UNIVERSITY OF PADOVA

Department of Land, Environment, Agriculture, and Forestry—TESAF

Master course in Food and Health

Valorization of Grape Pomace Extracts as Natural Antioxidants for Enhancing the Oxidative Stability of Edible Oils

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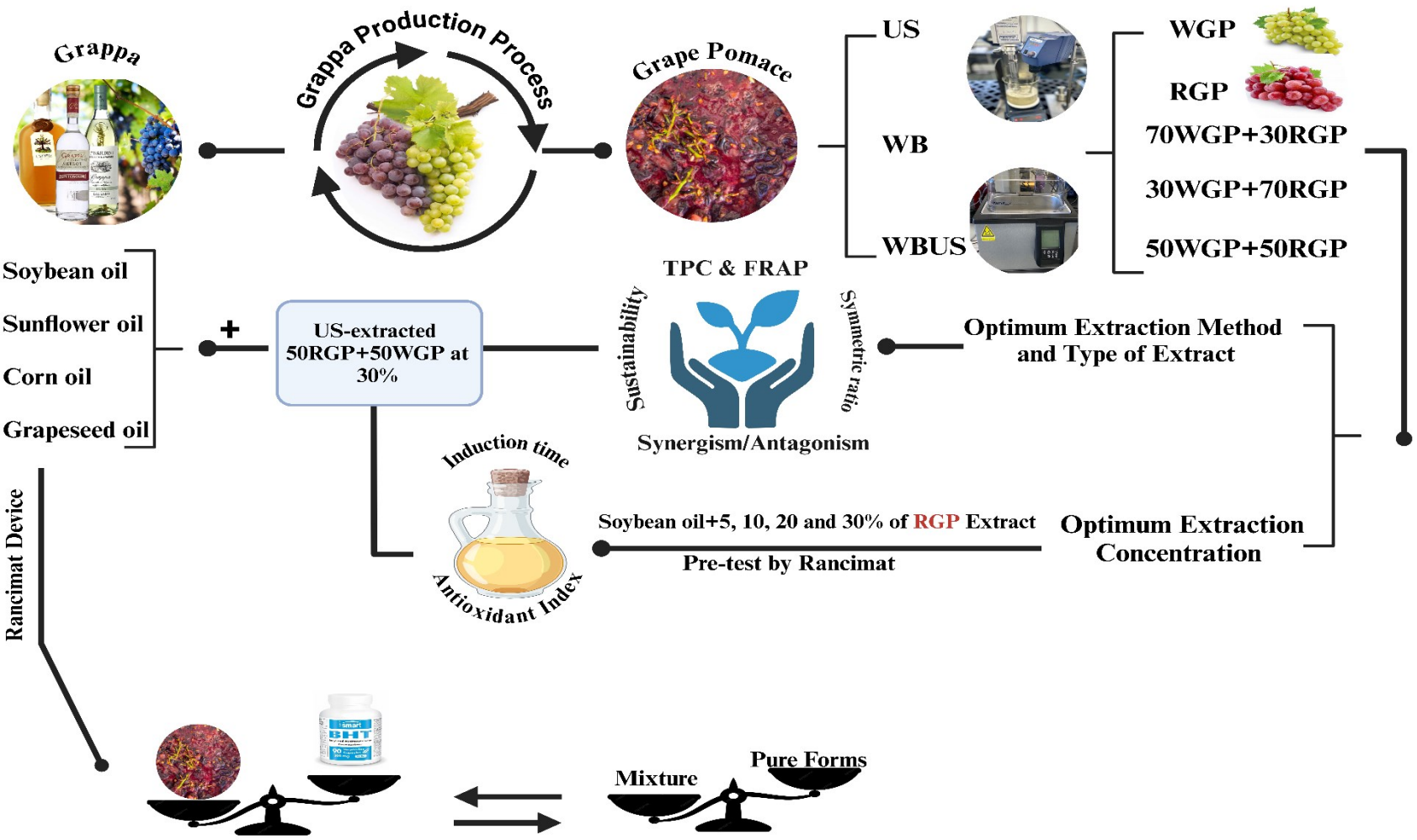
Contents

Abstract

Grape pomace is the main by-product of the winemaking process. This study aimed at recovering natural antioxidants from white and red grape pomace (WGP and RGP, respectively) to inhibit lipid oxidation in different vegetable oils, compared to butylated hydroxytoluene (BHT), a synthetic antioxidant. To obtain ethanolic extracts of the pomace samples, water bath (WB) and ultrasonic extraction (US) methods and a combination of them (WBUS) were used. After the extraction, three mixtures with different WGP:RGP ratios, including 50:50, 70:30, and 30:70 %, were prepared and, along with pure WGP and RGP, were subjected to total phenolic content (TPC) and antioxidant activity (AOA) evaluations, aiming at finding the optimal extraction method and extract concentration. The TPC and FRAP tests revealed that there is a significant difference among the yield of the methods ($p \leq 0.05$). Although WBUS showed the highest TPC values in TPC evaluation, the US method was chosen as optimal due to its sustainability and the RGP50+WGP50 mixture with the highest value was selected as the optimum sample. The same in FRAP, where the US method was selected as it is a sustainable method; nevertheless, instead of RGP30+WGP70 mixture, RGP50+WGP50 mixture was selected, highlighting the importance of the symmetric ratio in the sample. To determine the optimum concentration to be added to the oil sample, a preliminary test was done by applying RGP at 5, 10, and 30% on soybean oil, followed by subjecting the extract-added oil to the Rancimat test. The results revealed that for both Induction Time (IT) and Antioxidant Activity Index (AAI), according to the obtained significance levels (0.05), there is a significant difference between 30%RGP extract with other lower concentrations of RGP extract ($p \leq 0.05$). Determining the 50RGP+50WGP mixture at 30% concentration derived through the US as the optimum extraction, it was applied to the four edible oils, namely sunflower oil, soybean oil, corn oil, and grape seed oil, and the results were compared to BHT antioxidant. In addition, pure forms of RGP and WGP extracts at the same concentration were derived, and the same extraction method was used in the experiment to evaluate the performance of the mixture with pure forms, control, and BHT samples. Taking the value of AAI and IT into consideration, mixed grape pomace extracts significantly improve the oxidative stability of different edible oils more effectively than either individual grape pomace extracts or BHT at 200 ppm, suggesting a synergistic effect when red and white grape pomace are combined. The results of this study highlight the point that grape pomace-derived phenolic compounds possess considerable antioxidant potential, which can function as natural additives and produce functional foods.

Keywords: Grape pomace, Ethanolic extract, Phenolic content, Oxidative stability, Edible oil

Graphical abstract



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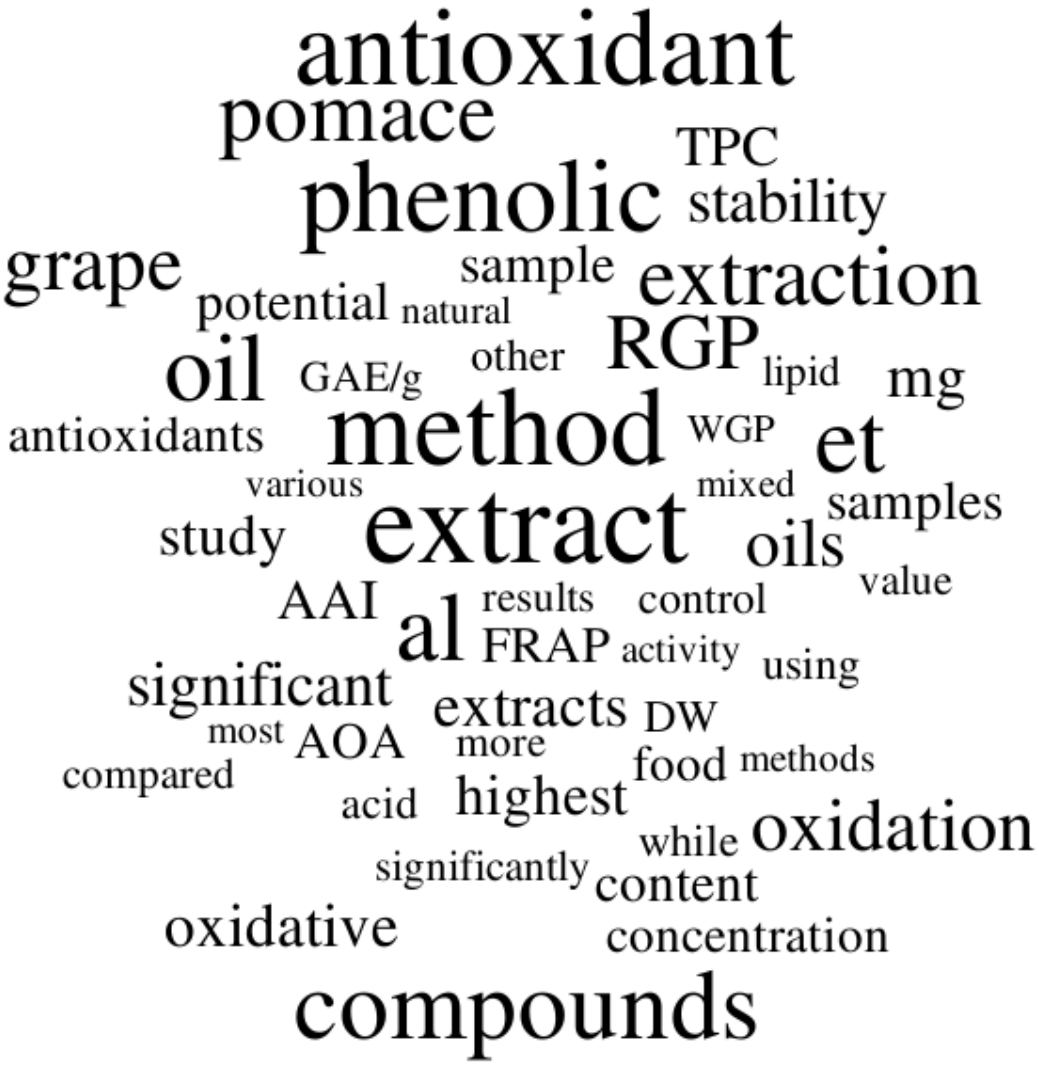


*The word Cloud was obtained according to the manuscript, and tables and figures were excluded based on 100 total words with a minimum frequency of 20. Variations in word sizes correspond to frequency (created by *worditout.com*).

Abbreviations

A
AAE : Ascorbic Acid Equivalents
AAI : Antioxidant Activity Index
AOA : Antioxidant Activity
ABTS : 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

B
BHT : Butylated Hydroxytoluene
BHA : Butylated Hydroxyanisole

C
CE : Catechin Equivalents
°C : Degrees Celsius

D
DPPH : 2,2-Diphenyl-1-picrylhydrazyl
DW : Dry Wight

E
None

F
FCI : Free Radical Scavenging Capacity Index
Fe^{2+} : Iron (II) or Ferrous Iron
Fe^{3+} : Iron (III) or Ferric Iron
FRAP : Ferric Reducing Antioxidant Power

G
GAE : Gallic Acid Equivalents
GPE : Grape Pomace Extract
GPP : Grape Pomace Powder
GP : Grape Pomace

H
HAT : Hydrogen Atom Transfer
HCl : Hydrochloric Acid
HHP : High Hydrostatic Pressure
HPLC-DAD : High-Performance Liquid Chromatography with Diode Array Detector
HPLC-PDA : High-Performance Liquid Chromatography with Photodiode Array Detector

I
IC50 : Half Maximal Inhibitory Concentration
IP : Potential Ionization
IT : Induction Time

L
LC-ESI-QTOF-MS/MS : Liquid Chromatography Electrospray Ionization Quadrupole Time-of-Flight Mass Spectrometry/Mass Spectrometry
LOX : Lipoxygenase

M
MAE : Microwave-Assisted Extraction
MUFAs : Monounsaturated Fatty Acids

N
$NaCO_3$: Sodium Carbonate

O
ORAC : Oxygen Radical Absorbance Capacity

P
PDA : Photodiode Array
PPM : Parts Per Million
PUFA : Polyunsaturated Fatty Acids

Q
None

R
RGP : Red Grape Pomace
RNS : Reactive Nitrogen Species
RSO : Refined Soybean Oil

S
SAR : Structure-Activity Relationship
SE : Soxhlet Extraction
SEM : Soxhlet Extraction Method
SET-PT : Sequential Electron Transfer-Proton Transfer
SFAs : Saturated Fatty Acids

T
TBHQ : Tertiary Butylhydroquinone
TEAC : Trolox Equivalent Antioxidant Capacity
TE : Trolox Equivalent
TPC : Total Phenolic Content
TPTZ : 2,3,5-Triphenyltetrazolium

U
US : Ultrasonic
UAE : Ultrasound-Assisted Extraction

V
None

W
WB : Water Bath
WBUS : Water Bath and Ultrasonic (combined technique)
WGP : White Grape Pomace

X
None

Y
None

Z
None

Chapter 1:

Introduction

The origins of food industrial by-products vary, encompassing a diverse array of components such as peel, stem, leaf, seed, shell, bran, kernel, pomace, and oil cake, as documented in studies. The incorporation of functional ingredients derived from distinct by-products exerts a discernible influence on the technological, nutritional, and health-enhancing attributes of bakery products (Martins & Ferreira, 2017). One common use of food by-products is as animal feed. For example, spent grains from breweries can be used as livestock feed (Brewers Association, 2021). Similarly, the pulp and peels left over from juicing can be used to feed animals like pigs and cows (FoodPrint, 2021). Another use of food by-products is in the production of biofuels. For example, the oils extracted from used cooking oil can be converted into biodiesel (USDA, 2021). This not only provides a renewable source of energy but also helps to reduce waste. Finally, some food by-products can even be used to create new food products. For example, the whey left over from cheese production can be used to make protein powders and other food supplements (FoodPrint, 2021). This not only reduces waste but also provides a new source of income for food producers.

In this relevance, grape pomace can also be mentioned, and its production process can be seen in Figure 1. Wine pomace has historically been undervalued due to a lack of alternative applications offering economic benefits. Traditionally, it has been subjected to distillation processes, yielding various forms of "wine alcohol" (Silva et al., 2000), which are employed in the production of esteemed distilled spirits, liquors, and liqueurs (González-SanJosé, 2014), as well as for fortifying wines. Conventional uses of wine pomace also extend to its utilization as fertilizer or animal feed (Arvanitoyannis et al., 2006). For example, Diaz et al. (2002) advocated for the composting of wine pomace to enhance the organic matter, nitrogen, and mineral content of vineyard soils. However, these practices are not without drawbacks, particularly concerning the presence of antinutritive compounds that may adversely impact crop yields and animal weight gain. Furthermore, these approaches fall short of fully capitalizing on the comprehensive market potential inherent in this by-product (Dwyer et al., 2014).

Grapes rank among the most extensively cultivated crops globally, with an annual production approaching approximately 63 million tons worldwide. Notably, a significant proportion of this total grape yield, precisely 75%, is dedicated to wine production. The by-product derived from the winemaking process is of particular significance, constituting approximately 20% of the grape weight. Termed either grape pomace or wine pomace, this residual material is composed of remnants from pressed grapes, including small stalk fragments and yeast cells resulting from the wine fermentation process (García-Lomillo et al., 2017). Post-distillation grape pomace is also a by-product of the winemaking process that consists of the solid remains of grapes after they have been pressed and fermented. This pomace contains a variety of compounds, including grape skins, seeds, and stems, as well as residual sugars and acids (Vázquez-Araújo et al., 2018).

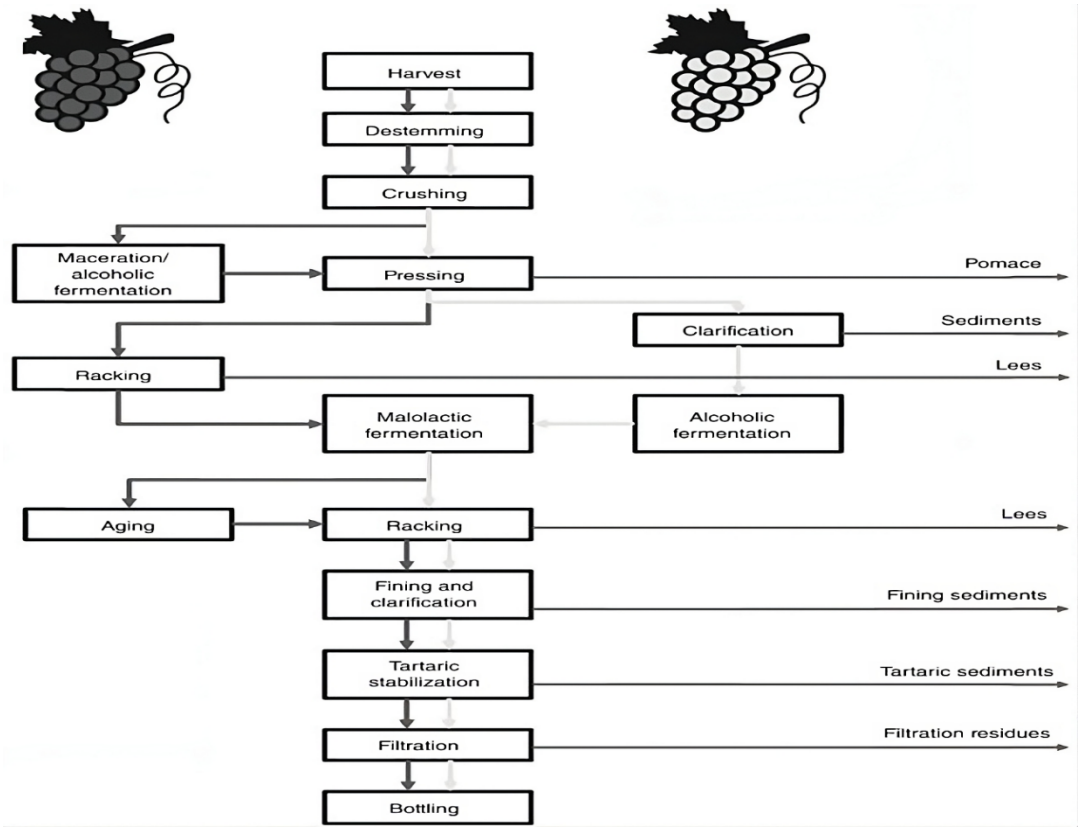


Figure 1: Generation of grape pomace in the process of white and red wine (Galanakis, 2017)

While grape pomace is often used as animal feed or compost, there has been growing interest in its potential for use in human food and beverage products. Pomace has been found to contain high levels of antioxidants, dietary fiber, and other beneficial compounds, making it an attractive ingredient for functional foods and supplements. In addition, pomace has been shown to have potential as a source of bioactive compounds for the pharmaceutical industry. Studies have found that pomace extracts have anti-inflammatory, anticancer, and antidiabetic properties (Galanakis, 2017; Llobera & Cañellas, 2007).

The concept of revalorizing wine pomace is not a recent endeavour, with diverse alternatives proposed since the 1970s, all centred on harnessing the valuable compounds contained within this by-product. One notable early proposal gaining international recognition in the food, pharmaceutical, and cosmetics industries was the production of "enocyanine." This innovation marked a significant milestone, leading to the development of several commercially available enocyanines, recognized in Europe as the food colourant E-163, derived from anthocyanins isolated from red wine pomace (Dwyer et al., 2014). While enocyanins have achieved notable success, other alternatives have also been explored, albeit without matching the prominence of enocyanins. For instance, proanthocyanidins extracted from grape seeds have been commercialized in France since the 1970s (e.g., Endotelon) for medical applications. However, the use of similar products in the food industry is not commonplace. Grape seed oil, another derivative of grape processing, has been produced for decades and is gaining traction in the market as a gourmet product (Dwyer et al., 2014).

Until the late 1990s, predominant approaches to wine pomace revalorization encompassed extraction processes followed by concentration and separation procedures, aiming to yield products enriched with specific compounds (e.g., tartaric acid or

proanthocyanidins). However, in recent decades, novel strategies have emerged, seeking to circumvent extraction phases and instead focus on the generation and utilization of minimally processed derivatives derived from wine pomace (Duque et al., 2011; García-Lomillo & González-SanJosé, 2013; Jang et al., 2015).

The ultimate goal for finding the most appropriate extraction method has been to characterize and identify compounds with biological activities, specifically antioxidants. Instance given, the antioxidant effectiveness in mitigating lipid oxidation by wine pomace has been investigated in conjunction with various natural extracts, including essential oils (Adams et al., 2002; Moradi et al., 2011; Tajik et al., 2015) and green tea extracts (Rababah et al., 2010, 2011a), with potentially objectionable additives such as sulphites (Bañón et al., 2007). To elaborate more, phenolic compounds present in the extract of grape pomace have been reported to improve the oxidative stability of oils and products containing oil. In Figure 2, various phenolic compounds which can be derived from different art of grape fruit can be seen.

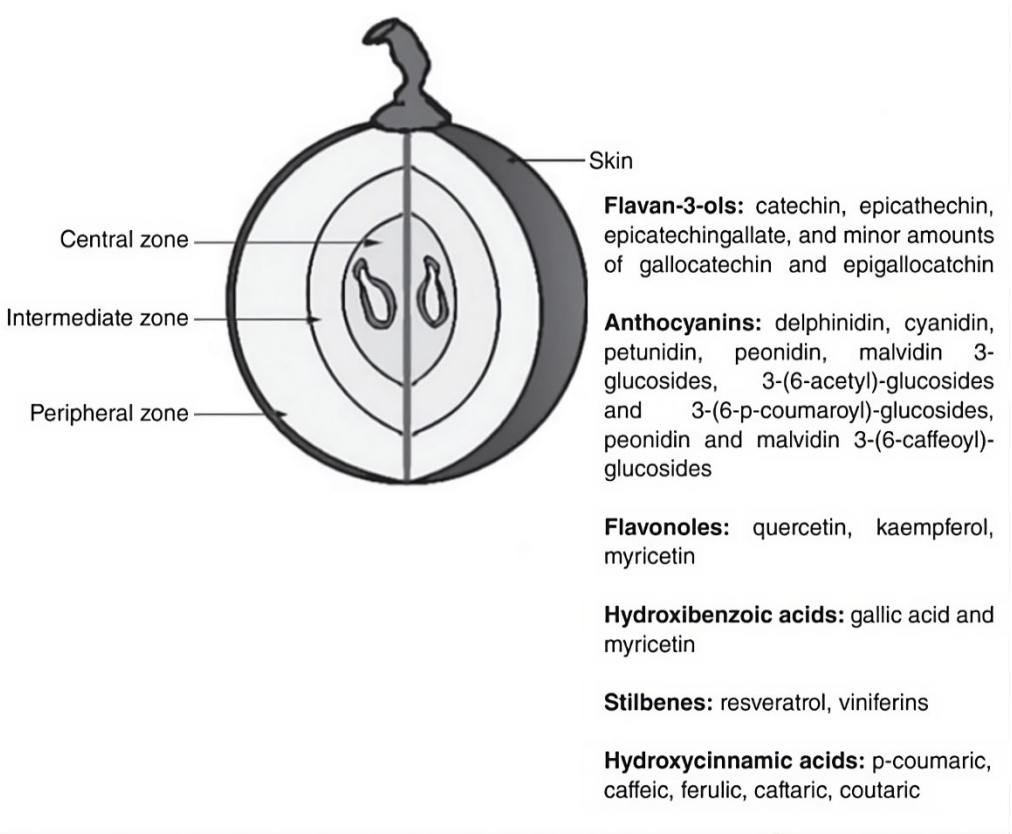


Figure 2: Various phenolic compounds present in the grape fruit (Galanakis, 2017)

In the context of preventing lipid oxidation in fats and oils, wine pomace products have been successfully integrated into oils with varying fatty acid profiles. This includes oils rich in linoleic acid, such as grapeseed oil (Jang et al., 2015), sunflower oil (Poiana, 2022), and soybean oil (Bakota et al., 2015), as well as oils abundant in oleic acid, such as olive oil (Bonilla et al., 1999) and canola oil (Schevey & Brewer, 2015). Grape seed extract has been incorporated into solid systems like pork lard and oil-water emulsions (Altunkaya et al., 2013), which holds particular relevance in food systems like mayonnaise and salad dressing (Tseng & Zhao, 2013).

Lipid oxidation is, with microbial spoilage, one of the main factors limiting the shelf-life of food products, causing large loses during storage. Lipid oxidation involves two phases: primary oxidation, which induces the formation of lipid hydroperoxides, diene and triene conjugates, and secondary oxidation, which leads to the formation of volatile compounds (Frankel 1983). Consequently, the sensory quality deteriorates, the nutritional value is reduced (due to the destruction of nutrients such as polyunsaturated fatty acid (PUFA) and vitamins), and the technological properties may also be affected (Kanner 1994). Moreover, some compounds derived from lipid oxidation, especially those from the primary oxidation, can present toxic effects. In broad terms, existing research indicates that products derived from wine pomace exhibit a more pronounced inhibitory effect on the secondary phase of lipid oxidation compared to the primary phase (Sánchez-Alonso et al., 2006). While individual phenolic compounds may possess noteworthy antioxidant activity (AOA), the collective activity of wine pomace products typically surpasses that of isolated compounds (Shaker, 2006; Maestre et al., 2010). This observation implies a synergistic impact among phenolic compounds. Concerning concentration, relatively modest levels of wine pomace extracts suffice to impart an antioxidant effect, albeit with variations in published data depending on the specific product utilized. Consequently, various wine pomace products have demonstrated effectiveness at concentrations as low as ten ppm to as high as 10% (Rojas and Brewer, 2007; Shirahigue et al., 2010; Hasani and Alizadeh, 2015).

In the present study, we aimed to find the best-performing, most sustainable extraction method for deriving phenolic compounds from white and red grape pomace to finally yield extract with the highest phenolic compounds form comparing its lipid oxidation effect on shelf life extension of four edible oils, namely soybean, sunflower, corn, and grape seed oil, as a natural antioxidant and its competitiveness with BHT syntactic antioxidant.

Phenolic compounds

Polyphenols, also known as phenolic compounds, constitute significant phytochemical substances found in plants (Bravo, 1998). They show a large diversity of structures, including relatively simple molecules (e.g. vanillin, gallic acid, caffeic acid) and polyphenols such as stilbenes, flavonoids, and polymers derived from these various groups. For example, over 8,000 molecules have been reported in the flavonoid family alone, and the list continues to expand (Andersen & Markham 2006). Phenolic chemicals have significant antioxidant properties and are often present in fruits and vegetables (Heim, 2002). These compounds include flavonoids, flavanols, anthocyanins, anthraquinones, benzoyl acetyl, and their derivatives. Polyphenols are divided into two classes: flavonoids and phenolic acids. Certain parts of the plant contain phenolic chemicals, which are natural antioxidants. In Figure 3, the chemical structure of the most simple phenolic compounds can be seen.

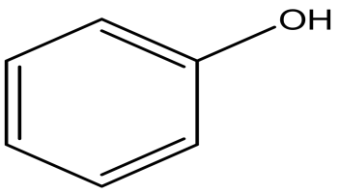


Figure 3: Phenol, the most straightforward component of phenolic compounds

Phenolic compounds possess a high potential for AOA, and plants have always been excellent food sources for the consumption of valuable bioactive compounds. These natural antioxidants have been extracted from plants in the form of essential oils and extractions from various sources such as fruits (grapes, pomegranates, dates, etc.), vegetables (broccoli, potatoes, drumstick, pumpkin, Indian turmeric, nettle), medicinal plants, and spices (tea, rosemary, oregano, cinnamon, common sage, thyme, peppermint, ginger, clove) and have been investigated for reducing fat oxidation (Maleki et al., 2022). Phenolic compounds are discovered in combination with saccharides (monosaccharides and polysaccharides) bonded to one or more phenolic groups. Although some phenolic compounds are ubiquitous, others are specific to particular plant families and are found in particular plant organs or at specific stages of plant growth (Maleki et al., 2022).

Phenols may shield biomolecules (proteins, nucleic acids, polyunsaturated lipids, and carbohydrates) from oxidative damage caused by free radical-mediated procedures (Heleno et al., 2015). Antioxidants perform these functions through two primary mechanisms: free radical inactivation and electron transfer (Valko et al., 2007). The first process involves the free radical ($R^{\bullet}$) removing a hydrogen atom from the antioxidant (ArOH), causing it to become radical. The lower the bond dissociation energy of the O-H bonds, the easier it is to inactivate the free radical and, hence, the more significant the AOA.

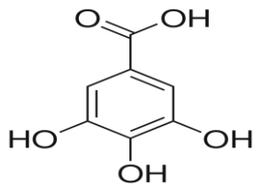
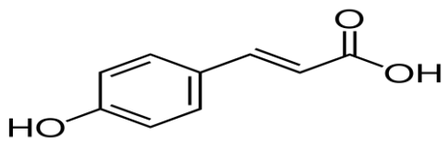
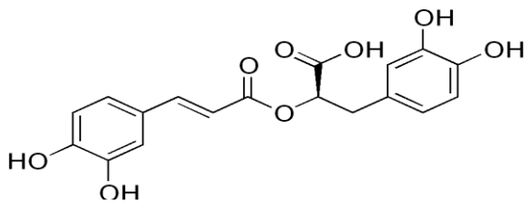
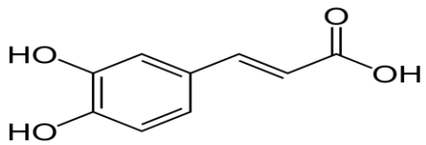
$$R^{\bullet}+ArOH \rightarrow RH+ARO^{\bullet}$$

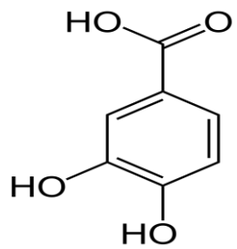
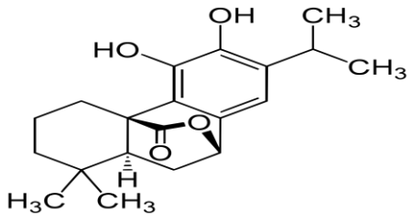
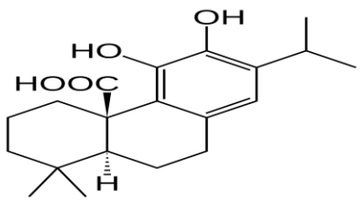
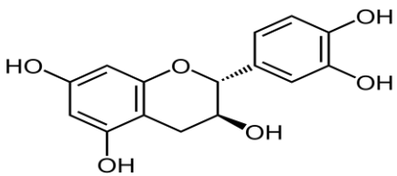
The second technique involves the antioxidant donating an electron to the free radical, causing it to become a cation radical. In this process, the lower the potential ionization (IP), the easier the electron abstraction, resulting in increased AOA.

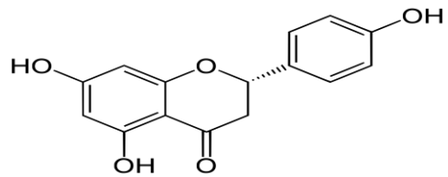
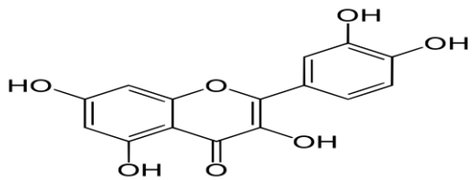
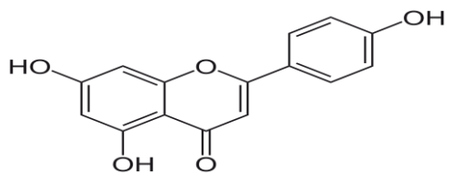
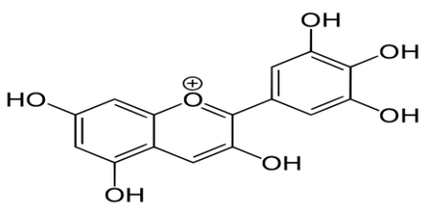
$$R^{\bullet}+ArOH \rightarrow RH^{-}+ARO^{\bullet +}$$

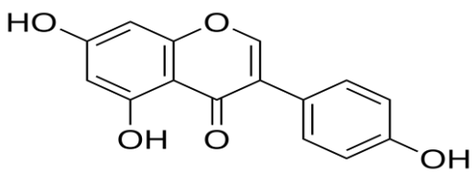
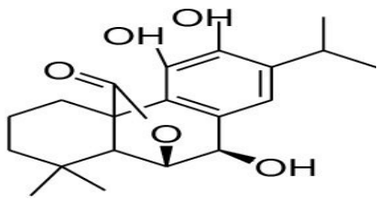
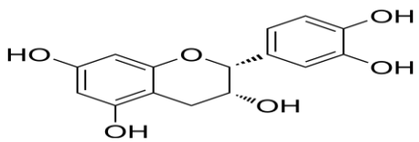
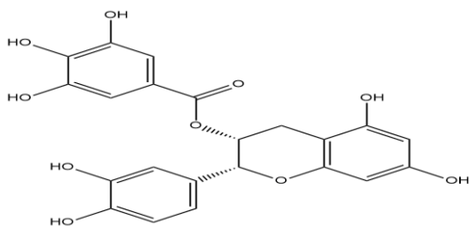
The structure-activity relationship (SAR) (Bendary et al., 2013) establishes the antioxidant effect of phenolics, including the number and locations of the hydroxyl group (-OH), the existence of double bonds (C2=C3), glycosylation, and the presence of substituents in the rings (Wang et al., 2018). As previously proven, hydroxyl groups transfer hydrogens and electrons, resulting in stable radicals. Thus, the location and hydroxylation number of a flavonoid correlate with its antioxidative properties. The presence of two hydroxyl groups in the ring implies an enhanced antioxidant impact, whereas the presence of 3-OH contributes to inhibiting AOA. Hydrophilicity is improved by increasing the amount of hydroxyls, retaining the nucleus of the flavonoid in the hydrophobic cavity, which may establish a link with the enzymatically relevant active site (Wang et al., 2018). The presence of a C2=C3 double bond with a 4-carbonyl group enhances antioxidant action by providing planarity, electron expansion, and displacement between rings. The 4-carbonyl group can produce electron shift through resonance effects, altering the dissociation constant of the hydroxyl groups and the stability of radicals. In Table 1, the chemical structure of the most common phenolic compounds has been presented (Wang et al., 2018).

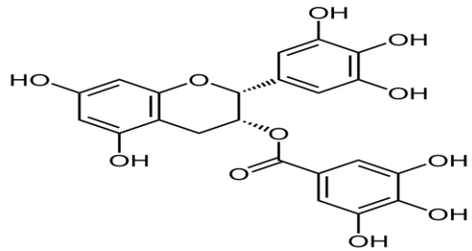
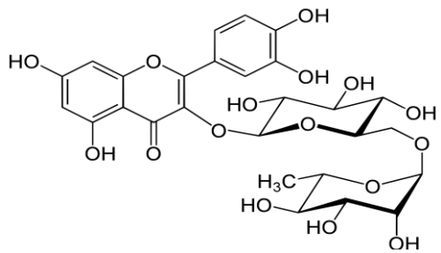
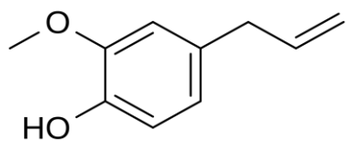
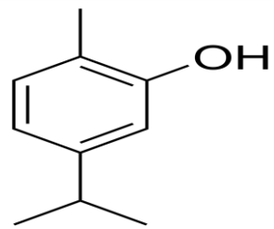
Table 1: The phenolic compounds with high antioxidant activity (By Maleki et al., (2022) with modifications)

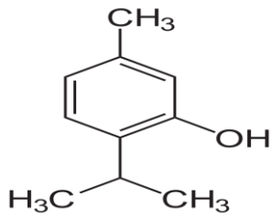
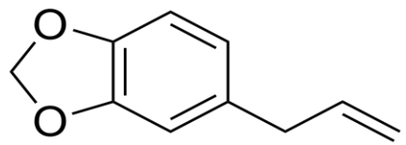
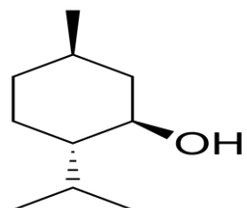
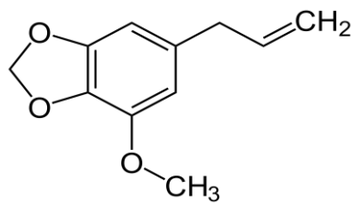
Name	Chemical formula	Structure	Reference(s)
Gallic acid	$C_7H_6O_5$		Gallic acid prevents the melanogenesis, rancidity, and spoilage of fats and oils due to its antioxidant nature, facilitating its application as a food additive in various eatable materials like baked goods, candy, chewing gums, and cosmetics (Su et al., 2013).
p-Coumaric acid	$C_9H_8O_3$		*p*-coumaric acid occurs widely in the cell walls of graminaceous plants. It decreases low-density lipoprotein peroxidation, shows antioxidant and antimicrobial activities, and plays a vital role in human health. It is a good antioxidant and a good antimicrobial; therefore, it is a natural alternative instead of synthetic additives (Boz et al., 2015).
Rosmarinic acid	$C_{18}H_{16}O_8$		A significant number of herbal preparations and food supplements containing rosmarinic acid are marketed with claims for beneficial health effects. Due to lipid peroxidation and bacterial growth inhibition, rosmarinic acid is approved for use as a natural antioxidant and/or preservative in the food industry (Marchev et al., 2021).
Caffeic acid	$C_9H_8O_4$		Natural phenolic antioxidants, including caffeic and ferulic acids, gained attention as photoprotective agents. Caffeic acid is an α-tocopherol protective in low-density lipoprotein. Furthermore, their combination with other products, such as chlorogenic and caftaric acids, showed more potent AOA in various systems (Magnani et al., 2014).

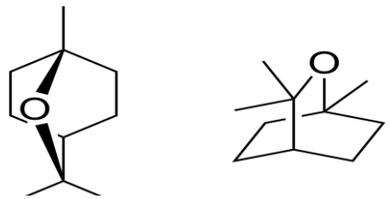
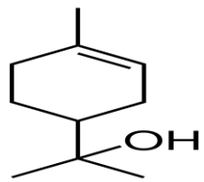
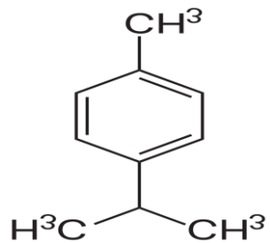
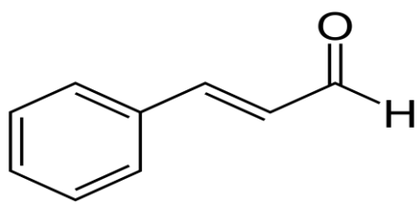
Protocatechuic acid	$C_7H_6O_4$		Protocatechuic acid is a type of widely distributed naturally occurring phenolic acid. Protocatechuic acid has structural similarities with gallic acid, caffeic acid, vanillic acid, and syringic acid, which are well-known antioxidant compounds. Hence, it could therefore be used in the pharmacological or food industry as a natural antioxidant (Kakkar, & Bais, 2014).
Carnosol	$C_{20}H_{26}O_4$		Several *in vitro* studies were reviewed regarding the AOA of the carnosol and carnosic acid. Using the DPPH method, these bioactive compounds were validated for their AOA. Also, using the thiobarbituric acid, superoxide anion, and lipid-free radicals scavenging activity assays and Rancimat methods (determinationof oxidative stability of fat), carnosol has been reported to inhibit lipid peroxidation through the lipid-free radical scavenging mechanism. These studies have shown the antioxidant potential of carnosol and carnosic acid, whose properties are closely related to other biological activities, such as cytoprotective and anticancer, primarily due to their ability to neutralizereactive oxygen species (Andrade et al., 2018).
Carnosic acid	$C_{20}H_{28}O_4$		
Flavanol (catechin)	$C_{15}H_{14}O_6$		It has been revealed that catechin is an antioxidant at all concentrations, and the antioxidant behaviour is attributed to iron chelation. Consequently, catechin has been a widely used antioxidant in combination with other biopolymers for the development of active food packaging (Sivakanthan et al., 2020).

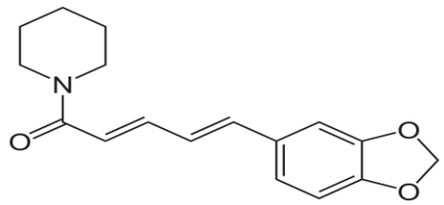
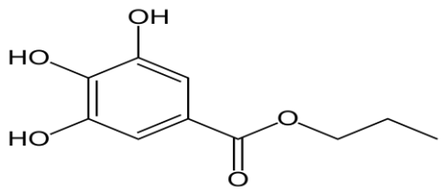
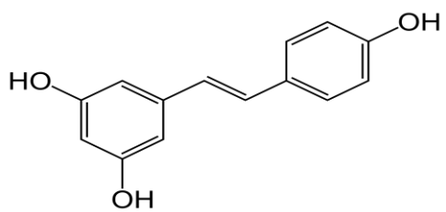
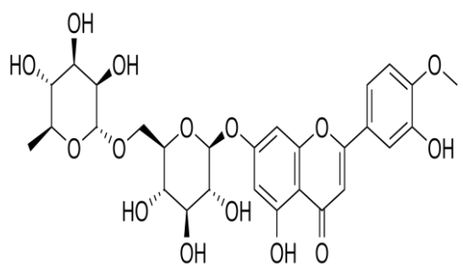
Flavanone (naringenin)	$C_{15}H_{12}O_5$		Naringenin has exhibited higher antioxidant capacity and hydroxyl and superoxide radical scavenger efficiency than naringin. Additionally, naringenin has shown greater effectiveness in the protection against oxidative damage to lipids in a dose-dependent manner (Cavia-Saiz et al., 2010).
Flavonol (quercetin)	$C_{15}H_{10}O_7$		Due to the capacity of quercetin to scavenge free radicals and bind transition metal ions, quercetin is considered a powerful antioxidant. Because of these qualities, quercetin can prevent lipid peroxidation (Baghel et al., 2012).
Flavone (Apigenin)	$C_{15}H_{10}O_5$		Apigenin is a widely distributed flavonoid responsible for antioxidant potential and chelating redox-active metals. The antioxidant mechanism of apigenin includes oxidant enzyme inhibition, modulation of redox signalling pathways, reinforcing enzymatic and nonenzymatic antioxidants, metal chelation, and free radical scavenging (Kashyap et al., 2022).
Anthocyanin (delphinidin)	$C_{15}H_{11}O_7^+$		Anthocyanins are polyphenols with known AOA, which can be responsible for various biological activities. The antioxidant capacity of plant-derived anthocyanins has been demonstrated with various assay methods, including ORAC, FRAP, TEAC, and DPPH (Miguel, 2011).

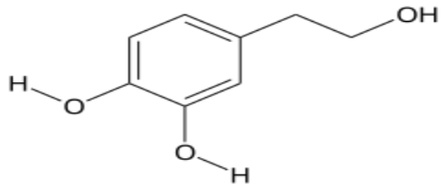
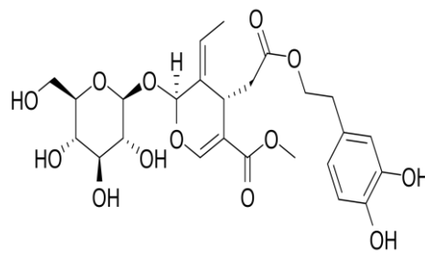
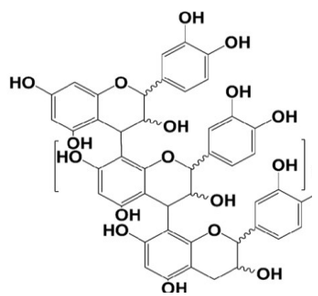
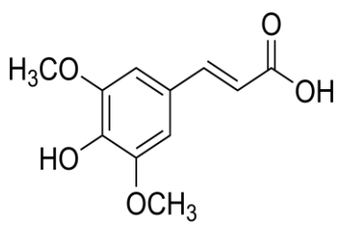
Isoflavone (genistein)	$C_{15}H_{10}O_5$		Genistein has been widely studied as an antioxidant, with some research claiming that it may have the ability to scavenge reactive oxygen and nitrogen species (ROS and RNS) effectively (Weng et al., 2019).
Rosmanol	$C_{20}H_{26}O_5$		A comparison between rosmanol and synthetic antioxidants has shown that rosmanol possesses an antioxidant capacity four times higher than synthetic antioxidants of BRT and BRA (Nieto et al., 2018).
Epicatechin	$C_{15}H_{14}O_6$		Catechin and epicatechin are two flavan-3-ol stereoisomers that, according to their structure, have comparable radical scavenging and AOA. Both have the ability to bind the iron ion and scavenge DPPH radicals (Yilmaz, 2006).
Epicatechin gallate(ECG)	$C_{22}H_{18}O_{10}$		Evaluation of radical scavenging activity of some catechins *in vitro* revealed that in each of the assays, including DPPH, ABTS, and FRAP after epigallocatechin gallate, epicatechin gallate had the highest potential (He et al., 2018).

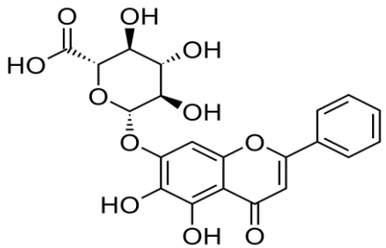
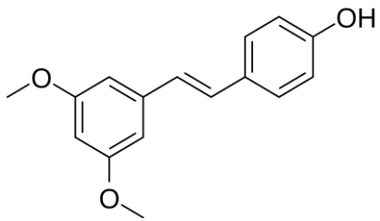
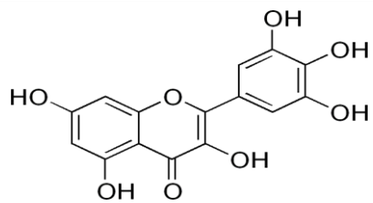
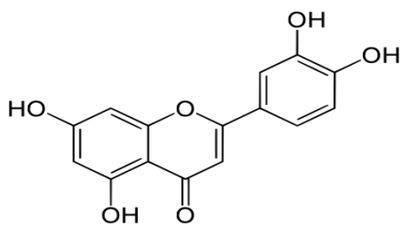
Epigallocatechin gallate (EGCG)	$C_{22}H_{18}O_{11}$		Epigallocatechin gallate effectively protected against supercoiled DNA nicking caused by peroxyl and hydroxyl radicals. In scavenging the DPPH radical, epigallocatechin gallate had a synergistic impact with -tocopherol, demonstrating a direct free radical scavenging potential (Hu & Kitts, 2001).
Rutin	$C_{27}H_{30}O_{16}$		Rutin has exhibited vigorous DPPH radical scavenging activity. In addition, rutin possesses an effective inhibition property of lipid peroxidation. The AOA of rutin is competitive with various antioxidants such as BHT and ascorbic acid (Yang et al., 2008).
Eugenol	$C_{10}H_{12}O_2$		The AOA of eugenol has been evaluated with different assays such as DPPH, ABTS, and DMPD, which are competitive with inhibiting potential for lipid peroxidation of standard antioxidants including BHA, BHT, α-tocopherol, and Trolox (Gülçin, 2011).
Carvacrol	$C_{10}H_{14}O$		The AOA of carvacrol has been shown through a series of *in vitro* assays, including hydroxyl radical, superoxide, nitric oxide, DPPH, andABTS radical scavenging assays (Aristatile et al., 2015).

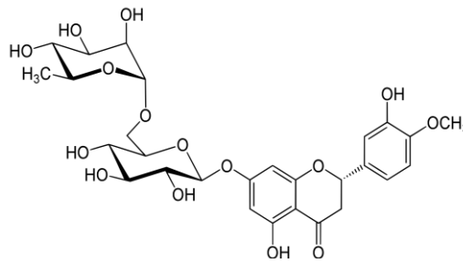
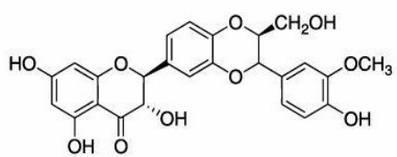
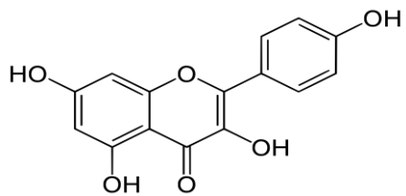
Thymol	$C_{10}H_{14}O$		Different reports regarding the antioxidant properties of thymol are due to its ability to donate H-atoms from phenol hydroxyl groups, which could react with peroxyl radicals to produce stabilized phenoxyl radicals and, consequently, terminate lipid peroxidation chain reactions (Viuda-Martos et al., 2010).
Safrole	$C_{10}H_{10}O_2$		The AOA of safrole oil has been reported to be comparatively lower than the Trolox reference standard, which is known to have high AOA. However, free radicals scavenging activity of safrole is still considered eminent (Eid et al., 2021).
Menthol	$C_{10}H_{20}O$		Different studies have reported that the AOA of some of the plant-derived essential oils is related to menthol; for instance, the radical scavenging activity of peppermint essential oil is associated with the presence of menthol and menthone, which contains the hydroxyl radical (Wu et al., 2019).
Myristicin	$C_{11}H_{12}O_3$		While evaluating the myristicin with DPPH and ABS methods revealed its AOA inadequacy compared to BHA, the synergetic effect of myristin with other antioxidants can be considered (Lim & Shin, 2012).

Eucalyptol	$C_{10}H_{18}O$		Previous research has shown that there might be a direct relationship between the high scavenging activity of essential oil over BHT and the high content of Eucalyptol in the essential oil (Larayetan et al., 2017).
Terpineol	$C_{10}H_{18}O$		Due to various biological activities, terpineol is a proper compound to improve the shelf life of food products, as it has been shown to be effective in enhancing the AOA of chitosan biopolymer to inhibit free radicals mediated deterioration (Chaudhari et al., 2020).
p-Cymene	$C_{10}H_{14}$		Previous investigations on DPPH free radical scavenging by some plant-derived essential oils have revealed that *p*-Cymene, as one of the main constituents, plays a prominent role in the AOA of the essential oils (Reza & Nejad, 2014).
Cinnamaldehyde	C_9H_8O		Cinnamaldehyde enhances the antioxidant defence against reactive oxygen species and, reduces the levels of lipid peroxidation products, and increases the activities of antioxidant enzymes (Subash-Babu et al., 2014).

Piperine	$C_{17}H_{19}NO_3$		Piperine interacts readily with highly oxidizing radicals and binds redox-active metal ions, indicating that it could potentially function as an effective antioxidant (Carp et al., 2021).
Propyl gallate	$C_{10}H_{12}O_5$		As it has become determined by free radical scavenging assays, the propyl gallate compound has the potential to inhibit lipid oxidation which is competitive with TBHQ (Alamed et al., 2009).
Resveratrol	$C_{14}H_{12}O_3$		In different studies, resveratrol has exhibited a robust reducing power, chelating activity on Fe^{2+}, free radical-scavenging, hydrogen peroxide scavenging, and hydroxyl radical scavenging activities (Hussein, 2011).
Diosmin	$C_{28}H_{32}O_{15}$		The *in vitro* studies on diosmin showed its free radical scavenging activity. The hydroxyl-free radical scavenging and superoxide-free radical scavenging activities of diosmin have been evidenced by the *in vitro* studies (Queenthy & John, 2013).

Hydroxytyrosol	$(HO)_2C_6H_3CH_2CH_2OH$		Researchers synthesized two novel lipophilic derivatives of hydroxytyrosol, which exhibited significantly stronger antioxidant activities at high temperatures compared to hydroxytyrosol and tert-butylhydroquinone, making them suitable for industrial applications requiring robust antioxidants (Olajide et al., 2020).
Oleuropein	$C_{25}H_{32}O_{13}$		It has been reported that oleuropein can be efficiently extracted and purified from olive leaves of different varieties, with genetic factors significantly affecting its yield, and the purified compound exhibits enhanced antioxidant and antimicrobial activities, suggesting its potential for improving the functional properties and shelf life of food products (Topuz & Bayram, 2022).
Proanthocyanidins	$C_{30}H_{26}O_{12}$		A study found that proanthocyanidins from the rhizome of *F. dibotrys* are the primary antioxidant and antidiabetic compounds, showing similar AOA and more vigorous antidiabetic activity than grape seed proanthocyanidins, with further research needed to fully understand their mechanisms and ensure safe and effective use (Li et al., 2021).
Sinapic acid	$C_{11}H_{12}O_5$		Yang et al., (2021) evaluated the AOA and mechanism of sinapic acid (SA) and related phenolic acids, finding that SA has significant antioxidant potential, mainly through radical adduct formation and mechanisms such as the Hydrogen Atom Transfer (HAT) in the gas phase and SETPT/SPLET in polar solvents, with methoxyl groups on benzene rings playing a crucial role in their activity.

Baicalin	$C_{21}H_{18}O_{11}$		In an experiment, high-purity baicalin (95.5%) was successfully obtained from huangqin root with a yield rate of 5.8%, demonstrating its potent antioxidant activities in three different assays and showing an excellent linear correlation between its concentration and AOA, indicating its potential as a natural antioxidant for various applications (Peng-Fei et al., 2013).
Pterostilbene	$C_{16}H_{16}O_3$		Wang et al., (2023b) demonstrate for the first time that pterostilbene can enhance the color, pH, tenderness, water-holding capacity, and nutritional value of post-mortem chicken meat, mainly through improved antioxidant capacity and muscle protein stability.
Myricetin	$C_{15}H_{10}O_8$		Myricetin is identified as one of the most effective natural antioxidants for preserving omega-3-rich edible oils from autoxidation due to its low bond dissociation enthalpy, presence of pyrogallol moieties, capacity to form active dimers, and high radical-trapping efficiency, making it a superior alternative to α-tocopherol and synthetic antioxidants (Guitard et l., 2016).
Luteolin	$C_{15}H_{10}O_6$		Luteolin, similar to hesperetin, hesperidin, and hesperidin glucoside, exhibits potent antioxidant, anti-inflammatory, and antibacterial activities, with higher efficacy in radical scavenging and antibacterial assays, particularly in its aglycone form, indicating that its biological activity may be enhanced compared to less soluble forms (Choi et al., 2022).

Hesperidin	$C_{28}H_{34}O_{15}$		Hesperidin enhances the antioxidant capacity and stability of whey protein, forming complexes through hydrophobic interactions that improve the resistance of oil-in-water emulsions to environmental changes, making them more effective as antioxidant emulsifiers in food products (Wang et al., 2023c).
Silymarin	$C_{25}H_{22}O_{10}$		Silymarin, a liver-protective supplement derived from Silybum marianum seeds, contains seven significant components, with taxifolin being identified as the most effective antioxidant, demonstrating superior free radical scavenging and oxygen radical antioxidant capacity compared to the other components (Anthony & Saleh, 2013).
Kaempferol	$C_{15}H_{10}O_6$		Kaempferol, along with luteolin, apigenin, and quercetin, exhibits significant anti-inflammatory and antioxidant activities in vitro, with kaempferol demonstrating effective NO reduction and phagocytosis inhibition, and notable DPPH and ABTS radical scavenging abilities, suggesting its potential as an adjuvant treatment for inflammatory diseases and oxidative stress (Tian et al., 2021).

Antioxidant potential of phenolic compounds: recent studies

In recent years, research on the identification and quantification of antioxidant compounds of phenolic compounds derived from food by-products and wastes has expanded significantly, exploring their mechanisms, health benefits, and potential applications in food production.

In a study conducted by Costa et al. (2023), it was aimed to evaluate the phenolic content and antioxidant capacity of hydroethanolic extracts from grape stems, showing that these extracts are rich in phenolic compounds, including phenolic acids, flavonols, flavanols, a flavone, anthocyanins, stilbenes, and proanthocyanins. Among the varieties studied, *Tinta Roriz* exhibited the highest phenolic content and antioxidant capacity, as identified by HPLC-DAD. These findings suggest that grape stems, particularly from the *Tinta Roriz* variety, are a valuable source of natural bioactive compounds with significant antioxidant potential.

Khemakhem et al. (2021) have reported that pomegranate seed oils obtained via UAE from three Tunisian pomegranate cultivars yielded significant amounts of oil, particularly from the *Testouri* ecotype. In addition, the phytochemical composition, antioxidant properties, and phenolic profiles of the defatted pomegranate seeds also differed, with the *Gabsi* ecotype showing the highest levels of polyphenols and flavonoids and the most substantial antioxidant potential.

A research on the pulp of dragon fruit, revealed a higher total phenolic content and more robust antioxidant capacity compared to the peel; the peel contained higher levels of flavonoids and tannins, and using liquid chromatography-electrospray ionization quadrupole time-of-flight mass spectrometry (LC-ESI-QTOF-MS/MS), a total of 80 phenolic compounds were identified, including 25 phenolic acids, 38 flavonoids, six lignans, three stilbenes, and eight other polyphenols, with high-performance liquid chromatography with a photodiode array detector (HPLC-PDA) quantifying these phenolics and revealing that the peel had higher concentrations of phenolics than the pulp (Chen et al., 2021).

A study characterizing six Australian banana cultivars at various ripening stages for their phenolic compounds using LC-ESI-QTOF-MS/MS, quantified polyphenols with HPLC-PDA, and measured antioxidant capacity, revealing that ripe *Ducasse* peel and pulp had the highest total polyphenols (1.32 and 1.28 mg GAE/g), tannins (3.34 mg CE/g), and free radical scavenging capacity (106.67 mg AAE/g), while ripe *Plantain* peel had the highest flavonoids (0.03 mg QE/g), unripe *Ladyfinger* pulp exhibited the greatest total AOA (1.03 mg AAE/g), and a total of 24 phenolic compounds, including six phenolic acids, 13 flavonoids, and five other polyphenols were identified, confirming that banana peel and pulp possess significant AOA and potential for use in human food and animal feed for health enhancement (Bashmil et al., 2021).

Extracts from *Veronica* species were studied to identify and quantify phenolic compounds in three species (*Veronica anagallis-aquatica* L., *Veronica persica Poir*., and *Veronica polita* Fr.) using Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS), tested for AOA via DPPH and ORAC methods, with methanol, 80% ethanol, and water used as solvents, revealing that *V. anagallis-aquatica* had the highest phenolic content, major compounds including *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, gentisic acid,

and apigenin, and that caffeic acid exhibited the highest AOA (IC50 value for DPPH activity of 1.99 μg/mL), with methanolic/ethanolic extracts generally showing higher activity than water extracts, indicating the potential application of Veronica extracts for antioxidant purposes (Vrca et al., 2024).

In a study by Ali at al., (2021) on characterizing the phenolic compounds and antioxidant potential of 12 widely used spices including allspice, black cardamom, black cumin, black pepper, cardamom, cinnamon, clove, cumin, fennel, nutmeg, star-anise, and turmeric using LC-ESI-QTOF-MS/MS, revealing that clove and allspice had the highest total polyphenol content (215.14 and 40.49 mg GAE/g), clove and turmeric had the highest flavonoid (5.59 mg QE/g) and tannin contents (23.58 mg CE/g), black cumin and black pepper had the highest phosphomolybdate activity (15.61 and 15.43 mg AAE/g), and clove exhibited the highest free radical scavenging capacity, with a positive correlation observed between phenolic compounds and antioxidant activities, identifying a total of 79 phenolic compounds including 26 phenolic acids, 33 flavonoids, 16 other polyphenols, and 4 lignans, and HPLC-PDA quantification showing higher phenolic acids, highlighting the powerful antioxidant potential of these spices for use in food and animal feed supplements for health promotion.

Another study on polyphenol content and antioxidant potential in twenty fruit peel samples using LC-MS/MS and HPLC, found that mango peel had the highest total phenolic content (27.51 ± 0.63 mg GAE/g) and total flavonoid content (1.75 ± 0.08 mg QE/g), avocado peel had the highest total tannin content (9.01 ± 0.20 mg CE/g), grapefruit peel exhibited the highest antioxidant capacities across various assays, and LC-ESI-QTOF-MS/MS identified 176 phenolic compounds, highlighting fruit peels as significant sources of polyphenols for potential use in food, feed, and nutraceutical products (Suleria et al., 2020).

Zhong et al., (2020) investigated the phenolic compounds in eight seaweeds, including *Chlorophyta*: *Ulva* sp., *Caulerpa* sp., *Codium* sp.; *Rhodophyta*: *Dasya* sp., *Grateloupia* sp., *Centroceras* sp.; *Ochrophyta*: *Ecklonia* sp., *Sargassum* sp., using LC-ESI-QTOF-MS/MS, and assessed their total phenolic content, total flavonoid content, and total tannin content as well as antioxidant potential through DPPH, ABTS, and FRAP assays, finding that brown seaweeds exhibited the highest polyphenol content and antioxidant potential, with 54 phenolic compounds identified, the most phenolics in *Centroceras* sp., *Ecklonia* sp., and *Caulerpa* sp., and *p*-hydroxybenzoic acid as the most abundant phenolic in *Ulva* sp., highlighting seaweed as a valuable source of polyphenols for food, pharmaceutical, and nutraceutical applications.

In a study where the synthesis and *in vitro* antioxidant capacity of phenolic compounds produced by the microalgae *P. boryanum* grown in six different culture media was evaluated, finding that the highest biomass concentration (1.75±0.01 $g.L^{-1}$) and phenolic content (3.18±0.00 $mg.g^{-1}$) were achieved in MBG11, identifying phenolic acids such as gallic, protocatechuic, chlorogenic, hydroxybenzoic, and vanillic acids, with all extracts showing scavenger activity in the ABTS assay and inhibiting peroxidase, while those grown in BG11 and MBG11 exhibited the most potent scavenger activity in the DPPH assay, suggesting that *P. boryanum* is a promising new source of free phenolic compounds with potential AOA when cultivated in MBG11 (Corrêa da Silva et al., 2022).

All these research have highlighted the *in vitro* antioxidant potential of phenolic compounds, which can range in spectra considering the applied extraction methods and treatments on the sample. Hence, these outcomes lay the groundwork for future research on the utilization of the antioxidant potential of the phenolic compounds in the food sector to preserve food products and promote their nutritional value.

Lipid oxidation

Lipid oxidation is the most crucial quality parameter in foods, and it will deteriorate the nutrition, flavour, texture, and visual appeal of food, diminish the quality of lipid-containing food, shorten the shelf life, and result in significant economic losses (Barden & Decker, 2016). Lipid oxidation in edible oils is a complex process influenced by various factors such as temperature, exposure to light, the presence of oxygen, and the composition of fatty acids in the oil. On the other hand, food lipids involve more lipid oxidation products than biological lipids, given the heat treatment techniques necessary to extract and refine oils (Jackson & Penumetcha, 2019). There are three main mechanisms which cause lipid oxidation.

Autoxidation is the principal mechanism by which unsaturated fatty acids interact with oxygen, resulting in the oxidative degradation of foods. This has been previously defined as oxygen-mediated oxidation events in the food system. Based on Figure 4, this process is divided into three stages: initiation (the production of radicals), propagation (the rise in the number of reactive compounds), and termination (the degradation or reaction of reactive compounds to generate non-reactive chemicals) (Wang et al., 2023a).

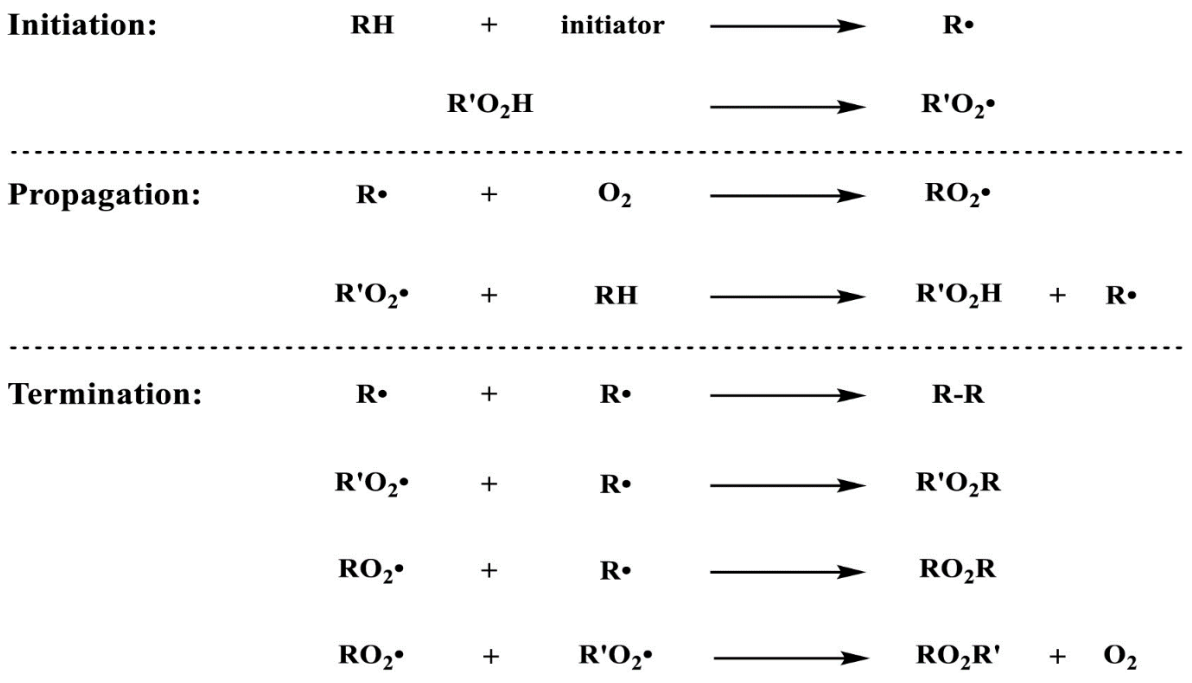


Figure 4: Different stages of autoxidation (Wang et al., 2023a)

As shown in Figure 5, in photooxidation, unsaturated fatty acids are oxidised by non-radical processes using singlet oxygen ($^1O_2$), which interacts directly with C=C bonds (Diaz-Uribe et al., 2022). Singlet oxygen, which is produced from triplet oxygen via chemical, photochemical, and enzymatic techniques (Van Dyck, 2010), reacts 1500 times quicker than triplet oxygen. Photosensitizers like chlorophyll and riboflavin are required for this process, as they absorb light and activate oxygen. These excited photosensitizers subsequently return to their ground state by either interacting with lipids (type I) or converting triplet oxygen to singlet oxygen (type II), therefore triggering lipid oxidation.

Type I photooxidation occurs through the radical or radical ion pathway, resulting in the formation of hydroperoxides. In Type II photooxidation, however, the highly electrophilic singlet oxygen reacts directly with the Cdouble bondCs of the unsaturated fatty acid, rather than through the radical intermediate. When singlet oxygen attacks lipid molecules, it produces hydrogen peroxide and moves C-double bonds (Ito et al., 2017).

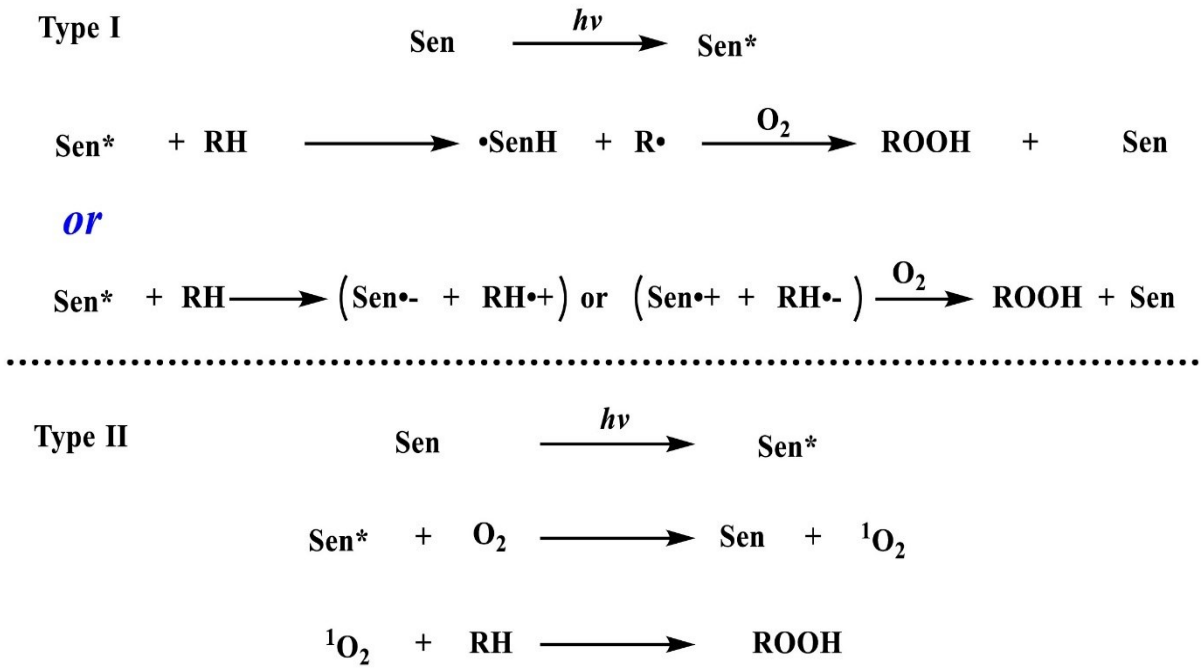


Figure 5: Lipid photooxidation pathway in which photosensitizers participate (Wang et al., 2023a)

Enzymatic oxidation includes the oxidation of lipids by enzymes, most notably lipoxygenase (LOX) and hydroperoxidase (Lampi et al., 2020). LOX is a single-stranded polypeptide protein with a high-spin non-heme iron atom that can be inactive (Fe^{2+}) or active (Fe^{3+}). It catalyses the oxidation of the cis-1,4-pentadiene structure in polyunsaturated fatty acids, resulting in hydroperoxides, but not saturated or monounsaturated fatty acids (Ito et al., 2015). The acknowledged LOX oxidation mechanism is a radical oxidation reaction. As indicated in Figure 6, this includes three steps: (1) LOX dehydrogenates linoleic acid, producing radicals and reducing Fe^{3+} to Fe^{2+}. (2) Oxygen combines with the radical, producing peroxy-radical and $O_2^{\cdot-}$. (3) Fe^{2+} reduces the peroxy-radical, making hydroperoxide and returning LOX to the active Fe^{3+} state (Hatcher et al., 2007).

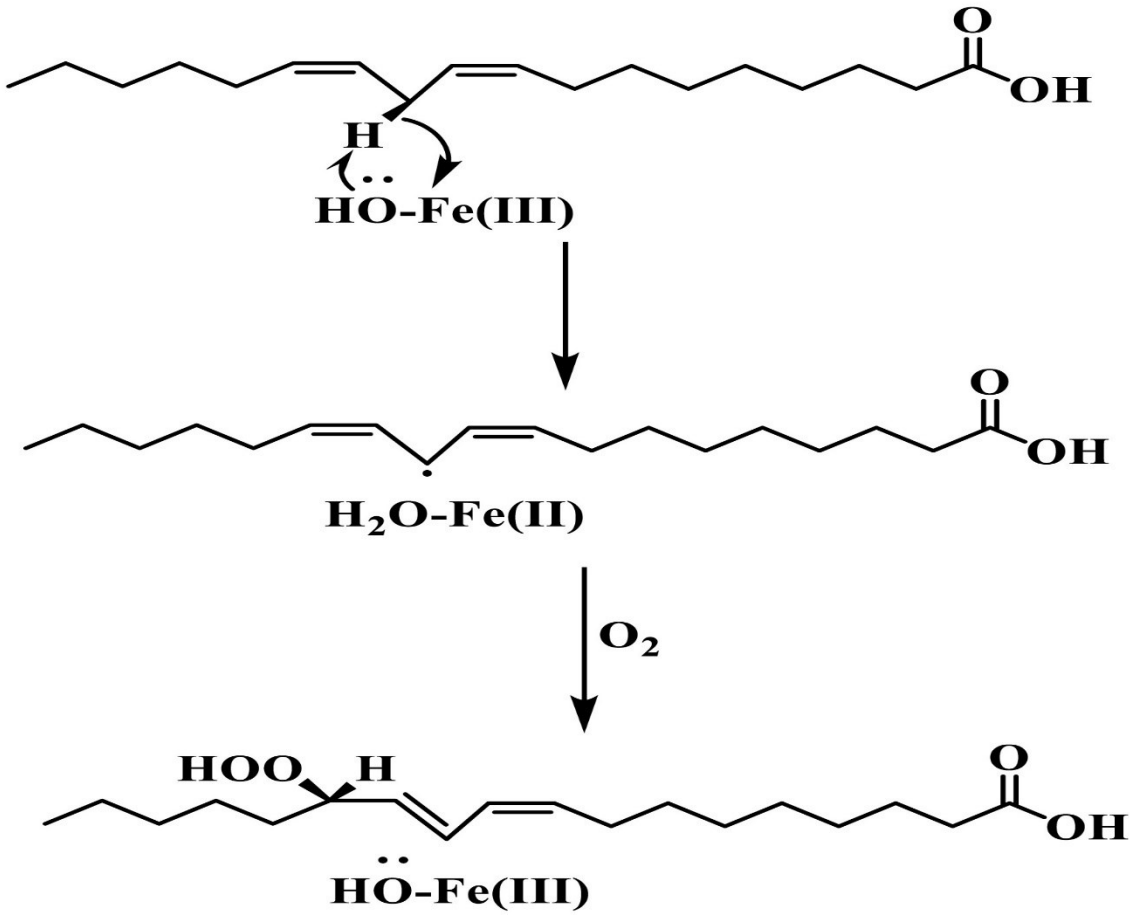


Figure 6: Mechanism of lipid oxidation in the presence of LOX (Wang et al., 2023a)

Antioxidants prevent lipid oxidation by scavenging free radicals and limiting the production of lipid hydroperoxides. Some oils include natural antioxidants such as tocopherols (vitamin E) and phenolic compounds, which assist in stabilising them against oxidation. Additionally, packaging and storage circumstances, such as utilising opaque containers and keeping oils in cold, dark locations, help to reduce oxidation (Pokorny & Pokorny, 2012). Besides the process of the oils, their susceptibility to oxidation varies according to their fatty acid composition; for example, oils high in PUFAs, such as soybean and sunflower oils, are more prone to oxidation than oils high in monounsaturated fatty acids (MUFAs) or saturated fatty acids (SFAs), like olive or coconut oil (Frankel, 2005). Understanding and regulating lipid oxidation is critical for preserving the quality and nutritional integrity of edible oils across their shelf life. Manufacturers frequently use a mix of antioxidants, correct packaging, and storage procedures to guarantee that customers obtain oils that are not only safe but also keep the ideal sensory and nutritional properties (Shahidi and Zhong, 2010).

Chapter 2:

Materials and methods

Materials and chemicals

White and red spent grape pomace, derived primarily from Chardonnay and Valpolicella cultivars (*Vitis vinifera*) post-Grappa production, along with grape seed oil, were procured from the industrial distillery Bonollo located in Padova, Italy. Ethanol, Folin-Ciocalteu's phenols reagent, gallic acid, sodium carbonate, sodium hydroxide, hydrochloric acid, acetic acid, iron chloride, 2,3,5-Triphenyltetrazolium (TPTZ), and Tetramethylchromane-2-Carboxylic Acid (Trolox) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All solvents and other chemicals meeting analytical or MS grade standards were acquired from Merck (Darmstadt, Germany) and Fischer Scientific (Fair Lawn, NJ, USA).

Spent grape pomace powder preparation

Both Solid Grape Products (SGPs) underwent a drying process in an oven maintained at 50 °C for a duration of 48 hours. Subsequently, the dried materials were ground using a water-cooled laboratory mill (IKA Werke M20, Germany) to achieve a particle size smaller than 500 μm. The resulting powders were stored in dark conditions at -18°C until further analysis. Moisture content was assessed in all samples, and all analytical determinations were expressed based on dry matter.

Extraction procedure

The extraction process involved the comparison of three distinct techniques. A conventional method, consistent with prior research (Cisneros-Yupanqui et al., 2021a), was implemented. In this procedure, dried and ground SGP samples were dissolved in a 50% aqueous ethanol solution (1:10, w/v) at 50°C for 45 minutes in a water bath (WB) with agitation (140 rpm). Subsequently, solid residues were eliminated through centrifugation (10,000 g for 5 minutes at 4 °C) and filtration using Whatman® paper N°1.

Additionally, an Ultrasonic (US) extraction was conducted utilizing the SONOPULS ultrasonic homogenizer operating at 20 kHz±500Hz Frequency. The KE76 tip was employed for sonication with the same solvent and ratio as the conventional method for 30 minutes at 25% with 200 W output power (60 seconds on, 10 seconds off). Solid residues were eliminated through centrifugation (10,000 g for 5 minutes at 4 °C) and filtration with Whatman® paper N°1. Furthermore, a combination of both techniques (WBUS) was executed, wherein the samples underwent treatment for 15 minutes with the WB and 15 minutes with the US, under the same conditions as previously described. As reported in Table 2, prior to the evaluation of the extracts, three types of mixture were made by mixing various ratios of each extract with the other. In addition, the pure form of each extract was also evaluated.

Table 2: Experimental plan of sample preparation

Sample ratio (%)	Red Grape Pomace (RGP)	White Grape Pomace (WGP)
Mixed type 1	30	70
Mixed type 2	70	30

Mixed type 3	50	50
Individual type	100	100

After assessment of the extracts, the selection of the optimal extraction technique and the best extract was based on the quantity of Total Phenolic Content (TPC) and the values recorded for the Antioxidant Activity (AOA) as well as considering the sustainability of the technique and importance of the symmetric ratio of extract for marketing reasons and consumers perspective. Subsequently, the extract underwent vacuum concentration to determine oxidative stability. The concentrated extract was obtained through vacuum concentration using a rotary evaporator to assess oxidative stability.

Analytical determinations

Total Phenolic Compounds (TPC)

TPC was determined in accordance with the methodology outlined in the literature (Campos et al., 2022) utilizing the Folin-Ciocalteau method. In brief, 500 µL of the diluted sample was mixed with 250 µL of 1N Folin-Ciocalteau reagent and 1250 µL of $NaCO_3$ 7.5%. A blank solution, substituting water for the sample, was also prepared. Following a 30-minute incubation period in darkness, the absorbance was measured at 755 nm using a Varian Carry 50 Bio UV/Vis spectrophotometer. The results were expressed as milligrams of gallic acid equivalent per gram of dry matter (mg GAE/g).

Antioxidant Activity (AOA)

The assessment of AOA was conducted utilizing the ferric reducing antioxidant potential (FRAP) method, as described in the literature (Cisneros-Yupanqui, Rizzi, et al., 2021a). The FRAP solution was prepared by combining 2.5 mL of 0.01 M 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) in 40 mM HCl, 2.5 mL of an aqueous solution containing 0.02 M FeCl3, and 25 mL of 0.2 M sodium acetate buffer. Subsequently, 100 µL of the sample was mixed with 900 µL of the FRAP solution and incubated at 37°C for 30 minutes. Additionally, a blank solution was prepared using the dilution solvent. The absorbance was measured at 593 nm using a UV/Vis Varian Carry 50 Bio spectrophotometer. The results were expressed in milligrams of Trolox Equivalent per gram of dry matter (mg TE/g).

Oxidative Stability

The oxidative stability capacity was evaluated in a blend of four edible oils, namely sunflower, soybean, corn and grape seed oil, with grape pomace extract. Butylated hydroxytoluene (BHT) at 0.02% (w/w) was also added to the oil samples to evaluate the effectiveness of the extract and the difference with the control samples. All treatments underwent ultrasound treatment at an amplitude of 25% for 2.30 minutes (in intervals of 30 seconds). Following sonication, the samples, placed in an ice bath, were homogenized using an Ultra Turrax T-25 (Janke and Kunkel, Germany) operating at 12,000 rpm for 5 minutes. Subsequently, 3 g samples were subjected to the official Rancimat method at 110 °C with an insufflation of 20 L/h of air. Oxidative stability was assessed based on the Induction Time (IT), representing the duration (in hours) before a rise in water conductivity (µS/min) occurs due to compounds resulting from lipid oxidation. The Antioxidant Activity Index (AAI) was calculated using the following equation.

Eq.1: $$AAI = \frac{\text{OIT of oil with antioxidants}}{\text{OIT of oil without antioxidant}}$$

Statistical analysis

The experimental results are presented as the mean ± standard deviation from three replications of each experiment. Statistical analysis was conducted using one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post hoc test to assess pairwise comparisons, with a significance level of 0.05 considered statistically significant (P<0.05). The analysis was performed using SPSS Statistics (Ver. 27.0), and graphical representations of the data were created with Excel software (Ver. 2020).

Chapter 3:

Results and Discussions

Effect of different extraction techniques on TPC of the samples

Total phenolic content (TPC) serves as a quantifiable metric for the concentration of phenolic compounds within a given sample. Phenolic compounds, being secondary metabolites in plants, play a pivotal role in bolstering plant defence mechanisms against various environmental stressors and pathogens. Moreover, these compounds are recognized for their antioxidant properties, which hold potential health benefits for humans (Matsumura et al., 2023). In this study, we investigated the efficacy of different extraction methodologies, including WB, US, and a hybrid approach combining both methods, in elucidating their influence on TPC yield.

Table 3: The performance of three extraction methods on the TPC value of two samples and their mixtures based on mg GAE/g dry sample

Method / Sample	US	WB	WBUS
WGP	38.11 ± 3.18^{Db}	7.89 ± 0.55^{Ba}	45.59 ± 2.48^{Cc}
RGP	16.50 ± 0.17^{Ab}	4.76 ± 0.42^{Aa}	23.49 ± 3.70^{Ac}
30RGP+70WGP	24.23 ± 0.23^{Bb}	20.17 ± 0.77^{Ea}	35.28 ± 1.31^{Bc}
70RGP+30WGP	20.48 ± 0.37^{Bb}	14.49 ± 0.92^{Ca}	30.52 ± 2.35^{Bc}
50RGP+50WGP	28.87 ± 2.18^{Cb}	16.43 ± 0.42^{Da}	33.29 ± 4.64^{Bb}

Results are reported as mean ± standard deviation (n=3).

Different capital letters in each column and different small letters in each row indicate significant differences ($P\leq 0.05$), as assessed by one-way ANOVA and Tukey's honestly significant difference tests.

Table 3 presents the performance of three extraction methods—US, WB, and WBUS—on the TPC value of WGP, RGP, and their mixtures. The TPC value is expressed in mg GAE/g dry sample. The data reveals significant differences in the effectiveness of these methods and the impact of sample mixtures on the TPC values.

The US extraction method employs high-frequency sound waves to create cavitation bubbles in a liquid medium, which collapse and generate intense local pressure and temperature. This mechanical action disrupts the cell walls of plant materials, facilitating the release of intracellular compounds, including phenolics. In this study, the US method was

particularly effective for all samples tested. For WGP extract, the TPC value was 38.11±3.18 mg GAE/g DW, indicating that the ultrasonic waves efficiently broke down cell structures, releasing a high amount of phenolic compounds. The RGP extract had a TPC value of 16.50±0.17 mg GAE/g DW. While this value is lower than that of WGP extract, it still reflects a substantial extraction, demonstrating that the US method can effectively extract phenolic compounds even from samples that naturally contain lower phenolic content. The results of the mixtures were also promising; the 30RGP+70WGP mixture yielded a TPC of 24.23±0.23 mg GAE/g DW, showing that blending different proportions of RGP and WGP extracts can optimize the extraction of phenolic compounds. Similarly, the 70RGP+30WGP mixture had a TPC of 20.48±0.37 mg GAE/g DW, while the 50RGP+50WGP mixture showed a TPC of 28.87±2.18 mg GAE/g DW. These results indicate that the US method is not only effective for pure samples but also for mixed samples, maintaining a high level of phenolic extraction regardless of the sample composition. This consistency across different samples suggests that the US method is robust and versatile.

The WB method involves immersing the samples in hot water, which facilitates the extraction of phenolic compounds through diffusion and convection mechanisms. Heat can enhance the solubility of phenolic compounds, but it may also lead to the degradation of heat-sensitive compounds and proteins. In this study, the WB method showed lower efficiency compared to the US method. For the WGP extract, the TPC value was 7.89±0.55 mg GAE/g DW, significantly lower than that obtained with the US method. This indicates that while the heat might have facilitated some extraction, it was not sufficient to release a large amount of phenolic compounds effectively. RGP extract had an even lower TPC value of 4.76±0.42 mg GAE/g DW, the lowest across all methods and samples. This suggests that the phenolic compounds in RGP extract are either less accessible or more prone to degradation under heat. The mixtures exhibited slightly improved results compared to pure RGP extract, but they still lagged behind the US method: 30RGP+70WGP had a TPC of 20.17±0.77 mg GAE/g DW, 70RGP+30WGP showed 14.49± 0.92 mg GAE/g DW, and 50RGP+50WGP yielded 16.43±0.42 mg GAE/g DW. These findings highlight the limitations of the WB method in terms of both efficiency and extraction capacity, particularly for samples rich in RGP. The lower temperatures and longer exposure times might not be as effective in disrupting cell walls as the mechanical action of ultrasound.

The combined method, the WBUS method, aims to leverage the strengths of both approaches: the mechanical disruption of ultrasonic waves and the enhanced solubility provided by heat. This method produced the highest TPC values among all methods, indicating a synergistic effect. For the WGP extract, the TPC value was 45.59±2.48 mg GAE/g DW, the highest observed in this study, suggesting that the combined action of ultrasound and heat can maximize the release of phenolic compounds from this type of sample. For RGP extract, the TPC was 23.49±3.70 mg GAE/g DW, higher than using either method alone, demonstrating the benefit of combining these extraction techniques for samples with inherently lower phenolic content. The mixed samples also showed substantial improvements with the WBUS method, 30RGP+70WGP had a TPC of 35.28± 1.31 mg GAE/g DW, 70RGP+30WGP showed 30.52± 2.35 mg GAE/g DW, and 50RGP+50WGP yielded 33.29±4.64 mg GAE/g DW. These results indicate that the combination of ultrasonic waves and heat can significantly enhance the extraction of phenolic compounds across different sample compositions. The high TPC values suggest that the WBUS method is highly effective, likely due to the combined mechanical and thermal effects that enhance cell disruption and phenolic compound release.

Given the sustainability considerations, the US method was selected for further studies. The US method, while slightly less effective than the WBUS method, still provides significantly higher phenolic content than the WB method, making it a robust choice for extraction. Furthermore, ultrasound-assisted extraction is recognized for being more energy-efficient, faster, and environmentally friendly compared to traditional methods. Since we aim to explore the impact of both WGP and RGP extracts on oils, and given the importance of using a symmetric ratio in our formulations, the 50RGP+50WGP mixture with a TPC value of 28.87±2.18 GAE/g DW was selected for its balanced composition and its promising phenolic content. Noteworthy is the fact that for this ratio, there is no significant difference between the US method and WBUS, which was reported to be equal to 33.29±4.64 GAE/g DW. This selection ensures that we can maximize the health benefits and antioxidant properties imparted by both types of grape pomace while adhering to sustainable practices.

In a study by Karabegović et al., (2014), various extraction techniques, including microwave-assisted extraction (MAE), UAE, classical extraction (CE), and Soxhlet extraction (SE), were explored for cherry laurel leaf and fruit extraction based on extractive yields, antioxidant activities, and extract composition. In consistence with the results of the current study, their results showed that extraction techniques and plant material significantly influenced extractive yields, so the SE method achieved the highest yield but required eight times longer extraction time compared to the MWAE method, which provided 70% of the maximal yield. CE method yielded the lowest. The highest total phenol, flavonoid content, and AOA were observed in cherry laurel leaf extracts obtained through the MWAE method. In another study where High Hydrostatic Pressure (HHP), UAE, and MAE methods were compared for extracting phenolic content and AOA from sour cherry pomace, results were in line with the findings of the present study, showing that these novel techniques outperform conventional solvent extraction, yielding higher phenolic content and AOA (Okur et al., 2019). The results of a study conducted by Das et al., (2019) also align with our findings since they reported that by comparing extraction methods for polyphenols and antioxidants in Piper betle leaves, sonication extract showed the highest AOA and polyphenol content, followed by maceration and SE methods. Another study, which compared conventional and UAE techniques, reported that the UAE method notably reduces extraction time and increases polyphenolic content and antioxidant capacity in the extracts of Chamomile dried flowers (Žlabur et al., 2020). In the study by Zainal et al., (2022), a combination of maceration and ultrasound-assisted extraction with 70% aqueous ethanol and longer extraction times yielded the highest TPC for *Tetrigona apicalis* Malaysian propolis. In the present study based on TPC results and sustainability goals, the US method was selected as the best extraction method, and although, based on other studies, the MAE method might be a slightly better method from various aspects, Espada-Bellido et al., (2019) reported that although MAE method showed slightly more remarkable recoveries in less time for extracting antioxidant compounds from blackberries. While the current study did not investigate the utilized solvents ratio's impact on TPC; so that Castro-López et al., (2017) also reported that total phenolic content and AOA of an extract could be influenced by the solid: liquid ratio.

Ultimately, according to the results of the TPC evaluation as well as the sustainability goals, although the US method performed lower than the WBUS method, it was still better than the WB performance. Considering our attempt to select the most sustainable approach, the US method is selected as the optimum extraction as it is a more sustainable method.

Regarding the mixture and aiming at taking advantage of both types of pomaces and exploring the possible synergic or antagonistic impact of them on each other while dispersed in an oil sample, 50%WGP+50%RGP is selected as it possesses hights TPC among other samples extracted through the US method, as well as providing consistency in the formulation and importance of symmetric ratios of extract for marketing reasons and consumer perception.

Effect of different extraction techniques on FRAP values of the samples

FRAP (Ferric Reducing Antioxidant Power) is a commonly used method to measure the antioxidant capacity of different compounds. The FRAP assay measures the ability of a compound to reduce Fe^{3+} to Fe^{2+} in the presence of a reducing agent. This reduction leads to a change in absorbance at 593 nm, which is directly proportional to the antioxidant capacity of the compound being tested (Gonzalez-Rivera et al., 2018).

Table 4: The performance of three extraction methods on the FRAP value of two samples and their mixtures based on mg TE/g dry sample.

Method / Sample	US	WB	WBUS
WGP	88.61 ± 12.00^{Cb}	29.79 ± 1.96^{ABa}	71.39 ± 3.95^{Bb}
RGP	45.75 ± 4.94^{Ab}	24.39 ± 4.98^{Aa}	42.99 ± 2.49^{Ab}
30RGP+70WGP	67.52 ± 9.97^{BCb}	$30.13\pm0.79A^{Ba}$	105.51 ± 10.28^{Cc}
70RGP+30WGP	55.04 ± 4.13^{ABb}	41.30 ± 1.09^{Ca}	63.84 ± 5.82^{Bb}
50RGP+50WGP	72.62 ± 6.06^{BCc}	33.10 ± 3.38^{Ba}	56.37 ± 6.82^{ABb}

Results are reported as mean ± standard deviation (n=3).

Different capital letters in each column and different small letters in each row indicate significant differences ($P\leq 0.05$), as assessed by one-way ANOVA and Tukey's honestly significant difference tests.

Table 4 presents the performance of three extraction methods— US, WB, and WBUS—on the FRAP (Ferric Reducing Antioxidant Power) values of white grape pomace WGP, RGP, and their mixtures. The FRAP values are expressed in mg TE/g dry sample.

The US method is highly effective, delivering consistently high FRAP values across various samples, indicating its robust ability to extract antioxidants. For the WGP sample, it yields the highest FRAP value of 88.61±12.00 mg TE/g DW among the standalone methods, demonstrating that ultrasonic energy is particularly efficient at disrupting cell structures and releasing antioxidants. The effectiveness of the US method is also evident in mixed samples, such as the 50RGP+50WGP blend, where it achieves a notable FRAP value of 72.62±6.06 mg TE/g DW. The success of the US method can be attributed to the cavitation process, where ultrasonic waves create microbubbles that implode, generating localized high pressure and temperature, thereby enhancing the extraction of antioxidants. This method's consistency across different sample compositions highlights its reliability and versatility in extracting bioactive compounds.

The WB method, in contrast, shows the least effectiveness in extracting antioxidants, as reflected in the consistently low FRAP values it produces. For WGP, the FRAP value is

significantly lower at 29.79±1.96 mg TE/g DW, indicating that heat alone, as used in the WB method, is not sufficient to disrupt the cellular matrix and efficiently extract antioxidants. This trend is consistent in the RGP sample, where the FRAP value drops further to 24.39±4.98 mg TE/g DW. Although the WB method does show a slightly higher FRAP value in the 70RGP+30WGP mixture, 41.30±1.09 mg TE/g DW, it remains less effective than the other methods. The WB method's reliance on thermal energy might also lead to the degradation of sensitive compounds, making it less ideal for preserving antioxidant capacity. Overall, while the WB method might have some limited application in specific mixtures, it generally underperforms in terms of antioxidant extraction efficiency.

The WBUS method, which combines the mechanical effects of ultrasonic waves with the thermal energy of a water bath, emerges as the most effective technique, particularly in certain sample mixtures. For instance, in the 30RGP+70WGP mixture, the WBUS method achieves the highest FRAP value recorded in the study at 105.51±10.28 mg TE/g DW, suggesting that the combined effects of cavitation from ultrasonic waves and the gentle heating provided by the water bath create a synergistic environment that maximizes antioxidant extraction. In the WGP sample, the WBUS method also produces a high FRAP value of 71.39±3.95 mg TE/g DW, which is higher than what is achieved by WB alone but slightly lower than the US method, demonstrating that while the WBUS method can significantly enhance extraction in certain mixtures, its effectiveness can vary depending on the specific composition of the sample. Nonetheless, the WBUS method represents a powerful tool in situations where maximum extraction efficiency is required.

Despite the high efficacy of the WBUS method, the US method was selected as the optimum extraction technique due to its sustainability and operational efficiency. The US method is more energy-efficient and has a lower environmental impact, making it a more sustainable option for industrial-scale applications. Moreover, the US method effectively maximizes antioxidant extraction while maintaining a more straightforward operational process compared to the combined WBUS method. Additionally, the 50RGP+50WGP mixture with FRAP value of 72.62±6.06 mg TE/g DW was chosen for further study because it provides a balanced representation of both pomace types, allowing for the investigation of their combined effects on the stability and quality of oil samples. The symmetric ratio of 50RGP+50WGP is critical in formulation studies as it ensures an equal contribution of both pomace types, facilitating the understanding of their individual and combined impacts on the target application. This balanced approach not only contributes to optimizing the functional properties of the final product but also supports the sustainable utilization of both grape pomace varieties.

A study conducted by Musa et al., (2011) reported similar outcomes to the current study where variations in AOA depended on the extraction techniques employed; specifically, the Free Radical Scavenging Capacity Index (FCI), DPPH radical scavenging activity, FRAP differ-based on the extraction method used; so that, in the case of pink-flesh guava fruit, both US method and homogenization exhibit significantly higher efficiency for FCI and DPPH values compared to other techniques. Additionally, US extraction surpasses other methods, including homogenization, in terms of FRAP values. In another study, the free fraction of Chilean papaya exhibited the highest FRAP value under high hydrostatic pressure, while the combined methods of high hydrostatic pressure-ultrasound and high hydrostatic pressure-agitation showed the highest FRAP values in bound fractions (Uribe et al., 2015). Exploring

different solvent and extraction methods for *Nepeta leucophylla* also showed that the FRAP results varied according to the extraction method, with the SE method yielding the highest FRAP values for methanol extracts, followed by the maceration method (MM) and UAE method, whereas for chloroform and hexane extracts, SEM yielded the highest FRAP values followed by UAE method and MM (Sharma & Cannoo, 2017).

Ultimately, similar to TPC results, according to the result of the FRAP and consideration of selecting the most sustainable method of extraction and mixture, although there is a significant difference between US and WBUS, US is selected as the optimum extraction as it is the more sustainable. Regarding the mixture and aiming at taking advantage of both types of pomaces and exploring the possible synergic or antagonistic impact of them on each other while dispersed in an oil sample, 50WGP+50RGP is selected since it not only does not significantly differ from other mixtures but also provides consistency in the formulation and highlights the importance of symmetric ratios for marketing reasons and consumer perception.

Selection of optimum concentration of the extract

To determine the optimum concentration of extract for extending the shelf life of edible oils, a preliminary test was conducted using soybean oil, incorporating RGP extract concentrations of 5, 10, 20, and 30%, as well as involving a control sample. The primary objective was to measure the effectiveness of these concentrations in delaying oil oxidation by considering the IT and AAI for the oil samples with each concentration of the RGP extract and the mixture.

The IT in the Rancimat method serves as a pivotal measure of the oxidative stability of oils and fats, crucial for assessing their shelf life and suitability for various applications in the food and oil industries. This method involves subjecting a sample of oil or fat to accelerated oxidation conditions, typically around 100°C, while continuously passing air or oxygen through it. Throughout the test, the conductivity of the reaction mixture is monitored. The IT represents the duration between the start of the test and the onset of a significant increase in conductivity, indicating the initiation of oxidative degradation. This period, usually reported in hours (h), reflects the resistance of the sample to oxidation. A longer IT suggests higher oxidative stability, while shorter times indicate increased susceptibility to oxidation. By measuring IT, manufacturers can optimize processing conditions, select suitable antioxidants, and enhance packaging to prolong product shelf life. Ultimately, this method aids in ensuring the quality and safety of oils and fats for consumption and various industrial applications (Frankel, 2012).

In addition to the IT, the AAI was also taken into consideration when selecting the optimum concentration of the extract. The AAI in the Rancimat method offers a quantitative assessment of the effectiveness of antioxidants in delaying the onset of oxidation in oils and fats. This index provides valuable insight into the ability of antioxidants to protect these substances from oxidative degradation, which is crucial for extending shelf life and preserving product quality. During the Rancimat test, antioxidants are incorporated into the oil or fat sample before subjecting it to elevated temperatures and continuous air or oxygen flow, simulating accelerated oxidation conditions. The conductivity of the reaction mixture is continuously monitored, with an increase indicating oxidation product formation. By comparing the IT of the sample containing antioxidants to that of a control sample without

antioxidants, the AOA index is calculated. A higher index signifies greater effectiveness in inhibiting oxidation, reflecting more potent AOA. This information assists manufacturers in selecting and formulating antioxidant additives to enhance product stability and quality. Ultimately, the AOA index serves as a valuable tool in developing products with extended shelf life and improved resistance to oxidative degradation (Dobarganes & Carmen Pérez-Camino, 2007).

Based on the information provided in Figure 7, the blank oil (no RGP extract added) has an IT of approximately 5.7 h, indicating that the blank oil possesses the most basic level of oxidative stability among all samples. When 5% RGP extract is added, the IT seems to experience a minor reduction, suggesting that adding 5% RGP does not significantly affect the oxidative stability of the oil, keeping it comparable to the blank oil. As the RGP extract concentration increases to 10%, the IT slightly increases but is partially within the same category of control and 5% RGP extract. This implies that even though there is an increase in stability, it is not entirely statistically significant compared to the blank oil and the 5% RGP-containing sample. With 20% RGP extract, the IT further increased slightly but now falls into the same category as 10%RGP. This indicates that this sample is also not wholly statistically significant in oxidative stability compared to the blank oil and the 10% RGP sample. However, this highlights a trend towards increasing stability with increasing RGP extract concentration. Finally, at 30% RGP extract, the IT drops to rise to approximately 5 h, showing a significant increase, which indicates that the highest concentration of RGP extract leads to a substantial elevation in the oil's oxidative stability compared to both the blank oil and the 5% RGP containing-sample, showing that at this concentration, the protective effect against oxidation is promoted, possibly due to antioxidant content at higher RGP extract levels or a balance in the antioxidant system. Although with 30% RGP extract, there is still performance overlap with 20% and 10% of RGP, we select the highest percentage of the extract to maximize the presence of antioxidant compounds dispersed in the oil.

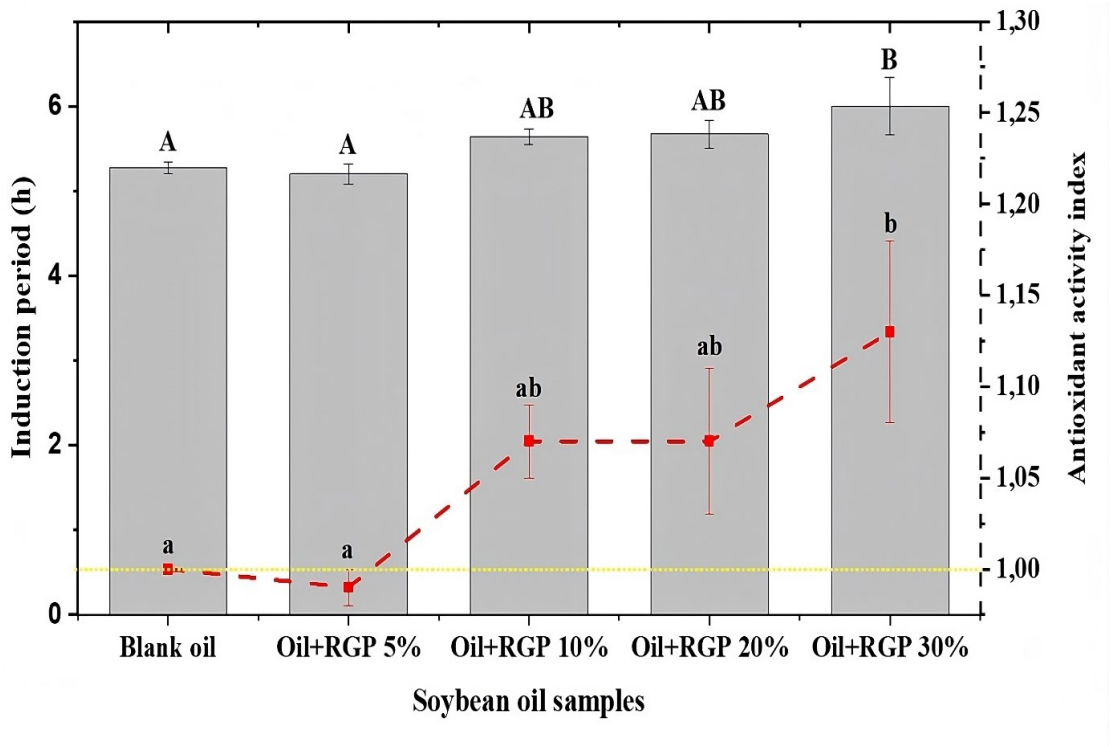


Figure 7: The impact of different concentrations of red grape pomace extract on the induction period and antioxidant activity index. Bars show the induction period, and the red dashed line shows the antioxidant activity index. Results are indicated as mean ± SD (n = 3). Different characters show a significant difference ($P \leq 0.05$) among the samples, as assessed by one-way ANOVA and Tukey's honestly significant difference tests.

The red dashed line in Figure 7 is also an indicator of the Antioxidant Activity Index (AAI), which quantifies the antioxidant capacity of the soybean oil samples, with higher values indicating more robust antioxidant activity. The blank oil (without any RGP extract) has an AAI of around 1.00, labelled with a lowercase 'a'. This value serves as the baseline, corresponding to the yellow line, indicating the inherent antioxidant activity of the oil without any added RGP extract. When 5% RGP extract is added, the AAI slightly increases, indicating no statistically significant enhancement in antioxidant activity. This suggests that at this low concentration, RGP extract does not substantially boost the antioxidant properties of the oil. At 10% RGP extract, the AAI increases to approximately 1.08. This slight increase marks the beginning of a significant enhancement in antioxidant activity compared to the blank oil and the 5% RGP extract-containing sample, though the improvement is still moderate. As the RGP concentration increases to 20%, the AAI continues to rise, reaching around 1.15, revealing further increase and enhancement in antioxidant capacity, though the difference between 10% and 20% RGP extract is not statistically significant, showing a consistent but gradual improvement in antioxidant activity as the concentration of RGP extract is elevated. The most substantial increase is observed at 30% RGP extract, where the AAI reaches approximately 1.23. This significant jump indicates a substantial and statistically significant improvement in antioxidant activity compared to all lower concentrations of RGP extracts and the blank oil. The high TPC content in RGP at this concentration likely contributes to this pronounced antioxidant effect, making the oil significantly more effective at neutralizing free radicals.

In summary, the addition of RGP extract to soybean oil has a dual effect; it enhances antioxidant activity as indicated by the AAI, particularly at higher concentrations (30% RGP extract), and it also tends to enhance the oxidative stability of the oil, with the most significant increase at the 30% concentration. This suggests a positive direct correlative trend between increasing antioxidant capacity and stability of the oil samples with extract concentration. Hence, the highest concentration, 30%, was selected to be applied to the different edible oils aiming at extending their oxidative stability.

Effect of the extract on oxidative stability of edible oils

Table 5 provides detailed data on the oxidative stability of various oils fortified with GPE, assessed using the Rancimat test. The oils tested include soybean, sunflower, corn, and grapeseed oils. For each oil type, three samples were evaluated: control oil, oil with BHT (200 ppm), and oil fortified with a 1:1 ultrasonic mixed grape pomace extract at a 30% concentration. The critical metrics measured are IT (hours), which indicates how long the oil resists oxidation, and the Antioxidant Activity Index (AAI), which quantifies the oil's antioxidant effectiveness.

Table 5: Oxidative stability of different oils fortified with the grape pomace extracts and BHT, measured by the Rancimat test

Samples	Soybean Oil		Sunflower Oil		Corn Oil		Grapeseed Oil	
	Induction time (h)	**AAI**	**Induction time (h)**	**AAI**	**Induction time (h)**	**AAI**	**Induction time (h)**	**AAI**
Control Oil	5.27±0.07^{a}	1.00±0.00^{a}	3.92±0.07^{a}	1.00±0.00^{a}	10.55±0.27^{ab}	1.00±0.00^{a}	4.31±0.01^{a}	1.00±0.00^{a}
Oil + RGP extract 30%	6.00±0.34^{bc}	1.14±0.05^{bc}	4.30±0.28^{ab}	1.10±0.07^{ab}	11.17±0.17^{b}	1.06±0.02^{b}	5.07±0.02^{b}	1.18±0.01^{b}
Oil + WGP extract 30%	6.01±0.22^{bc}	1.14±0.05^{bc}	4.50±0.10^{b}	1.15±0.03^{b}	11.87±0.12^{c}	1.13±0.02^{c}	5.06±0.10^{b}	1.18±0.02^{b}
Oil + Mixed grape pomaces (RGP:WGP; 50:50) extract 30%	6.29±0.08^{c}	1.19±0.02^{c}	4.43±0.04^{b}	1.13±0.03^{b}	11.92±0.38^{c}	1.13±0.03^{c}	5.49±0.10^{c}	1.27±0.02^{c}
Oil + BHT 200 ppm	5.76±0.39^{ab}	1.09±0.00^{b}	4.32±0.19^{ab}	1.10±0.07^{ab}	10.60±0.14^{a}	0.95±0.04^{a}	4.92±0.16^{b}	1.14±0.04^{b}

Results are reported as mean ± standard deviation (n=3).

Different capital letters in each column and different small letters in each row indicate significant differences ($P \leq 0.05$), as assessed by one-way ANOVA and Tukey's honestly significant difference tests.

AAI stands for Antioxidant Index.

For soybean oil, the control sample showed an IT of 5.27 h and an AAI of 1.00, indicating its baseline oxidative stability. The addition of RGP extract increased the IT to 6.00 h and the AAI to 1.14, showing a significant improvement in oxidative stability. Similarly, the WGP extract resulted in an IT of 6.01 h and an AAI of 1.14, also demonstrating a significant enhancement over the control. The mixed grape pomace extract provided the highest IT of 6.29 h and an AAI of 1.19, indicating a significantly more significant improvement in oxidative stability compared to the individual extracts and the control. The BHT treatment at 200 ppm increased the IT to 5.76 h and the AAI to 1.09, which was better than the control but less effective than the grape pomace extracts. These results show that mixed grape pomace extract significantly outperforms other treatments in enhancing the oxidative stability of soybean oil.

For sunflower oil, the control sample had an IT of 3.92 h and an AAI of 1.00. Adding RGP extract increased the IT to 4.30 h and the AAI to 1.10, showing a significant improvement over the control. WGP extract led to a further increase in IT to 4.50 h and an AAI of 1.15, indicating a significant enhancement compared to the control and RGP-treated oils. The mixed grape pomace extract resulted in an IT of 4.43 h and an AAI of 1.13, which was significantly better than the control and comparable to the WGP extract. The BHT treatment showed an IT of 4.32 h and an AAI of 1.10, indicating a significant improvement over the control but similar to the RGP extract. These findings suggest that both WGP and mixed extracts significantly enhance the oxidative stability of sunflower oil, with the WGP extract slightly outperforming the others.

Corn oil, which had a high initial oxidative stability with a control IT of 10.55 h and an AAI of 1.00, showed further improvements with the addition of extracts. The RGP extract increased the IT to 11.17 h and the AAI to 1.06, demonstrating a significant enhancement over the control. The WGP extract led to an even higher IT of 11.87 h and an AAI of 1.13, indicating a significant improvement compared to both the control and RGP-treated oils. The mixed grape pomace extract provided the highest IT of 11.92 h and an AAI of 1.13, showing it was significantly more effective than the control and comparable to the WGP extract. The BHT treatment resulted in an IT of 10.60 h and an AAI of 0.95, which did not offer a significant improvement over the control. These results highlight that mixed grape pomace extract is highly effective in enhancing the oxidative stability of corn oil, whereas BHT is not significantly effective.

Grapeseed oil control had an IT of 4.31 h and an AAI of 1.00. The addition of the RGP extract increased the IT to 5.07 h and the AAI to 1.18, showing a significant improvement over the control. The WGP extract yielded similar results with an IT of 5.06 h and an AAI of 1.18, indicating a comparable enhancement to the RGP extract and significantly better than the control. The mixed grape pomace extract provided the most substantial increase, with an IT of 5.49 h and an AAI of 1.27, showing a significant improvement over both the control and the other extracts. The BHT-treated oil had an IT of 4.92 h and an AAI of 1.14, which showed significant improvement over the control but was less effective than the grape pomace extracts. These results indicate that mixed grape pomace extract offers the most significant enhancement in oxidative stability for grapeseed oil.

The results showed that grape pomace extracts, mainly the mixed variety, significantly enhance the oxidative stability of various edible oils. This is evidenced by increased ITs and

AAIs across all tested oils, suggesting that these natural extracts offer superior protection compared to synthetic antioxidants like BHT at 200 ppm concentration. It should also be mentioned that this concentration for BHT is equal to the maximum permissible level established by Codex Alimentarius (Codex Alimentarius, 2023). The result can also be an indicator of the synergistic effect of the pomaces on each other when mixed in equal ratios, as the mixture performed better than pure extract in oxidation inhibition. These findings underscore the potential for using natural grape pomace extracts as effective antioxidants to improve the shelf life and quality of edible oils, providing a natural and potentially healthier alternative to synthetic options.

In a study grape pomace powder (GPP) was tested as a natural antioxidant in delaying corn oil oxidation using Rancimat equipment. Results align with the result of the current study, so significant differences were observed between the corn oil treated with GPP and the control, suggesting the potential of GPP as a natural antioxidant to mitigate corn oil oxidation(Cisneros-Yupanqui et al., 2021b). In another study, Poiana et al. (2022) explored the potential of lyophilized *Pinot Noir* grape pomace extract as a natural antioxidant to enhance the stability of sunflower oil against thermo-oxidative damage compared to BHT, showing significant inhibitory effects on oil oxidation, concentration-dependently. Also, Zhu et al. (2018) examined grape polyphenols' antioxidant potential in seven Chinese edible oils, offering a natural alternative to synthetic antioxidants like BHT and TBHQ, with optimal efficacy observed at a concentration of 0.02% and in sesame oil and demonstrating synergy with Vitamin C at a 1:4 ratio. In another research the AOA of Thompson GPE, rosemary extract (RE), and tocopherols mix (TM) on the oxidative stability of refined soybean oil (RSO) was compared, finding that GPE at 0.3% and 0.5% (w/w) exhibited superior AOA to TM, resulting in an oxidative stability index higher than 48 h at 110°C when added to RSO, while citric acid did not enhance the effect of GPE but showed synergistic effects with TM at specific concentrations and with RE at all concentrations tested (Gamez-meza et al., 2009).

The extract from grape pomace may not only lead to higher oxidative stability in edible oils but also enhance oxidative stability of other foodstuff such as meat and their products. In a study where the effects of feeding lambs a diet supplemented with GP was evaluated, it was reported that meat characteristics showed no significant differences, and GP supplementation influenced fatty acid composition, increasing beneficial acids. Additionally, it enhanced meat's oxidative stability, suggesting GP as a promising dietary additive for improving animal production quality (Bennato et al., 2023). Microencapsulated phenolic-rich extract from GP demonstrated superior AOA compared to synthetic antioxidants in inhibiting lipid and protein oxidation in raw and precooked beef burgers during cold storage, showcasing its potential as a natural antioxidant for the meat industry while preserving product color (dos Santos Silva et al., 2022). Another study it was aimed to assess the impact of dietary GP intake on the nutritional quality, lipid oxidation, and volatile profile of chicken meat, finding that GP supplementation induced variations in drip loss, meat yellowness, and redness while increasing PUFA, particularly linoleic acid, and decreasing lipid oxidation, suggesting potential benefits for both meat quality and shelf-life, thus offering a sustainable approach for the valorization of agricultural by-products (Bennato et al., 2020). The results of another experiment showed that adding grape GP to chicken feed increased polyphenol intake and digestibility without affecting growth, while both GP and vitamin E enhanced AOA in

diets and excreta, though vitamin E proved more effective in reducing meat oxidation and increasing liver α-tocopherol concentration (Goñi et al., 2007).

Although in the current study, the nutritional impact of the extract on edible oils was not explored, in some other cases, utilization of the GP extracts in food products can be associated with improved nutritional attributes without significantly enhancing the products' shelf life or even deteriorating the shelf life. In this regard, a study assessed the impact of GPP addition in breadsticks formulations on AOA and shelf-life. While GP increased the polyphenol content and AOA of breadsticks, it also accelerated oxidation, reducing shelf-life; although GP fortification enhances nutritional value, it compromised commercial shelf-life due to increased oxidation (Bianchi et al., 2021).

Chapter 4:

Conclusion

This study demonstrates that the extraction method significantly impacts the yield of TPC and antioxidant activity, as measured by FRAP, in grape pomace samples. Among the methods tested—US, WB, and a combined (WBUS) approach—the US and WBUS methods emerged as the most effective for extracting phenolic compounds and antioxidants. The US method provided a robust, efficient, and sustainable extraction process, yielding high TPC and FRAP values across different grape pomace samples. However, the WBUS method yielded the highest TPC and FRAP values, particularly in mixed samples, indicating a synergistic effect of combining ultrasonic waves with heat. Despite this, the US method was selected for further studies due to its operational simplicity and energy efficiency, making it a more sustainable option for industrial-scale applications. Hence, after selecting the optimal extraction method, it is pivotal to select the type of pure extract or mixture. As the interest of the study is centred around taking the most advantage of the pomaces, plus exploring potential synergic or antagonistic effects of RGP and WGP on each other, the mixture is preferred over the pour forms. Among the mixture, considering the importance of symmetric ratio in the regulatory process and consumers' perspective, 50RGP+50WGP was selected.

The findings also revealed that in the pre-test stage for determination of the optimal concentration, increasing the concentration of RGP in soybean oil enhances its oxidative stability, as evidenced by increased IT and AAI. Oils fortified with 30% RGP showed the highest oxidative stability, suggesting a direct correlation between RGP concentration and antioxidant protection. Additionally, the mixed grape pomace extract, 50RGP+50WGP, significantly outperformed synthetic antioxidants like BHT in enhancing the oxidative stability of various edible oils. This highlights the potential of using natural grape pomace extracts as effective, environmentally friendly antioxidants to improve the shelf life and quality of edible oils.

Moreover, when optimal extract, 30% of 50RGP+50WGP derived through the US method, tested across various edible oils—soybean, sunflower, corn, and grapeseed—the mixed grape pomace extract consistently demonstrated superior antioxidant capacity compared to synthetic antioxidants of BHT at 200 ppm. The study found that mixed grape pomace extracts significantly enhance the oxidative stability of various edible oils more

effectively than individual grape pomace extracts or the synthetic antioxidant BHT. The mixed extracts increased IT and AAI across soybean, sunflower, corn, and grapeseed oils, suggesting a synergistic effect when red and white grape pomace are combined.

These findings highlight the potential of using natural grape pomace extracts as effective, environmentally friendly alternatives to synthetic antioxidants for improving the shelf life and quality of edible oils. The ability of these natural extracts to outperform traditional synthetic options not only aligns with the growing consumer preference for natural ingredients but also supports sustainable practices by utilizing agricultural by-products.

In conclusion, the study underscores the importance of selecting appropriate extraction methods and concentrations to maximize the antioxidant properties of grape pomace extracts. These findings contribute to the growing body of evidence supporting the use of natural antioxidants as a sustainable alternative to synthetic options, aligning with the increasing consumer demand for natural and health-promoting food additives. Future research should focus on optimizing extraction parameters, exploring different ratio of the extract on the oils, and exploring the application of grape pomace extracts in other food systems to fully realize their potential benefits.

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