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Co-milling process of olives and oleaginous matrices with high nutritional value: a preliminary characterisation of the obtained oils

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ABSTRACT

Extra virgin olive oil (EVOO), grape seeds (GS) and pomegranate seeds (PS) are very popular for human consumption because of their nutraceutical properties. A co-milling of olives with GS or PS was carried out with the aim of a preliminary study of the lipidic and phenolic characteristics of the obtained vegetable oils, also during their shelf life. Results show that the use of GS and PS in the olive co-milling enriches the oil in healthy compounds, such as punicic acid and γ-tocopherol. However, the co-milling process must be re-evaluated, since the compositional profile of the co-milled oils is just slightly different from EVOO. The oxidative state of the oils obtained suggests the use of a protective packaging combined with small containers in order to ensure a rapid consumption. With an appropriate formulation the co-milled oils could bring health-positive compounds and, simultaneously, raw materials could be valorised.

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Extra virgin olive oil; grape seed; pomegranate seed; lipid characterisation; phenolic content

Introduction

Over the last three decades, the global production of olive oil reaches 3.3 million tons (International Olive Council 2022) because of its well-recognized health-promoting effect thanks to the presence of polyunsaturated fatty acids (PUFA), sterols, tocopherols and phenolic compounds (Eguaras et al. 2015; Farinetti et al. 2017; Salas-Salvadó et al. 2018). This evidence led the European Union, in 2006, to approve nutritional and health claims for virgin olive oil, to be included on the label.

Nowadays also grape seeds (GS) and pomegranate seeds (PS), by-products of several food industry processes (including juices ad jams), have become very popular in nutritional supplement production because of their high bioactive compound content with interesting biological properties. These benefits are mainly due to their fatty acid profile: in particular, GS, which report a fat percentage of about 7%, is rich in linoleic acid, a polyunsaturated essential fatty acid (Bombai et al. 2017), whereas PS present 7–16% of fat, of which 65–80% is represented by conjugated fatty acids and the most important is punicic acid (C18:3 n5), a conjugated linolenic acid (CLnA) (Verardo et al. 2014). Besides, these seeds present high level of tocopherols and tocotrienols, important phytosterols and polyphenols, conferring biological properties and stability towards oxidation. Because of that, GS and PS are associated with various health benefits such as cardioprotective effect (Orsavova et al. 2015), low-density lipoprotein down regulation (Lutterodt et al. 2011), anti-atherosclerotic activity and antioxidant activity (Shinagawa et al. 2015). Literature reports a lot of different food applications of these waste seeds. Xiong et al. (2020) developed a chitosan–gelatin edible coating system, using nisin and GS extract, for fresh meat; GS extract enhanced the antioxidant activity and the quality and safety of the food. Marchante et al. (2019) used GS extract as alternative source of SO_2 in white wine making found that it could inhibit the oxidation of wine without affecting its characteristic features. In a study conducted by Lydia et al. (2020), gold nanoparticles were synthesised from PS oil and incorporated in commercial yogurt and the functional yogurt exhibited antioxidant and in vitro cytotoxic properties against lung and colon cancer. In addition, it was demonstrated that PS oil could be a natural additive to prevent oxidative deterioration of canola and sunflower oils during storage and, also, improve their nutritional attributes (Siraj et al. 2019).

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Co-milling is a technique that allow to obtain flavoured and enriched oil starting from two or more matrices during the same process. It consists, in fact, of the addition of ingredients during milling or in the malaxation step in order to obtain a production with higher antioxidant activity or to enrich and change the acidic composition or part of the unsaponifiable fraction of oil (Peres et al. 2021). Many studies report the co-extraction of olive oil with the addition of matrices with positive sensory notes like lemon, bergamot, rosemary, thyme and basil (Baiano et al. 2009; Clodoveo et al. 2016; Sacchi et al. 2017). Other studies were intended to increase the antioxidant activity of olive oil with the addition of citrus fruits peels (Cerretani et al. 2008; Ergönül and Sánchez 2013; Ascrizzi et al. 2018), spices (Caponio et al. 2016) and tomato (Bendini et al. 2015).

The health promotion properties of grape and PS make them valuable oleaginous by-products that can be utilised to produce a co-processed olive oil enriched in functional molecules, which remain intact thanks to minimal handling of the starting fruit and then stored in the finished food.

According to our knowledge, this is the first study on olives co-milled with these oleaginous by-products in order to evaluate the possibility to improve bioactivity and shelf life of the olive oil. In particular, the aim of the present study was the evaluation of the fatty acid profile, tocochromanols, sterols and oxidative stability of grape and PS, widely available on the Italian territory, and also a preliminary characterisation of the obtained co-milled vegetable oils. Finally, in order to evaluate the shelf life of co-milled oil, oxidative stability, tocochromanols and phenolic compounds of oils were determined along the shelf life up to 18 months.

Materials and methods

Samples

GS and PS were supplied and co-milled with olives (cultivar Coratina, cultivated in Bari and harvested at full maturity) by an Italian oil mill, Basso Fedele e figli s.r.l. (Avellino, Italy, 40.88723, 14.83856). For both the co-milled oils, a continuous low-scale plant equipped with a hammer crusher, a horizontal malaxator and a two-phase decanter, was used. Temperature of 27 °C and 30 min of malaxation were the process parameters set for the production of all samples. Olives and oleaginous matrices were mixed together before process in a proportion of about 80:20, respectively. In particular, 60 kg of olives were co-milled with 14 kg of grape seeds (O-GS) (1:4 w/w) and 70 kg of olives were co-milled with 17 kg of pomegranate seeds (O-PS) (1:4 w/w). From the co-milling process, 8 kg of O-GS oil and 11 kg of O-PS oil were achieved, with a yield of 10.8 and 12.6%, respectively. A control sample of EVOO was obtained in order to evaluate the co-milling process effect on the considered parameters and, in particular, on bioactive enrichment. Sensory analysis was performed by a group of trained panellists; positive sensory descriptors (fruity, bitter and pungent) and defects were evaluated according to the official procedure (European Community, Commission Regulation 2568/91, 1991 and following amendments). In addition, the oxidative state was monitored during shelf life. The samples were stored in greenish glass bottles in the dark at room temperature (25 °C), evaluating the antioxidant compounds content (tocochromanols and phenols) and the resistance to forced oxidation (Oxitest® analysis). The analyses were carried out every six months for the 18 months of storage (t0, t6, t12 and t18, respectively).

Lipid extraction

According to AOAC Official Method, the lipid fraction of grape and PS was extracted from ground samples (10 g) with ed *n*-hexane in a Soxhlet apparatus (Behr Labor-Technik, Fischer Scientific Italia, Milano). The oil was taken up with *n*-hexane/isopropanol (4/1 v/v) solution and stored at −18 °C until use. Each extraction was carried out two time ($n = 2$) (AOAC 2000).

Fatty acids analysis

The fatty acid composition was determined as fatty acid methyl esters (FAMEs) by capillary gas chromatography analysis after alkaline treatment according to Marzocchi et al. (2018). Methyl tridecanoate (C13:0, 2 mg/mL) was used as internal standard and FAMEs were measured on a GC 2010 Plus gas chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a flame ionisation detector (FID) and an AOC-20s auto sampler (Shimadzu Corporation). Peak identification was accomplished by comparing peak retention time with GLC-463 standard mixture from Nu-Check (Elysian. MN. USA) and FAME 189-19 standard mixtures from Sigma-Aldrich Chemicals (St. Louis, MO, USA) and expressed as weight percentage of total FAMEs. FAMEs composition was measured in two replicates for each lipid extract ($n = 4$).

Tocochromanols analysis

For the tocochromanols determination of oleaginous matrices and co-milled oils along the shelf-life (t0, t6, t12 and t18), approximately 0.05 g of fat was dissolved in 0.5 mL of *n*-hexane. The solutions were filtered through a 0.45 µm nylon filter. The tocochromanols were determined by HPLC (Agilent 1200 series, Palo Alto, CA, USA) equipped with a fluorimeter detector (Agilent, Palo Alto, CA, USA) according to Ben Lajnef et al. (2017). The excitation wavelength was 290 nm and the emission one was 325 nm. The separation of tocopherols was performed by a HILIC Poroshell 120 column (100 mm × 3 mm and 2.7 µm particle size; Agilent Technologies, USA), in isocratic conditions, using an n-hexane/ethyl acetate/acetic acid (97.3: 1.8: 0.9 v/v/v) mobile phase. The flow rate was 0.8 mL/min. Calibration curve was constructed with α-tocopherol standard solution (from 1 to 100 µg/mL) and it was used for quantification. Analysis was achieved in two replicates for each extract ($n = 4$).

Sterols analysis

In order to determine the phytosterols content, 0.5 mL of dihydrocholesterol (c = 2 mg/mL) was added to 250 mg of oil and a cold saponification was performed (Sander et al. 1989). Before injection, samples were silylated (Sweeley et al. 1963) and the sterol separation was performed by GC-MS (GCMS-QP2010 Plus, Shimadzu, Tokyo, Japan) in the same chromatographic conditions reported by Cardenia et al. (2012). Phytosterol identification was achieved by comparing peak mass spectra with peaks of standard mixture and by comparing them to the GC-MS data reported in literature (Pelillo et al. 2003). An internal standard was used to quantify all the sterols identified. Analysis was conducted in two replicates for each lipid extract ($n = 4$).

Determination of peroxide value (PV)

The International Dairy Federation method of Shantha and Decker (1994) was used to determinate peroxide values (PV) in oleaginous matrices. Specifically, 0.05 g of fat was added to an Fe(II) and ammonium thiocyanate solution, and the intensity of a red–violet complex at 500 nm was evaluated. The analysis was repeated twice for each lipid extract ($n = 4$).

Determination of total phenolic compounds (TPC)

The co-milled oils, during shelf-life (t0, t6, t12 and t18) were analysed after a liquid–liquid extraction as previously described by Pirisi et al. (2000) with some modifications. Two grams of oil were mixed with 1 mL *n*-hexane and 2 mL of 60% methanol. The mixture was vigorously stirred for 1 min, centrifuged at 3000 rpm for 5 min and the supernatant was recovered. The extraction was repeated twice, and 2 mL *n*-hexane was added to the combined methanolic fractions to remove the residual oil. The *n*-hexane was discarded and the methanolic solution evaporated to dryness with a rotary evaporator at 35 °C. The dry extract was dissolved in 2 mL of a methanol/water (50/50, v/v) solution and filtered through a 0.2 µm nylon filter. Each extraction was achieved twice and the extracts were stored at −18 °C until use. The total phenolic content (TPC) of the oils was assessed by means of the Folin–Ciocalteu method (Singleton and Rossi 1965). Briefly, 100 µL of each extract was shaken with 500 µL Folin–Ciocalteu reagent and 6 mL of distilled water. Two millilitres of 15% Na_2CO_3 were added and the mixture was shaken once again for 0.5 min. Finally, the solution was brought up to 10 mL by adding distilled water. After 2 h, the absorbance at 750 nm was measured. The phenolic content was calculated on the basis of the gallic acid calibration curve (from 25 to 1000 µg/mL). Analysis was achieved in two replicates for each extract ($n = 4$) and the results were expressed as milligram gallic acid equivalents (GAE)/100 g of fat.

Determination of oxidation stability with OXITEST®

Ten grams of oils at the different time of shelf life (t0, t6, t12 and t18) were placed in the appropriate oxidation reactors in OXITEST® (Velp Scientific, Usmate Velate-MB-Italy); the analysis was carried out at 100 °C and 6 bar of oxygen pressure until the instrument did not show a collapse of the pressure, the signal which is the maximum possible oxidation achieved in the sample. The analysis was repeated twice for each replicate.

Statistical analysis

Relative standard deviation was obtained, where appropriate, for all data collected. One-way analysis of variance (ANOVA) was evaluated using Statistica 8 software (2006, StatSoft, Tulsa, OK, USA). p Values lower than 0.05 were considered statistically

significant using Tukey honest significant difference (HSD) test. All chemical analyses were carried out two replicates for each extract ($n = 4$ for each sample), and the analytical data were used for statistical comparisons.

Results and discussion

The oil obtained only from the pressing of the olives showed values of acidity (0.24 ± 0.03), PV (7.40 ± 0.64), K_{232} (2.21 ± 0.03), K_{270} (0.19 ± 0.03) and Delta-K (≤0.01) in accordance with EU Regulation 1604/2019 for the extra virgin olive oil (EVOO). Besides, the sensory analysis presented the median of the defects and the median for fruity 0 and >0, respectively, confirming the category.

In agreement with previous studies (Bombai et al. 2017; Verardo et al. 2014), GS and PS reported a fat concentration of about 11.7 and 15.0%, respectively. The PV of GS was 10 meqO_2/kg of fat, in agreement with literature (Yousefi et al. 2013); whereas the PV of PS was 16.9 meqO_2/kg of fat, a value higher than the result reported by Alfekaik and AL-Hilfi (2016). These differences may be affected by the different cultivation climate, cultivar conditions and fruit ripening (Alfekaik and AL-Hilfi 2016). Besides, it is important to take into account that the PV may be also influenced by the storage conditions of the seeds. Nevertheless, these values were both under the legal limit (20 meqO_2/kg of fat) imposed for foods.

Determination of fatty acids

As showed in Table 1, a total of 18 fatty acids were identified and quantified in samples. About oleaginous matrices, PUFAs were the principal class with a content of approximately 66% and 88% for GS and PS, respectively; monounsaturated fatty acids (MUFAs) were the second class, followed by saturated fatty acids (SFAs). The two seed samples showed also qualitative differences in their fatty acid pattern. In particular, in GS sample almost all of the PUFA content was represented by linoleic acid (C18:2 n6) and a small amount by linolenic acid (C18:3 n3), as already reported by Bombai et al. (2017). Conversely, in PS more than 80% of the total FAME were represented by the punicic acid (C18:3 n5), in accordance with literature (Verardo et al 2014; Charalampia and Koutelidakis 2017). Punicic acid belongs to the group of CLnA and its functional and health properties are well known and widely discussed (Verardo et al. 2014; Guerra-Vázquez et al. 2022). Unlike the oleaginous matrices, MUFA was the principal class of fatty acids both in EVOO and co-milled oils (Table 1). This class represented about the 70% of total FAME in all oil samples and the most characterising compound was oleic acid (C18:1 cis9). This result was expected because oleic acid is the main fatty acid of EVOO (Boskou et al. 2006), which represents the basis oil in the analysed samples. Nevertheless, oleic acid was present with significative different percentages ($p < 0.05$) in the three oils (72%, 70% and 69% in EVOO, O-GS oil and O-PS oil, respectively). SFA was the second

Table 1. Fatty acid composition and content (mg FA/100 mg of FAME) of oleaginous matrices and co-milled oils.

FA	GS	PS	EVOO	O-GS oil	O-PS oil
C8:0	0.51 ± 0.08a	0.36 ± 0.15a	0.31 ± 0.05a	0.31 ± 0.05a	0.28 ± 0.14a
C14:0	0.06 ± 0.01a	0.03 ± 0.00b	n.d.	0.02 ± 0.00b	0.02 ± 0.00b
C15:0	0.04 ± 0.01a	0.03 ± 0.00a	n.d.	0.02 ± 0.00a	0.02 ± 0.00a
C16:0	6.38 ± 0.06c	2.84 ± 0.01d	13.38 ± 0.01a	13.34 ± 0.00a	13.17 ± 0.05b
C16:1 *trans*	0.13 ± 0.01a	0.02 ± 0.00c	0.09 ± 0.01b	0.10 ± 0.00ab	0.09 ± 0.01b
C16:1 *cis*	0.13 ± 0.00d	0.03 ± 0.00e	1.20 ± 0.01a	1.00 ± 0.00b	0.98 ± 0.00b
C17:0	0.08 ± 0.00a	0.05 ± 0.00b	0.04 ± 0.01b	0.04 ± 0.00b	0.05 ± 0.00b
C17:1	n.d.	n.d.	0.09 ± 0.00a	0.07 ± 0.00a	0.08 ± 0.01a
C18:0	5.62 ± 0.02a	1.75 ± 0.01e	2.22 ± 0.02d	2.60 ± 0.01b	2.52 ± 0.00c
C18:1 *cis*9	20.24 ± 0.06d	5.57 ± 0.34e	71.93 ± 0.08a	69.86 ± 0.03b	68.57 ± 0.12c
C18:2 n6	65.96 ± 0.01a	6.36 ± 0.06e	8.56 ± 0.01d	10.59 ± 0.03b	9.35 ± 0.00c
C18:3 n3	0.38 ± 0.01b	0.06 ± 0.01c	0.69 ± 0.00a	0.70 ± 0.00a	0.70 ± 0.00a
C18:3 n5	n.d.	81.15 ± 0.57a	n.d.	n.d.	2.74 ± 0.02b
C20:0	0.18 ± 0.01c	0.38 ± 0.01b	0.40 ± 0.00b	0.44 ± 0.00a	0.46 ± 0.01a
C20:1	0.15 ± 0.00c	0.43 ± 0.01a	0.26 ± 0.00b	0.26 ± 0.02b	0.28 ± 0.00b
C22:0	n.d.	0.09 ± 0.01b	0.11 ± 0.00ab	0.13 ± 0.01a	0.13 ± 0.01a
C22:2	n.d.	0.19 ± 0.01c	0.65 ± 0.00a	0.45 ± 0.03b	0.49 ± 0.02b
C24:0	n.d.	0.09 ± 0.00a	0.06 ± 0.01b	0.07 ± 0.00b	0.07 ± 0.00b
SFA	12.87 ± 0.02c	5.63 ± 0.16d	16.46 ± 0.07b	16.91 ± 0.05a	16.65 ± 0.10ab
MUFA	20.64 ± 0.06d	6.04 ± 0.33e	73.58 ± 0.07a	71.29 ± 0.01b	70.00 ± 0.10c
PUFA	66.34 ± 0.02b	87.76 ± 0.50a	9.90 ± 0.01e	11.73 ± 0.06d	13.27 ± 0.00c

GS: grapeseed; PS: pomegranate seed; EVOO: extra virgin olive oil; O-GS oil: olives and grape seed co-milled oil; O-PS oil: olives and pomegranate seed co-milled oil; n.d.: not determined. Results of the analysis of variance by Tukey's test are shown: $p < 0.05$; letters in the same row show significantly different values within each fatty acid.

most abundant class with a similar percentage in all samples, which was around 17%. With regard to this class, the three oils analysed had very similar compositional characteristics, except for small traces of myristic acid (C14:0) and pentadecanoic acid (C15:0) present only in the co-milled oils. The most representative SFA was palmitic acid (C16:0) with values of about 13%, followed by stearic acid (C18:0, ∼2%) and traces of long chain fatty acids such as C20:0, C22:0 and C24:0, as already found in the literature for EVOO (Boskou et al. 2006). PUFA was the poorest class in all oil samples with some significant differences ($p < 0.05$) among them. The control sample (EVOO) showed the lowest content (9.9%), followed by the O-GS oil where PUFA were 11.7%, almost 2 percentage points more than EVOO, due to a slightly higher content of linoleic acid (C18:2 n6) added by GS. Finally, O-PS oil showed more than 13% of PUFA, the highest recorded content ($p < 0.05$) due mainly to the contribution of the punicic acid present in PS, even if its percentage significantly decrease ($p < 0.05$) in the final co-milled oil (Table 1). Nevertheless, considering the few natural sources of CLnA in foods and their importance for human health (Jose and Joseph, 2020), the possibility of using by-products, such as PS, as a source of these bioactive compounds is a promising alternative for the development of functional foods. EVOO is one of the valuable component of the Mediterranean diet, widely consumed for its beneficial and sensory properties but it is lacking in CLnA. For these reasons, the co-milled vegetable oil could help to increase the dietary CLnA intake in the human diet, especially for vegan and vegetarian people. Besides, the co-milled oils showed a higher PUFA/SFA ratio compared to EVOO and several studies report how an higher ratio have more positive effect mainly concerning cardiovascular health (CVH) (Chen and Liu, 2020; Winiarska-Mieczan et al. 2020).

Determination of tocochromanols

Table 2 shows the tocochromanols concentration of oleaginous matrices and co-milled oils. GS, although reported the significant lowest ($p < 0.05$) total content of tocochromanols (19.5 mg/100 g of fat), was the samples with the highest number of individual compounds, that are α-tocopherol (α-T), γ-tocopherol (γ-T), α-tocotrienol (α-T3) and γ-tocotrienol (γ-T3), with the latter as the preponderant one (7.3 mg/100 mg of fat). Bombai et al. (2017) identified in GS only α-T, α-T3 and γ-T3 but with higher content than ours (170–230 mg/100 mg of fat). Crews et al. (2006) also found higher levels of tocochromanols, reaching a concentration of 63–1208 mg/100 g of fat. This variability could be due to the different origin of seeds, grape cultivar as well as the cultivation and processing techniques. PS analysed did not show tocotrienols but it had the highest ($p < 0.05$) content of γ-T (266.7 mg/100 g fat) and it was also the only one with a presence of δ-tocopherol (δ-T), compared to the other samples. These results are in line with what has already been reported in literature about PS (Habibnia et al. 2012; Verardo et al. 2014).

As regard oil samples, the tocochromanol concentrations are in line with those already reported in literature for EVOOs, where the α-T is the main exponent (Cayuela and García 2017). Obviously, as reported by various studies, the pattern in tocochromanols, as well as their content, in EVOO can vary depending on various factors such as cultivar, harvesting and extraction technologies. In some cases, in fact, other isomers such as β-, γ- or δ-T (Boskou 2006) may also be present, but they have been totally absent in our samples.

O-PS oil was the only one with a presence of γ-T because it was the principal tocochromanol in PS with a high concentration (266.7 mg/100 g of fat). As already seen for FAME, also in this case the content of this principal compound significantly decrease ($p < 0.05$) in the final co-milled oil (Table 2). However, the γ-T content improves the tocochromanol pattern of EVOO, increasing its health and antioxidant effects. In particular, some epidemiological studies suggest that γ-T may be superior to α-T as chemiopreventive agent. The unsubstituted 5-position on the chromanol ring of γ-T makes it superior as scavengers of the reactive nitrogen species, like nitrogen oxides or Nox, with the ability to form stable nitro adduct. This ability to trap highly reactive compounds seems to be helpful for protection against prostate cancer (Christen et al. 1997; Sato et al. 2017).

Table 2. Tocochromanol composition and content (mg/100g of fat) of oleaginous matrices and co-milled oils.

	GS	PS	EVOO	O-GS oil	O-PS oil
α-T	3.2 ± 0.7b	8.5 ± 0.6b	90.8 ± 3.2a	80.4 ± 4.1a	82.3 ± 4.7a
α-T3	4.8 ± 0.0	n.d.	n.d.	n.d.	n.d.
γ-T	4.2 ± 0.3c	266.7 ± 4.1a	n.d.	n.d.	35.1 ± 2.2b
γ-T3	7.3 ± 0.0	n.d.	n.d.	n.d.	n.d.
δ-T	n.d.	10.0 ± 0.8	n.d.	n.d.	n.d.
Total	19.5 ± 0.9c	285.6 ± 4.3a	90.8 ± 3.2b	80.4 ± 4.1b	117.4 ± 3.4b

GS: grapeseed; PS: pomegranate seed; EVOO: extra virgin olive oil; O-GS oil: olives and grape seed co-milled oil; O-PS oil: olives and pomegranate seed co-milled oil; α-T: α-tocopherol, α-T3: α-tocotrienol, γ-T: γ-tocopherol γ-T3: γ-tocotrienol, δ-T: δ-tocopherol; n.d.: not determined. Results of the analysis of variance by Tukey's test are shown: $p < 0.05$; letters in the same row show significantly different values within each tocochromanol.

Table 3. Sterol composition and content (mg/100g of fat) of oleaginous matrices and co-milled oils.

	GS	PS	EVOO	O-GS oil	O-PS oil
Campesterol	28.1 ± 0.7b	49.8 ± 3.6a	3.2 ± 0.0c	3.4 ± 0.3c	4.1 ± 1.0c
Campestanol	0.7 ± 0.1	n.d.	n.d.	n.d.	n.d.
Stigmasterol	30.0 ± 0.8a	13.8 ± 1.3b	0.9 ± 0.0c	1.2 ± 0.0c	1.4 ± 0.4c
Clerosterol	5.8 ± 0.3b	9.0 ± 0.3a	n.d.	n.d.	n.d.
β-Sitosterol	187.2 ± 7.5b	320.0 ± 39.4a	89.0 ± 0.2c	92.7 ± 1.8c	107.3 ± 2.8c
Sitostanol	8.4 ± 0.7b	45.5 ± 7.7a	n.d.	n.d.	n.d.
Δ^5-Avenasterol	4.9 ± 0.1c	28.7 ± 3.3a	15.5 ± 0.0b	19.4 ± 0.1b	18.9 ± 2.5b
Δ^7-Sitosterol	2.0 ± 0.3b	10.0 ± 1.0a	n.d.	n.d.	n.d.
Total	267.1 ± 8.9b	476.8 ± 56.0a	108.3 ± 0.4c	116.7 ± 3.3c	131.7 ± 9.6c

GS: grapeseed; PS: pomegranate seed; EVOO: extra virgin olive oil; O-GS oil: olives and grape seed co-milled oil; O-PS oil: olives and pomegranate seed co-milled oil; n.d.: not determined. Results of the analysis of variance by Tukey's test are shown: $p < 0.05$; letters in the same row show significantly different values within each sterol.

The other isomers identified in GS and PS were present in concentrations too low (less than 10 mg/100 g of fat) to make a further contribution to the total tocochromanol pattern of the co-milled oils.

Determination of sterols

A total of eight sterols were identified and quantified in matrices and co-milled oils (Table 3). The β-sitosterol was the main compound present in all samples; in GS, it was followed by stigmasterol, campesterol and sitostanol (30, 28 and 8 mg/100 g of fat, respectively); whereas in PS, β-sitosterol was followed by campesterol, sitostanol and Δ^5-avenasterol (50, 46 and 29 mg/100 g of fat, respectively). In addition, GS was the only sample that reported campestanol (0.7 mg/100 g of fat). These results reflect completely what is already reported in literature for these matrices (Verardo et al 2014; Bombai et al 2017). The three oil samples showed the same sterol composition and content ($p < 0.05$), presenting a pattern typical of EVOO. In fact, confirming what is reported in the literature for EVOO (Demirag and Konuskan 2021), the main sterol in the three oils was the β-sitosterol, with the highest concentration in the O-PS oil, but with values significant lower ($p < 0.05$) compared to the oleaginous matrices. Δ^5-avenasterol was the second main sterol present in all co-milled oils (16, 19 and 19 mg/100g of oil in EVOO, O-GS oil and O-PS oil, respectively), followed by campesterol and stigmasterol (Demirag and Konuskan 2021). The other sterols identified in GS and PS were not detected in the corresponding final co-milled oils.

Study of shelf life

Tocochromanols

As already reported in Table 2, only α-T and γ-T were registered in all oil samples and significantly decrease ($p < 0.05$) along the shelf-life (Table 4). α-T had the same trend in EVOO and O-GS oil with a strong decrease from t0 to t6 equal to 37% and 40%, respectively, and then later, from t6 to t12, both samples showed a constant decrease but not significative from the statistical point of view. Finally from t12 to t18, the α-T concentration decreases significantly ($p < 0.05$) again reaching a similar value in both samples (25.8 and 25.3 mg/100 g of fat in EVOO and O-GS oil, respectively). In O-PS oil, both α-T and γ-T showed a significant decrease during shelf life. From t0 to t6 both the compounds decrease of 60%, then, from t6 to t12 α-T showed a decrease still of the 60% and γ-T only of the 20%. Finally, at t18, α-T and γ-T reached a final concentration equal to 2.1 and 6.1 mg/100 g of fat, respectively, corresponding a decrease of 97% and 83%, respectively. This behaviour is confirmed by literature (Lobo-Prieto et al. 2020; Mancebo-Campos et al. 2022) which reported the same decreasing trend due to their antioxidant properties to counteract the oxidative phenomena.

Table 4. Tocochromanol composition and content (mg/100g of fat) of co-milled oils during shelf life.

	EVOO	O-GS oil	O-PS oil	
	α-T	α-T	α-T	γ-T
t0	90.8 ± 3.2a	80.4 ± 4.1a	82.3 ± 4.7a	35.1 ± 2.2a
t6	57.2 ± 2.5b	48.2 ± 2.3b	32.9 ± 3.2b	14.0 ± 0.9b
t12	48.6 ± 3.1b	33.8 ± 2.5b	13.2 ± 0.9c	11.2 ± 1.1c
t18	25.8 ± 0.1c	25.3 ± 0.2c	2.1 ± 0.1d	6.1 ± 0.1d

EVOO: extra virgin olive oil; O-GS oil: olives and grape seed co-milled oil; O-PS oil: olives and pomegranate seed co-milled oil, α-T: α-tocopherol, γ-T: γ-tocopherol. t0, t6, t12 and t18: shelf life after 0, 6, 12 and 18 months. Results of the analysis of variance by Tukey's test are shown: $p < 0.05$; letters in the same column show significantly different values within each tocochromanol.

Total phenolic content (TPC)

As reported in Table 5, at the beginning EVOO showed higher phenolic content compared to O-GS oil and O-PS oil (57, 33 and 19 mg GAE/100 g). Nevertheless, during the first 6 month of storage, all the samples oils showed the highest and significant ($p < 0.05$) decrease in phenols of about 20%, 19% and 25%, for EVOO, O-GS oil and O-PS oil, respectively.

Table 5. Total phenolic content (TPC, mg GAE/100 g) of co-milled oils during shelf life.

	EVOO	O-GS oil	O-PS oil
t0	57.0 ± 0.9a	33.0 ± 0.5a	19.4 ± 0.5a
t6	45.6 ± 1.2b	26.7 ± 0.5b	14.6 ± 0.9b
t12	43.7 ± 1.6b	25.9 ± 0.9b	13.8 ± 0.7b
t18	42.4 ± 1.4b	25.4 ± 0.8b	11.4 ± 0.4c

EVOO: extra virgin olive oil; O-GS oil: olives and grape seed co-milled oil; O-PS oil: olives and pomegranate seed co-milled oil. t0, t6, t12 and t18: shelf life after 0, 6, 12 and 18 months. Results of the analysis of variance by Tukey's test are shown: $p < 0.05$; letters in the same column show significantly different values within each TPC value.

Table 6. Oxidative stability (IP value, h) of co-milled oils during shelf life.

	EVOO	O-GS oil	O-PS oil
t0	28.2 ± 0.2a	18.1 ± 0.5a	3.1 ± 0.1a
t6	21.2 ± 0.8b	13.6 ± 0.3b	2.2 ± 0.1b
t12	20.1 ± 0.4b	12.9 ± 0.3b	2.0 ± 0.1b
t18	17.6 ± 0.0c	10.6 ± 0.2c	1.6 ± 0.0c

EVOO: extra virgin olive oil; O-GS oil: olives and grape seed co-milled oil; O-PS oil: olives and pomegranate seed co-milled oil. t0, t6, t12 and t18: shelf life after 0, 6, 12 and 18 months. Results of the analysis of variance by Tukey's test are shown: $p < 0.05$; letters in the same column show significantly different values within each IP value.

From the sixth month to the end of the shelf-life, EVOO and O-GS oil did not show significant changes in TPC value reaching a final phenolic content equal to 42.4 and 25.4 mg GAE/100 g, respectively; conversely, O-PS oil showed a further significant reduction from t12 to t18, reaching a final TPC value of 11.4 mg GAE/100 g that was the lowest phenolic content recorded among samples. This behaviour is confirmed by different studies (Fadda et al. 2012; De Leonardis et al. 2021) which reported an initial TPC decrease and a consequent stabilisation; this is due to the polyphenol oxidase action which needs oxygen and, at the same time, to the orientation of phenols in the air–oil interface. So, the significant TPC decrease in the first six months of shelf life is mainly due to the presence of air in the headspace of the storage bottle; differently, the consequent stabilisation could be related to the oxygen dissolved in the oil. Therefore, these results show how both tocochromanols and phenols decrease during the storage of oils probably due to the antioxidant action that these molecules perform in particular against lipid oxidation. Besides, tocochromanols seem to have a higher antioxidant potential because their higher concentration decreases than phenols.

Oxidative stability

Finally, all the oil samples were also tested in order to evaluate their oxidative stability, using the OXITEST® instrument. The results are expressed as IP (Induction Period) value that is the time required to reach the starting point of oxidation, corresponding to either a level of detectable rancidity or a sudden change in the rate of oxidation. The longer the IP value, the higher the stability against oxidation over time. As reported in Table 6, the highest oxidative stability at t0 was registered for EVOO with an IP value (h) of 28 h. Conversely, O-GS oil and O-PS oil presented significantly lower IP values compared to the control amounting to 18 and 3 h, respectively. These results are consistent with the PV of the oleaginous matrices, where PS reported the highest PV ($p < 0.05$) of about 17 meqO_2/kg of fat and GS had a PV of 10 meqO_2/kg of fat; in addition, O-PS oil showed also a very low TPC value, of about 65 and 40% less than the other two samples (Table 5). Comandini et al. (2009) reported a lower IP value for EVOO (6–12 h) but the analysis was performed at higher temperature (110 °C) and probably the oil was obtained from olives of different cultivar; whereas, Lutterodt and collaborators (2011) reported IP for GS oil of about 19 h, in accordance with our results. About PS oil, Costa et al. (2019) reported an IP value very lower than ours (0.1–0.2 h). Obviously these differences are also due to the greater quantity of EVOO in the final co-milled oil.

Along the shelf-life, the trend of the three samples was the same, with a significant ($p < 0.05$) decrease of the IP value during the first six months, followed by a stabilisation between t6 and t12 and again a significant ($p < 0.05$) IP value decrease at the end of the storage (t18). At the end of the shelf life, EVOO still has the highest IP value (17.6 h) compared to the other oils with a decrease in resistance to forced oxidation equal to 38%. Despite, O-GS presented an IP decrease slightly higher (41%), the final IP value was much lower compared to EVOO (Table 6); whereas O-PS oil showed the most significant loss of oxidation resistance (50%) reaching a very low IP equal to 1.6 h.

These results show how the oleaginous matrices are good sources of bioactive compounds but the enrichment of oil was not as satisfactory as expected, except of the increment of punicic acid and γ-T in O-PS oil sample, and this could be partly due to the lower extractive power of co-milling process rather than the phenolic extraction with specific solvent. Most of phenols are polar compounds and the co-milling process, where the solvent is represented by the olive oil, had probably a dilution effect rather than a concentration. In fact, literature reports that only about the 2% of the TPC of the olive fruit are present in the final extracted oil, while the largest amount is unfortunately lost in wastes (olive mill wastewater and pomace) (Solomakou and Goula, 2021). The lowest oxidative

stability of EVO-PS compared to the other two samples, could be partly linked to the lower content of phenolic compounds together with the higher content of PUFA.

Conclusion

Industrial by-products such as GS and PS are large sources of bioactive compounds with important healthy effects. The co-milling is an economically advantageous process for industry, because a single process produces an optimal extractive yield in oil from different raw materials. Results show that the by-product seeds are undoubtedly still rich in bioactive substances that could further increase the nutritional value of EVOO, making it an even more interesting and valuable product. Co-milling with oleaginous matrices allow, undoubtedly, an enrichment of compounds with beneficial and biological effects, such as punicic acid and γ-T from PS. However, the results obtained suggest to better re-evaluate the percentage ratios between olives and matrices, since the compositional profile of the oil samples is just slightly different from EVOO. Besides, it could also be useful to improve the extraction technique, in particular, the malaxation process in order to obtain a better and enriched final product. On the other hand, the results of the oxidative state of the oil samples recommend the need to slow down and prevent the oxidation of these co-milled oils. Definitely, it is important to better preserve the quality and management of the raw by-products (i.e. a rapid drying of the seeds) and could also be useful as a protective and a small container to ensure the highest quality of the final oil until it is completely consumed. Since in a pilot plant the number of tests must necessarily be limited, this study can be considered a preliminary approach that needs further investigations taking into account the great variability of the EVOO composition. Nevertheless, this process represents definitely a technological alternative with remarkable health benefits and the co-milled oils are potentially very interesting for "noble" cold uses, finding the consent of the most demanding consumers because they are product naturally enriched in antioxidants avoiding the use of solvents or chemicals. This new vegetable oils might be marketed as enriched "dressing" produced with olives and by-products. With an appropriate formulation, these vegetable oils could increase the intake of beneficial compounds for health, as well as raise awareness of environmental sustainability for the use of by-products of the food industry.

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Author contributions

Conceptualization, S.M., F.P. and M.F.C.; Methodology, S.M., F.P. and M.F.C.; Validation, S.M., F.P. and M.F.C.; Formal Analysis, S.M., F.P.; Investigation, S.M., F.P. and M.F.C.; Resources, X.X.; Data Curation, S.M., F.P. and M.F.C.; Writing – Original Draft Preparation, S.M.; Writing – Review & Editing, S.M., F.P. and M.F.C.; Supervision, M.F.C.; Project Administration, M.F.C.; Funding Acquisition, M.F.C.

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Data availability statement

Data sharing not applicable.

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