%), followed by St. John's Wort (21.9%), and basil (21.4%), while those with the lowest bioavailability were orange peel and pomegranate peel with 4.8% and 6.4%, respectively. The mean values of total phenolic content before digestion varied significantly ($p<0.05$) from 17.38 to 64.35 mg GA/L. Orange peel has the highest phenolic content, followed by Basil with a non-significant difference. The values for pomegranate, pomace and St. John's Wort were observed to be lower, with 42.31±4.77, 20.27±4.86, and 17.38±8.59 mg GAE/L, respectively. Total phenolic content after in vitro digestion ranged from 9.23±8.47 to 20.95±13.93 mg GAE/L. St. John's Wort displayed the highest predicted bioavailability (53.1%), followed by pomegranate peel (49.5%) and pomace (48.6%), while orange peel (20.6%) and basil (19.4%) presented the lowest predicted bioavailability.

Regarding correlations between the samples before and after in vitro digestion experimental process, four samples (refined olive oils fortified with orange peel, pomegranate peel, pomace, and Basil) suggest a significant correlation ($p<0.05$), while Saint John's Wort illustrates significant correlation at the 0.001 level. Consequently, as for the phenolic concentration pomace displayed a significant correlation ($p<0.05$) while pomegranate peel, and Basil presented a significant correlation at 0.01 level.

4. Discussion

The study of the antioxidant effects of bioactive compounds is supported by the current interest in natural products and the ongoing replacement of synthetic antioxidants with natural antioxidants from plant sources [25] Numerous studies regarding food fortification with herbs, and by-products as well as the contribution of bioactive compounds to human health have been conducted in recent years [26]. The fortification of processed foods with natural bioactive compounds has a dual role: maintaining the quality characteristics of the product and promoting human health [27,28]. Refined olive oil is a food product that due to the refining process has much lower content of bioactive compounds such polyphenols, compared to extra virgin or virgin olive oil [29]. However, although refined olive oil has a lower nutritional value it is regularly consumed by a large part of the global population due to its low cost [30]. Since there is limited research on refined oil fortification with natural additives, our study aims to explore the most suitable extraction method amongst conventional, incubation-assisted maceration and ultrasound-assisted maceration for the fortification of refined olive oil with herbs and by-products. The different methods used in the study for the extraction process of olive oils were used in different studies [36–39]. The most used in the food industry is conventional maceration followed by incubation shaking and ultrasound-assisted maceration, since the different days of extraction can play an important role in oils' fortification [36,37]. According to current research data, the above methods are commonly used for flavoring olive oil products[31], while results regarding fortification with bioactive compounds, especially in refined olive oils, are limited [32,33].

Conventional maceration is the method most commonly used in the food industry, as it is a technologically simple low cost method [34]. Olive oil by-products have been examined as a protentional way for olive oil fortification, using conventional maceration methods. Olive leaves have been used for the fortification of olive oil, using conventional maceration in different concentrations between 1-10% for 7 days, and the fortified final products presented a higher percentage of phenolic components compared to the unfortified samples[35]. A similar study examined the fortification of refined olive oil with olive leaves, using conventional maceration for 5 days and the phenolic content was significantly improved in the final fortified product [36]. Above data comes in accordance with our study results, while the short extraction time used in the study proposes that we can achieve high

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percentage of phenolic compounds in shorter intervals of extraction than those examined in the present study. Moreover, similar results were obtained in the study of Issaoui et al. 2020, which utilized dry lemon in various concentrations to enrich olive oil (mixture of virgin (VOO) and refined olive oil (ROO)) by conventional maceration for a 2-month duration at room temperature [37]. This method increased the phenolic compounds in the fortified olive oil which is in line with the above observations regarding lemon peel at all conditions of the conventional maceration [37]. On the contrary, Ayadi et al. 2009 observed a decreased phenolic capacity when utilizing dry lemon zest instead of dried lemon at 5% concentration and 15-day duration at room temperature. This result of conventional maceration does not confirm our findings regarding lemon peel but this may be expected due to variations between different cultivars of the samples [38]. Furthermore, similar results were presented in 2015 by Khemakhem et al. that performed a conventional maceration of 10-day duration to enrich olive oil with fresh instead of dried orange peel in the same concentration of 5%, at room temperature. The total antioxidant capacity of the final samples was significantly increased in comparison to the control [39].

In the case of the conventional maceration methods for oil fortification with bioactive compounds from herbs, there is a large field of research on extra virgin and virgin olive oil, while research on refined olive oil is limited. The most common herbs that have been used to flavor or fortify olive oils are rosemary and sage [40]. Nevado et al. 2012, Ayadi et al. 2009, and Kaismoglu et al. 2018 performed a conventional maceration on virgin olive oil, by enriching it with a 5% dry sample for a duration of 10, and 15 days. The first observed an increase in the total antioxidant activity of the enriched olive oil, while the second observed a decrease in the phenolic capacity [38,41,42]. Moreover, Ayadi et al. 2009 used 5% and 15% w/w concentration of dry Basil to enrich EVOO by conventional maceration at room temperature for 15 days with no phenolic increase in both concentrations, while the results of the present study indicated that refined olive oil fortification with 8% and 17% of dry Basil resulted to an increased phenolic content in both the 15 and 30 day period during conventional maceration[38]. The difference may be attributed to the unique chemical composition of EVOO compared to refined olive oil and also to the initial difference in the phenolic content.

Regarding the incubator shaking maceration, there are not many studies that followed the same methodology for olive oil fortification with herbs or by-products. In the present study, it was observed that the phenolic content of most fortified refined oils with herbs and by-products was increased while basil, sage, dittany, pomace, olive leaves, orange, and mandarin peel showed significant antioxidant capacity levels in all concentrations and times of extraction. According to literature data, Penalvo et al. 2016 performed a similar shaking process to fortify virgin olive oil with oregano and resulted in an of the phenolic content of the enriched olive oils, which agrees with our findings when performing the incubator shaking maceration [43]. Therefore, this method has promising results that may benefit from more research on fortifying olive oils using an incubator-assisted method.

Finally, the present study examined the ultrasound-assisted extraction maceration by using different times, temperatures, and concentrations of the herbs and by-products presented significant results towards antioxidant capacity and polyphenolic content to the refined olive oil. More specifically, sage and orange peel showed the highest antioxidant capacity during ultrasound-assisted maceration compared to the other samples ($p<0.05$). Moreover, different temperatures ($30^{o}C/40^{o}C$) and concentrations (5%w/w nad 10%w/w) of the sample during the extraction process did not play an important role in enriching the antioxidant capacity of the refined oil. As for the phenolic content it seems that the quantity of the herbs and by-products in the olive oil during extraction does play an important variable in the enrichment of the refined olive oil. Additionally, refined olive oil with sage, lemon, and orange peel presented the highest content among others in 10%w/w concentration, at all time periods and temperature conditions. Some differences observed comparing the above results with other studies; Japon-Lujan et al. 2008 used an ultrasound-assisted maceration, with a 10%w/w concentration of dry olive leaves for 20 min at room temperature of ultrasound-assisted maceration, that eventually resulted in increased phenolic contents [44]. These results were in line with the study by Achat et al. 2012, which also used olive leaves to fortify olive

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oil by ultrasound-assisted maceration for 45 min at 16°Cresulting in increased final phenolic content [45]. The above results also align with those outcomes since an increase in both phenolic content and antioxidant capacity was observed in refined olive oil with olive leaves using an ultrasound-assisted method in all conditions. However, for both measurements, all parameters presented non-significant differences which may mean that either the sample used is not ideal for this extraction method, or that the method itself is not as effective for this specific purpose.

To sum up, the conventional maceration, incubation-assisted and ultrasound-assisted maceration water baths could be alternative methods to accelerate the fortification process of refined olive oil with compounds rich in antioxidants and polyphenols, as well as to improve the stability and induction time for some compounds. According to the data of this research and from other studies it can be suggested that all the above methodologies can be used for the fortification of refined olive oils. It is important to underline that there is limited research in refined olive oil fortification compared to virgin/extra virgin enrichment with the above methods and herbs/by-products, so more research is needed [28]. As of the results according to the food chosen to fortify the refined olive oil, pink savory, sage, Basil, St. John's Wort, pomegranate, lemon, and orange peel showed the highest total antioxidant capacity and polyphenolic content among all three methods, that is comparable to the total antioxidant capacity and polyphenolic content of virgin and extra virgin olive oil.

Following the in vitro digestion simulation, the values of total phenolic content and antioxidant capacity significantly decreased. The predicted bioavailability of polyphenols was found to vary among the selected samples, with refined olive oil enriched with pomace having the highest bioavailability, followed by St. John's Wort (53.11%) and Basil the lowest (19.42%). Meanwhile, refined olive oil with orange peel (4.84%) and pomegranate peel (6.40%) showed the lowest predicted antioxidant bioavailability. These findings are promising compared to other studies that have shown the importance of fortifying olive oils with polyphenols. More specifically, Alberdi-Cedeño J. et al. 2020, presented that the enrichment of olive oil with phenolic bioactive compounds can increase the in vitro bioavailability and bioaccessibility of olive's oil main components that could be absorbed from the intestinal wall. By adding phenolic and antioxidant compounds to the oil could be observed an increase in the shelf life of the food because of the decrease in the lipid oxidation process. It has been reported that the oil-generated aldehydes, may react with nitrogen compounds, decreasing the lipolysis extent, which is frequently produced in vitro digestion process of most oils [45].

Finally, the three extraction methods examined led to an increase of phenolic and antioxidant content of the studied samples. Examining different concentrations of herbs or by-products, as well as extraction times, makes it difficult to propose a methodology for fortifying oil products. Each herb and byproduct examined showed different optimal extraction conditions. This leads to the conclusion that each proposed enrichment sample must be examined individually to identify the conditions under which it will achieve the optimum enrichment yield. In the present study, a wide range of samples were examined, which provides valuable information for the research community; however, further studies are needed to improve our knowledge of the behavior of the proposed enrichment samples as well as their phenolic and antioxidant profile. Moreover, further studies are needed to accurately identify whether the refined olive oil fortification with natural bioactive components can have beneficial aspects towards health, especially interventional human studies for the bioactivity and bioavailabity of the bioactive compounds. Also, consumer preferences and organoleptic sensory tests of enriched refined olive oils are considered important to understand consumers' acceptance over the proposed products.

5. Conclusions

In conclusion, the fortification of refined olive oil with herbs and by-products using different enrichment methods could lead to the creation of a novel, possibly functional products, rich in antioxidants and polyphenols that may be a competitive addition to the agri-food sector. With parallel promote of sustainable development. In the current study, different methodologies (conventional, incubation shaking maceration, and ultrasound-assisted maceration) were used to enhance refined olive oils that were then evaluated for their antioxidant capacity and phenolic

content. All methods showed that different parameters such as time of maceration, temperature, and sample concentration play an important role during the extraction process of fortified olive oil with herbs and by-products. Furthermore, the olive oil fortified with pomace, basil, st. john's wort, and pomegranate peel presented the highest antioxidant and phenolic predicted bioavailability during in vitro digestion process. Concluding, even if the data is promising, future research on different variations of the fortified olive oils should be examined to evaluate, organoleptic characteristics and consumer preferences for these products, while nutritional interventional studies are need for investigation of their possible health on human health.

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