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The Non-additive Effect of Food and Wine in the Spanish Diet on Total Antioxidant Capacity of the Spanish Mediterranean Diet

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Abstract

With an increase in mortality rates related to obesity, cardiovascular disease, heart disease, and other related diseases, a greater understanding of the Mediterranean diet may be important to dietary treatment of these illnesses and comorbidities. While the foods in the Mediterranean diet have been studied in depth for their great health benefits, the potential role of wine in this diet is under examined. Interest in wine and its health benefits has increased within the past decade. While wine is a common component of the Mediterranean diet, very few studies have looked at the possible effects and outcomes of consuming wine in tandem with the foods in this diet. Studies which have explored the possible health benefits of consuming red wine with food have provided little to no examination of the chemical interactions that may be occurring, or the impact on antioxidant potential. **PURPOSE:** To explore the possible interaction effects on antioxidant capacity levels when pairing common foods and wines in the Spanish Mediterranean diet. **METHODS:** Three monovarietal wines and three individual foods common to the Spanish Mediterranean diet were evaluated. The foods and wines were tested alone and in each possible pairing (9 total pairings) for Trolox Equivalency Antioxidant Capacity against dilutions of Trolox using DPPH. The expected summative value for each pairing was compared to the actual value for each pairing. The actual antioxidant capacity of each pairing was then compared to the expected value using a one-way ANOVA. **RESULTS:** All data tested showed significant relevance when tested with one-way ANOVA in SPSS ($p<0.05$). Tomatoes had significantly negative (antagonistic) interaction effects in all three wine combinations. Spanish onions and persimmons had significantly positive (synergistic) interaction effects in all three wine pairings. **CONCLUSION:** The results indicated that antioxidants from different foods have distinct

interactions on a molecular level that are non-additive. These results suggest that further research to identify food interactions is warranted.

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The Non-additive Effect of Food and Wine in the Spanish Diet on Total Antioxidant Capacity of the Spanish Mediterranean Diet

by

Carol Majkrzak

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1. INTRODUCTION

1.1 Wine and the Mediterranean Diet

Antioxidants are compounds naturally found in foods, as well as most other biological systems, that relieve oxidative stress and mitigate damage to molecules caused by free radicals (Freeman & Crapo, 2018). This damage can lead to or be caused by several diseases and illness that are food-related (Valko, Leibfritz, Moncol, Cronin, Mazur, and Telser, 2007). Many diets that are geared towards improving overall health aim to increase the intake of foods high in antioxidants. The Mediterranean diet in particular has been touted as particularly beneficial because of the high antioxidant capacity of foods typical to the diet (Panagiotakos, Pitsavos, and Stefanadis, 2006). It has been prescribed by registered dietitians and doctors for treatment of heart disease, obesity, hypertensions, and other cardiovascular diseases due to these health benefits (Ortega, 2006). Wine, while not extensively studied as part of the Mediterranean diet, is commonly consumed with meals in Mediterranean countries such as France, Spain, and Italy - all of which are known for their relatively low mortality rates which are attributed to their dietary habits (Willett, Sacks, Trichopoulou, Drescher, Ferro-Luzzi, Helsing, and Trichopoulos, 1995).

There are many types of antioxidants found in food (Rice-Evans, Miller, and Paganga, 1997). Red wines have higher concentrations of antioxidants than other wines because they are fermented with the fruit skins, twigs and leaves attached (Vinson and Hontz, 1995). The flavor of the wine, the color or darkness, and the number of antioxidants are affected by the length of time the wine is left to ferment with the skins, twigs, and leaves (Yilmaz & Toledo, 2006). The amount of tannins gained from the seeds, skin, and leaves can directly impact flavor and antioxidant levels

(Mattivi, Zulian, Nicolini, and Valenti, 2002). Traceable amounts of wine polyphenols can be found in human plasma after both acute and chronic wine intake (Bub, Watzl, Heeb, Rechkemmer and Briviba, 2001). The polyphenols that are most common in wine are broken into two categories, flavonoids, such as quercetin and catechin, and non-flavonoids, such as gallic acid, caffeic, and resveratrol (German and Walzem, 2000).

Some research has shown that pairing certain high antioxidant foods together can increase the observed total antioxidant capacity beyond the sum of its parts (Cavallini, Straniero, Donati, and Bergamini, 2015). This makes the antioxidants more potent within the body, potentially allowing for greater health benefit. A recent study looking at the antioxidant capacity of red wine, found that the resveratrol in red wine is more powerful as an antioxidant because of the other polyphenols found in the wine and the synergistic effect they have on each other (Cavallini, Straniero, Donati and Bergamini, 2015).

Additionally, wine also contains alcohol in the form of ethanol which has shown to increase HDL production and protect LDL from oxidative stress (Covas, Gambert, Fitó and de la Torre, 2010). Some epidemiological studies show drinking one to two glasses of alcohol a day may help with cardiovascular disease, all-cause mortality, and cognitive function (Artero, Artero, Tarín and Cano, 2015). However, not all alcohols are equally beneficial. A recent meta-analysis found that while beer and wine consumption have similar ability to reduce cardiovascular risk, other hard liquors and spirits do not have the same benefit. The same study found that it is the polyphenolic compounds found in beer and wine that are responsible for the health effects found, and that wine, but not beer, reduced all-cause mortality (Costanzo, Di Castelnuovo, Donati, Iacoviello and de Gaetano, 2011). These studies looked at alcoholic drinks alone and not how they interacted with food.

Despite a diet full of saturated fat and cholesterol, the French have low mortality rates due to coronary heart disease. This has been coined the French Paradox (Gresele, Cerletti, Guglielmini, Pignatelli, de Gaetano, and Vivoli, 2011). One possible explanation is that the French are known for consuming wine. While the French Mediterranean diet has been heavily studied, there is a lack of research on the diet of other Mediterranean regions such as Greece, Spain, and Southern Italy. Furthermore, research on the health benefits of regional Mediterranean dietary staples paired with wine consumption is largely lacking. A review of literature was done in 2004 to examine the fats consumed in the Mediterranean diet and red wine for the impact on cardiovascular incidents. When only including the observations performed in Mediterranean countries, red wine when consumed with meals was more protective as part of the diet than when consumed alone (Martínez-González and Sánchez-Villegas, 2003).

Countries that are considered part of the Mediterranean share many common characteristics when it comes to their diets. However, location, temperature, growing regions, and climate can cause staple foods to vary between each country and its regions. This will affect the overall antioxidant consumption from foods for each regional diet. For this study, the focus will be on Spain. The three wines and foods will be typical to Spain, which is part of the Mediterranean collective. The wine being chosen will be Spanish monovarietals, i.e. wines made completely from a single grape varietal found in Spain. The three foods will be fruit and vegetables common to everyday Spanish eating.

Previous research has found that wines such as cabernet sauvignon, cabernet franc, and pinot noir have higher total amounts of phenolic compounds due to their grape varietals (Van Leeuw, Kevers, Pincemail, Defraigne & Dommes, 2014), with the highest amount in Cabernet Franc (Gris, Mattivi, Ferreira, Vrhovsek, Filho, Pedrosa, Bordignon-Luiz, 2013). Phenolic compounds come from the skins and leaves of grapes during the fermentation process (Pérez-Magariño, González-San José, 2004). Due to this, the three Spanish wines chosen (Tempranillo, Monastrell, and Grenache) have similar tannin levels to that of cabernet sauvignon, cabernet franc, and pinot noir (Puckette and Hammack, 2015).

Recent studies done on adherence to the Mediterranean diet in Spain shows that the majority of the population who follow the diet have lower incidence for cardiovascular events (Marisca-Arcas, Caballero-Plasencia, Monteagudo, Hamden, Pardo-Vasquez, and Olea-Serrano, 2011). The three foods chosen (raw tomatoes, Spanish onion, and persimmons) are all common in the everyday Spanish Mediterranean diet (Bach-Faig, Berry, Lairon, Reguant, Trichopoulou, and Dernini, 2011).

1.2 Free Radicals, Antioxidants, and Health Implications

Free radicals are molecules with an electron or electrons in the outer layer, the valence layer, of the molecule that is not paired. This causes the molecule to be unstable and causes damage to neighboring molecules because it will try to pull an electron from elsewhere to stabilize itself. By doing so the molecule that the electron is pulled from is now also unstable, repeating the process. This causes extreme damage to the cell and possibly even premature death of the cell. Free radicals are naturally occurring in the body (Halliwell and Gutteridge, 2015). They can form due to everyday life, being caused by a range of activities including but not limited to physical exercise and energy conversion and digestion (Sen, Chakraborty, Sridhar, Reddy and De, 2010). They can also form due to environmental factors like sunlight UV rays or air pollution (Chun, Frei, Gardner, Alekel and Killen, 2013). This damage to cells is called oxidative stress and antioxidants have been shown to help reduce it. Antioxidants do this due to their specific structure. Phenolic antioxidants' molecular structure includes a hexagonal ring that contains three double bond and three single bond sides. They are able to safely donate an electron because the newly unstable electron that forms in the antioxidant is trapped with the antioxidant ring structure, bouncing from double bond to double bond but never leaving or taking outside electrons (Flora, 2009). This traps the electron and stops the free radicals from doing harm and damage to the body (Lobo, Patil, Phatak, and Chandra, 2010).

Antioxidants can protect against a number of illnesses, diseases, and mortalities such as cardiovascular disease, hypertension, coronary heart disease, cholesterol plaque buildup, diabetes, osteoporosis, and possibly even cancer (Artero, Artero, Tarín and Cano, 2015). In the United States, in 2015, over six hundred thousand people die from heart disease, with coronary heart disease being the most common cause of death, killing over three hundred thousand people a year (Bach-Faig, Berry, Lairon, Reguant, Trichopoulou, and Dernini, 2011). Comorbidities for heart disease include diabetes, overweight and obesity, and poor metabolic function (Roger, Lloyd-Jones, Benjamin, Berry, and Borden, 2011). Over thirty three percent of adults in the United States have hypertension (Benner, Smith, Petrilla, Klingman, Goel, Tang, and Wong, 2008). In 2016, it was recorded by the CDC that over twenty nine million adults in the United States have diabetes, and ninety to ninety five percent of them have type two (Brault, 2012). For all of these diseases, diet plays a huge role in managing or fixing the illness.

Scientists have attempted to replicate the abilities of naturally occurring antioxidants by creating lab formulated supplements. Antioxidants can be created in a lab setting, but studies show that they are not able to duplicate the effect of antioxidants when absorbed from food (German, and Walzem, 2000). While antioxidants are found in almost all biological systems, free radicals occur naturally as well. Oxidative stress in the body is linked to many different human diseases and illnesses (Lobo, Patil, Phatak, and Chandra, 2010). Thus, eating foods that are rich in antioxidants in order to balance the amount of free radicals in the body is speculated to improve human health. If a way to increase antioxidant capacity from antioxidant containing foods can be found, then it may become easier to avoid excessive oxidative stress.

There are many health benefits documented in relation to the Mediterranean diet and to chronic wine consumption. A study done on female rats with hypertension found that continual treatment of polyphenols specifically found in red wine reduced oxidative stress in the rats as well as lowered systolic blood pressure and slowed the development of endothelial dysfunction (Roger, Lloyd-Jones, Benjamin, Berry, and Borden, 2011). Moderate long term red wine consumption has been found to have beneficial effects on cardiovascular disease, diabetes, blood pressure, osteoporosis, neurological diseases, and mortality rates (Artero, Artero, Tarín and Cano, 2015). Red wine is often consumed with meals in the Mediterranean diet (Martínez-González and Sánchez-Villegas, 2003). The foods of the Mediterranean diet, such as garlic, onion, tomatoes, whole grains, fish, nuts and seeds, are high in antioxidants (Lopez-Sepulveda, Jimenez, Romero, Zarzuelo, Sanchez, and Gomez-Guzman, 2008). If red wine polyphenols have a synergistic effect with the antioxidants in foods common in the Mediterranean diet, it may suggest it is not just the food alone, but also the addition of red wine, that makes the diet so beneficial to health. This outcome can further the understanding of why people who follow the Mediterranean diet have such low cardiovascular incidence, increased endothelial function, low incidence of coronary heart disease, reduced rates of hypertension, and overall lower amounts of all-cause mortality (de Lorgeril, Salen, Martin, Monjaud, Delaye, and Mamelle, 1999).

1.3 Research Objectives

This research and following thesis is planned to be an exploratory study to observe the possible interactions between pairings of three red wines and three individual foods common to the Spanish Mediterranean diet. The study is designed to test the thesis that pairing foods and wines together may cause the antioxidants in each to interact and have a non-additive effect greater or lesser than the sum of their parts. From a speculative health standpoint, if pairing food and wine has any effect on antioxidant capacity for either the food, the wine, or both, we can better understand the effects of drinking red wine with a meal. Previous studies have shown that antioxidants can interact, which means we may see that certain foods have different interactions with the same wines due to the different polyphenols present. There may also be no interaction and the tested values for the pairs are simply the sum of the food and wine present in the combination. This would lead to the belief that the food and wine do not have to be consumed together for the greatest possible health benefits. However, if this is not true and interactions are present, the outcomes may lead to a better understanding of the Mediterranean diet, particularly that of Spain, and explain how we can better influence public health and the human condition. Understanding the interactions between food and wine antioxidants can allow doctors and registered dieticians to create better interventions for those with certain health conditions and diseases that can possibly benefit by following a Mediterranean diet.

2. MANUSCRIPT #1: "A COMPARISON OF ANTIOXIDANT CAPACITY OF FOODS AND WINES IN THE SPANISH MEDITERRANEAN DIET"

JOURNAL OF NUTRITION AND FOOD SCIENCES

2.1 Abstract:

Objective: The Mediterranean diet has been shown through recent health and nutritional studies to help ease or cure many illnesses affected by food including obesity, hypertension, coronary heart disease, and other cardiovascular diseases. Studies have shown that those who follow a Mediterranean diet have lower all-cause mortality. This is attributed to the high antioxidant capacity of many of the foods in the diet. However, while red wine is traditionally a part of the Mediterranean diet, it is not included in most of the studies done. While many mention that wine may play a part, they do not look at the potential antioxidant capacity that drinking wine may contribute to the diet and overall health of the population. This study is designed to look at and compare the antioxidant potential of foods and wines common to the Spanish Mediterranean diet to see if the wines may offer a significant level of antioxidants to the diet. Methods: Three foods (raw tomatoes, Spanish onion, and persimmons) and three wines (Tempranillo, Monastrell, and Grenache) common to the Spanish diet were assessed by Trolox Equivalency Antioxidant Capacity. Results: Raw tomatoes and Spanish onions had the highest antioxidant capacity of all the samples tested. Monastrell had the highest antioxidant capacity out of the wines and was significantly higher than the persimmons tested. The other two wines were not statistically significantly different from the persimmons in antioxidant capacity. Conclusion: The Spanish onions and raw tomatoes exhibited approximately 200% the antioxidant potential of the wines, while the difference between the wines and the persimmons was much smaller. The wines alone demonstrated substantial antioxidant capacity. Further research should be done to examine the comparative antioxidant values of more foods and wines in the Mediterranean diet.

Keywords: Trolox Equivalency Antioxidant Assessment (TEAC), Antioxidants, Polyphenols, Wine, Mediterranean Diet, Mortality Rate, Oxidation, Free Radicals

2.2 Introduction:

Antioxidants' main role in the body is protection from free radicals. Free radicals cause harm by stealing electrons from the valence layer of a molecule within the body's cells, causing the cell damage (Lobo, Patil, Phatak, and Chandra, 2010). This damage has been linked to several diseases and dangerous health conditions including cardiovascular diseases and cancer (Artero, Artero, Tarín and Cano, 2015). A diet high in antioxidants, such as the Mediterranean diet, has been shown to help prevent this damage from occurring (Dai, Jones, Goldberg, Ziegler, Bostick, and Wilson, et al. 2008). Foods common in the Mediterranean diet are naturally high in antioxidants and have been studied extensively. Many doctors and registered dieticians suggest following the Mediterranean diet to help fight illnesses such as cardiovascular disease (Carluccio, 2003), heart disease (de Lorgeril, Renaud, Salen, Monjaud, Mamelle, and Martin, 1994), hypertension, and obesity (Schröder, Marrugat, Vila, Covas & Elosua, 2004). However, most studies that look at the antioxidants in the Mediterranean diet only look at the foods consumed. Wine, red wine in particular, is a large part of the traditional Mediterranean diet (Martínez-González and Sánchez-Villegas, 2003) and is also a source of antioxidants which may contribute to the overall antioxidant capacity and healthiness of the diet.

The Mediterranean diet is traditionally high in foods rich in antioxidants such as garlic, onion, tomatoes, whole grains, fish, nuts and seeds (Lopez-Sepulveda, Jimenez, Romero, Zarzuelo, Sanchez, and Gomez-Guzman, 2008). This typical style of eating has been studied extensively for its health benefits. A ten year study done in Europe with over two thousand participants between the ages of seventy and ninety years old found that following a typical Mediterranean diet decreased mortality rates by fifty percent for both all cause and cause specific incidences (Knoops, de Groot, Kromhout, Perrin, Moreiras-Varela, Menotti, and van Staveren, (2004). Traditionally, the Mediterranean diet is prescribed for patients with or at risk for cardiovascular disease due to the high antioxidant levels having protective properties for the heart and surrounding organs (Marisca-Arcas, Caballero-Plasencia, Monteagudo, Hamden, Pardo-Vasquez, and Olea-Serrano, 2011).

Wine is high in antioxidants, specifically resveratrol (Siemann & Creasy, 2018). These antioxidants are highest in red wine because, unlike white wine, red wine is fermented with skins and leaves attached (Vinson and Hontz, 1995). Resveratrol has been studied in depth and has shown to have many health benefits. A recent study found that resveratrol can help with wound healing as it directly impacts endothelial cell growth by increasing the rate at which cells in the inner layer of blood cells reproduce (Yurdagul, Kleinedler, McInnis, Khandelwal, Spence, Orr, and Dugas, 2014). Another found that red wine antioxidants, when used during angioplasty surgery for heart disease patients, can be directly administered to the arteries and help healing while decreasing the risk of further complications (Barr, 2018). Resveratrol has also been linked by several studies to decreased growth of cancer cells. One study shows that resveratrol that is specifically derived from grapes works to heal and inhibit human breast cancer cells (Lu & Serrero, 1999). The resveratrol found in red wine and grapes blocks estrogen receptors and inhibits cell growth, thus limiting the growth on cancerous cells in the breasts (Levenson, Gehm, Pearce, Horiguchi, Simons, and Ward, 2003).

The purpose of this study is to look at and compare antioxidant levels of three foods (raw tomatoes, Spanish onion, and persimmons) and three wines (Tempranillo, Monastrell, and Grenache) typical to the Spanish Mediterranean diet and assess the antioxidant levels of each in order to see how great of an impact wine may have on the antioxidant levels of a traditional Spanish Mediterranean diet.

2.3 Materials and Methods:

2.3.1 Materials and Equipment

Filtered water, Methanol, Acetone, Formic Acid, Trolox, DPPH (2,2-diphenyl-1-picrylhydrazyl), Labconco FreeZone 4.5 Freeze Dryer, Rotary Evaporator, Molecular Devices VERSAmax microplate reader and SoftMax Pro Insider software.

2.3.2 Sample preparation

A total of six different samples that were tested. Three Spanish red wines (Tempranillo, Monastrell, and Grenache) were measured out into three separate test tubes. Samples measured to 25 milliliters exactly. Three common Spanish foods (raw tomatoes, Spanish onion, and persimmons) were pureed into liquid form individually with three separate blenders. Samples of the individual foods were measured out into three separate test tubes in 25 milliliters amounts. All samples were tested at the sample volume of 25 milliliters to set a standard. Each individual food or wine was tested in triplicate. All volumes of samples used (25 milliliters) will be the same in order to compare on a one-to-one scale during analysis. The samples were first pre-frozen at negative 80 degree C° in a specialized freezer to help the freeze drying process. The samples were then freeze dried for twenty four hours overnight. This removed the water from the samples leaving behind solids, which include the antioxidant compounds, allowing for full control of the amount of water during wet chemical processing through hydrophilic phenolic extraction. The extracted antioxidants were then assessed through Trolox Equivalency Antioxidant Assessment (TEAC) with a microplate reader at 517 nm. After antioxidant capacities for all samples are recorded individually, food sample antioxidant levels will be compared to the wine sample antioxidant levels.

2.3.3 Hydrophilic phenolic extraction

The freeze dried samples were mixed with a four to one acetone to water solution. The sample was sonicated for fifteen minutes to fully mix the dried solids with the solution in order to extract the antioxidants. The samples were then centrifuged for ten minutes to separate the antioxidants and other solids. The liquid that the antioxidants are in is called supernatant, which was then extracted to another test tube. More acetone/water solution was added to the remaining solids, sonicated for ten minutes, and centrifuged for ten minutes to ensure all antioxidants are extracted. The second supernatant was then extracted and added to the first.

The supernatant was then treated to rotary evaporation. It was placed in special glass vials and lowered into a hot water bath at 40 degrees C°. The glass vials rotated through the bath so as to not overheat. The temperature is specific to acetone and will evaporate the acetone, not the water and water soluble antioxidants. The acetone was pulled off and condensed into a separate test tube to be discarded. Over three hours the vacuum pressure was increased so that all acetone and acetone solubles were separated from the water soluble antioxidants. Once this process was done the sample was purely water and the water-soluble molecules, including the antioxidants.

2.3.4 Antioxidant Capacity Testing: Trolox Equivalent Antioxidant Capacity (TEAC)

Trolox Equivalent Antioxidant Capacity (TEAC) was evaluated as described in the study of Brand-Williams, Cuvelier, & Berset (1995), with minor modifications. TEAC testing was done by taking the extracted antioxidants and adding a mild formic acid solution, then diluting. The formic acid was used to stabilize the polyphenols. In a microplate sample tray ten microliters of the wine or food sample was mixed with 290 microliters of a 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution. This solution was created by mixing one milligram of DPPH powder with 80/20 methanol/water. DPPH (2,2-diphenyl-1-picrylhydrazyl) is a known compound of stable free-radicals that cause measurable oxidation. By mixing the sample solution and the DPPH solution, there should be a measurable change in the color of the DPPH as the antioxidants in the sample work on the free radicals. DPPH is a deep lilac purple color when mixed in methanol/water. The sample plus DPPH mixture was compared to Trolox and DPPH mixtures at different concentrations of Trolox. Trolox is a known antioxidant and by using several dilutions we can create a scale to compare the sample mixture to. The dilutions used were 0.2 mM Trolox, 0.4 mM Trolox, 0.6 mM Trolox, 0.8 mM Trolox, 1.0 mM Trolox, 1.2mM Trolox, 1.4 mM Trolox and 1.6 mM Trolox. By using small increments of Trolox solution we can figure out where the sample falls and understand the Trolox equivalents the sample represents. By comparing the sample to a known antioxidant, we can see the true strength of the antioxidant and its capacity. This was then run through a microplate reader for 30 minutes at 37 °C. After thirty minutes a reading was taken to measure the change in color of each mixture.

2.3.5 Data Analysis

All samples were evaluated in triplicate. Antioxidant levels were measured using Molecular Devices VERSAmax microplate reader and SoftMax Pro Insider software. The data was analyzed using SPSS-15 (SPSS Inc., Chicago, IL, USA) through linear regression modeling. Significance was set at $\alpha = 0.05$ for all testing.

2.4 Results and Discussions:

2.4.1 Antioxidant Potential of Food Samples

Figure 1: Trolox Equivalents Individual Food Samples

Food/Wine	umol Trolox/1 L wet sample (mean±SD; n=9, 3 each)
Raw Tomatoes	637 ± 34
Spanish Onion	637 ± 23
Persimmons	331 ± 15

Of the three foods tested, raw tomatoes and Spanish onions had very similar levels of antioxidants. Raw tomatoes, however, had a larger standard deviation than Spanish onions (SD=34, SD=23). Persimmons measured the lowest level of antioxidant capacity out of the three foods tested, and was significantly lower than both the raw tomatoes and the Spanish onions ($p<0.05$). All foods were tested raw, so cooking had no impact on available antioxidant levels. More foods should be tested in future studies in order to have a better understanding of the range of antioxidant levels available in the full scope of the Mediterranean diet. With the three foods we tested we can speculate there to be a range between 315 umol Trolox/1 L wet sample and 670 umol Trolox/1 L wet sample, taking into account the standard deviation and possibility for higher or lower antioxidant level outcomes for each individual food.

2.4.2 Antioxidant Potential of Wine Samples

Figure 2: Trolox Equivalents Individual Wine Samples

Food/Wine	umol Trolox/1 L wet sample (mean±SD; n=9, 3 each)
Tempranillo	309 ± 11
Monastrell	408 ± 23
Grenache	302 ± 34

Of the three wines tested, the Monastrell had the highest antioxidant capacity, being significantly greater than the other two wines ($p<0.05$). Monastrell tested to also have a significantly higher antioxidant capacity than that of the persimmons (408 ± 23 umol Trolox/1 L wet sample, 331 ± 15 umol Trolox/1 L wet sample; $p<0.05$). The Tempranillo wine and the Grenache wine were similar in antioxidant levels (309 ± 11 umol Trolox/1 L wet sample, 302 ± 34 umol Trolox/1 L wet sample). These levels of antioxidants were not much lower than the level of persimmons and may contribute significant amounts of antioxidants. A wider range of red wines should be tested in future studies in order to have a better understanding of the range of antioxidant levels available in Spanish red wines. With the three wines we tested we can speculate there to be a range between 268 umol Trolox/1 L wet sample and 431 umol Trolox/1 L wet sample, taking into account the standard deviation and possibility for higher or lower antioxidant level outcomes for each individual food.

2.5 Conclusions:

Overall, raw tomatoes and Spanish onions contribute the most antioxidants to the diet out of the six individual foods and wines tested in this study. While expecting the wine antioxidant levels to be much lower and contribute much less to the diet than the foods, this was found not to be true. By looking at the mean antioxidant capacity levels of both the foods and the wines that were individually tested, it is clear that while the majority of the wines do not have an antioxidant capacity level as high as the antioxidant capacity levels of the majority of the foods tested, they are at least half or more. This leads to the belief that they are contributing significant amounts of antioxidants to the diet if consumed on a regular basis. With wine being a standard part of the Mediterranean diet and culture, it can be inferred that drinking one to two glasses of Spanish red wine a day does increase the overall antioxidant levels of the Spanish Mediterranean diet. Further research is needed to see if similar results occur with foods and wines from other Mediterranean regions, and to what extent the foods and wines contribute to the overall antioxidant levels consumed in those regions.

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3. MANUSCRIPT #2: "INVESTIGATION OF INTERACTION EFFECTS ON ANTIOXIDANT CAPACITY OF PAIRING FOODS AND WINES OF THE SPANISH MEDITERRANEAN DIET"

JOURNAL OF AGRICULTURE AND FOOD CHEMISTRY

3.1 Abstract:

Statement of the Problem: Recent research has shown that antioxidants may be vital to human health. Both foods and wines can be high in polyphenols, which inhibit oxidation in the body. However, no studies have looked at the possible interactions when foods and wines high in antioxidants are consumed together. Studies have shown that antioxidants can interact, but these interactions are not yet well understood. There is a specific interest in the possible interaction effects among components of the Mediterranean Diet, as prior health studies have shown this diet to be associated with lower-than-expected mortality rates. The typical Mediterranean diet includes consumption of wine, but no studies have examined how these wines interact with the health properties of other components of the diet. Methodology and Theoretical Orientation: Three foods (raw tomatoes, Spanish onion, and persimmons) and three wines (Tempranillo, Monastrell, and Grenache) common to the Spanish diet were assessed by Trolox Equivalency Antioxidant Capacity, both individually and in pairing combinations. Each food was paired with each wine and evaluated in triplicate. Interaction effects were assessed by one-way ANOVA (α=0.05). Findings: Tomatoes showed negative interaction effects with all three wines. Spanish onions and persimmons showed a positive interaction effect with all three wines. The greatest interaction effect was observed between Persimmons and Tempranillo, which demonstrated antioxidant potential ~200% the value predicted for no interaction. Conclusion and Significance: Consuming

persimmons and Spanish onions with wine may increase the antioxidant potential, allowing for greater oxidation inhibition *in vivo*. Consuming raw tomatoes with wine may decrease the antioxidant potential. Further studies examining the interaction effects between antioxidant-rich foods and wines may be warranted.

3.2 Introduction:

The Mediterranean diet has been extensively studied in the past decade and has been shown through human observations to help decrease mortality rates from coronary heart disease, cardiovascular diseases, and cancer as well as comorbidities such as hypertension, obesity, and metabolic syndrome (Knoops, de Groot, Kromhout, Perrin, Moreiras-Varela, Menotti, and van Staveren, 2004). However, many countries in the Mediterranean diet eat foods high in fats. Counter-intuitively, countries like France have low cardiovascular related mortality rates despite a diet high in saturated fats. This has been coined as "The French Paradox" (Gresele, Cerletti, Guglielmini, Pignatelli, de Gaetano, and Vivoli, 2011). Many studies into why this may be have linked the answer to a diet high in antioxidants.

Antioxidants are molecular structures that are able to freely donate an electron without becoming volatile in the body (Flora, 2009). They play an important role of preventing cell damage from free radicals. Free radicals are naturally occurring in the body due to environmental exposure to UV rays and pollution and natural aging (Chun, Frei, Gardner, Alekel and Killen, 2013). Free radicals are molecules that lack an electron so they take it from other molecules. In the human body, they steal these from other molecules within the cell. This causes damage to the cell leading to disease and premature death. Antioxidants readily donate electrons to free radicals to avoid such damage (Lobo, Patil, Phatak, and Chandra, 2010).

Recent studies have found that when different antioxidants are present together in the body, they may work together or against each other. Resveratrol, a powerful antioxidant in grapes and wine, has augmented antioxidant capacity due to its interactions with other polyphenolic antioxidants in the wine (Cavallini, Straniero, Donati and Bergamini, 2015). This interaction may take place outside the wine as well. The antioxidants in food may interact with the antioxidants in wine when consumed together, potentially allowing for increased or decreased antioxidant capacity and potential health benefits.

More recently, the interaction of wine and food has begun to be studied and questioned. A 2011 study looking at the health differences between an alcoholic and nonalcoholic Mediterranean diets found that moderate consumption of red wine while consuming a typical Mediterranean diet has complimentary effects and lowers the risk of cardiovascular disease (Mezzano, Leighton, Martínez, Marshall, Cuevas, and Castillo, 2001). Human trials of red wine consumption have also shown that the inclusion of red wine in the diet is a powerful form of protection against oxidative stress, heart disease, cellular aging, atherogenesis, and several chronic diseases (Perez, Strobel, Foncea, Diez, Vasquez, and Urquiaga, 2018). Another study looking at the effect of red wine consumption of the high fat Mediterranean diet found that including a moderate amount of red wine while consuming a typical Mediterranean diet increases plasma antioxidant capacity, allowing for greater absorption, and decreases DNA oxidative damage (Leighton, Cuevas, Guasch, Pérez, Strobel, and San, 2018).

The purpose of this study is to examine the interactions and compare antioxidant levels of three foods (raw tomatoes, Spanish onion, and persimmons) and three wines (Tempranillo, Monastrell, and Grenache) common in the Spanish Mediterranean diet when paired together. Expected values were determined for each pair by assessing the antioxidant capacity of each food or wine alone and adding those values together. The expected value was then compared to the actual values generated by testing the pairings via wet chemical extraction and TEAC. This approach allows us to determine if there is a positive or negative interaction taking place. From this information we can then infer the potential effects of food and wine pairing on antioxidant capacity of the overall diet and potential health effects of the pairing.

3.3 Materials and Methods:

3.3.1 Materials and Equipment

Filtered water, Methanol, Acetone, Formic Acid, Trolox, DPPH (2,2-diphenyl-1-picrylhydrazyl), Labconco FreeZone 4.5 Freeze Dryer, Rotary Evaporator, Molecular Devices VERSAmax microplate reader and SoftMax Pro Insider software.

3.3.2 Sample preparation

A total of six different samples that were tested. Three Spanish red wines (Tempranillo, Monastrell, and Grenache) were measured out into three separate test tubes. Samples measured to 25 milliliters exactly. Three common Spanish foods (raw tomatoes, Spanish onion, and persimmons) were pureed into liquid form individually with three separate blenders. Samples of the individual foods were measured out into three separate test tubes at 25 milliliters amounts. A total of nine paired samples were also tested. Each food was paired with each wine and tested in triplicate. Each paired sample was measured to exactly 25 milliliters (12.5 milliliters wine sample and 12.5 milliliters food sample). All samples were tested at the sample volume of 25 milliliters to set a standard. Each individual food or wine was tested in triplicate. All volumes of samples used (25 milliliters) will be the same in order to compare on a one-to-one scale during analysis. The samples were first pre-frozen at negative 80 degree C° in a specialized freezer to help the freeze drying process. The samples were then freeze dried for twenty four hours overnight. This removed the water from the samples leaving behind solids, which include the antioxidant compounds, allowing for full control of the amount of water during wet chemical processing through hydrophilic phenolic extraction. The extracted antioxidants were then assessed through Trolox Equivalency Antioxidant Assessment (TEAC) with a microplate reader at 517 nm. After antioxidant capacities for all samples are recorded individually, food sample antioxidant levels will be compared to the wine sample antioxidant levels.

3.3.3 Hydrophilic phenolic extraction

The freeze dried samples were mixed with a four to one acetone to water solution. The sample is sonicated for fifteen minutes to fully mix the dried solids with the solution in order to extract the antioxidants. Sonication uses sound waves in liquid to churn the sample and mix it thoroughly. The samples were then centrifuged for ten minutes to separate the antioxidants and other solids. The liquid that the antioxidants are in is called supernatant, which was then extracted to another test tube. More acetone/water solution was added to the remaining solids, sonicated for ten minutes, and centrifuged for ten minutes to ensure all antioxidants are extracted. The second supernatant was then extracted and added to the first.

The supernatant was then treated to rotary evaporation. It was placed in special glass vials and lowered into a hot water bath at 40 degrees C°. The glass vials rotated through the bath so as to not overheat. The temperature is specific to acetone and will evaporate the acetone, not the water and water soluble antioxidants. The acetone was pulled off and condensed into a separate test tube to be discarded. Over three hours the vacuum pressure was increased so that all acetone and acetone solubles were separated from the water soluble antioxidants. Once this process was done the sample was purely water and the water-soluble molecules, including the antioxidants.

3.3.4 Antioxidant Capacity Testing: Trolox Equivalent Antioxidant Capacity (TEAC)

Trolox Equivalent Antioxidant Capacity (TEAC) was evaluated as described in the study of Brand-Williams, Cuvelier, & Berset (1995), with minor modifications. TEAC testing was done by taking the extracted antioxidants and adding a mild formic acid solution, then diluting. The formic acid was used to stabilize the polyphenols. In a microplate sample tray ten microliters of the wine or food sample was mixed with 290 microliters of a 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution. This solution was created by mixing one milligram of DPPH powder with 80/20 methanol/water. DPPH (2,2-diphenyl-1-picrylhydrazyl) is a known compound of stable free-radicals that cause measurable oxidation. By mixing the sample solution and the DPPH solution, there should be a measurable change in the color of the DPPH as the antioxidants in the sample work on the free radicals. DPPH is a deep lilac purple color when mixed in methanol/water. The sample plus DPPH mixture was compared to Trolox and DPPH mixtures at different concentrations of Trolox. Trolox is a known antioxidant and by using several dilutions we can create a scale to compare the sample mixture to. The dilutions used were 0.2 mM Trolox, 0.4 mM Trolox, 0.6 mM Trolox, 0.8 mM Trolox, 1.0 mM Trolox, 1.2mM Trolox, 1.4 mM Trolox and 1.6 mM Trolox. By using small increments of Trolox solution we can figure out where the sample falls and understand the Trolox equivalents the sample represents. By comparing the sample to a known antioxidant, we can see the true strength of the antioxidant and its capacity. This was then run through a microplate reader for 30 minutes at 37 degrees C°. After thirty minutes a reading was taken to measure the change in color of each mixture.

3.3.5 Data Analysis

All samples have been done in triplicate. Antioxidant levels were measured using Molecular Devices VERSAmax microplate reader and SoftMax Pro Insider software. The data was collected in Microsoft Excel and analyzed.

In order to compare expected and actual outcomes, expected outcomes have been calculated using the baseline numbers from the individual samples. The Trolox equivalency of Tempranillo, when tested alone, is added to the Trolox equivalency of raw tomatoes, when tested alone, for the expected outcome of the Tempranillo plus raw tomatoes pairing. The Trolox equivalency of Tempranillo, when tested alone, is added to the Trolox equivalency of Spanish onion, when tested alone, for the expected outcome of the Tempranillo plus Spanish onion pairing. The Trolox equivalency of Tempranillo, when tested alone, is added to the Trolox equivalency of persimmons, when tested alone, for the expected outcome of the Tempranillo plus persimmons pairing.

The Trolox equivalency of Monastrell, when tested alone, is added to the Trolox equivalency of raw tomatoes, when tested alone, for the expected outcome of the Monastrell plus raw tomatoes pairing. The Trolox equivalency of Monastrell, when tested alone, is added to the Trolox equivalency of Spanish onion, when tested alone, for the expected outcome of the Monastrell plus Spanish onion pairing. The Trolox equivalency of Monastrell, when tested alone, is added to the Trolox equivalency of persimmons, when tested alone, for the expected outcome of the Monastrell plus persimmons pairing.

The Trolox equivalency of Grenache, when tested alone, is added to the Trolox equivalency of raw tomatoes, when tested alone, for the expected outcome of the Grenache plus raw tomatoes pairing. The Trolox equivalency of Grenache, when tested alone, is added to the Trolox equivalency of Spanish onion, when tested alone, for the expected outcome of the Grenache plus Spanish onion pairing. The Trolox equivalency of Grenache, when tested alone, is added to the Trolox equivalency of persimmons, when tested alone, for the expected outcome of the Grenache plus persimmons pairing.

The calculated expected outcomes have been recorded in a chart. In the same chart the actual tested outcomes have been recorded. These numbers are analyzed for statistical significance. Significance was evaluated by linear regression modeling procedures using SPSS-15 (SPSS Inc., Chicago, IL, USA). Interaction was evaluated by making a composite variable of each of the food pairings and testing for its significance in linear regression models. Significance was set at $\alpha=0.05$ for all testing.

3.4 Results and Discussions:

3.4.1 Antioxidant Potential of Individual Samples

Figure 1: Trolox Equivalents Individual Food Samples

Food/Wine	umol Trolox/1 L wet sample (mean±SD; n=9)
Raw Tomatoes	637 ± 34
Spanish Onion	637 ± 23
Persimmons	331 ± 15
Tempranillo	309 ± 11
Monastrell	408 ± 23
Grenache	302 ± 34

When the individual foods and wines were tested for baseline antioxidant levels, Spanish onions and raw tomatoes were found to have the highest mean antioxidant capacity (637 ± 34 umol Trolox/1 L wet sample, 637 ± 23 umol Trolox/1 L wet sample) out of all six samples. Out of the three wines, Monastrell had the highest mean antioxidant capacity level, higher than what persimmons tested (408 ± 23 umol Trolox/1 L wet sample, 331 ± 15 umol Trolox/1 L wet sample). This would infer that the raw tomato and Monastrell wine combination pairing along with the Spanish onion and Monastrell wine combination pairing will have the highest tested actual values for antioxidant capacity if the effects are additive. That would mean no synergistic or antagonistic have taken place and there are no interactions being viewed between the food and wine antioxidants when they are placed together.

3.4.2 Antioxidant Potential of Paired Samples

Figure 2: Antioxidant Potential of Paired Samples

<u>Food/Wine</u>	umol Trolx/1 L wet sample (mean±SD; n=9)
Persimmon and Tempranillo	636 ± 13
Tomatoes and Monastrell	625 ± 6
Onions and Grenache	532 ± 8
Onions and Monastrell	498 ± 56
Tomatoes and Grenache	470 ± 21
Persimmons and Grenache	426 ± 11
Persimmons and Monastrell	417 ± 9
Onions and Tempranillo	395 ± 9
Tomatoes and Tempranillo	301 ± 9

The chart in figure 2 lists the mean and standard deviation of actual tested antioxidant capacity levels for each food and wine combination pairing from highest antioxidant capacity to lowest antioxidant capacity. The persimmon and Tempranillo tested to have the highest antioxidant capacity level out of all nine combination pairings. When tested alone, persimmons had the lowest antioxidant capacity level out of the three individual foods and Tempranillo had the second highest antioxidant capacity level out of the three individual wines. This would allude that if there is no synergistic effect this pairing should have tested as a much lower antioxidant capacity level. Similarly, the lowest antioxidant capacity level for combination pairings tested was raw tomatoes and Tempranillo. Raw tomatoes was tied with Spanish onions for the highest antioxidant capacity levels out of all six individual samples tested. Because Tempranillo was second to last of the six individual samples, the pairing would be expected to fall somewhere in the middle of the range of antioxidant capacity levels for the nine pairings if there is no antagonistic effect taking place. Further research is needed to better understand what antioxidants are unique to persimmons, tomatoes, and Spanish onions that cause these synergistic or antagonistic effects to occur when paired with the polyphenolic antioxidants present in the Spanish red wines.

In the following chart, figure three, it is clear to see the differences between the expected additive values for antioxidant capacity of the combination pairings and the actual tested values for antioxidant capacity of the combination pairings. In all cases, raw tomatoes paired with red wine had an antagonistic effect on the total mean antioxidant capacity levels. Both Spanish onion and persimmons presented synergistic effects of varying degrees when paired with any of the three Spanish red wines. This would suggest the antioxidants in the tomatoes affect or are affected negatively by the antioxidants in the Spanish red wines and that the antioxidants in the persimmons and Spanish onion affect or are affected positively by the antioxidants in the Spanish red wines.

Figure 3: Expected Averages vs Actual Equivalents of Pairings

	umol Trolx/1 L wet sample for sample			
Food/Wine	Expected Average	Actual Average	Difference	Non-additive Effect
Tomatoes and Tempranillo	473	301	-172	Antagonistic***
Tomatoes and Monastrell	523	325	-198	Antagonistic***
Tomatoes and Grenache	504	470	-34	Antagonistic***
Onions and Tempranillo	311	395	+84	Synergistic***
Onions and Monastrell	360	498	+138	Synergistic***
Onions and Grenache	307	532	+225	Synergistic***
Persimmons and Tempranillo	320	636	+316	Synergistic***
Persimmons and Monastrell	370	417	+47	Synergistic***
Persimmons and Grenache	317	426	+109	Synergistic***

***: statistically significant ($p<0.05$)

3.5 Conclusions:

When looking at the actual and expected values for antioxidant capacity of the nine food and wine combination pairings it is clear that there is a non-additive effect, synergistic or antagonistic, taking place. The impact this could have on health and antioxidant absorption in the diet could be an important step needed in properly treating patients who would benefit from a high antioxidant diet such as the Mediterranean diet. Further research would need to be done on a greater range and variety of foods and wines common to the whole Mediterranean diet, not just the Spanish Mediterranean diet, to fully understand the possible health impact of the interactions of antioxidants between the two groups. Further research is also warranted that looks into the actual antioxidants unique to each food and wine and how the individual antioxidants interact on a molecular level. If scientists can pinpoint which antioxidants are causing the antagonistic or synergistic effects taking place, then food recommendations and supplementation can be safely administered to the public with full scientific backing. There are definitely interactions taking place between the different antioxidants in foods and wines common to the Spanish Mediterranean diet.

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