ZITA MEDICAL MANAGEMENT

www.hsmc.gr/en/pharmakeftiki-journal/

ΦΑΡΜΑΚΕΥΤΙΚΗ, 36, I, 2024 | 10-26 ΕΡΕΥΝΗΤΙΚΗ ΕΡΓΑΣΙΑ

PHARMAKEFTIKI, 36, I, 2024 | 10-26 RESEARCH ARTICLE

Measurement of Antioxidants and Phenolics in Wines and Tsipouro Enriched with Powerful Antioxidants such as Vitamins and Resveratrol

Eirini Lagoutari[1], Maria Liouni[1], Maria Lagoutari[3], Anastasia Detsi[4], Panagiotis Zoumpoulakis[5], and Charalampos Proestos[2*]

[1]Department of Chemistry, Industrial Chemistry Laboratory, School of Sciences, National and Kapodistrian University of Athens, Zografou,15771, Athens, Greece.

[2]Department of Chemistry, Food Chemistry Laboratory, School of Sciences, National and Kapodistrian University of Athens, Zografou,15771, Athens, Greece.

[3]Department of Chemical Sciences, Laboratory of General Chemistry, School of Chemical Engineering, National Technical University of Athens, Heroon Polytechniou 9, Zografou Campus, 15780, Athens, Greece.

[4]Department of Chemical Sciences, Laboratory of Organic Chemistry, School of Chemical Engineering, National Technical University of Athens, Heroon Polytechniou 9, Zografou Campus, 15780, Athens, Greece.

[5]Department of Food science and Technology, University of West Attica, Ag. Spyridonos Str. Egaleo, 12243, Athens, Greece.

KEYWORDS:
Wine; Tsipouro; Retinol (vitamin A); Tocopherol (vitamin E); Ascorbic Acid (vitamin C); Resveratrol (RVT); Enrichment; DPPH; ABTS; Folin-Ciocalteu.

ARTICLE INFO:
Received: November, 6, 2023
Revised:February, 28, 2024
Accepted : February, 29, 2024
Available on line: April 3, 2024

*** CORRESPONDING AUTHOR:**
harpro@chem.uoa.gr
Tel.: +302107274160.

ABSTRACT

The subject of this article is the incorporation of powerful antioxidants such as resveratrol (RVT), retinol (vitamin A), tocopherol (vitamin E) and ascorbic acid (vitamin C) into alcoholic beverages such as red, white. , and rosé wine, and spirits such as tsipouro with the aim of enriching them and increasing the added nutritional value of the final products. Enrichment was achieved with substances, such as resveratrol, and the vitamins mentioned above, encapsulated in suitable food and beverage compatible matrices, such as cyclodextrins. This integration is expected to increase the antioxidant properties of alcoholic beverages and, by extension, the nutritional and economic value of the final product. The substances used were placed in wines and spirits (tsipouro) at a specific concentration of 200mg of antioxidant in 1000ml of alcoholic beverage and sampling was done at specified time intervals. The final-maximum values were evaluated and thus found

the antioxidant activity measured by two methods DPPH, ABTS and the total content of phenolic compounds measured by the Folin-Ciocalteau method. Finally, the enriched alcoholic beverages were subjected to an organoleptic test in order to determine any alterations due to the addition of antioxidants.

1. Introduction

According to recent research, France has low rates of cardiovascular events compared to other European countries despite consuming large amounts of fat in their daily diet. This is due to the regular but moderate consumption of wine, the so-called French paradox, which helps us understand how these low rates are explained[1,2]. It has been observed that the phenolic compounds abundant in red wine in particular may be responsible for the good health of the people of France. Although the mechanisms of action are not yet fully understood, it is known that phenolics behave as free radical scavengers due to being powerful antioxidants. It has also been shown that they can protect the human body from bad cholesterol[3,4].

Substances such as resveratrol and vitamins that have high antioxidant, antimicrobial and other properties should be taken daily in specific amounts to strengthen the human body. Alcoholic beverages, such as wine, contain certain amounts of these substances, such as resveratrol, but for the average person to get the specific amounts they need of these substances they would have to consume large amounts of beverages, and this is something that is not we want. So the uniqueness of this research is to try to enhance these products by adding these substances to them, so that with the consumption of about one to two glasses, as stated as the daily consumption of wine according to the World Health Organization (WHO), the average man receives, if not the whole, at least half the amount he needs from these substances.

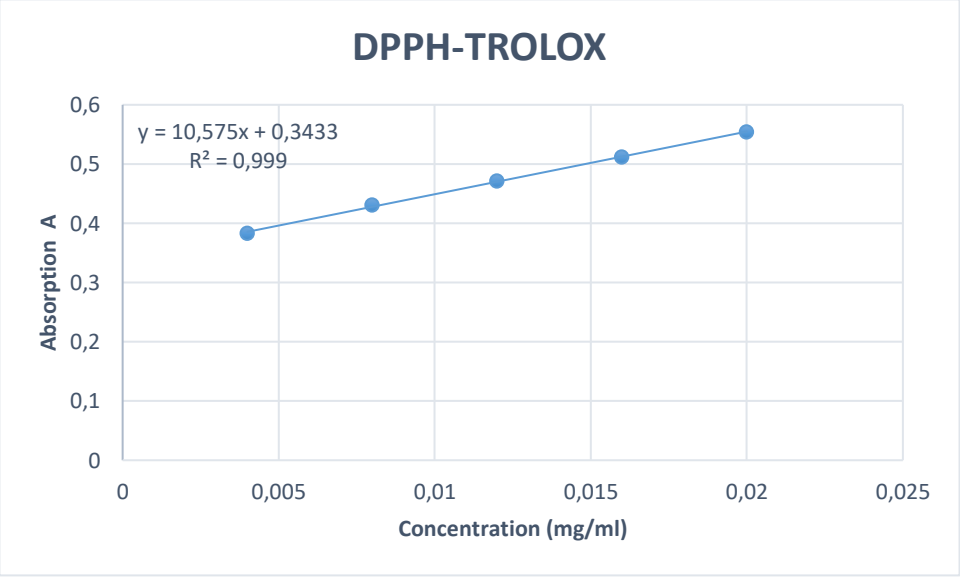
The substances used are resveratrol, a natural substance of the class of phytoalexins with the basic structure of stilbenes, and vitamins such as vitamin E (tocopherol), vitamin C (ascorbic acid) and vitamin A (retinol).

1.1 Wine

Esters are qualitatively the main components of wines in addition to water, ethanol and alcohols, main quantitative components. Epidemiological studies from many different populations reveal that people with a habit of moderate daily wine consumption enjoy significant reductions in all-cause and especially cardiovascular mortality compared to abstainers or heavy drinkers. Researchers are working to explain this observation in molecular and nutritional terms. Moderate ethanol intake from any type of beverage improves lipoprotein metabolism and reduces the risk of cardiovascular mortality. The question now is whether wine, particularly red wine with its abundant content of phenolic acids and polyphenols, offers additional health benefits. Discovering the nutritional properties of wine is a challenging task, requiring the biological actions and bioavailability of >200 individual phenolic compounds to be documented and interpreted within the social factors that stratify wine consumption and the myriad effects of alcohol. A further challenge arises because the health benefits of wine relate to the prevention of slowly developing diseases for which validated biomarkers are rare. Scientific research has shown that molecules present in grapes and wine alter cellular metabolism and signaling, which is mechanistically consistent with reducing arterial disease[5,6].

1.2 Tsipouro

Tsipouro has from 36 to 45 alcoholic degrees. Its main difference with Cretan raki is that tsipouro is



Scheme 1. Standard reference curve with trolox for the DPPH method

usually double-distilled and often in some regions different spices such as anise are added. It should not be confused with ouzo, the popular Greek drink with a different preparation method.

The production of tsipouro is lost in the depths of time, but it is said to have started in the 14th century on Mount Athos by monks who lived there. Over the years it spread to different parts of Greece, mainly in Macedonia, Epirus, Thessaly, Peloponnese and Crete.

The raw material for the production of spirits is the stem, i.e. the mass that remains after the pulp of the grapes, from which the wine is produced.

Tsipouro can be produced from stems from red winemaking with a smaller or larger amount of wine in them. In addition, they can be used separately, from the main volume of must, which comes from white grapes, but also from red grapes, which have been used to produce rosé or white wine by direct pressing.

Often tsipouro is distilled a second time, as in this way its quality is improved.

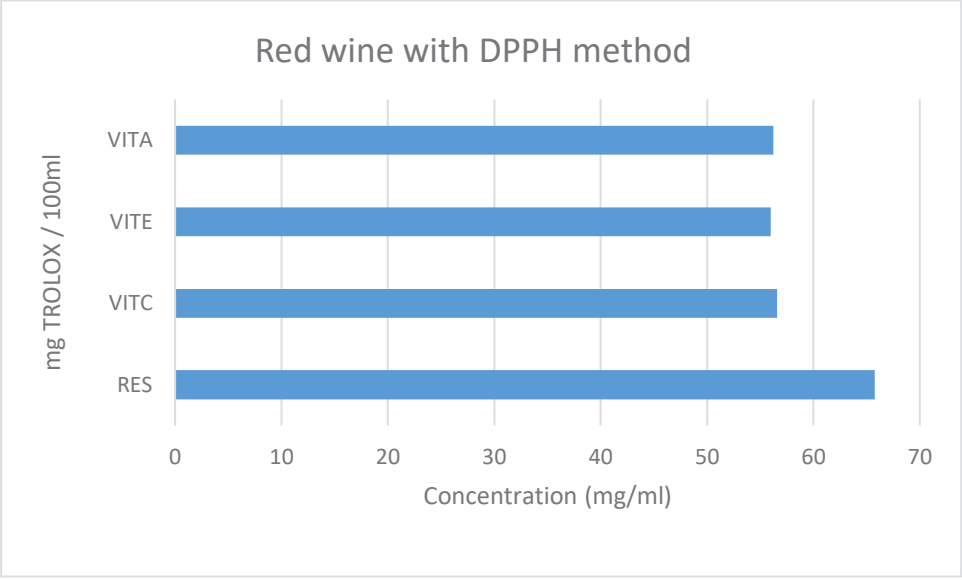
1.3 Vitamins and resveratrol

Vitamin C is a water-soluble vitamin, a white to off-white "powder", which has a carbohydrate chemical structure and takes part in metabolic processes mainly of animal organisms[7].

Vitamin E is a fat-soluble vitamin, of a light yellow color and an oil-like form with a very slight characteristic odor. A substance practically insoluble in water and miscible in any proportion with oils, acetone, alcohol, chloroform, ether and other fatty solvents, it is the main fat-soluble antioxidant agent of the antioxidant defense system of cells[8].

Vitamin A is a fat-soluble vitamin, yellow-orange in color and of "sticky" texture. It is necessary for the proper functioning of the retina of the eye[9].

The compound resveratrol (resveratrol, RVT) is a natural substance of the class of phytoalexins with a basic structure of stilbenes. Resveratrol (RVT) is mainly found in high concentrations in the skin of grapes and especially red grapes, nuts, berries (mulberries, blueberries, cranberries, bilberries) and in smaller quantities has been found in 70 more plant products. The content of resveratrol (RVT) in the skin of fresh, red grapes is 50-100 μg/g, while in red wine it can be found in concentrations of 1.5-3 mg/L. In white wine it can be found in smaller quantities because the fermentation of the wine is carried out after removing the skins of the grapes. Resveratrol



Scheme 2. Final results (after 1500 hours) of red wine ...with Resveratrol (RES), Viatmin C (VITC), Vitamin E (VITE),Vitamin A (VITA), for the DPPH method

(RVT), like other phytoantioxidants, is a natural component of plants with antibiotic activity to protect against fungi and oxidative damage. It is a colorless to pale yellow crystalline substance (depending on its purity). It is a natural substance of the class of phytoalexins and has the basic structure of stilbenes. With a strong antioxidant effect[10].

1.4 Antioxidants

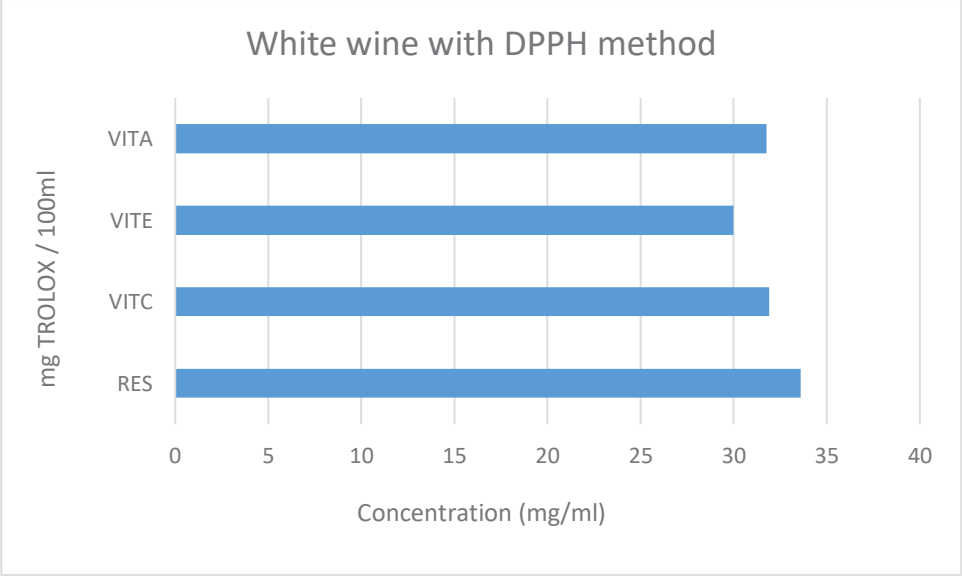
The more we burn, and therefore the more oxygen we use, the greater our need for antioxidant protection. All cells need protection from free radicals and more so the brain which, despite weighing only 2% of our weight, uses 20% of the oxygen we breathe. The body produces antioxidant enzymes that neutralize free radicals, but with age the amount of these enzymes decreases. So we need to get antioxidant molecules from the diet which easily provide the electrons to the free radicals. Antioxidant substances are an ally in protecting the body against aging and serious diseases. Antioxidants are substances that inhibit - prevent the oxidation (destruction) of cells. The oxidation process causes cell damage through the production of free radicals. Air pollution, solar radiation, stress, smoking and some foods (fried foods) are some of the most important factors in the formation of free radicals. Free radicals are particularly harmful to cells and are very likely to cause irreversible damage (i.e. changes in either cell morphology or function, resulting in diseases such as cancer, heart disease, neurodegenerative conditions, etc.). Antioxidants are substances that prevent or slow down the oxidation of food components caused by atmospheric oxygen. Most antioxidants are aromatic compounds, possessing at least one free hydroxyl or amine group[11,12].

2. MATERIALS AND METHODS

2.1 Materials

Specific methods were used to conduct the experiments, and below are mentioned all the machines, and reagents that were used to conduct the experiments. The machines and reagents used for the analyzes are:

- ASCORBIC ACID A.G. (vitamin C), 99, 5%,



Scheme 3. Final results (after 1500 hours) of white wine ..with Resveratrol (RES), Viatmin C (VITC), Vitamin E (VITE),Vitamin A (VITA), for the DPPH method

MW=176, 13 gr/mol, PENTA;

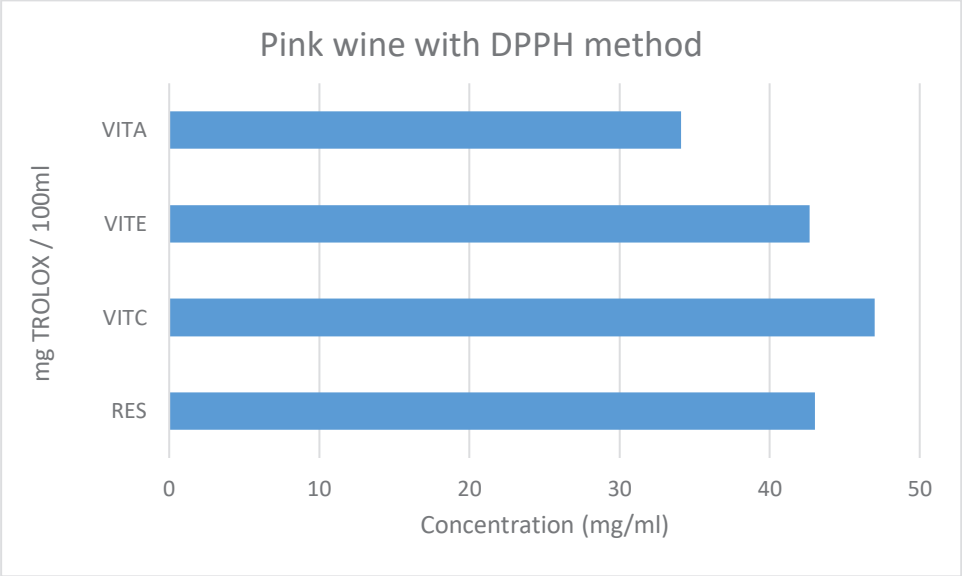
- DL-a-Tocopherol (vitamin E), 96%, MW=430, 72 gr/mol, TCI;
- all-trans-Retinol (vitamin A), 95%, MW=286, 4516 gr/mol, ACROS ORGANICS;
- Resveratrol (RVT), 99%, MW=228, 25 gr/mol, TCI;
- 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 95%, MW=394, 32 gr/mol, Alfa Aesar GmbH Co KG;
- Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylicacid), 97%, C14H18O4, MW=250,29 g/mol CAS: 53188-07-01, Sigma-Aldrich, Germany.
- Sodium Carbonate anhydrous(Na2CO3): Assay 99,5-100,5%, MW=105,99 g/mol, CAS: 497-19-8, Carlo ΕΓ eagents, Italy
- Gallic Acid: 3,4,5-Trihydroxybenzoic acid anhydrous 99%, C7H6O5, MW=170,12 g/mol, CAS: 149-91-7, Alfa Aesar GmbH&Co KG, Germany
- Potassium Persulfate (K_{2S2O8})
- Methanol 99,9%
- UV-Visible spectrophotometer, Novaspec III;
- Analytical balance: KERN ADJ, TechnoLab;
- Water bath, Heraus

2.2 Analysis Methods

2.2.1 Measurement of antioxidants activity with the DPPH method

2.2.1.1 DPPH mother solution: The balance is set to zero with the beaker to be used and an amount of 0.0060g of DPPH is added to it with the help of a special spatula. Then, a small amount of methanol is added to the beaker at a time while stirring until the DPPH granules dissolve. Finally, place the contents of the beaker in a 250 ml volumetric flask and add methanol up to the mark. The solution should not be used immediately after preparation. Leave the solution in the dark for 30 minutes. It can be kept in the fridge for up to one day [13-16].

2.2.1.2 Trolox solution: The balance is zeroed with the beaker used and 0.0250g trolox is added to it with the help of a special spatula. These are dissolved in 90ml of methanol and the solution is transferred to a 100ml volumetric flask and made up to



Scheme 4. Final results (after 1500 hours) of pink wine with Resveratrol (RES), Viatmin C (VITC), Vitamin E (VITE),Vitamin A (VITA), for the DPPH method

volume with deionized water (10ml).

2.2.1.3 Dilution of samples: The initial concentration of the substances is 300 mg in 1000 ml of alcoholic beverage. At a specified time, an amount of samples was taken, diluted and then measured. Red wine was diluted with 100 μl of red wine in 99.9 ml of methanol, rosé wine was diluted with 100 μl of rosé wine in 49.9 ml of methanol. The white wine and tsipouro were not diluted.

2.2.1.4 Experiment procedure

50μl of sample (standard or sample) and 1450μl of DPPH mother solution are added to an eppendorf with the help of pipettes.

After vortexing for 30 sec absorbance at a wavelength of 517nm (t=0) is measured in wells. After 30 minutes that the samples remained in the dark, a vortex is performed and the absorbance is measured again (t=30 sec). The spectrophotometer is zeroed with methanol.

From the two absorbances, the % difference in absorbance is calculated according to the formula:

%ΔA (517nm) = [A(0) – A(30)/ A(0)] × 100.

Through the trolox reference curve, the antioxidant capacity is expressed in trolox equivalents.

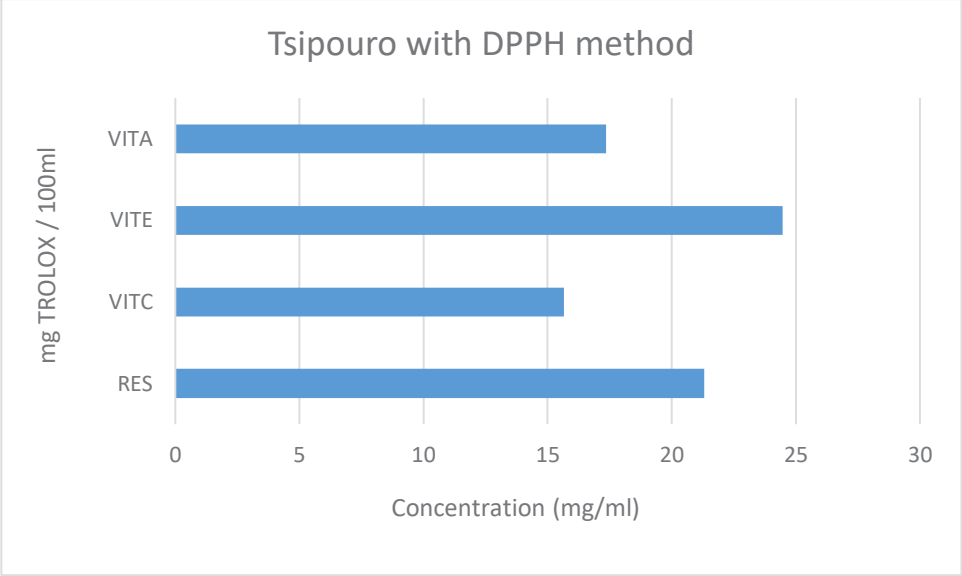
Trolox solution: 0.0250g of trolox is dissolved in 90ml of methanol and 10ml of deionized water.

For the standard reference curve, a Trolox solution with a concentration of 2mM (solution A) is prepared.

In 10 ml volumetric flasks, the corresponding amounts of 8, 6, 4, 2 ml of solution A are added and the volume is made up with pure methanol.

2.2.2 Measurement of antioxidants activity with the ABTS method

2.3.2.1 ABTS mother solution: The balance is set to zero with the beaker to be used and with the help of a special spatula the amount of 0.1801g ABTS and 0.0331g K_{2S2O8} is added to it and we first dilute them with 20ml of deionized water. The solution is then added to a 50ml volumetric flask and deionized wa-



Scheme 5. Final results (after 1500 hours) of tsipouro with Resveratrol (RES), Viatmin C (VITC), Vitamin E (VITE),Vitamin A (VITA), for the DPPH method

ter is added up to the mark. Leave the solution for 16 hours in the dark at room temperature to acquire an intense deep blue color. Finally, we take 3.6ml of the initial solution prepared above, place it in a 250ml volumetric flask and dissolve it with 246.4ml of methanol. Measure the absorbance which should be close to 0.700 when spectrophotometered at 734nm. The solution can be used immediately after its preparation. Keeps in the fridge for many days[18-20].

2.2.2.2 Trolox solution: The scale is zeroed with the beaker to be used and 0.0250g of trolox is added to it with the help of a special spatula. These are dissolved in 90ml of methanol and the solution is transferred to a 100ml volumetric flask and made up to the mark with deionized water (10ml).

2.2.2.3 Dilution of samples: The initial concentration of the substances is 300 mg in 1000 ml of alcoholic beverage. At a specified time, an amount of samples was taken, diluted and then measured. Red wine was diluted with 100μl of red wine in 99.9ml of methanol, rosé wine was diluted with 100μl of rosé wine in 49.9ml of methanol. The white wine was diluted with 100μl of white wine in 49.9ml of methanol and the tsipouro was not diluted.

2.2.2.4 Experiment procedure

50μl of sample (standard or sample) and 1450μl of ABTS mother solution are added to an eppendorf with the help of pipettes.

Strong vortexing is carried out for 30 sec and left in the vials to rest for 6 minutes, the absorbance is measured at a wavelength of 734 nm. The spectrophotometer is zeroed with methanol.

From the two absorbances, the % difference in absorbance is calculated according to the formula:

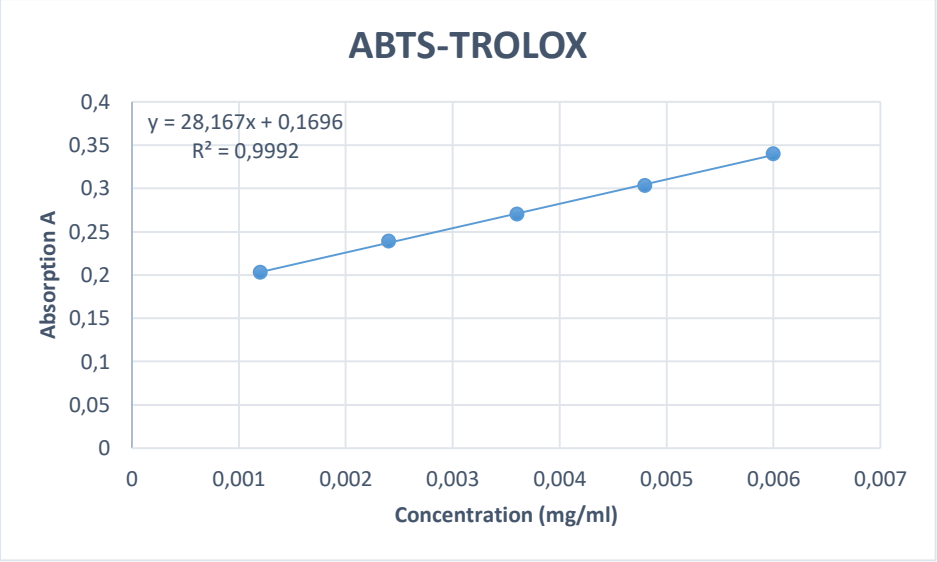
%ΔA (734nm) = [A(δ) – A(6min)/ A(δ)] × 100.

Through the trolox reference curve, the antioxidant capacity is expressed in trolox equivalents.

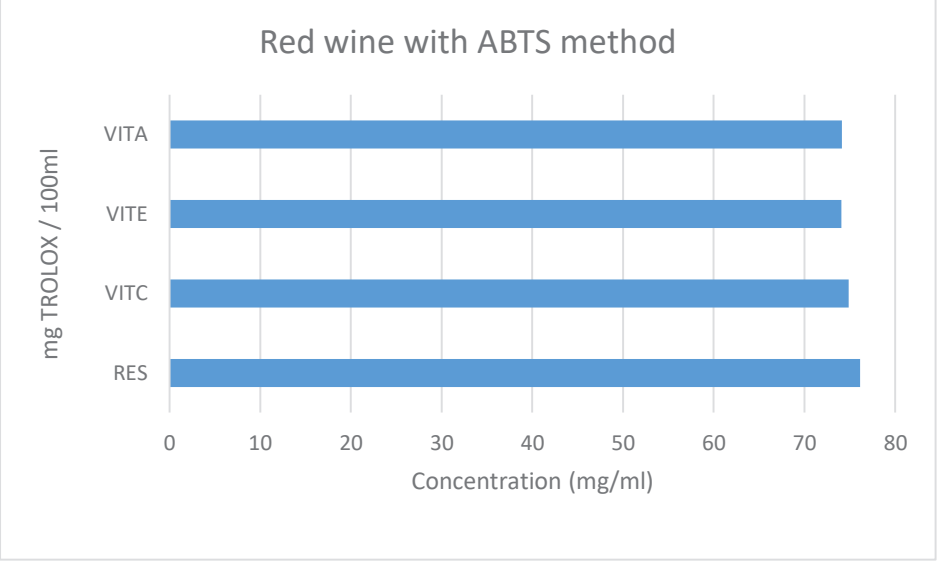
Trolox solution: 0.0250g of trolox is dissolved in 90ml of methanol and 10ml of deionized water.

For the standard reference curve, a Trolox solution with a concentration of 2mM (solution A) is prepared.

In 10 ml volumetric flasks, the corresponding amounts of 8, 6, 4, 2 ml of solution A are added and the volume is made up with pure methanol.



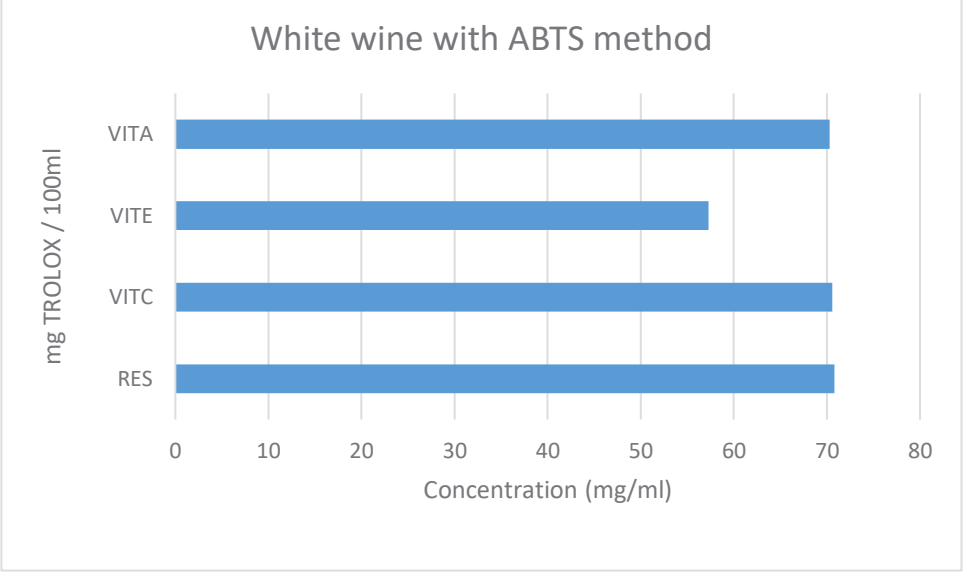
Scheme 6. Standard reference curve with trolox for the ABTS method



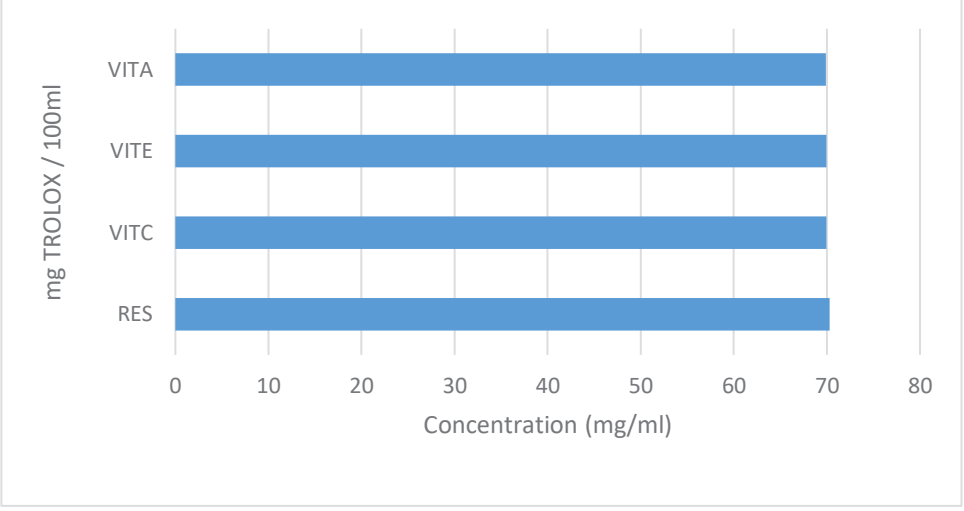
Scheme 7. Final results (after 1500 hours) of red wine with 1) resveratrol, 2) vitamin C, 3) vitamin E, 4) vitamin A, for the ABTS method

2.2.3 Measurement phenolic compounds by the Folin-Ciocalteau method

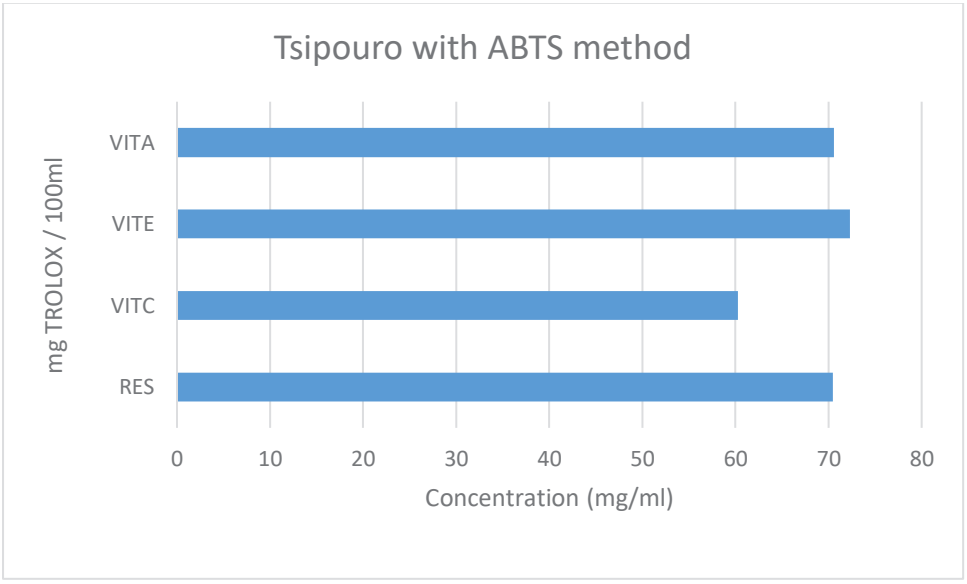
2.2.3.1 Anhydrous Sodium Carbonate Solution: The scale is set to zero with the beaker to be used and with the help of a special spatula, 50.00g of anhydrous sodium carbonate is added to it, which is dissolved in deionized water using very mild heating and simultaneous stirring. Once dissolved, it is placed in a 250ml volumetric bottle and the bottle is filled with deionized water up to the mark. It is then left at room temperature and in the absence of light for 24 hours. Then by using filter paper the content is filtered and not diluted. The solution can be used



Scheme 8. Final results (after 1500 hours) of white wine with Resveratrol (RES), Viatmin C (VITC), Vitamin E (VITE),Vitamin A (VITA), for the ABTS method



Scheme 9. Final results (after 1500 hours) of pink wine with Resveratrol (RES), Viatmin C (VITC), Vitamin E (VITE),Vitamin A (VITA), for the ABTS method



Scheme 10. Final results (after 1500 hours) of tsipouro with Resveratrol (RES), Viatmin C (VITC), Vitamin E (VITE),Vitamin A (VITA), for the ABTS method

immediately after its preparation. It keeps for many days[16,17].

2.2.3.2 Folin-Ciocalteu solution:

Folin-Ciocalteu solution is commercially available, not prepared by us.

2.2.3.3 Gallic Acid Solution:

The balance is set to zero with the beaker to be used and 0.50g of gallic acid is added to it with the help of a special spatula. These are dissolved in 90ml deionized water and the solution is transferred to a 100ml volumetric flask and made up to the mark with ethanol (10ml). The solution stays for two weeks.

2.2.3.4 Dilution of samples:

The initial concentration of the substances is 300 mg in 1000 ml of alcoholic beverage. At a specified time, an amount of samples was taken, diluted and then measured. Red wine was diluted 100μl of red wine in 99.9ml of deionized new, rosé wine was diluted with 100μl of rosé wine in 49.9ml of deionized water. White wine was diluted with 100μl of white wine in 49.9ml of deionized water and tsipouro was diluted with 100μl of tsipouro in 24ml of deionized water.

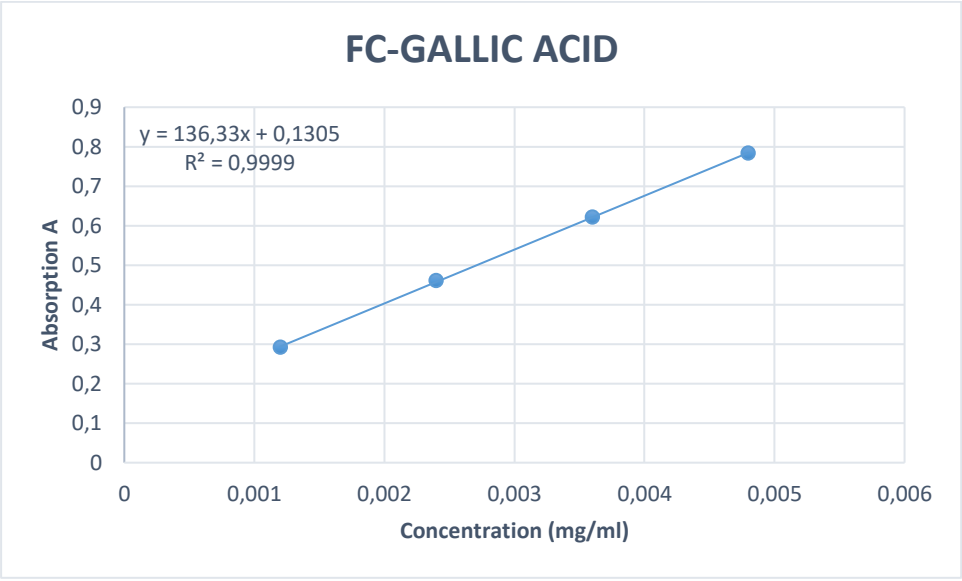
2.2.3.5 Experiment procedure

20μl of sample (standard or sample) and 1450μl of deionized water are added to an eppendorf and 100μl with the help of pipettes. We vigorously stir for 30sec in the vortex and let it rest for 8 minutes, add 300μl of anhydrous sodium carbonate and again vigorously stir for 30sec in the vortex.

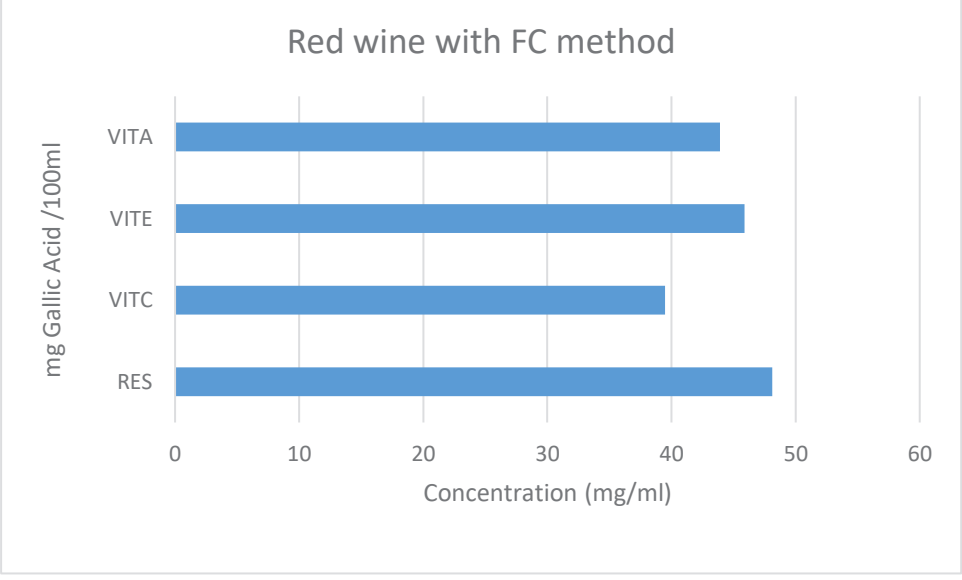
We place the sample in the wells and cover them with parafilm and place them for 30 minutes in the water bath at 40oC. As soon as they acquire a blue color and have returned to room temperature, spectrophotometer at 750nm. The spectrophotometer is zeroed with deionized water.

Phenolics are expressed through the reference curve in gallic acid.

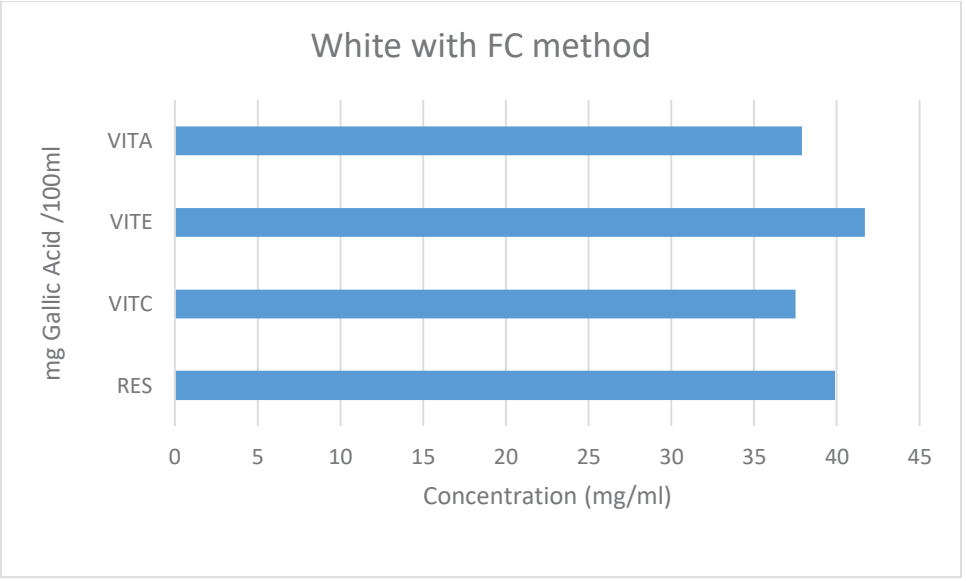
Gallic Acid Solution: 0.50g gallic acid is dissolved in 90ml deionized water and 10ml ethanol.



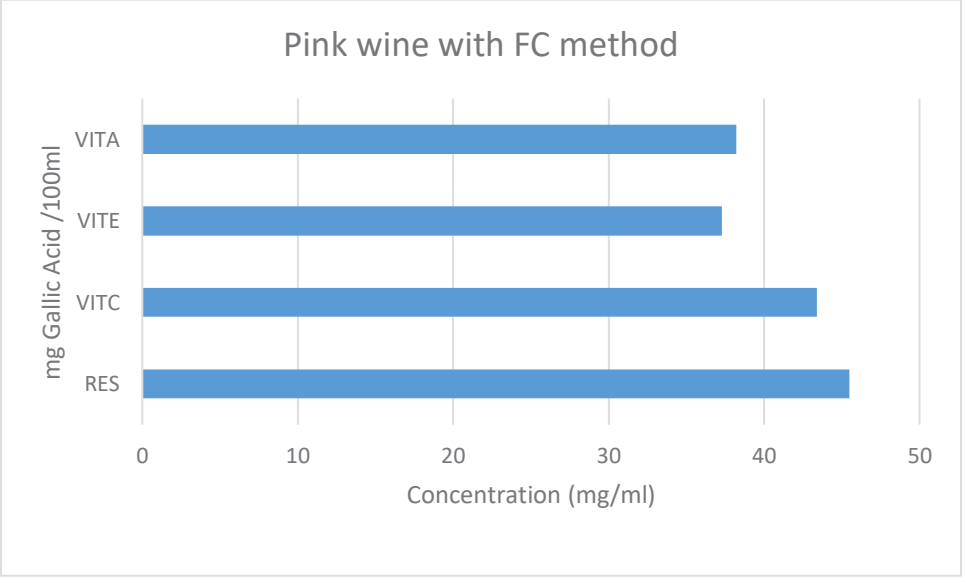
Scheme 11. Standard reference curve with gallic acid for the FC method



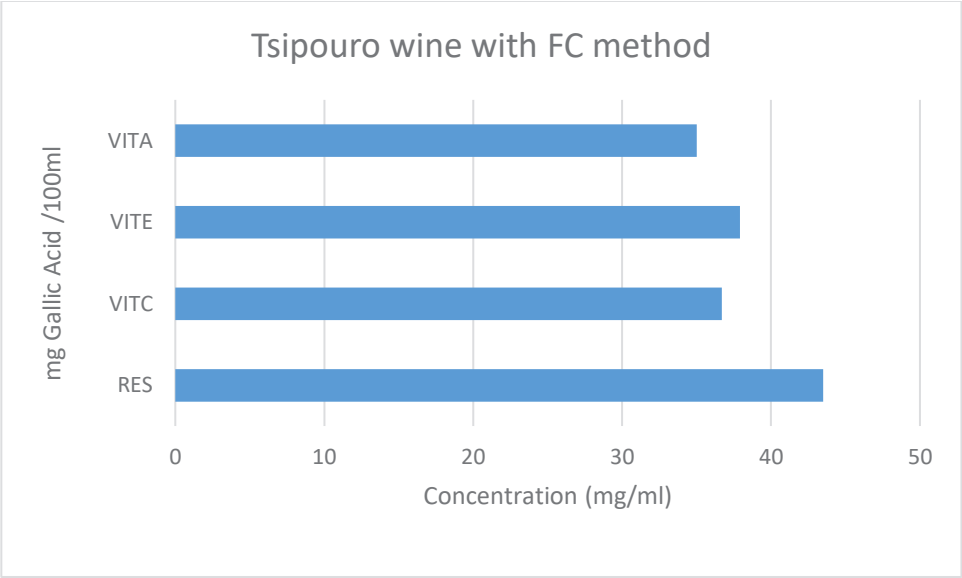
Scheme 12. Final results (after 1500 hours) of red wine with Resveratrol (RES), Viatmin C (VITC), Vitamin E (VITE),Vitamin A (VITA), for the FC method



Scheme 13. Final results (after 1500 hours) of white wine with Resveratrol (RES), Viatmin C (VITC), Vitamin E (VITE),Vitamin A (VITA), for the FC method



Scheme 14. Final results (after 1500 hours) of pink wine with Resveratrol (RES), Viatmin C (VITC), Vitamin E (VITE),Vitamin A (VITA), for the FC method



Scheme 15. Final results (after 1500 hours) of tsipouro with Resveratrol (RES), Viatmin C (VITC), Vitamin E (VITE),Vitamin A (VITA), for the FC method

A gallic acid solution (solution A) is prepared for the standard reference curve.

In 10 ml volumetric flasks, add the proportional amounts of 2.5/2.0/1.5/1.0/0.5 ml of solution A and make up the volume with deionized water.

3. Results and discussion

The same treatment and the measurement of their antioxidant activity were applied to all samples.

However, each method applied (DPPH, ABTS, Folin-Ciocalteu) has a different photometric response from the samples, while the dilutions made were found experimentally and applied for each sample separately.

3.1 Evaluation of DPPH radical scavenging capacity of wine and tsipouro samples

Assessment of the DPPH radical scavenging capacity of samples of vitamins A, E, C and resveratrol is a method widely used to assess the antioxidant capacity of samples of plant origin. To estimate the antiradical capacity of the samples, mg Trolox equivalents per 100ml of solution were calculated based on a standard Trolox curve.

Based on the diagram above, we notice that all four substances we added to the wine had positive effects. More specifically, resveratrol gives the best rates of antioxidant capacity based on the reference curve and trolox equivalents, 65.77mg/100 ml of solution, followed by vitamins C, A, and E, whose results are 55.99mg/100 ml of solution 56.24mg /100ml of solution 56.57mg/100ml of solution.

Based on the diagram above, we notice that all four substances we added to the wine had positive effects. More specifically, resveratrol gives the best rates of antioxidant capacity based on the reference curve and trolox equivalents, 33.60mg/100 ml of solution, followed by vitamin C with 31.90mg/100 ml of solution, vitamin A with 31.75mg/ 100ml of solution and finally vitamin E 30.00mg/100ml of solution.

Based on the diagram above, we notice that all four substances we added to the wine had positive effects. More specifically, vitamin C gives the best rates of antioxidant capacity based on the reference curve and trolox equivalents, 46.99mg/100 ml of solution, followed by the sample with resveratrol

with 43.02mg/100 ml of solution and vitamin E with 42.67mg/100ml of solution which give very good results and finally vitamin A with 34.01mg/100ml of solution whose results are also satisfactory.

Based on the diagram above, we notice that all four substances we added to the wine had positive effects. More specifically, vitamin E gives the best rates of antioxidant capacity based on the reference curve and trolox equivalents, 24.46mg/100ml of solution, followed by resveratrol with 21.31mg/100ml of solution, vitamin A with 17.35mg/100ml solution and finally vitamin C with 15.66mg/100ml of solution.

3.2 Estimation of ABTS free radical scavenging of wine and tsipouro samples

An estimate of the antioxidant capacity of the studied samples is given by means of the ABTS+ radical inhibition test. The results are expressed as mg trolox equivalents per 100ml of solution and with the percentages of root inhibition from each sample. The concentration of the samples is calculated using a trolox standard curve.

Based on the diagram above, we notice that all four substances we added to the wine had positive results and their values are all very close. More specifically, resveratrol with 76.16mg/100ml of solution is followed by vitamin C with 74.90mg/100ml of solution, next is vitamin A 74.15mg/100ml of solution and finally vitamin E with 74.05mg/100ml of solution.

Based on the diagram above, we notice that all four substances we added to the wine had positive results and their values are all very close. More specifically, resveratrol with 70.82mg/100ml of solution is followed by vitamin C with 70.57mg/100ml of solution, next is vitamin A 70.28mg/100ml of solution and finally vitamin E with 57.28mg/100ml of solution.

Based on the diagram above, we notice that all four substances we added to the wine had positive results and their values are all very close. More specifically, resveratrol with 70.28mg/100ml of solution is followed by vitamin C with 69.93mg/100ml of solution, next is vitamin E 69.91mg/100ml of solution and finally vitamin A with 69.90mg/100ml of solution.

Based on the diagram above, we notice that all four substances we added to the wine had positive results and their values are all very close. More specifically, vitamin E with 72.28mg/100ml of solution is followed by vitamin A with 70.57mg/100ml of solution, next is resveratrol with 70.48mg/100ml of solution and finally vitamin C with 60.28mg/100ml of solution.

3.3 Calculation of the total phenolic components (Total Phenolic Content, TPC) determined by the FOLIN-CIOCALTEU method

The estimation of the total phenolic content was done with the Folin-Ciocalteu method. To extract the results, the standard curve is constructed graphically, through which the concentration of the phenolic components of the samples is calculated, expressed in gallic acid equivalents (Gallic Acid Equivalents, GAE).

Based on the diagram above, we notice that all four substances we added to the wine had positive results and their values are all close. More specifically, the largest amount is resveratrol with 48.1mg, followed by vitamin E with 45.9mg, next is vitamin A 43.9mg and finally vitamin C with 39.5mg.

Based on the diagram above, we notice that all four substances we added to the wine had positive results and their values are all close. More specifically, the largest amount is vitamin E with 41.7mg, followed by resveratrol with 39.9mg, next is vitamin A 37.9mg and finally vitamin C with 37.5mg.

Based on the diagram above, we notice that all four substances we added to the wine had positive results and their values are all close. More specifically, resveratrol has the largest amount with 45.5mg, followed by vitamin C with 43.4mg, next is vitamin A 38.2mg and finally vitamin E with 37.3mg.

Based on the diagram above, we notice that all four substances we added to the wine had positive results and their values are all close. More specifically, resveratrol has the largest amount with 43.5mg, followed by vitamin E with 37.9mg, the next is vitamin C 36.7mg and finally vitamin A with 35mg.

3.4 Organoleptic test

The organoleptic evaluation was done by 22 people aged 18 to 30, 12 women and 10 men. Based on their evaluations, we have the following results.

The color in all four complexes that have been produced, when we integrated them into the wines and the tsipouro has not changed at all.

The aroma in all four complexes that have been produced, when we incorporated them into the wines and the tsipouro did not affect it at all.

Finally, the taste of all four complexes that have been produced, when we integrated them into the wines and the tsipouro, has not been affected at all.

All of the above applies to all the concentrations we have placed in wines and tsipouro.

From the above, it follows that the wines and tsipouro produced at the end can be accepted by consumers, since their organoleptic characteristics have not undergone any changes.

4. Conclusions

The recorded results are the final ones after 1500 hours of experiments. We notice that with the DPPH and ABTS methods red, white and rosé wine as well as tsipouro with the addition of the four substances that are resveratrol, vitamin C, vitamin E and vitamin A show us that the increase has been achieved of their antioxidant capacity.

In more detail we see that there are increases in white wine, rosé and red wine for all four substances incorporated. The darker red and rosé wines show a greater increase in antioxidant capacity than white wine and tsipouro. The positive effect of the four substances included in them in terms of increasing their antioxidant property is proven.

Total phenolics were measured by the Folin Ciocalteu method. The increase of phenolics was also observed and we see that the results are the same as those of antioxidants, the highest percentages are observed in red wines, followed by rosés and then whites and tsipouros. Darker wines also show a greater increase than white wine and tsipouro.

The correlation of the results for the total phenolics of the wine and its antioxidant capacity is high.

It is also observed that between the two antioxidant methods dppi and avcs the results have a very high correlation.

It should also be mentioned that the wines and the tsipouro have not undergone oxidation during the experiment or any other alteration because the experiment was completed in a relatively short time after the incorporation of the substances and with great care so that they did not come into contact with the air, protected from the light and at an appropriate temperature (ambient temperatures).

Red wine has the largest percentage increase in all three methods, followed by rosé, white wine and tsipouro. The results of the DPPH and ABTS methods are identical, which means that the results are correct, as are the results of the Folin Ciocalteu method. Finally, from the organoleptic control it appears that the final products are of high quality and absolutely safe and stable and suitable for consumption by consumers.

Funding: This research was funded by IKY. **«The implementation of the doctoral thesis was co-financed by Greece and the European Union (European Social** Fund-ESF) through the Operational Programme «Human Resources Development, Education, and Lifelong Learning» in the context of the Act "Enhancing Human Resources Research Potential by undertaking a Doctoral Research" Sub-action 2: IKY Scholarship Programme for PhD candidates in the Greek Universities».

Data Availability Statement: Not applicable.

Acknowledgments: The authors warmly thank the National, and Kapodistrian University of Athens, and the National Technical University of Athens for the availability of the laboratories, and reagents that were used. Finally, we warmly thank the PhD candidate Konstantina Papastavropoulou of the National, and Kapodistrian University of Athens for her help.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results. □

References

1. Nikfardjam Pour M.S. La´zlo Gy., Dietrich, H., Resveratrol-derivatives and antioxidative capacity in wines made from botrytized grapes. *Food Chem*., 96, 74–79, 2006. *https://doi:10.1016/j.foodchem.2005.01.058*
2. Gorelick-Feldman J., Maclean D., Ilic N., Poulev A., Lila M.A., Cheng D., Raskin I. Phytoecdysteroids increase protein synthesis in skeletal muscle cells. *J. Agric. Food Chem.* 56(10), 3532-7, 2008. *https://doi.org/10.1021/jf073059z*
3. Brouillard R., George F., Fougerousse A., Polyphenols produced during red wine ageing. *Biofactors.* 6(4),403-10 1997. *https://doi.org/10.1002/biof.5520060406*
4. Ceryova N., Bajcan D., Lidikova J., Snirc M., Trebichalsky P., Beresecka J., Horvathova J., Phenolic Contents, Antioxidant Activity and colour density of Slovak pinot noir Wines. *Acta Agriculturae Slovenika* 117(3):1, 1-8, 2021. *https://doi:10.14720/aas.2021.117.3.2043*
5. German B.J., Walzem R.L., The Health Benefits of Wine. *Annual reviews.* 20, 561-593, 2000. *https://doi.org/10.1146/annurev.nutr.20.1.561*
6. Simpson J. (2012) Creating Wine, The Emergence of a World Industry. *Pricenton Univercity Press,* 36, 1840-1914. *https://doi.org/10.1515/9781400838882*
7. Arrigoni O., De Tullio M.C., Ascorbic acid: much more than just an antioxidant. *Elsevier.* Biochim. Biophys. Acta.1569, 1-9, 2002. *https://doi.org/10.1016/S0304-4165(01)00235-5*
8. Azzi A., Molecular mechanism of α-tocopherol action. *Elsevier.* Free Radic. Biol. Med. 43, 16-21, 2007. *https://doi.org/10.1016/j.freeradbiomed.2007.03.013*
9. Newcomer M.E., Jones T.A., Aqvist J., Sundelin J., Eriksson U., Rask L., Peterson P.A., The three-dimensional structure of retinol-binding protein. *The EMBO journal.* 3, 1451-1454, 1984. *https://doi.org/10.1002/j.1460-2075.1984.tb01995.x*
10. Nikfardjam Pour M.S., Laszlo G., Dietrich H., Resveratrol-derivatives and antioxidative capacity in wines made from botrytized grapes. *Food chemistry,* 96, 74-79, 2006. *https://doi.org/10.1016/j.foodchem.2005.01.058*
11. Dasgupta A., Klein K., Antioxidants in Food, Vitamins and Supplements, Prevention and Treatment of Disease. *Oxford: Elsevier.* 58, 2014.
12. Mandal M., Antioxidants in food. *News – Medical.* 2015.
13. Clarke G., Ting K.N., Wiart C., Fry J., High Correlation 2,2-diphenyl-1- picrylhydrazyl (DPPH) Radical Scavenging, Ferric Reducing Activity Potential and Total Phenolics Content Indicates Redundancy in Use of all Three Assays to Screen for Antioxidant Activity of Extracts of Plants from the Malaysian Rainforest, Antioxidants. *National library of medicine.* 2(1), 1-10, 2013. *https://doi.org/10.3390/antiox2010001*
14. Enujiugha V.N., Talabi J.Y., Malomo S.A., Olagunju A.I., DPPH Radical Scavenging Capacity of Phenolic Extracts from African Yam Bean (Sphenostylis stenocarpa). *Food Nutrition Sci..* 3, 7-13 2012. *http://dx.doi.org/10.4236/fns.2012.31002*
15. Marinova, G., Batchvarov V., Evaluation of the method for determination of the free radical scavenging activity by DPPH. *Bulg. J. Agric. Sci.* 17(1), 11-24, 2011.
16. Danilewicz J. C., The Folin-Ciocalteu, FRAP, and DPPH, Assays for Measuring Polyphenol Concentration in White Wine. *Am. J. Enol. Vitic..* 66, 463-471, 2015. *http://dx.doi.org/10.5344/ajev.2015.15025*
17. Blainski A., Lopes G., Pallazo De Mello J.C., Application and analysis of the Folin – Ciocalteu Method for the Determination of the Total Phenolic Content from Limonium Brasiliense, L. *National library of medicine.* 18(6), 6852-6865, 2013. *https://doi.*

org/10.3390%2Fmolecules18066852

18. Re R., Pellegrini N., Proteggente A., Pannala A., Yang M., Rice-Evans C., Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*. 26(9-10), 1231-1237, 1999. *https://doi.org/10.1016/s0891-5849(98)00315-3*
19. Villano D., Fernandez-Pachon M.S., Troncoso A.M., Garcia- Parrilia M.C., The Antioxidant Activity of Wines Determined by the ABTS $^{+}$ Method: Influence of Sample Dilution and Time. *Elsevier. Talanta* 64, 501-509, 2004. *https://doi.org/10.1016/j.talanta.2004.03.021*
20. Walker R., Everette J., Comparative reaction rates of various antioxidants with ABTS radical cation. *J. Agric. Food Chem.* 57, 1156-1161, 2009. *https://doi.org/10.1021/jf8026765*