

University of Nevada, Reno

**Predominant Antioxidants of the Mediterranean Diet and Their Influence on
Lipid Oxidation**

A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science in Nutrition

by

Anne Kristine Etherton

Dr. Stanley Omaye/Thesis Advisor

May, 2014



University of Nevada, Reno
Statewide • Worldwide

THE GRADUATE SCHOOL

We recommend that the thesis
prepared under our supervision by

Anne Kristine Etherton

entitled

**Predominant Antioxidants Of The Mediterranean Diet And Their Influence
On Lipid Oxidation**

be accepted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE

Stanley T. Omaye, Ph.D., Advisor

Chris Pritsos, Ph.D., Committee Member

Michael Crognale, Ph.D., Graduate School Representative

Marsha H. Read, Ph.D., Dean, Graduate School

May, 2014

Oxidative stress can be defined as an imbalance that favors the production of reactive oxygen species over the ability of a biological system to detoxify or reduce such species. Antioxidants have the ability to inhibit oxidation but depending on the circumstances (concentration, presence of other oxidants), a compound may exhibit pro- or antioxidant activity. A balanced diet abundant in foods rich in fruits and vegetables likely contains mixtures of various antioxidants working in concert and synergistically. Polyphenolic compounds as mixtures have synergistic, sometimes additive effects in preventing human low-density lipoprotein (LDL) oxidation *in vitro* [1]. Our studies focused on examining the potential synergistic effects of other antioxidant mixtures (cocktails) in preventing copper (Cu^{2+}) mediated oxidative stress induced in isolated olive oil. Such information will be useful in determining optimal doses and combinations of antioxidants for reducing rancidity and perhaps models that could be used to modulate various chronic diseases that are associated with oxidative stress.

Table of Contents

Literature Review.....	1
Mediterranean Diet and Health.....	1
Mediterranean Diet: Mechanism of Action.....	1
Cardiovascular Profile.....	2
Inflammatory Response.....	2
Cholesterol.....	3
Dietary Mixtures.....	4
Mediterranean Diet: Fatty Acids.....	4
Mediterranean Diet: Antioxidants (Polyphenolic Compounds)	5
Mediterranean Diet: Coffee.....	6
Mediterranean Diet: Wine.....	6
Mediterranean Diet: Olive Oil.....	8
Purpose of the Current Study.....	9
Materials and Methods.....	10
Materials.....	10
Preparation Of Antioxidant-Stripped Olive Oil.....	11
Tocopherol Analysis.....	11
Total Phenolics Analysis.....	14
Determination of Malondialdehyde by Thiobarbituric Acid Reactive Products.....	16

Selection Of Polyphenolic Compounds Concentrations To Be Used <i>In Vitro</i> Evaluations.....	18
Polyphenolic Antioxidant Enrichment Of Olive Oil.....	25
HPLC Analysis to Determine Baseline Phenolic Concentration.....	26
Statistical Analysis.....	26
Results.....	27
Preliminary Results Of Non-Extracted Olive Oil Compared To Extracted Olive Oil.....	27
Oxidation of Antioxidant Stripped Olive Oil.....	31
Individual Compound Dose Response.....	33
Effect of Adding Polyphenolic compound mixtures on Copper Mediated Oxidation of Antioxidant Stripped Olive Oil.....	38
Total Phenolic Compounds Of Mixtures Who Have Undergone Significant Oxidation Changes Compared To Their Respective Controls.....	44
Discussion.....	46
Bibliography.....	52
Appendix.....	59

List of Tables

Table 1. Data retrieved from regression analysis from Bioavailability Data for Tyrosol and Hydroxytyrosol.....	23
Table 2. Data retrieved from regression analysis from Bioavailability Data for Caffeic Acid and Quercetin.....	24
Table 3. Mixtures that were significantly different more than 50% of the time intervals, $p < 0.05$, and their p value for every time interval are listed.....	43
Table 4. Initial amount of phenolic added and amount after oxidation.....	44
Table 5. Combinations of the four compounds of interest and the different added amounts (c, caffeic acid, q, quercetin, t, tyrosol and h, hydroxytyrosol).....	Appendix

List of Figures

Figure 1. Standard Curve Vitamin E.....	13
Figure 2. Standard Curve Utilizing Caffeic Acid Equivalentents.....	15
Figure 5.- Standard Curve Malondialdehyde.....	17
Figure 4. Determination Of Tyrosol Concentration For <i>In Vitro</i> Experiments.	19
Figure 5. Determination Of Hydroxytyrosol Concentration For <i>In Vitro</i> Experiments.	20
Figure 6. Determination Of Caffeic Acid Concentration For <i>In Vitro</i> Experiments..	21

Figure 7. Determination Of Quercetin Concentration For <i>In Vitro</i> Experiments.....	22
Figure 8. Reactive Substances Of Non-Extracted Oil Compared To Extracted Oil.....	28
Figure 9. Vitamin E concentration for non-extracted oil compared to extracted oil	29
Figure 10. Total phenolics.....	30
Figure 11. Copper Mediated Oxidation of Extracted Oil.	32
Figure 12. Caffeic Acid Dose Response Curve	34
Figure 13. Quercetin Dose Response Curve	35
Figure 14. Tyrosol Dose Response Curve	36
Figure 15. Hydroxytyrosol Dose Response Curve	37

Figure 16. Mixture 6.....	40
Figure 17. Mixture 27.....	41
Figure 18. Mixture 50	42
Figure 19. Mixture 70.....	42
Figure 20. Phenolic concentrations after enrichment (blue) and after oxidation (red)	45

Appendix

Figure 21. c 0.012 q 0.00025 t 0.03 h 0.2	
Figure 22. c 0.012 q 0.00025 t 0.03 h 0.1	
Figure 23. c 0.012 q 0.00025 t 0.03 h 0.05	
Figure 24. c 0.012 q 0.00025 t 0.015 h 0.2	
Figure 25. c 0.012 q 0.00025 t 0.015 h 0.1	
Figure 26. c 0.012 q 0.00025 t 0.0075 h 0.2	
Figure 27. c 0.012 q 0.00025 t 0.0075 h 0.1	
Figure 28. c 0.012 q 0.00025 t 0.0075 h 0.05	
Figure 29. c 0.012 q 0.000125 t 0.03 h 0.2	
Figure 30. c 0.012 q 0.000125 t 0.03 h 0.1	
Figure 31. c 0.012 q 0.000125 t 0.03 h 0.05	

- Figure 32. c 0.012 q 0.000125 t 0.015 h 0.2
- Figure 33. c 0.012 q 0.000125 t 0.015 h 0.1
- Figure 34. c 0.012 q 0.000125 t 0.015 h 0.05
- Figure 35. c 0.012 q 0.000125 t 0.0075 h 0.2
- Figure 36. c 0.012 q 0.000125 t 0.0075 h 0.1
- Figure 37. c 0.012 q 0.000125 t 0.0075 h 0.05
- Figure 38. c 0.012 q 0.0000625 t 0.03 h 0.2
- Figure 39. c 0.012 q 0.0000625 t 0.03 h 0.1
- Figure 40. c 0.012 q 0.0000625 t 0.03 h 0.05
- Figure 41. c 0.012 q 0.0000625 t 0.015 h 0.2
- Figure 42. c 0.012 q 0.0000625 t 0.015 h 0.1
- Figure 43. c 0.012 q 0.0000625 t 0.015 h 0.05
- Figure 44. c 0.012 q 0.0000625 t 0.0075 h 0.2
- Figure 45. c 0.012 q 0.0000625 t 0.0075 h 0.1
- Figure 46. c 0.006 q 0.00025 t 0.03 h 0.2
- Figure 47. c 0.006 q 0.00025 t 0.03 h 0.1
- Figure 48. c 0.006 q 0.00025 t 0.03 h 0.05
- Figure 49. c 0.006 q 0.00025 t 0.015 h 0.2
- Figure 50. c 0.006 q 0.00025 t 0.015 h 0.1
- Figure 51. c 0.006 q 0.00025 t 0.015 h 0.05
- Figure 52. c 0.006 q 0.00025 t 0.0075 h 0.2
- Figure 53. c 0.006 q 0.00025 t 0.0075 h 0.1

- Figure 54. c 0.006 q 0.00025 t 0.0075 h 0.05
- Figure 55. c 0.006 q 0.000125 t 0.03 h 0.2
- Figure 56. c 0.006 q 0.000125 t 0.03 h 0.1
- Figure 57. c 0.006 q 0.000125 t 0.03 h 0.05
- Figure 58. c 0.006 q 0.000125 t 0.015 h 0.2
- Figure 59. c 0.006 q 0.000125 t 0.015 h 0.1
- Figure 60. c 0.006 q 0.000125 t 0.015 h 0.05
- Figure 61. c 0.006 q 0.000125 t 0.0075 h 0.2
- Figure 62. c 0.006 q 0.000125 t 0.0075 h 0.1
- Figure 63. c 0.006 q 0.000125 t 0.0075 h 0.05
- Figure 64. c 0.006 q 0.0000625 t 0.03 h 0.2
- Figure 65. c 0.006 q 0.0000625 t 0.03 h 0.1
- Figure 66. c 0.006 q 0.0000625 t 0.03 h 0.05
- Figure 67. c 0.006 q 0.0000625 t 0.015 h 0.2
- Figure 68. c 0.006 q 0.0000625 t 0.015 h 0.05
- Figure 69. c 0.006 q 0.0000625 t 0.0075 h 0.2
- Figure 70. c 0.006 q 0.0000625 t 0.0075 h 0.1
- Figure 71. c 0.006 q 0.0000625 t 0.0075 h 0.05
- Figure 72. c 0.003 q 0.00025 t 0.03 h 0.2
- Figure 73. c 0.003 q 0.00025 t 0.03 h 0.1
- Figure 74. c 0.003 q 0.00025 t 0.03 h 0.05
- Figure 75. c 0.003 q 0.00025 t 0.015 h 0.2

- Figure 76. c 0.003 q 0.00025 t 0.015 h 0.1
- Figure 77. c 0.003 q 0.00025 t 0.015 h 0.05
- Figure 78. c 0.003 q 0.00025 t 0.0075 h 0.2
- Figure 79. c 0.003 q 0.00025 t 0.0075 h 0.1
- Figure 80. c 0.003 q 0.00025 t 0.0075 h 0.05
- Figure 81. c 0.003 q 0.000125 t 0.03 h 0.2
- Figure 82. c 0.003 q 0.000125 t 0.03 h 0.1
- Figure 83. c 0.003 q 0.000125 t 0.03 h 0.05
- Figure 84. c 0.003 q 0.000125 t 0.015 h 0.2
- Figure 85. c 0.003 q 0.000125 t 0.015 h 0.1
- Figure 86. c 0.003 q 0.000125 t 0.015 h 0.05
- Figure 87. c 0.003 q 0.000125 t 0.0075 h 0.1
- Figure 88. c 0.003 q 0.000125 t 0.0075 h 0.05
- Figure 89. c 0.003 q 0.0000625 t 0.03 h 0.2
- Figure 90. c 0.003 q 0.0000625 t 0.03 h 0.1
- Figure 91. c 0.003 q 0.0000625 t 0.03 h 0.05
- Figure 92. c 0.003 q 0.0000625 t 0.015 h 0.2
- Figure 93. c 0.003 q 0.0000625 t 0.015 h 0.1
- Figure 94. c 0.003 q 0.0000625 t 0.015 h 0.0
- Figure 95. c 0.003 q 0.0000625 t 0.0075 h 0.2
- Figure 96. c 0.003 q 0.0000625 t 0.0075 h 0.1
- Figure 97. c 0.00 q 0.0000625 t 0.0075 h 0.05

Literature Review

Mediterranean Diet and Health

Eating patterns can be negatively or positively associated with disease risks. Research that has investigated the various eating patterns has shown the Mediterranean Diet (MD) [2], to be one with possible health benefits. The 2010 Dietary Guidelines for Americans [3] suggests that the MD could be a means for an individual to meet their dietary needs, while reducing their health risks. In addition to these suggestions, recent research suggests that the composition of the MD can greatly reduce heart disease risks; an important example is with the recent findings of the *Primary Prevention of Cardiovascular Disease with a Mediterranean Diet (PREDIMED)*[4] study. The randomized primary prevention trial was ended early, after about 4.8 years, due to the 30% relative cardiovascular risk reduction of participants consuming the MD compared to a low-fat diet [5].

Mediterranean Diet: mechanism of action

When trying to determine the mechanism of action of the MD in preventing cardiovascular disease, it has been suggested that both the fatty acid composition of the diet and the antioxidants have beneficial potential. Many studies have focused on the MD and the potential health benefits associated with its consumption. Most have found similar results of improved cardiovascular profiles, decreased inflammatory conditions and reduced oxidative stress after MD intake for varying periods.

Cardiovascular profile

Participants of the PREDIMED consuming the MD had lower circulating Low Density Lipoproteins (LDL), decreased blood pressure readings and improved insulin sensitivity [6]. Additionally, a portion of the study *Adherence to the Mediterranean diet attenuates inflammation and coagulation process in healthy adults (ATTICA)* indicated that adherence to the MD can modestly improve insulin sensitivity, lower total cholesterol and improve blood pressure in overweight and obese participants [7]. Adherence to the MD has also been shown to significantly lower the odds ratio of having metabolic syndrome [4].

Inflammatory Response

During the PREDIMED study both MD groups showed an anti-inflammatory effect with reduced inflammatory molecules, while those were increased after the low-fat diet intervention [8]. A study completed by Camargo A, et al. showed that “ *consumption of a MD reduces the postprandial inflammatory response in mononuclear cells compared with the saturated fatty acid rich and carbohydrate-polyunsaturated fatty acid diets in elderly people* [9] ”. These studies indicate the anti-inflammatory power of the MD, but they do not provide evidence to which specific nutrients are contributing to the effect.

Cholesterol

The MD has shown to reduce circulating Low Density Lipoprotein (LDL); what is more significant is the ability to raise High Density Lipoprotein (HDL) levels significantly when compared to a low-fat diet, which has been associated with olive oil supplementation [6]. This, however, does not completely account for the reduced cardiovascular risks associated with MD consumption. A 3-month sub-study of the PREDIMED evaluated the effect of the MD supplemented with either nuts or virgin olive oil compared to a low fat diet on 372 high risk cardiovascular subjects. This study found that the MD supplemented with virgin olive oil had a significant reduction of circulating oxidized LDL when compared to the low-fat diet [2]. There were no significant differences in the LDL levels between the three groups which agreed with the previous research that low-fat

and monounsaturated fatty acids (MUFA) diets can lower circulating LDL. Since the only reduction was then seen in oxidized LDL, this suggests it is a factor of the antioxidant capacity of the diet that contributed to preventing the already lowered LDL from being oxidized [2]. Again, these results are supportive of the total diet composition contributing to the health benefits.

Dietary mixtures

Evaluation of the whole diet is starting to be a trend, yet there are many challenges to understanding the nutrients of action and the exact mechanism that is occurring. Several studies focus on select foods or drinks of interest, but evaluating a diet as a whole is a large-scale procedure. Many agree there are many beneficial components of the MD, but more epidemiological, *in vitro*, *in vivo* and control trials are needed.

Mediterranean Diet: Fatty Acids

Determining which dietary component contributes to good health is challenging and is an area of interest in current research. Many dietary components such as mono unsaturated fatty acid (MUFA) and polyunsaturated fatty acids (PUFA) have been researched to determine their possible benefit in atherosclerosis development. It is now common knowledge that PUFAs such as Omega-3 are known to reduce inflammation, while the Omega-6 tend to promote

inflammation, and both are necessary in the inflammatory response. Other research has shown MUFAs to be less prone to oxidation when compared to PUFAs. Massaro, M. *et al.* suggested that the Omega-3 contribution of the MD had much more beneficial effects compared to the phenolic antioxidant counterparts [10].

Mediterranean Diet: Antioxidants (Polyphenolic Compounds)

Several studies have investigated the link between the MD antioxidants and the decreased risk of disease. Specifically, it was recently shown that lower adherence to the MD and antioxidant consumption was associated to patients with atrial fibrillation [11]. Yet, a focus on the phenolic contribution of the diet has been limited.

The MD antioxidant results showed a broad array and increased levels of antioxidants present in the diet. The MD has been found to provide mostly a combination of polyphenols with roughly only 10% of the total daily antioxidant capacity (TDAC) coming from vitamins E and C [12]. This composition is actually an interesting development, since the majority of past antioxidant studies have been focused upon impact of Vitamin E and C. Additional studies have found that the MD acquires the majority of its TDAC from beverage consumption, roughly 68% [13], many of which are polyphenolic compounds in nature. Trials that have focused upon the MD for dietary prevention or treatment for coronary heart

disease have found a benefit to following the intake, but it is challenging to identify which nutrient is actually causing the impact. This same study also evaluated the total daily antioxidant capacity (TDAC) of the Spanish Mediterranean diet and found that coffee, the greatest contributor, contributed roughly 44.5% of the TDAC, and red wine had the second highest antioxidant capacity, even though it did not have the highest phenolic content [13].

Mediterranean Diet: Coffee

Coffee was found to be the greatest contributor 66% to the TDAC of the Spanish Mediterranean diet, when the ferric reducing ability of plasma (FRAP) of the Spanish diet was evaluated, [14]. Caffeic acid was found to be the predominate polyphenolic compound present after coffee consumption, peaking after about 1 hour [15]. Coffee has been a topic of interest for several years, and an ongoing debate of whether it has benefits to health is still being researched. The 2011 Singapore Chinese Health Study found that compared to non-coffee drinkers, people that consumed three or more cups of coffee a day had a 44% reduction of risk for developing hepatocellular carcinoma [16]. Coffee consumption greater than three cups a day has also been inversely associated with changes in blood pressure [17].

Mediterranean Diet: Wine

The health benefits of wine have been widely examined, most of the

findings point to the benefits of drinking one glass a day to reduce heart disease risk [18]. The benefits were shown to decline with increased wine consumption, and for people consuming more than two glasses a day there was a positive relationship to blood pressure [17]. Wine was found to contribute 14% of the TDAC, and red wine was consumed the most out of the different types of wine evaluated in the Spanish Mediterranean diet [13]. Up to 25 different phenolic compounds can be present in varying concentrations in aged wines [19]. Research directed at characterizing the bioavailability of wine phenolic compounds has had conflicting results. Since many studies used supplementation of phenolic compounds and not native phenolic compounds found in food or drink consumption, the bioavailability data did not translate to whole food consumption. Of the studies that did evaluate bioavailability after drinking wine with or without food there was a large variation in the results, and notable, resveratrol was never detected in 56% of the study participants [20]. Studies with quercetin resulted in mixed findings, as well. Quercetin additions to low-density lipoprotein (LD) suspensions *in vitro* acted as a prooxidant [1]. The half-life of quercetin is much longer than many other phenolic compounds, roughly 24 hours [21]. Additionally, in a study evaluating the phenolic compound content of different wine samples, quercetin was found to be at a higher content compared to resveratrol after oak and bottle aging [19]. In addition, diluted wine, 1000 fold, had a significant inhibition of LDL oxidation when compared to vitamin

E [22]. Frankel *et al.* [23] also found that the quercetin produced stronger protective effects against LDL oxidation when compared to resveratrol.

Mediterranean Diet: Olive Oil

Olive oil was not found to be a significant contributor to the TDAC of the Spanish Mediterranean diet, but it was found to be the main fat source [13]. Olive oil can come in a variety of fatty acid ratios, phenolic contents and qualities. When studies address the consumption of olive oil and the influences it may have on health, there are a lot of factors to consider, and many times this causes the different studies to be incomparable due to differences in the olive oil provided. The phenolic compound content of the olive oil can range from 50-800 mg/kg [24], thus it is important to take this into consideration when designing studies. A subsample of the EUROLIVE crossover study compared 3-week consumption of a virgin olive oil high in phenolic compounds (629 mg/L) with a refined olive oil with zero relative polyphenolic compounds. The results showed that subjects who consumed the virgin olive oil had a significant reduction in plasma oxidized LDL, $P= 0.001$ [25], indicating that the phenolic compounds of olive oil provide some contribution to the beneficial results of the MD. While there have been studies to show that a diet high in MUFAs can lead to a modified cellular membrane [26], a study comparing oils with similar MUFA content showed that after olive oil consumption there was a 10% increase in erythrocyte phospholipids, reducing the cholesterol to phospholipid ratio [27]. After a study of

4 week consumption of olive oil, there was a significant increase in erythrocyte membrane MUFA concentration, with a significant decrease in both PUFAs and saturated fatty acid concentrations [28]. This dietary modification could be contributing to the beneficial cardiovascular impact of olive oil intake, since MUFAs are less prone to oxidization. Olive oil polyphenolic compounds were found to significantly reduce copper mediated LDL oxidation [29]. Hydroxytyrosol is the most abundant polyphenolic compounds found in olive oil, yet conflicting data has been found about the antioxidant capacity of it and other olive oil phenols. When discussing olive oil stability, hydroxytyrosol has been shown to provide the majority of protection from oxidation, and tyrosol does not seem to offer any [30]. On the other hand, studies that have completed *in vitro* evaluation of the antioxidant capacity of olive oil phenols have found hydroxytyrosol and tyrosol to be providing most of the antioxidant defense [31]. Bioavailability of hydroxytyrosol and tyrosol has been addressed in several studies, most of which found bioavailability to be dose dependent in nature [32].

Purpose of the Current Study

After reviewing several studies, it was determined that, a study evaluating the protective effect of mixtures of polyphenolic compounds would be a notable contribution to the understanding of dietary polyphenolic protection in oxidative damage. We first evaluated which compounds to study and determined

appropriate concentrations to study. Many studies have evaluated the polyphenolic compounds of olive oil and their antioxidant capacity, but little has been to compare. between phenolic compounds found in difference sources. Hydroxytyrosol was found to inhibit reactive species generation better than caffeic acid, while tyrosol has very little antioxidant activity [33]. It was also found that alone, caffeic acid is a better inhibitor of LDL oxidation when compared to quercetin, and when the two are combined they have an additive effect on the inhibition almost equal to their combined individual potential [34].

This research study focused upon the sources (coffee, wine and olive oil) that are the primary sources of antioxidants in the MD. The phenolic compounds of interest were caffeic acid from coffee, quercetin from wine [20] and tyrosol and hydroxytyrosol from olive oil. These compounds will be utilized in combination at levels based on their bioavailability.

MATERIALS AND METHODS

Materials

Pure olive oil with an expiration date of 2014 was obtained from a commercial vendor (Kirkland Signature, Seattle, WA) and was stored in the dark refrigerated at 20 - 25°C. Methanol, thiobarbituric acid (TBA), hydrochloric acid, xylene, bathophenanthroline, ferric chloride, orthophosphoric acid, dl-alpha-

tocopherol, Folin-Ciocalteu reagent, caffeic acid, tyrosol and quercetin were purchased from Sigma-Aldrich (Milwaukee, WI). Trichloroacetic acid was purchased from Fisher Scientific (Rockingham, NH). Hydroxytyrosol was purchased from Cayman Chemicals (Ann Arbor, MI)

Preparation of antioxidant-stripped olive oil

Antioxidants including: tocopherols and phenolic compounds were removed (stripped) by liquid/liquid extraction procedures [35] [36] modified by subjecting 15 g of olive oil to vortex agitation (Fisher Vortex Genie 2, Rockingham, NH) with 15 ml of 80:20 methanol:water for 5 min followed by centrifugation at 6,000 xg at 4°C for 10 min (Bechman J2-21M refrigerated centrifuge, Beckman Instrument, Inc., Fullerton, CA). The supernatant was removed and subjected to the above extraction procedures an additional 4 times. Removal of tocopherol and phenolic compounds from the extracted olive oil was verified by spectrophotometric analysis [37] and high performance liquid chromatography (HPLC) and visible/UV detection [38] respectively.

Tocopherol Analysis

Tocopherol levels were determined in original commercial olive oil and olive oils stripped of antioxidants, which in turn were used for the selected phenolic mixtures studies [37]. Olive oil, 0.5 ml is combined with ethanol, 0.5 ml and vortexed for 1 min (Fisher Vortex Genie 2, Rockingham, NH) and centrifuged

at 4°C at 3,000 rpm (Sorvall RT 6000 B refrigerated centrifuge, Fullerton, CA). Supernatant (1.5 ml) was removed and added to test tubes containing 0.25 ml bathophenanthroline and mixed by vortexing. Ferric chloride solution ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$: EtOH; 15:25), (0.25 ml) was added to the sample and mixed by vortexing. This was followed by adding 0.25 ml orthophosphoric acid solution (87%), mixing by vortexing and held for 1 min and 1.2 ml was transferred to wells of a microtiter plate (Finstrument, MTX Lab Systems, Inc., Vienna, VA) for analysis at 539 nm.

Previous olive oil concentrations of tocopherol were utilized in determining the standard curve. Standard serial dilution was utilized to generate the standard curve present in figure 1 below, by using a stock solution concentration that was determined using previous research on tocopherol concentration in olive oil.

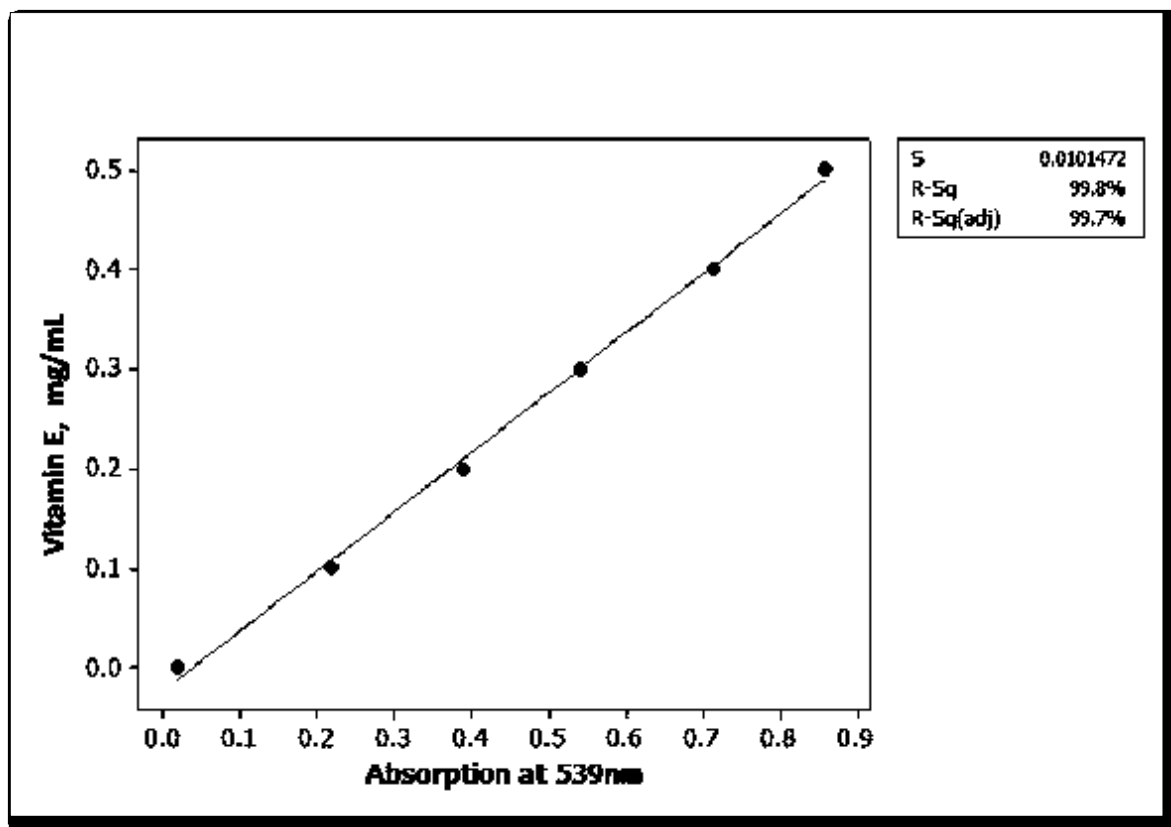


Figure 1. Standard Curve Vitamin E. Vitamin E = $-0.02290 + 0.5978$ (Absorption)

Total Polyphenolic Analysis

Total phenolic concentration was determined in original commercial olive oil and olive oils stripped of antioxidants, which in turn were used for the selected phenolic mixtures studies [39]. Olive oils were extracted following previously noted preparation of antioxidant-stripped olive oil and combined, and transferred to centrifuge tubes and brought to dryness using Savant vacuum rotary evaporating centrifuges (Savant, SC 110 SpeedVac Concentrator, Holbrook, NY) at 40°C. The remaining residue or phenolic standards, were diluted 1:10, v:v with methanol and mixed with 5 ml Folin-Ciocalteu reagent (1:10 diluted with deionized water and 4 ml saturated Na₂CO₃ and heated in a water bath at 45°C for 15 min. Total polyphenolic compounds were determined using a microplate reader at 690 nm (Finstruments, MTX Lab Systems, Inc., Vienna, VA). Total phenolic compounds values are expressed as caffeic acid equivalents represented in figure 2 (mg/15 ml extracted oil).

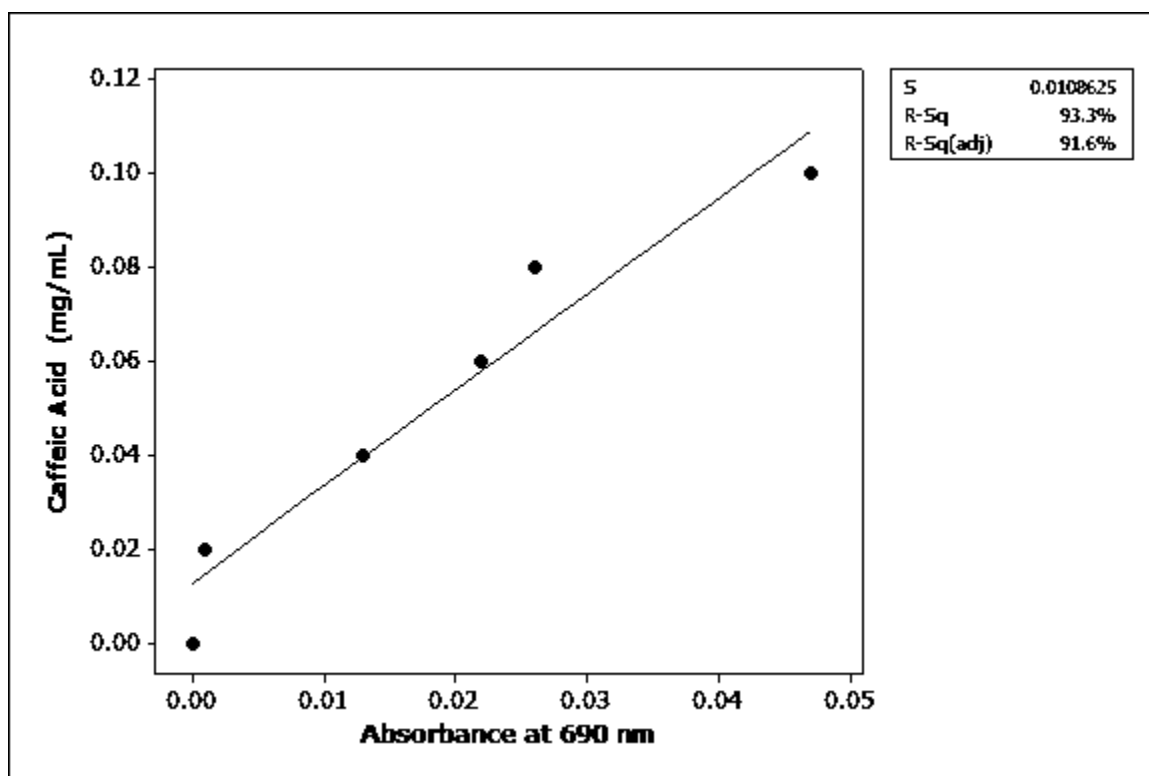


Figure 2. Caffeic Acid Equivalents Standard Curve. Caffeic Acid (mg/mL)=
 $0.01282+2.046$ (Absorbance at 690)

Determination of Malondialdehyde by Thiobarbituric acid reactive products

One ml of oil, antioxidant stripped oils (control) or antioxidant stripped oil mixed with phenolic compounds, were combined with 2 ml of TCA/TBA/HCl (15%/0.375%/0.25 N), mixed by vortex and heated at 37°C in a water bath for 15 min. Subsequently, the reaction was stopped by immersion of samples in ice bath and centrifugation for 10 min at 1000xg with refrigerated centrifuge (Sorvall RT 6000B, Fullerton, CA). The supernatant was transferred to wells of microplate reader (Finstruments, MTX Lab Systems, Inc., Vienna, VA) and read at 535 nm [40, 41]. MDA standard curve was determined utilizing previous oxidation studies and serial dilution. Figure 3 represents the curve utilized in this study.

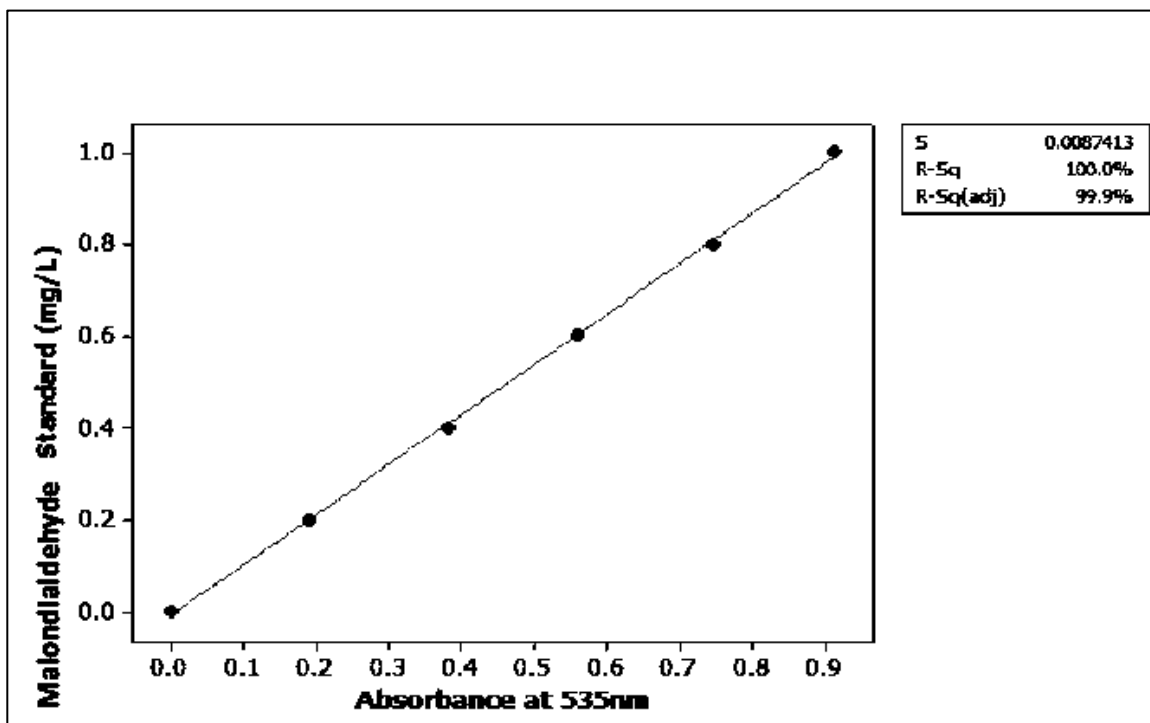


Figure 3. Standard curve of Malondialdehyde (MDA) as measured by TBA-reactive substances. TBA reactive substance(mg/L) = $-0.007429 + 1.092 (\text{Absorbance})$

Selection of polyphenolic compounds concentrations to be used *in vitro* evaluations.

Through a Pub Med (Medline) index search, plasma values for caffeic acid, tyrosol, hydroxytyrosol, and quercetin were found [15, 42-51]. Utilizing the literature values for plasma polyphenolic concentrations and linear regression we calculated physiological and at least one pharmacological concentration to be used in our *in vitro* studies (figures 4-7). For each polyphenolic compound, 3 concentrations were calculated. Tables 1 and 2 tabulate our calculated concentrations, for a total of 81 different mixture combinations.

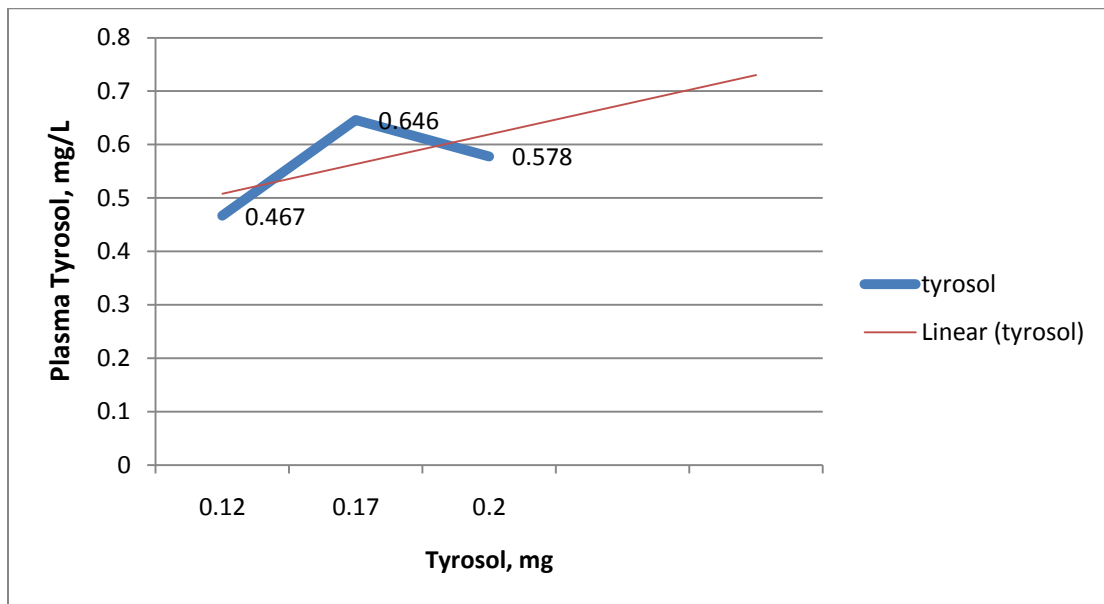


Figure 4. Determination of tyrosol concentrations to be used in *in vitro* experiments. Blue line = literature values of plasma tyrosol. Red line = r the linear regression analysis of Tyrosol determined through evaluation of bioavailability data of published research.

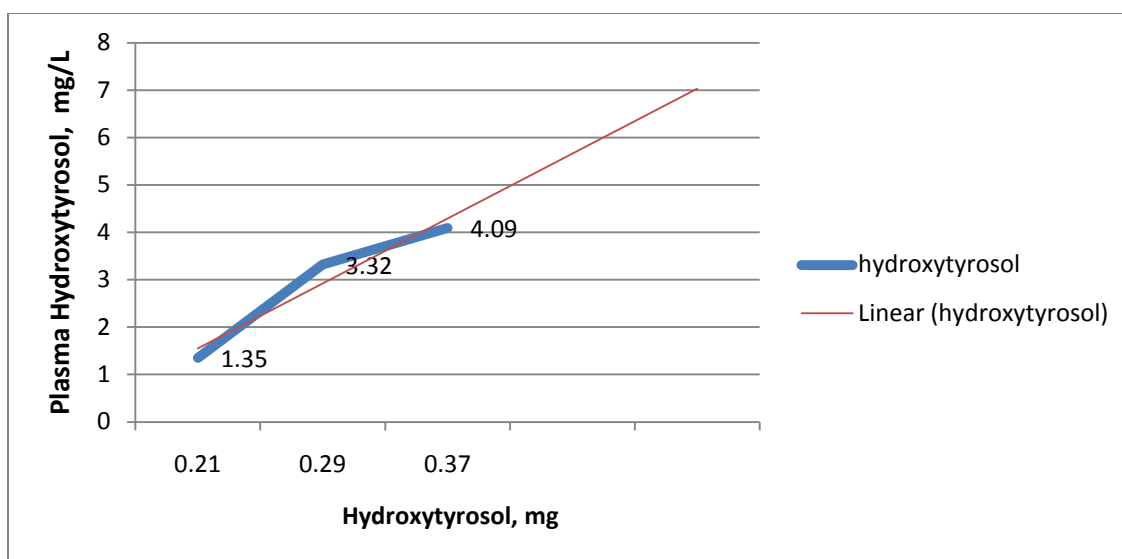


Figure 5. Determination of hydroxytyrosol concentrations to be used in *in vitro* experiments. Blue line = literature values of plasma hydroxytyrosol. Red line = r the linear regression analysis of hydroxytyrosol determined through evaluation of bioavailability of published research..

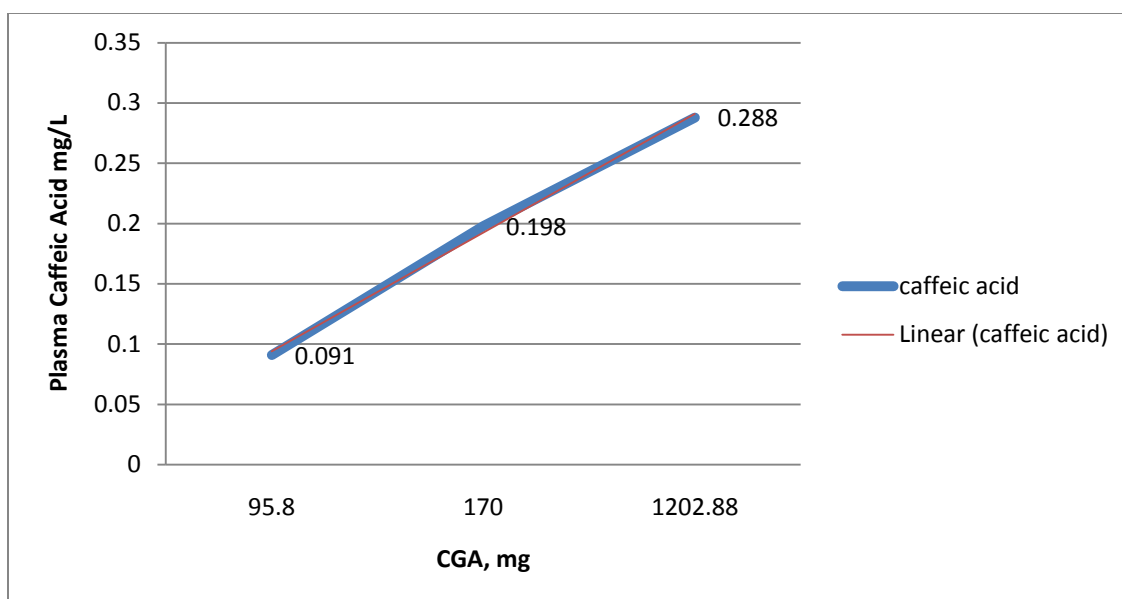


Figure 6. Determination of caffeic acid concentration for *in vitro* experiments. Blue= literature values of plasma caffeic acid, Red=r the linear regression analysis of caffeic acid completed through evaluation of bioavailability data of previous research. Regression lines correspond to the estimated plasma concentration.

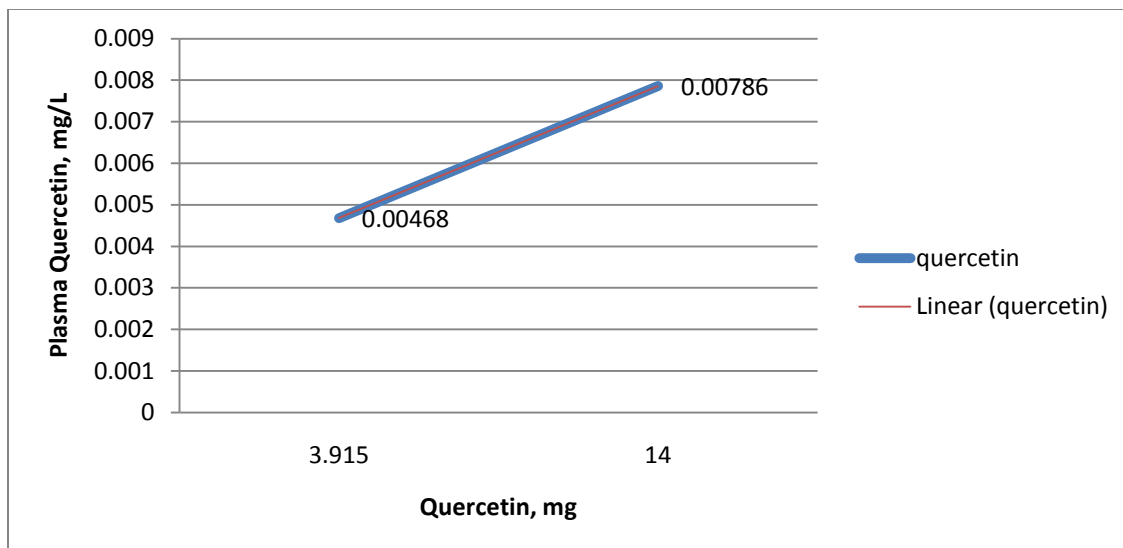


Figure 7. Determination of quercetin concentration for *in vitro* experiments. Blue= literature values of plasma quercetin, Red=r the linear regression analysis of quercetin completed through evaluation of bioavailability data of previous research. Regression lines correspond to the estimated plasma concentration.

Table 1. Data retrieved from regression analysis from Bioavailability Data for Tyrosol and Hydroxytyrosol

Compound	Amount of Food Consumed *	Concentration of phenolic from consumed amount (mg)	Estimated Plasma levels absorbed (mg/L)	Supplemented mg/15mL oil
Stock Tyrosol 3mg/100 μ L EtOH (0.03mg/microL) added amounts	30 mL of high phenolic olive oil	1.06	2.0	0.03
	30 mL of medium phenolic olive oil	0.43	1.0	0.015
	30 mL of low phenolic olive oil	0.12	0.5	0.0075
Stock Hydroxytyrosol 50mg/mL EtOH added amounts	30 mL of high phenolic olive oil	0.90	13.33	0.2
	30 mL of medium phenolic olive oil	0.51	6.667	0.1
	30 mL of low phenolic olive oil	0.31	3.33	0.05

** all olive oil studies provided the same amount of oil to participants during the studies*

Table 2 Data retrieved from regression analysis from Bioavailability Data for Caffeic Acid and Quercetin

Compound	Amount of Consumed Beverage *	Concentration of phenolic from consumed amount (mg)	Estimated Plasma levels absorbed (mg/L)	Supplemented mg/15mL oil
Stock Caffeic Acid 1.2mg/100microL (0.012mg/microL) Supplementation amounts	High Caffeic Acid 4.04 cups coffee	4863.31	0.8	0.012
	Medium Caffeic Acid 1.65cups coffee	1985.61	0.4	0.006
	Low Caffeic Acid 0.45 cups coffee	546.76	0.2	0.003
Stock Quercetin 2.5mg/10mL EtOH(00025mg/microL) Supplementation amounts	High Quercetin 8.75 cups wine	41.99	0.0167	0.00025
	medium Quercetin 3.24cups wine	15.53	0.0083	0.000125
	low Quercetin 0.48 cups wine	2.31	0.0042	0.0000625

* Studies provided varying amounts of beverages during experimentation.

Polyphenolic antioxidant enrichment of olive oil.

Caffeic acid, tyrosol, hydroxytyrosol, and quercetin at the predetermined concentrations were added to 15 ml (in duplicate) of olive oil previously stripped of antioxidant compounds and 200 μ l of 65 mmol CuSO_4 solution and incubated at 37°C constant temperature using a shaking water bath. At 0, 0.5, 1, 2, 4 and 6 hours, duplicate aliquots were removed, and further oxidation was stopped by adding 200 μ M of 0.6% butylated hydroxytoluene in ethanol and either frozen for later analysis or analyzed immediately for TBA-reactive products.

HPLC Analysis to Determine baseline Phenolic Concentration

A modified Tasioula-Margari and Okogeri [38] method of high-performance liquid chromatography (Agilent HPLC 1100) on a C18 reversed phase column was used to analyze Vitamin E and polyphenolics. Identification of compounds was achieved by comparing their retention time values with those of standards which were dissolved in methanol/isopropanol/hexane mixture (1:3:1, v/v/v). The column used was Agilent C18 (250x4.6mm ID) with 5- μm packing. Flow rate was 1 ml/min and run time, 70 min. The sample injection volume was 80 μl . Data was collected and processed using ChemStation A10.02 software.

Statistical Analysis

One-way ANOVA was used when comparing three or more groups, assuming that the data are sampled from Gaussian populations. A p-value <0.05 was considered statistically significant. The statistics between measurements of the same mixture or group were performed using a paired t test. Paired t test was used to determine significance of time on oxidation. Comparison of mixtures against the control was done using General Linear Model, utilizing the Dunnett comparison model, to determine significance at each time interval against the control set. A p-value <0.05 was considered statistically significant.

Results

Preliminary results of non-extracted olive oil compared to extracted olive oil

Baseline characteristics of extracted oil were significant when compared to non-extracted oil. Mean TBA reactive substances of extracted olive oil was 0.299 mg/L, which was significantly less than the TBA-reactive substance found in non-extracted oil, $p=0.037$, and the standard deviation was 0.01505 (figure 8). Mean Vitamin E for extracted olive oil was 0.125 mg/mL, which was significantly less than vitamin E content in non-extracted olive oil (figure 9). Mean polyphenolics of extracted olive oil was 0.0134 mg/mL, which was significantly less than the initial-extracted oil 0.0573 mg/mL, $p=0.001$ (figure 10). HPLC analysis indicated that tyrosol and hydroxytyrosol were effectively removed from the oil. Hydroxytyrosol was observed at 0.0027 mg/mL in initial extraction but was not observed in later extractions. Tyrosol was not observed in any extractions, the sensitivity to tyrosol is 0.0304 mg/mL. Vitamin E was also not observed in any extractions, the sensitivity being 0.0125 mg/mL, but it was indicated in spectrophotometric evaluation.

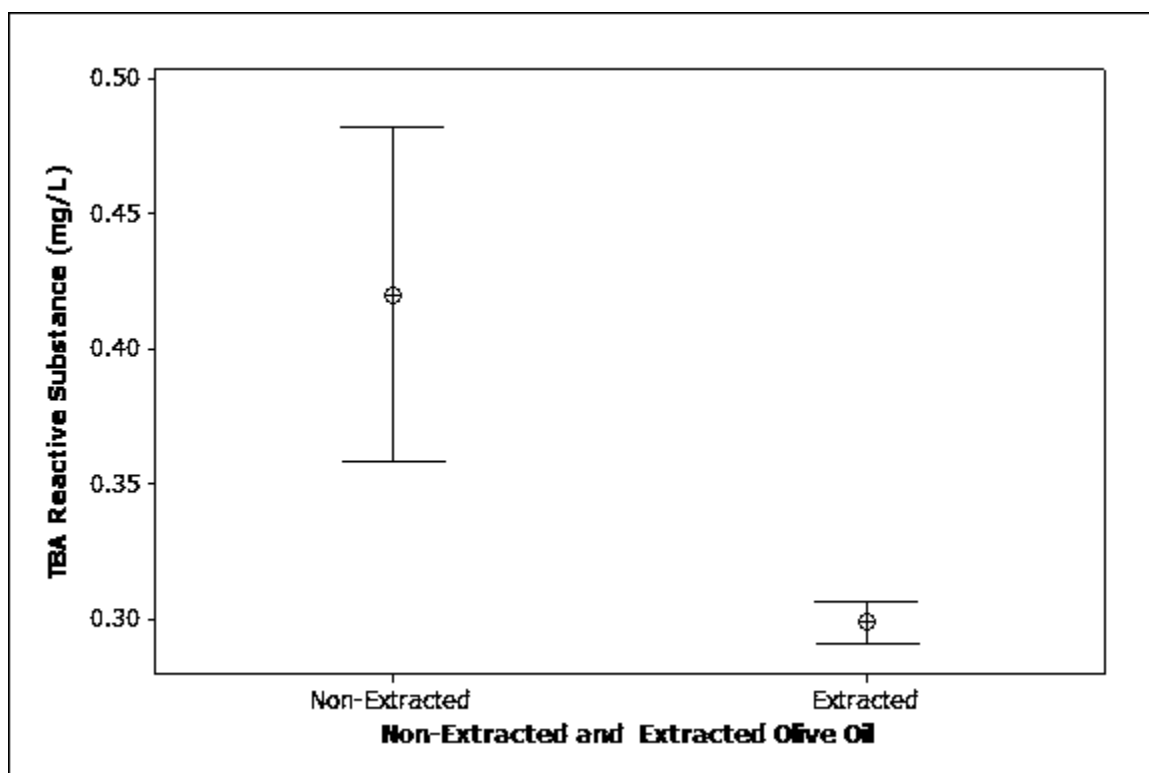


Figure 8. TBA reactive substances of non-extracted oil compared to extracted oil was significantly different, $P=0.037$. Bars are one standard error from the mean.

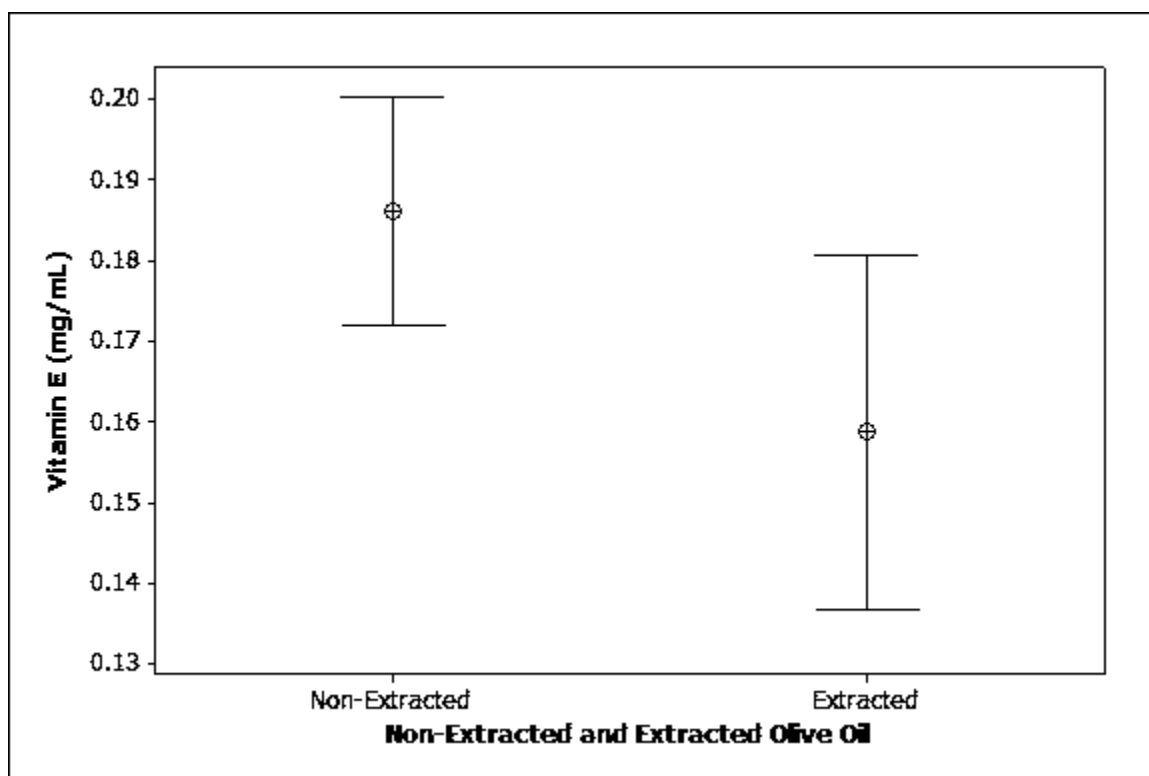


Figure 9. Vitamin E concentration for non-extracted oil compared to extracted oil was significant, $P=0.016$. Mean \pm Standard Deviation

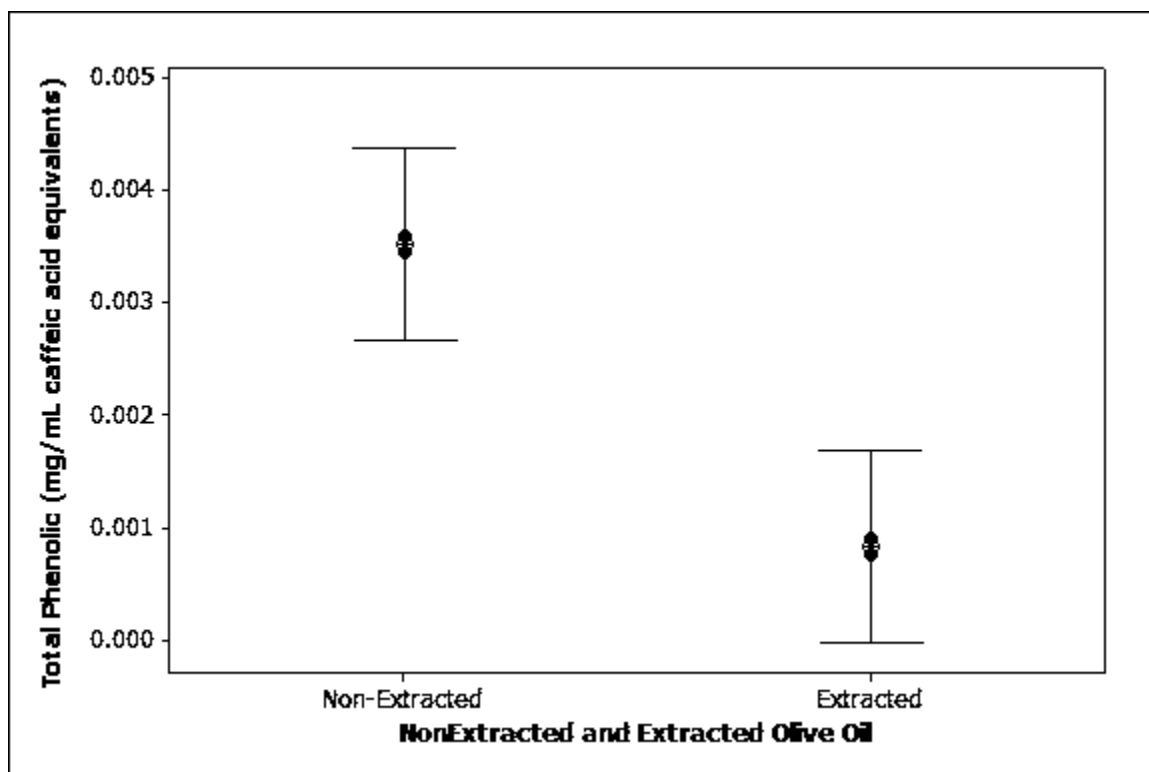


Figure 10. Total Phenolic concentration of non-extracted and extracted olive oil. Extracted oil was significantly less than non-extracted oil, $p=0.022$. Mean \pm Standard Deviation.

Oxidation of Antioxidant Stripped Olive Oil

There was a significant difference between the TBA reactive substances between time 0 and 4, 6 and 12 ($P=0.0021$, 0.0001 , 0.0000 respectively). There were significant differences from time 0.5 and times 6 and 12 ($P= 0.0042$, 0.0001 respectively). There were significant differences from time 1 and times 6 and 12 ($P= 0.0219$, 0.0001 respectively). The times 2, 4 did not have significant difference in later times, and there was no significant difference between the time 6 and 12 (figure 11).

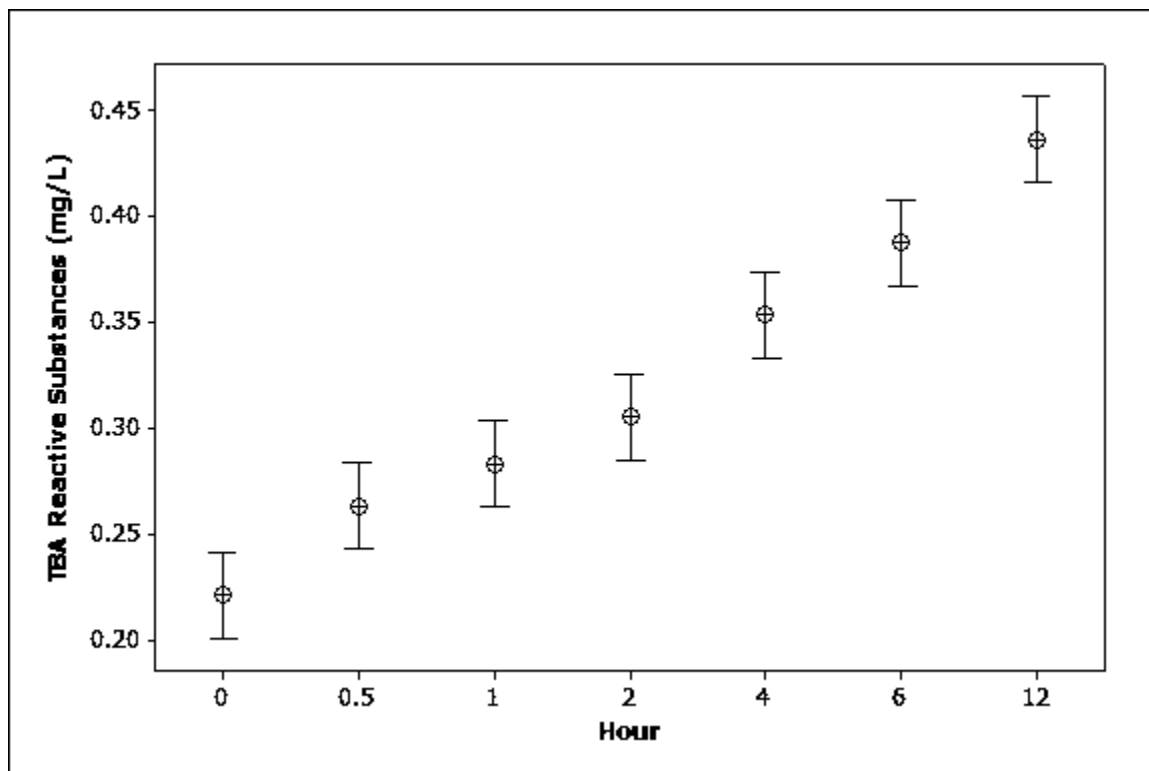


Figure 11. Copper mediated oxidation of extracted olive oil. Significant differences of TBA reactive substance were observed between the hours of 0,0.5,1 with times 6 and 12, $P>0.05$. Mean \pm Standard Deviation.

Individual Compound Dose Response

Figures 12-15 show the dose response curves for caffeic acid, quercetin, tyrosol and hydroxytyrosol. Of the four compounds, caffeic acid, displays a dose response tendency. Additionally, the figures show the TBA reactive substances of the dosages compared to the control. Production of TBA reactive substances is positively correlated to an increase in concentration of polyphenolic concentration. The lower concentration of caffeic acid, 0.003 mg, is associated to less TBA reactive substance than the control in addition to the other higher concentrations.

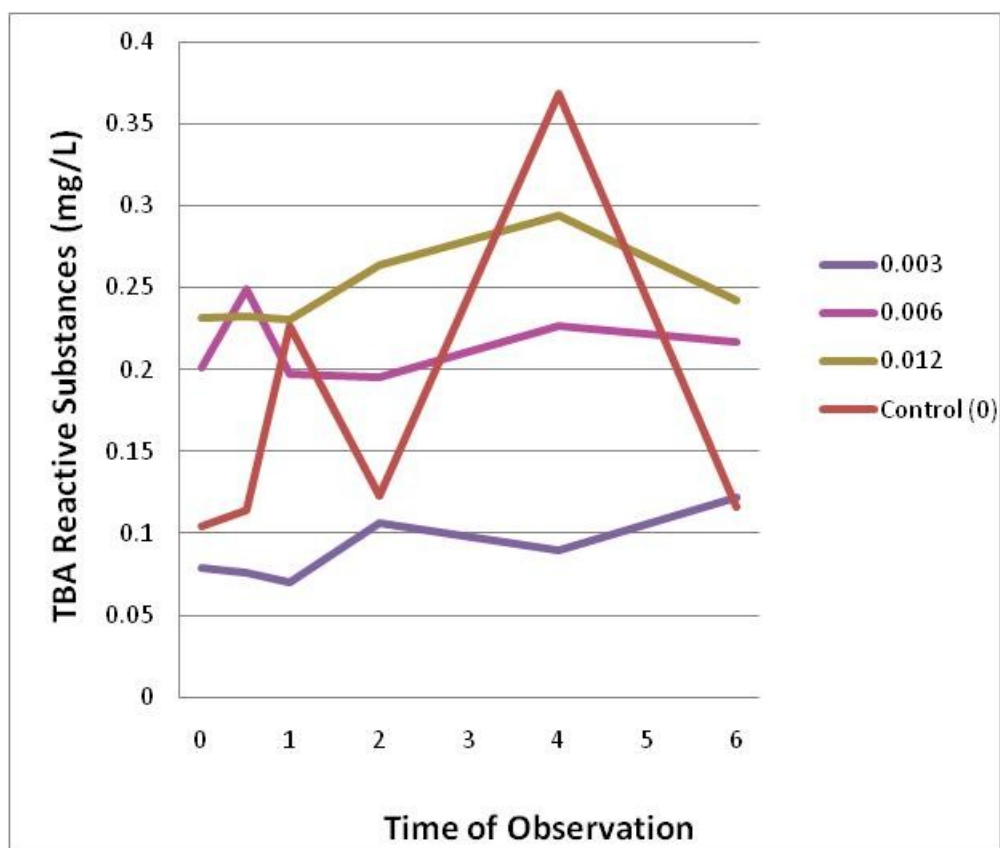


Figure 12. Caffeic acid dose response curve in hours of observation. Concentration of caffeic acid used in enrichment. Milligrams of caffeic acid added to 15 grams of stripped olive oil, control was striped olive oil without enrichment.

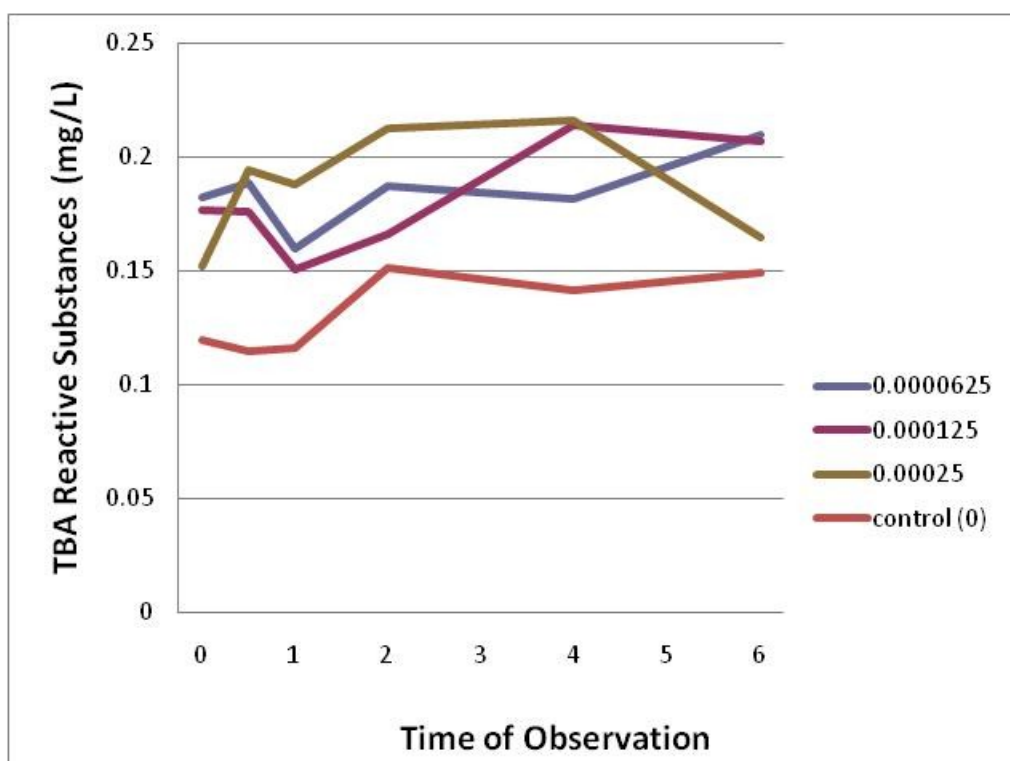


Figure 13. Quercetin dose response curve in hours of observation. Concentration of quercetin used in enrichment. Milligrams of quercetin added to 15 grams of stripped olive oil, control was striped olive oil without enrichment.

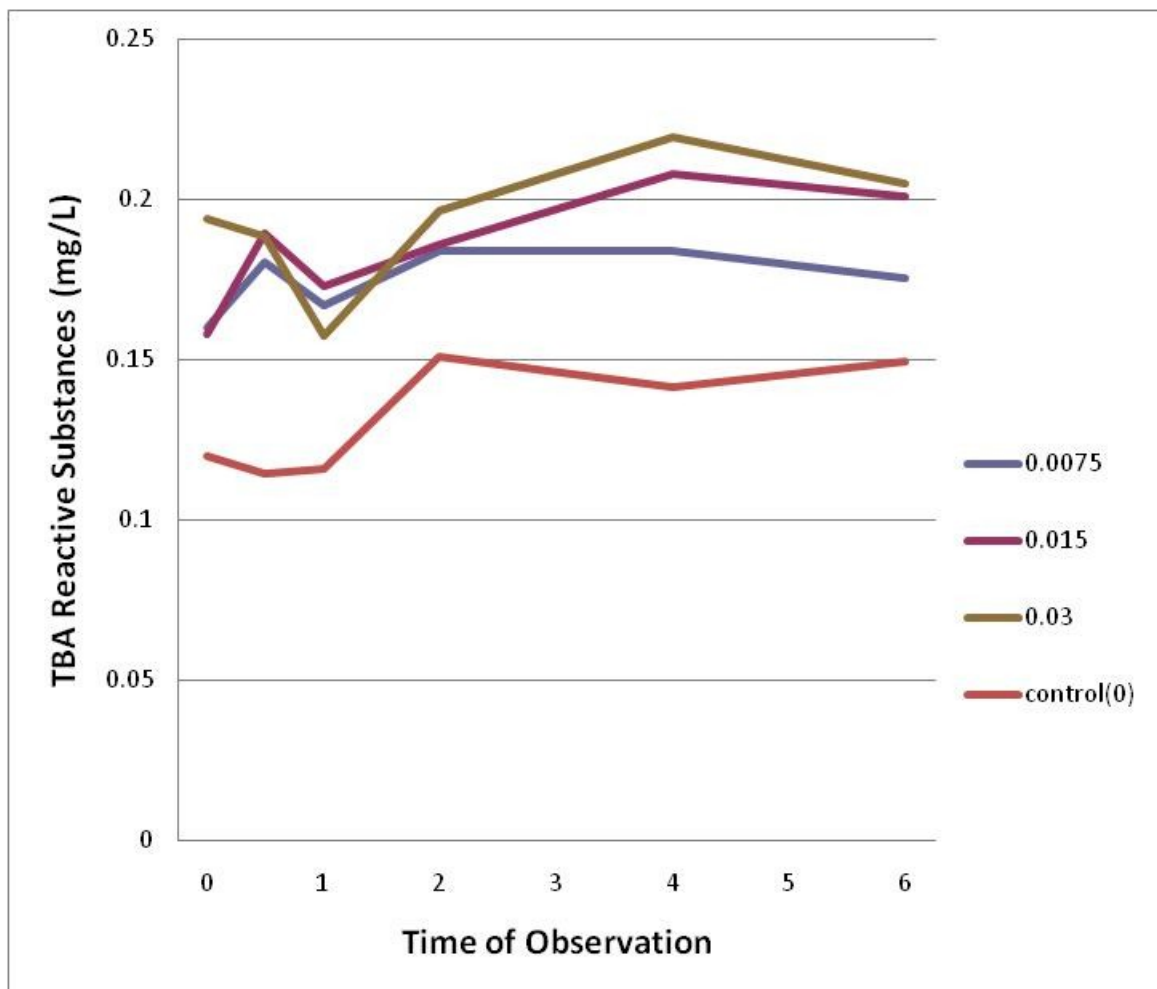


Figure 14. Tyrosol dose response curve in hours of observation. Concentration of tyrosol used in enrichment. Milligrams of tyrosol added to 15 grams of stripped olive oil, control was striped olive oil without enrichment.

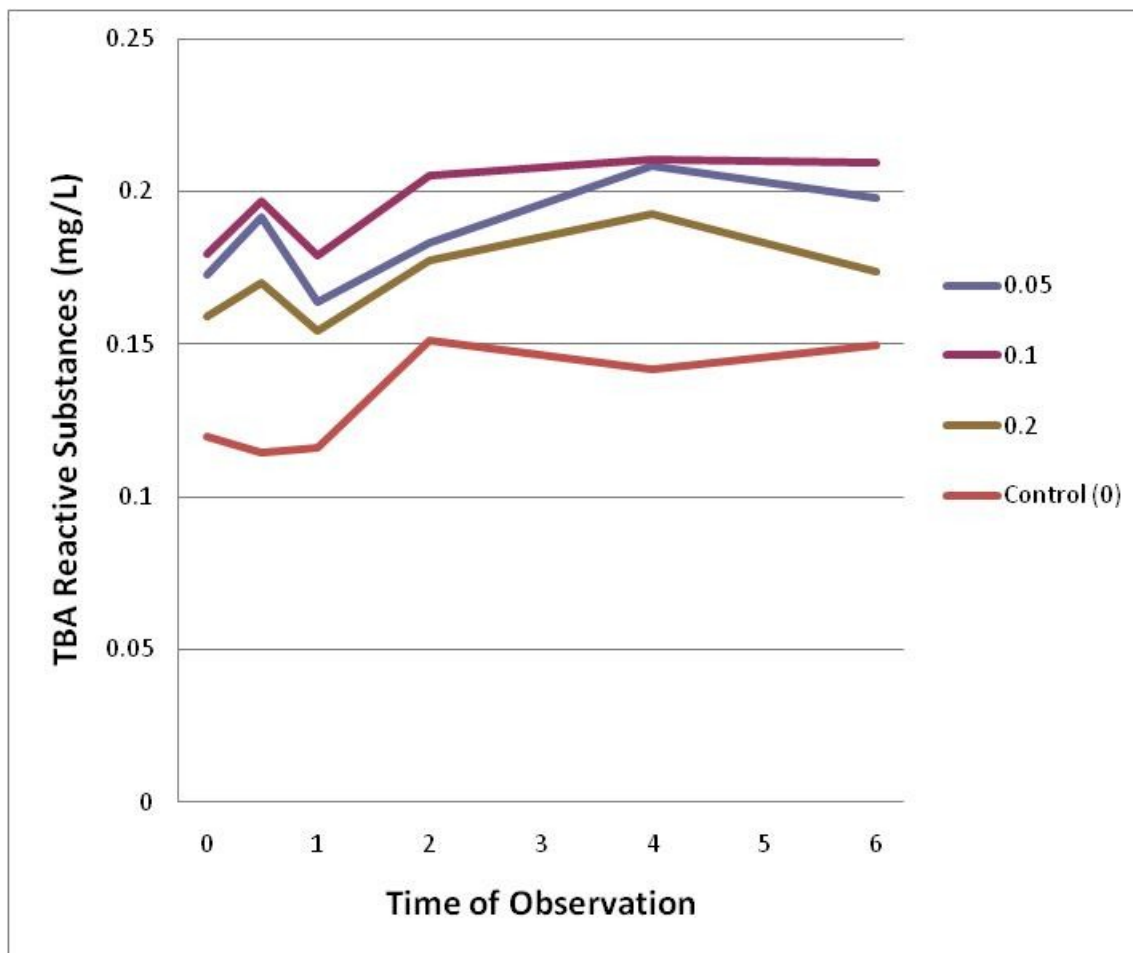


Figure 15. Hydroxytyrosol dose response curve in hours of observation. Concentration of hydroxytyrosol used in enrichment. Milligrams of hydroxytyrosol added to 15 grams of stripped olive oil, control was stripped olive oil without enrichment.

Effect of Adding Polyphenolic compound mixtures on Copper Mediated Oxidation of Antioxidant Stripped Olive Oil

A total of 81 polyphenolic mixtures were evaluated for their ability to modulate copper mediated oxidation of antioxidant stripped olive oil. Table 5 located in the appendix summarizes the 83 different combination of the four polyphenolic compounds studied. With the exception of the following polyphenolic compound mixtures; there was no statistical significance in oxidation compared to the respective controls. Figures 16-19 represents the mixture of polyphenolic compounds effect on copper-mediated oxidation compared to their respective controls. Mixture 6 contained 0.0012 mg of caffeic acid (c), 0.00025 mg of quercetin, 0.015 mg of tyrosol and 0.05 mg hydroxytyrosol shown in Figure 16. Mixture 6 was significant at 4 time intervals; p values are noted in table 3. There is a decrease in TBA-reactive products at time zero compared to control and a significant increase in TBA-reactive products at 1, 2, and 4 hours of incubation at 37°C compared to control. Mixture 27 contained 0.012mg of caffeic acid, 0.0000625 mg of quercetin, 0.0075 mg of tyrosol and 0.05 mg of hydroxytyrosol shown in figure 17. There as a decrease in TBA-reactive products at all time intervals compared to control and a significant decrease in TBA-reactive products at all hours of incubation at 37°C compared to control; p values are noted in table

3. Mixture 50 contained 0.006mg caffeic acid, 0.0000625mg quercetin, 0.015 mg tyrosol and 0.1mg hydroxytyrosol shown in figure 18. There is an increase in TBA-reactive products at all time intervals compared to control and a significant increase in TBA-reactive products at all hours of incubation at 37°C compared to control; p values are noted in table 3. Mixture 70 contained 0.003 mg caffeic acid, 0.000125mg quercetin, 0.0075 mg tyrosol and 0.2 mg hydroxytyrosol shown in figure 19. There is a decrease in TBA-reactive products at all time intervals, except time point 2, compared to control and a significant decrease in TBA-reactive products at hours 0,1 and 4 of incubation at 37°C compared to control; p values are noted in table 3.

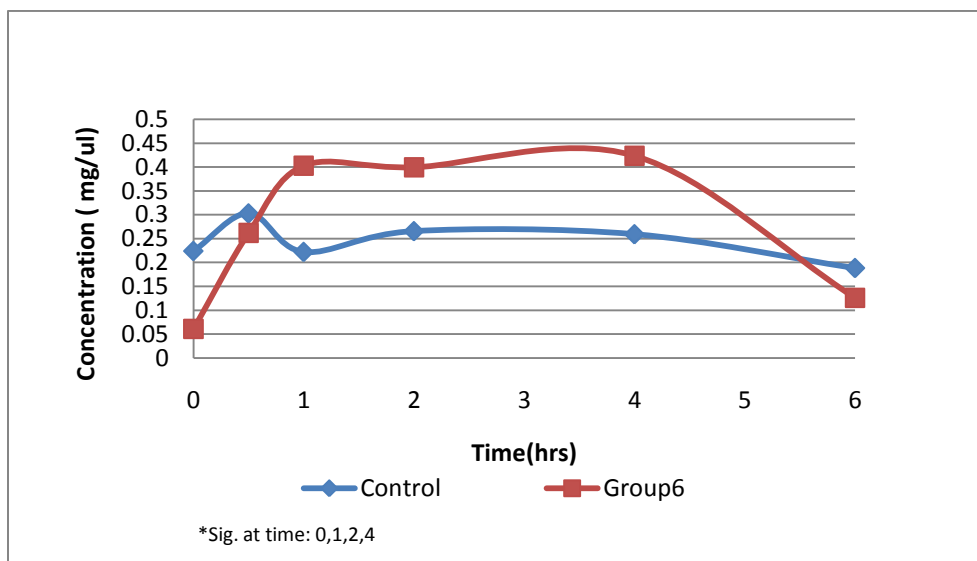


Figure 16. Mixture 6. Caffeic acid(c), quercetin (q), tyrosol (t) and hydroxytyrosol(h) were added in the following miligrams to 15 miligrams of stripped olive oil: c 0.012,q 0.00025,t 0.015,h 0.05. n=4

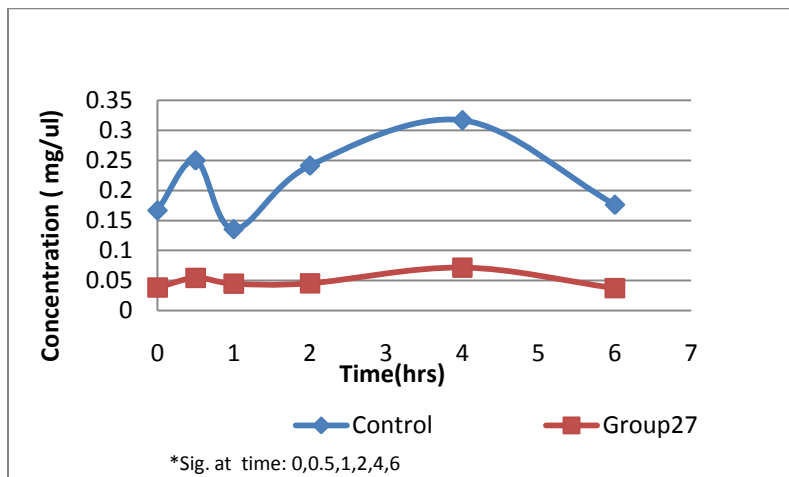


Figure 17. Mixture 27. Caffeic acid(c), quercetin (q), tyrosol (t) and hydroxytyrosol(h) were added in the following miligrams to 15 miligrams of stripped olive oil: c 0.012,q 0.0000625,t 0.0075 ,h 0.05. n=4

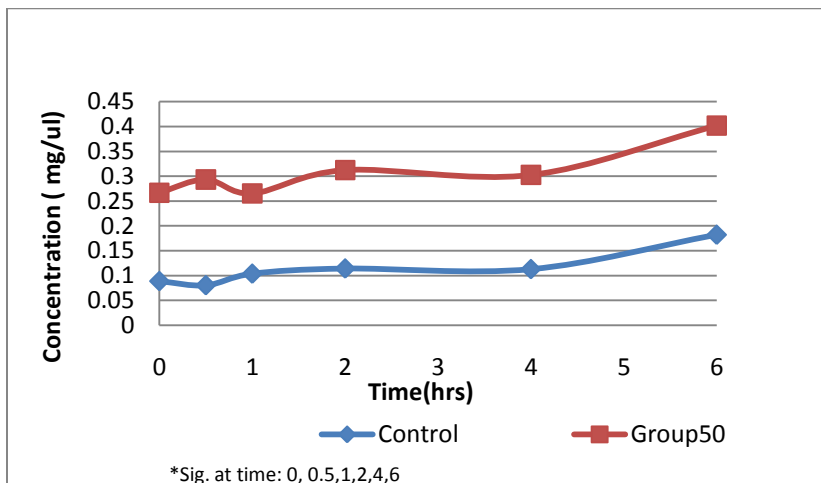


Figure 18. Mixture 50. Caffeic acid(c), quercetin (q), tyrosol (t) and hydroxytyrosol(h) were added in the following miligrams to 15 miligrams of stripped olive oil: c 0.006,q 0.0000625,t 0.015,h 0.1.n=4

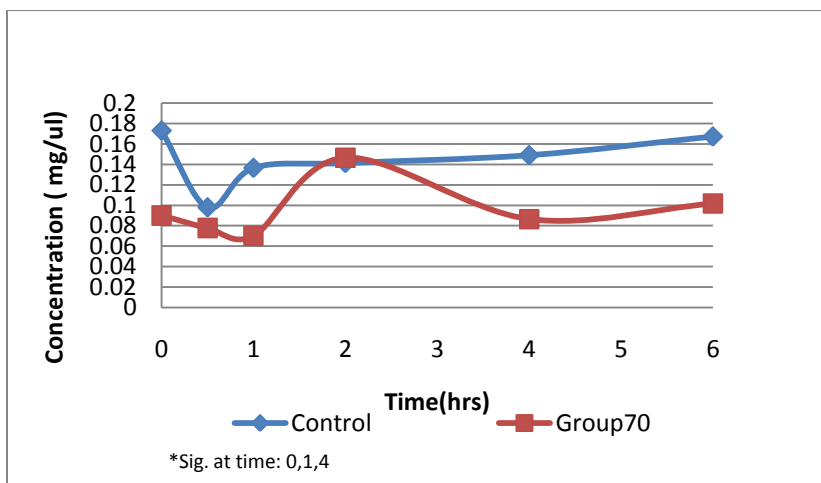


Figure 19. Mixture 70. Caffeic acid(c), quercetin (q), tyrosol (t) and hydroxytyrosol(h) were added in the following miligrams to 15 miligrams of stripped olive oil: c 0.003,q 0.000125,t 0.0075 ,h 0.2. n=4

In Table3, the relative levels of each are stated. Two acted as prooxidants, increasing the production of Malondialdehyde formation during the time trial, and two acted as antioxidant, decreasing the amount of MDA formed.

Table 3. Mixtures that were significantly different more than 50% of the time intervals, $p < 0.05$, and their p value for every time interval are listed.

Mixture	Hour 0	Hour 0.5	Hour 1	Hour 2	Hour 4	Hour 6
6	0	0.9293	0.0005	0.0033	0.028	0.2099
27	0	0	0.0032	0	0	0
50	0	0.0001	0	0	0.0002	0.0013
70	0.019	0.7125	0.0472	1	0.046	0.4127

* General Linear Model, Dunnett Comparison Against a Control, P Values for Each Time Interval

Total phenolic compounds of mixtures who have undergone significant oxidation changes compared to their respective controls

Mixtures 6 and 27 had a similar total phenolic content after oxidation to the mixture that was added to the oil initially. Mixture 70 was the only mixture to have a significant reduction in total polyphenolics after oxidation, $p=0.001$. The initial concentration of polyphenolics used for enrichment were calculated and utilized in paired t test evaluation against the post oxidation polyphenolic content, table 4. A graphic depiction of the polyphenolic changes after oxidation is represented in figure 20.

Table 4. Total phenolic compounds added to olive oil and after copper-mediated oxidation.

Mixture	Total Phenolic added (mg/mL)	Average Total Phenolics After Oxidation (mg/mL Caffeic Acid Equivalentents)	Paired T Test Adjusted P values
6	0.00474	0.00465	0.057
27	0.00427	0.00417	0.388
50	0.0074	0.00296	0.353
70	0.0154	0.00139	0.001

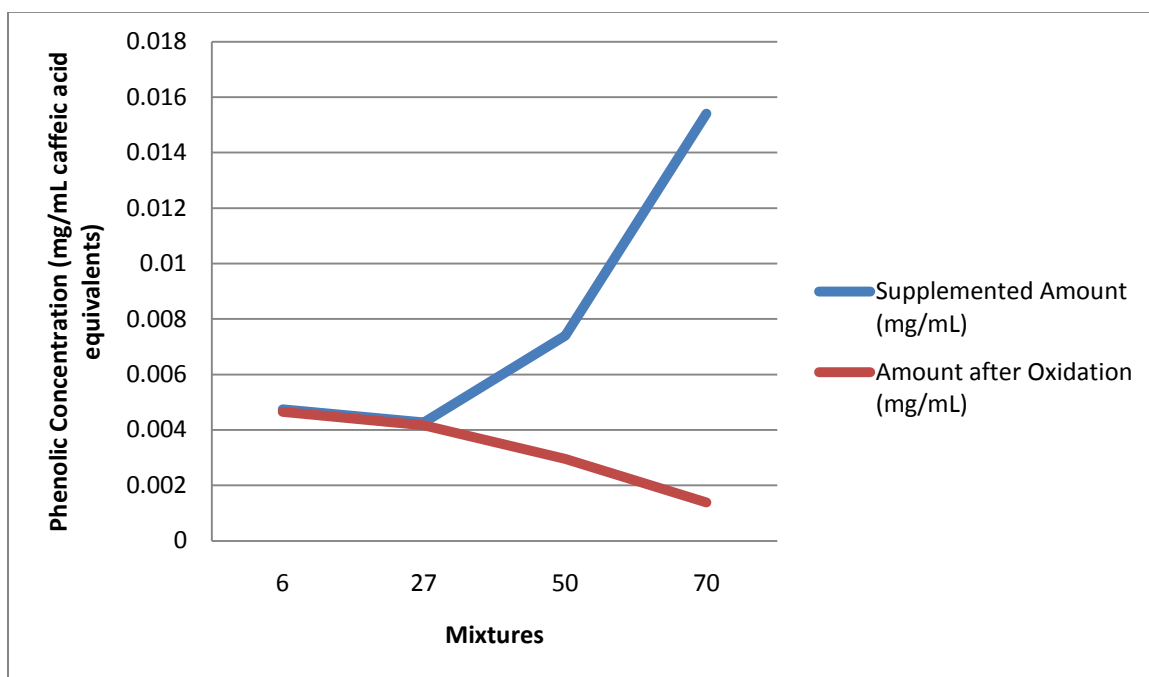


Figure 20. Phenolic Concentrations Added and After Oxidation

Discussion

Several dietary intake studies have investigated the consumption of the MD, coffee, wine and olive oil on decreasing health risks, and markers of disease [52-64]. The current study attempted to provide some insight to the possible antioxidant or prooxidant behaviors of certain combinations of polyphenolic compounds that come from the diet that would mimic the MD intake. In this study we evaluated the effect of polyphenolic compound mixtures in olive oil oxidized by copper using TBA reactive substance, as an indicator of lipid peroxidation. Studies evaluating the oxidation of LDL have also evaluated the formation of TBA-reactive substance *in vivo* [65]. Olive oil was selected as the medium because of its MUFA to PUFA ratio and innate capacity to resist oxidation, which was partially confirmed in this study. Since dietary olive oil can modify endogenous fats and reduce oxidation, diets such as the MD could be beneficial in modulation of LDL oxidation.

A dose dependent protective response was indicated when evaluating the compounds individually for caffeic acid. A low concentration of caffeic acid, 0.03 mg, was shown to produce lower amounts of TBA reactive substances, yet when evaluating the compounds according to their mixture it was determined that higher concentrations produced the lower TBA reactive substance amounts.

Tyrosol has been investigated extensively in previous research, and much has seen it to have little antioxidant capacity or requiring high dosages to provide protection [67]. Contrary to these results, our results indicate tyrosol as having influence in the oxidative process. The olive oil supplemented with the medium concentrations of tyrosol resulted in two of the four mixtures of significant oxidation, thus, acting as prooxidants, see mixtures 6 (figure 16) with caffeic acid(c), quercetin (q), tyrosol (t) and hydroxytyrosol(h) added in the following milligrams to 15 milligrams of stripped olive oil respectively: 0.012, 0.00025, 0.015, 0.05 and mixture 50 (figure 18) with caffeic acid(c), quercetin (q), tyrosol (t) and hydroxytyrosol(h) were added in the following milligrams to 15 milligrams of stripped olive oil respectively: 0.006, 0.0000625, 0.015, 0.1.

Mixture 27 (figure 17) acted as an antioxidant, inhibiting oxidation. This mixture is composed of the following compounds caffeic acid(c), quercetin (q), tyrosol (t) and hydroxytyrosol(h) added in the following milligrams to 15 milligrams of stripped olive oil respectively: 0.012 ,0.0000625,0.0075 , 0.05. This was a relative high caffeic acid and a relative low dose of the other three compounds. Our results are consistent with the findings of previous studies that investigated increased coffee consumption and reduced health risks. When looking at the reduction in polyphenolic compounds after oxidization, mixture 27 stayed relatively similar to the initial supplemented amount (figure 20), suggesting a synergistic relationship of the polyphenolic compounds is occurring. This mixture

would resemble a diet that consisted of 4 cups of coffee, half a glass of wine and olive oil that was low in tyrosol and hydroxytyrosol (or limited olive oil in the diet) [15, 42-51]. According to our findings, caffeic acid, in a high amount relative to 4 cups of coffee, when in combination with lower amounts of the other three phenolic compounds produces an antioxidant environment (figure 17).

Mixture 70 (figure 19) was the other mixture to act as an antioxidant. It was composed of the following compounds caffeic acid(c), quercetin (q), tyrosol (t) and hydroxytyrosol(h) in the following miligrams to 15 miligrams of stripped olive oil respectively: 0.003, 0.000125, 0.0075, 0.2. This was a relative high amount of hydroxytyrosol, with the other compounds being of low and medium levels. Our findings are consistent with much of the previous research showing that hydroxytyrosol is the major contributor to olive oil health benefits. It is important to note that this mixture is equivalent to a diet that would consist of an olive oil high in hydroxytyrosol with about 3 glasses of wine and less than half a cup of coffee. It would be easy to imagine a Mediterranean dinner consisting of this composition [15, 42-51].

These two antioxidant mixtures (27 and 70) are composed of relatively high concentration of one compound with the remaining compounds being at relatively medium and low concentrations. However, in the mixture with high caffeic acid concentration and other polyphenolic compounds in concentrations above a low dose, the mixture becomes prooxidant in nature. Indicating there is

a tendency of caffeic acid and the other compounds to become prooxidant. This would suggest there is a relationship to how much of these are consumed in a 6 hour period and possible antioxidant nature of the diet. Previous studies have linked an increase in wine consumption, 4 glasses a day or more, to an increased health risk [62], consistent with our current study. Research with coffee has provided conflicting information, with studies finding a low risk of Cardiovascular Disease (CVD) associated to 3-5 cups of coffee a day [68], while other studies have found increased risks with increased coffee consumption [69]. The findings from mixture 6 could suggest that consuming high amounts of coffee and wine, 4 and 9 cups respectively, in the same 6 hours time period can lead to a prooxidant environment (figure 16).

Mixture 50 has no relative high amounts of polyphenolic compounds, but it is acting as prooxidant, suggesting that there is a synergistic effect among the different antioxidants, where medium amounts are unable to prevent oxidation and possibly acts collaboratively to generate a prooxidant environment. The polyphenolic compound mixture 50 would resemble one that consists of 1.5 cups of coffee, half a cup of wine and an olive oil that contains both medium levels of tyrosol and hydroxytyrosol. This would suggest that there is a specific level at which the mixtures switch between antioxidant and prooxidant. Mixture 50 did show a reduction in total polyphenolic compounds, but it was not a significant reduction.

When comparing reduction in total polyphenolic compounds, mixtures 6 and 27 did not show much of any reduction, this could indicate there is a possible interaction among the different compounds. Mixtures 50 and 70 did have a reduction in total compounds, which indicates a depletion of polyphenolic compounds, yet mixture 70 was the only one to have a significant reduction in copper oxidation, $p < 0.05$.

Much of the results suggest that there could be antioxidants working complementary to one another, working well when one is high and the other is low to prevent oxidation. It would be necessary to investigate these possible relationships further in future research. In future experimentation, HPLC analysis can be completed to determine which antioxidants are being destroyed and which are being regenerated, if any. While we standardized for Vitamin E, we do not know the exact interaction the compounds had during oxidation.

It was found that patients who were given an olive oil dose equivalent to 5 times the hydroxytyrosol found in red wine had higher urinary hydroxytyrosol output [70]. The researchers suggested that there is an interaction between dopamine and ethanol that produces hydroxytyrosol. Therefore, it is important to recognize that *in vivo* plasma levels are dependent on metabolic pathways in

addition to the consumed amounts. Additionally, the selected polyphenolic compounds that were studied have been shown to be available from different sources, such as caffeic acid being present in olive oil, hydroxytyrosol in red wine and quercetin in several different food sources such as onions and tea. It was shown that caffeic acid was neutral in preventing DNA oxidation [67]; something that needs to be investigated further in future research.

In addition to the mixtures interaction of the polyphenolic compounds, it is also important to evaluate the other dietary influences on the antioxidant nature. This study did not address the fatty acid composition and the prevention of oxidation. There was only one source of olive oil so there is no comparison to other ratios of MUFA to PUFA, additionally further investigation to how the ratio is modified after oxidation would provide insight to the mechanism.

In conclusion, this study was able to show that there are some mixture combinations in which polyphenolic compounds can act as antioxidants; and as the concentrations are changed they can also become prooxidant in nature. The dynamic interaction between polyphenolic compounds and the importance of evaluating the effects of mixtures are important considerations for studying the consequences that develop from various diets. More research is needed to evaluate the different mixtures and the resulting antioxidant or prooxidant natures.

Bibliography

1. Cirico, T.L. and S.T. Omaye, *Additive or synergetic effects of phenolic compounds on human low density lipoprotein oxidation*. Food Chem Toxicol, 2006. **44**(4): p. 510-6.
2. Fito, M., et al., *Effect of a traditional Mediterranean diet on lipoprotein oxidation: a randomized controlled trial*. (0003-9926 (Print)).
3. United States. Dietary Guidelines Advisory Committee., et al., *Dietary guidelines for Americans*, in *HHS publication.*, U.S. Dept. of Health and Human Services : U.S. Dept. of Agriculture.: Washington, D.C.
4. Babio, N., et al., *Adherence to the Mediterranean diet and risk of metabolic syndrome and its components*. Nutr Metab Cardiovasc Dis, 2009. **19**(8): p. 563-70.
5. Estruch, R., et al., *Primary Prevention of Cardiovascular Disease with a Mediterranean Diet*. New England Journal of Medicine, 2013. **368**(14): p. 1279-1290.
6. Estruch, R., et al., *Effects of a Mediterranean-style diet on cardiovascular risk factors: a randomized trial*. (1539-3704 (Electronic)).
7. Tzima, N., et al., *Mediterranean diet and insulin sensitivity, lipid profile and blood pressure levels, in overweight and obese people; the Attica study*. Lipids Health Dis, 2007. **6**: p. 22.
8. Estruch, R., *Anti-inflammatory effects of the Mediterranean diet: the experience of the PREDIMED study*. (1475-2719 (Electronic)).
9. Camargo, A., et al., *Expression of proinflammatory, proatherogenic genes is reduced by the Mediterranean diet in elderly people*. (1475-2662 (Electronic)).

10. Massaro, M., et al., *Nutraceuticals and prevention of atherosclerosis: focus on omega-3 polyunsaturated fatty acids and Mediterranean diet polyphenols*. (1755-5922 (Electronic)).
11. Mattioli, A.V., et al., *Adherence to Mediterranean diet and intake of antioxidants influence spontaneous conversion of atrial fibrillation*. *Nutrition, Metabolism and Cardiovascular Diseases*, 2013. **23**(2): p. 115-121.
12. Saura-Calixto, F. and I. Goni, *Definition of the Mediterranean diet based on bioactive compounds*. (1549-7852 (Electronic)).
13. Saura-Calixto, F. and I. Goñi, *Antioxidant capacity of the Spanish Mediterranean diet*. *Food Chemistry*, 2006. **94**(3): p. 442-447.
14. Pulido, R., M. Hernandez-Garcia, and F. Saura-Calixto, *Contribution of beverages to the intake of lipophilic and hydrophilic antioxidants in the Spanish diet*. *Eur J Clin Nutr*, 2003. **57**(10): p. 1275-82.
15. Nardini, M., et al., *Absorption of phenolic acids in humans after coffee consumption*. *J Agric Food Chem*, 2002. **50**(20): p. 5735-41.
16. Johnson, S., et al., *Coffee consumption and reduced risk of hepatocellular carcinoma: findings from the Singapore Chinese Health Study*. *Cancer Causes Control*, 2011. **22**(3): p. 503-10.
17. Masala, G., et al., *Anthropometric and dietary determinants of blood pressure in over 7000 Mediterranean women: the European Prospective Investigation into Cancer and Nutrition-Florence cohort*. *J Hypertens*, 2008. **26**(11): p. 2112-20.
18. Szmítko, P.E. and S. Verma, *Red Wine and Your Heart*. *Circulation*, 2005. **111**(2): p. e10-e11.
19. Ginjom, I., et al., *Phenolic compound profiles in selected Queensland red wines at all stages of the wine-making process*. *Food Chemistry*, 2011. **125**(3): p. 823-834.
20. Vitaglione, P., et al., *Bioavailability of trans-resveratrol from red wine in humans*. (1613-4125 (Print)).
21. Hollman, P.C.H., et al., *Bioavailability of the dietary antioxidant flavonol quercetin in man*. *Cancer Letters*, 1997. **114**(1-2): p. 139-140.

22. Frankel, E.N., et al., *Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine*. The Lancet, 1993. **341**(8843): p. 454-457.
23. Frankel, E.N., A.L. Waterhouse, and J.E. Kinsella, *Inhibition of human LDL oxidation by resveratrol*. The Lancet, 1993. **341**(8852): p. 1103-1104.
24. Visioli, F. and C. Galli, *The role of antioxidants in the Mediterranean diet*. Lipids, 2001. **36 Suppl**: p. S49-52.
25. de la Torre-Carbot, K., et al., *Elevated Circulating LDL Phenol Levels in Men Who Consumed Virgin Rather Than Refined Olive Oil Are Associated with Less Oxidation of Plasma LDL*. The Journal of Nutrition, 2010. **140**(3): p. 501-508.
26. Ruiz-Gutierrez, V., et al., *Plasma lipids, erythrocyte membrane lipids and blood pressure of hypertensive women after ingestion of dietary oleic acid from two different sources*. (0263-6352 (Print)).
27. Ruiz-Gutiérrez, V., et al., *Olive oil and high-oleic sunflower oil on human plasma and erythrocyte membrane lipids*. Nutrition Research, 1997. **17**(9): p. 1391-1399.
28. Perona, J.S., et al., *Consumption of Virgin Olive Oil Influences Membrane Lipid Composition and Regulates Intracellular Signaling in Elderly Adults With Type 2 Diabetes Mellitus*. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences, 2007. **62**(3): p. 256-263.
29. Fito, M., et al., *Protective effect of olive oil and its phenolic compounds against low density lipoprotein oxidation*. Lipids, 2000. **35**(6): p. 633-8.
30. Bendini, A., et al., *Protective effects of extra virgin olive oil phenolics on oxidative stability in the presence or absence of copper ions*. J Agric Food Chem, 2006. **54**(13): p. 4880-7.
31. Di Benedetto, R., et al., *Tyrosol, the major extra virgin olive oil compound, restored intracellular antioxidant defences in spite of its weak antioxidative effectiveness*. Nutrition, Metabolism and Cardiovascular Diseases, 2007. **17**(7): p. 535-545.

32. de la Torre, R., *Bioavailability of olive oil phenolic compounds in humans*. Inflammopharmacology, 2008. **16**(5): p. 245-7.
33. Stupans, I., et al., *Comparison of radical scavenging effect, inhibition of microsomal oxygen free radical generation, and serum lipoprotein oxidation of several natural antioxidants*. J Agric Food Chem, 2002. **50**(8): p. 2464-9.
34. Meyer, A.S., M. Heinonen, and E.N. Frankel, *Antioxidant interactions of catechin, cyanidin, caffeic acid, quercetin, and ellagic acid on human LDL oxidation*. Food Chemistry, 1998. **61**(1-2): p. 71-75.
35. Brenes, M., et al., *Rapid and complete extraction of phenols from olive oil and determination by means of a coulometric electrode array system*. Journal of agricultural and food chemistry, 2000. **48**(11): p. 5178-5183.
36. Montedoro, G., et al., *Simple and hydrolyzable phenolic compounds in virgin olive oil. 1. Their extraction, separation, and quantitative and semiquantitative evaluation by HPLC*. Journal of agricultural and food chemistry, 1992. **40**(9): p. 1571-1576.
37. Rutkowski, M. and K. Grzegorzczuk, *Modifications of spectrophotometric methods for antioxidative vitamins determination convenient in analytic practice*. Acta Sci. Pol., Technol. Aliment, 2007. **6**(3): p. 17-28.
38. Tasioula-Margari, M. and O. Okogeri, *Simultaneous determination of phenolic compounds and tocopherols in virgin olive oil using HPLC and UV detection*. Food Chemistry, 2001. **74**(3): p. 377-383.
39. McDonald, S., et al., *Phenolic content and antioxidant activity of olive extracts*. Food Chemistry, 2001. **73**(1): p. 73-84.
40. Ramírez, M.R., et al., *Effects of the type of frying with culinary fat and refrigerated storage on lipid oxidation and colour of fried pork loin chops*. Food Chemistry, 2004. **88**(1): p. 85-94.
41. Badr El-Din, N.K. and S.T. Omaye, *Concentration-dependent antioxidant activities of conjugated linoleic acid and α -tocopherol in corn oil*. Journal of the Science of Food and Agriculture, 2007. **87**(14): p. 2715-2720.

42. Covas, M.I., et al., *Bioavailability of tyrosol, an antioxidant phenolic compound present in wine and olive oil, in humans*. *Drugs Exp Clin Res*, 2003. **29**(5-6): p. 203-6.
43. de Vries Jh Fau - Hollman, P.C., et al., *Red wine is a poor source of bioavailable flavonols in men*. (0022-3166 (Print)).
44. Farah, A., et al., *Chlorogenic Acids from Green Coffee Extract are Highly Bioavailable in Humans*. *The Journal of Nutrition*, 2008. **138**(12): p. 2309-2315.
45. Ferruzzi, M.G., *The influence of beverage composition on delivery of phenolic compounds from coffee and tea*. *Physiol Behav*, 2010. **100**(1): p. 33-41.
46. Manach, C., et al., *Quercetin is recovered in human plasma as conjugated derivatives which retain antioxidant properties*. *FEBS Letters*, 1998. **426**(3): p. 331-336.
47. Miro-Casas, E., et al., *Tyrosol and hydroxytyrosol are absorbed from moderate and sustained doses of virgin olive oil in humans*. *Eur J Clin Nutr*, 2003. **57**(1): p. 186-90.
48. Monteiro M Fau - Farah, A., et al., *Chlorogenic acid compounds from coffee are differentially absorbed and metabolized in humans*. (0022-3166 (Print)).
49. Rubió, L., et al., *Impact of olive oil phenolic concentration on human plasmatic phenolic metabolites*. *Food Chemistry*, 2012. **135**(4): p. 2922-2929.
50. Scalbert, A. and G. Williamson, *Dietary intake and bioavailability of polyphenols*. *J Nutr*, 2000. **130**(8S Suppl): p. 2073S-85S.
51. Visioli, F., et al., *Olive oil phenolics are dose-dependently absorbed in humans*. *FEBS Lett*, 2000. **468**(2-3): p. 159-60.
52. Hagfors, L., et al., *Antioxidant intake, plasma antioxidants and oxidative stress in a randomized, controlled, parallel, Mediterranean dietary intervention study on patients with rheumatoid arthritis*. *Nutr J*, 2003. **2**: p. 5.
53. Blum, S., et al., *Effect of a Mediterranean meal on postprandial carotenoids, paraoxonase activity and C-reactive protein levels*. *Ann Nutr Metab*, 2006. **50**(1): p. 20-4.

54. Conforti, F., et al., *In vivo anti-inflammatory and in vitro antioxidant activities of Mediterranean dietary plants*. J Ethnopharmacol, 2008. **116**(1): p. 144-51.
55. Franzini, L., et al., *Food selection based on high total antioxidant capacity improves endothelial function in a low cardiovascular risk population*. Nutr Metab Cardiovasc Dis, 2012. **22**(1): p. 50-7.
56. Gresele, P., et al., *Effects of resveratrol and other wine polyphenols on vascular function: an update*. J Nutr Biochem, 2011. **22**(3): p. 201-11.
57. Marin, C., et al., *Mediterranean diet reduces endothelial damage and improves the regenerative capacity of endothelium*. Am J Clin Nutr, 2011. **93**(2): p. 267-74.
58. Martinez-Gonzalez, M.A., et al., *Mediterranean diet and reduction in the risk of a first acute myocardial infarction: an operational healthy dietary score*. Eur J Nutr, 2002. **41**(4): p. 153-60.
59. Mitjavila, M.T., et al., *The Mediterranean diet improves the systemic lipid and DNA oxidative damage in metabolic syndrome individuals. A randomized, controlled, trial*. Clin Nutr, 2013. **32**(2): p. 172-8.
60. Pacheco, Y.M., et al., *Minor compounds of olive oil have postprandial anti-inflammatory effects*. (0007-1145 (Print)).
61. Rallidis, L.S., et al., *Close adherence to a Mediterranean diet improves endothelial function in subjects with abdominal obesity*. Am J Clin Nutr, 2009. **90**(2): p. 263-8.
62. Schrieks, I.C., et al., *Effect of red wine consumption on biomarkers of oxidative stress*. Alcohol Alcohol, 2013. **48**(2): p. 153-9.
63. Widmer, R.J., et al., *Beneficial effects of polyphenol-rich olive oil in patients with early atherosclerosis*. Eur J Nutr, 2013. **52**(3): p. 1223-31.
64. Yoo, Y.J., et al., *A cross-cultural study of wine consumers with respect to health benefits of wine*. Food Quality and Preference, 2013. **28**(2): p. 531-538.

65. Palinski, W., et al., *Low density lipoprotein undergoes oxidative modification in vivo*. Proc Natl Acad Sci U S A, 1989. **86**(4): p. 1372-6.
66. Huang, C.L. and B.E. Sumpio, *Olive oil, the mediterranean diet, and cardiovascular health*. J Am Coll Surg, 2008. **207**(3): p. 407-16.
67. Quiles, J.L., et al., *Olive oil phenolics: effects on DNA oxidation and redox enzyme mRNA in prostate cells*. (0007-1145 (Print)).
68. Ding, M., et al., *Long-Term Coffee Consumption and Risk of Cardiovascular Disease: A Systematic Review and a Dose-Response Meta-Analysis of Prospective Cohort Studies*. (1524-4539 (Electronic)).
69. Liu, J., et al., *Association of coffee consumption with all-cause and cardiovascular disease mortality*. (1942-5546 (Electronic)).
70. de la Torre, R., et al., *Is dopamine behind the health benefits of red wine?* (1436-6207 (Print)).

Appendix

Table 5. Combinations of the four compounds of interest and the different added amounts (c, caffeic acid, q, quercetin, t, tyrosol and h, hydroxytyrosol).

Mixture	Caffeic Acid (mg)	Quercetin (mg)	Tyrosol (mg)	Hydroxytyrosol (mg)
1	c 0.012	q 0.00025	t 0.03	h 0.2
2	c 0.012	q 0.00025	t 0.03	h 0.1
3	c 0.012	q 0.00025	t 0.03	h 0.05
4	c 0.012	q 0.00025	t 0.015	h 0.2
5	c 0.012	q 0.00025	t 0.015	h 0.1
6	c 0.012	q 0.00025	t 0.015	h 0.05
7	c 0.012	q 0.00025	t 0.0075	h 0.2
8	c 0.012	q 0.00025	t 0.0075	h 0.1
9	c 0.012	q 0.00025	t 0.0075	h 0.05
10	c 0.012	q 0.000125	t 0.03	h 0.2
11	c 0.012	q 0.000125	t 0.03	h 0.1
12	c 0.012	q 0.000125	t 0.03	h 0.05

13	c 0.012	q 0.000125	t 0.015	h 0.2
14	c 0.012	q 0.000125	t 0.015	h 0.1
15	c 0.012	q 0.000125	t 0.015	h 0.05
16	c 0.012	q 0.000125	t 0.0075	h 0.2
17	c 0.012	q 0.000125	t 0.0075	h 0.1
18	c 0.012	q 0.000125	t 0.0075	h 0.05
19	c 0.012	q 0.0000625	t 0.03	h 0.2
20	c 0.012	q 0.0000625	t 0.03	h 0.1
21	c 0.012	q 0.0000625	t 0.03	h 0.05
22	c 0.012	q 0.0000625	t 0.015	h 0.2
23	c 0.012	q 0.0000625	t 0.015	h 0.1
24	c 0.012	q 0.0000625	t 0.015	h 0.05
25	c 0.012	q 0.0000625	t 0.0075	h 0.2
26	c 0.012	q 0.0000625	t 0.0075	h 0.1
27	c 0.012	q 0.0000625	t 0.0075	h 0.05
28	c 0.006	q 0.00025	t 0.03	h 0.2

29	c 0.006	q 0.00025	t 0.03	h 0.1
30	c 0.006	q 0.00025	t 0.03	h 0.05
31	c 0.006	q 0.00025	t 0.015	h 0.2
32	c 0.006	q 0.00025	t 0.015	h 0.1
33	c 0.006	q 0.00025	t 0.015	h 0.05
34	c 0.006	q 0.00025	t 0.0075	h 0.2
35	c 0.006	q 0.00025	t 0.0075	h 0.1
36	c 0.006	q 0.00025	t 0.0075	h 0.05
37	c 0.006	q 0.000125	t 0.03	h 0.2
38	c 0.006	q 0.000125	t 0.03	h 0.1
39	c 0.006	q 0.000125	t 0.03	h 0.05
40	c 0.006	q 0.000125	t 0.015	h 0.2
41	c 0.006	q 0.000125	t 0.015	h 0.1
42	c 0.006	q 0.000125	t 0.015	h 0.05
43	c 0.006	q 0.000125	t 0.0075	h 0.2
44	c 0.006	q 0.000125	t 0.0075	h 0.1
45	c 0.006	q 0.000125	t 0.0075	h 0.05

46	c 0.006	q 0.0000625	t 0.03	h 0.2
47	c 0.006	q 0.0000625	t 0.03	h 0.1
48	c 0.006	q 0.0000625	t 0.03	h 0.05
49	c 0.006	q 0.0000625	t 0.015	h 0.2
50	c 0.006	q 0.0000625	t 0.015	h 0.1
51	c 0.006	q 0.0000625	t 0.015	h 0.05
52	c 0.006	q 0.0000625	t 0.0075	h 0.2
53	c 0.006	q 0.0000625	t 0.0075	h 0.1
54	c 0.006	q 0.0000625	t 0.0075	h 0.05
55	c 0.003	q 0.00025	t 0.03	h 0.2
56	c 0.003	q 0.00025	t 0.03	h 0.1
57	c 0.003	q 0.00025	t 0.03	h 0.05
58	c 0.003	q 0.00025	t 0.015	h 0.2
59	c 0.003	q 0.00025	t 0.015	h 0.1
60	c 0.003	q 0.00025	t 0.015	h 0.05
61	c 0.003	q 0.00025	t 0.0075	h 0.2
62	c 0.003	q 0.00025	t 0.0075	h 0.1

63	c 0.003	q 0.00025	t 0.0075	h 0.05
64	c 0.003	q 0.000125	t 0.03	h 0.2
65	c 0.003	q 0.000125	t 0.03	h 0.1
66	c 0.003	q 0.000125	t 0.03	h 0.05
67	c 0.003	q 0.000125	t 0.015	h 0.2
68	c 0.003	q 0.000125	t 0.015	h 0.1
69	c 0.003	q 0.000125	t 0.015	h 0.05
70	c 0.003	q 0.000125	t 0.0075	h 0.2
71	c 0.003	q 0.000125	t 0.0075	h 0.1
72	c 0.003	q 0.000125	t 0.0075	h 0.05
73	c 0.003	q 0.0000625	t 0.03	h 0.2
74	c 0.003	q 0.0000625	t 0.03	h 0.1
75	c 0.003	q 0.0000625	t 0.03	h 0.05
76	c 0.003	q 0.0000625	t 0.015	h 0.2
77	c 0.003	q 0.0000625	t 0.015	h 0.1
78	c 0.003	q 0.0000625	t 0.015	h 0.05
79	c 0.003	q 0.0000625	t 0.0075	h 0.2

80	c 0.003	q 0.0000625	t 0.0075	h 0.1
81	c 0.003	q 0.0000625	t 0.0075	h 0.05

*n=4 for figures 21-97

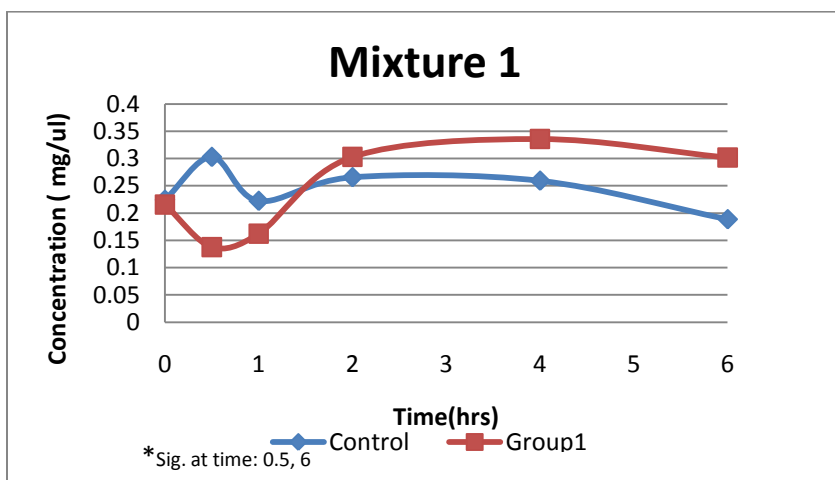


Figure 21. c 0.012 q 0.00025 t 0.03 h 0.2

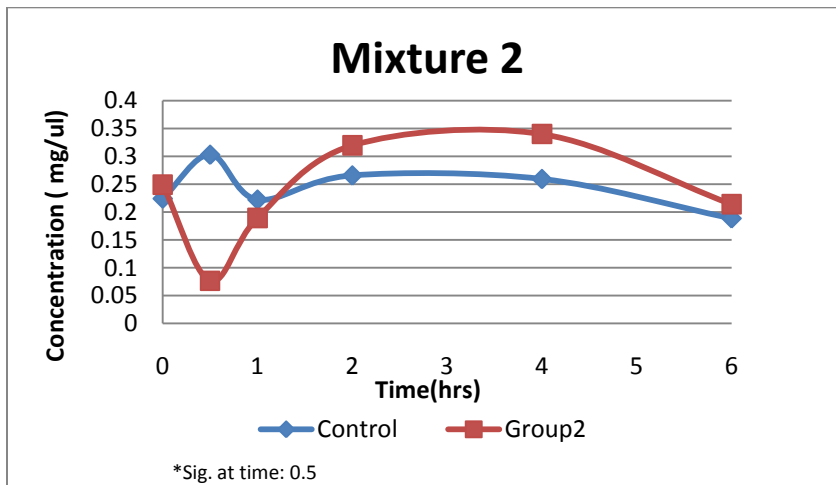


Figure 22. c 0.012 q 0.00025 t 0.03 h 0.1

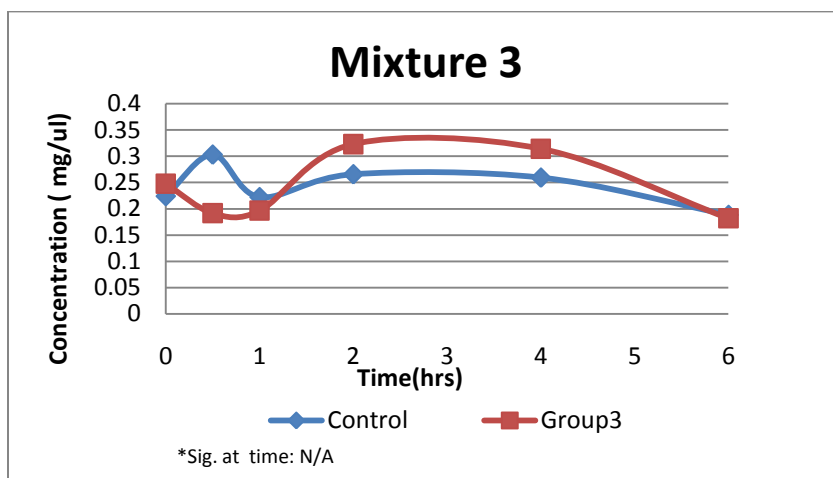


Figure 23. c 0.012 q 0.00025 t 0.03 h 0.05

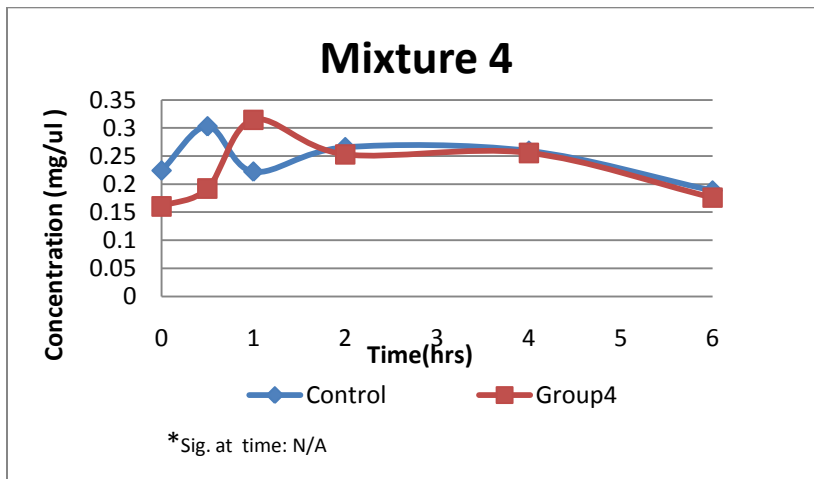


Figure 24. c 0.012 q 0.00025 t 0.015 h 0.2

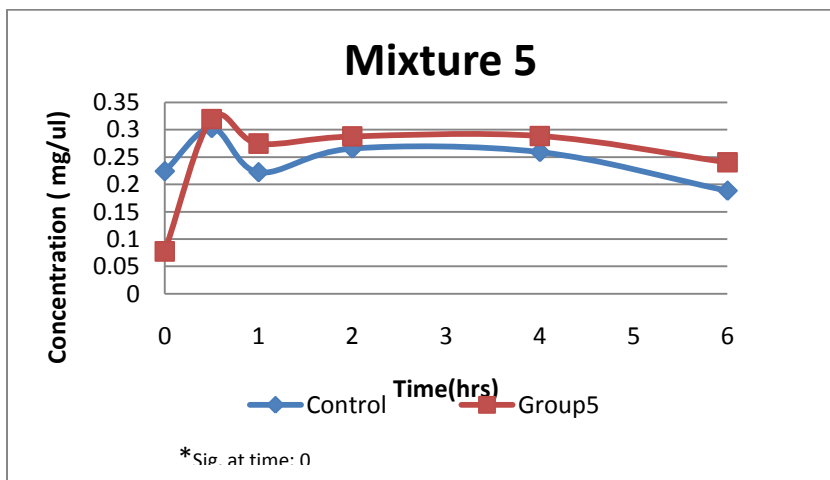


Figure 25. c 0.012 q 0.00025 t 0.015 h 0.1

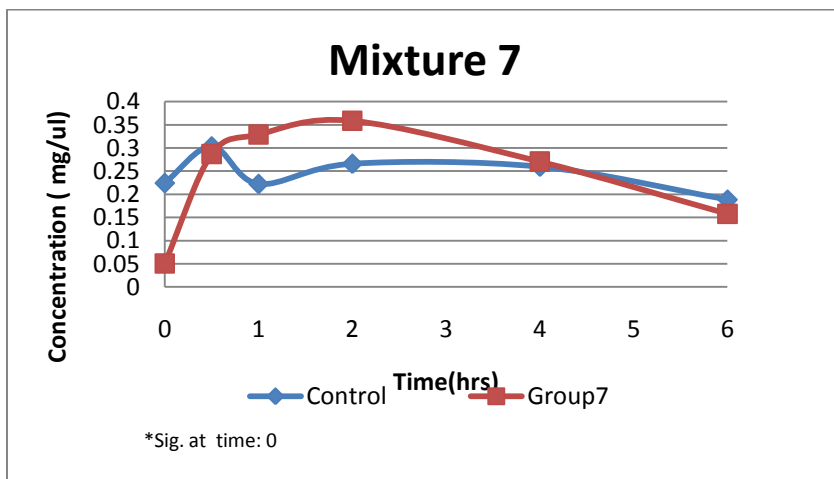


Figure 26. c 0.012 q 0.00025 t 0.0075 h 0.2

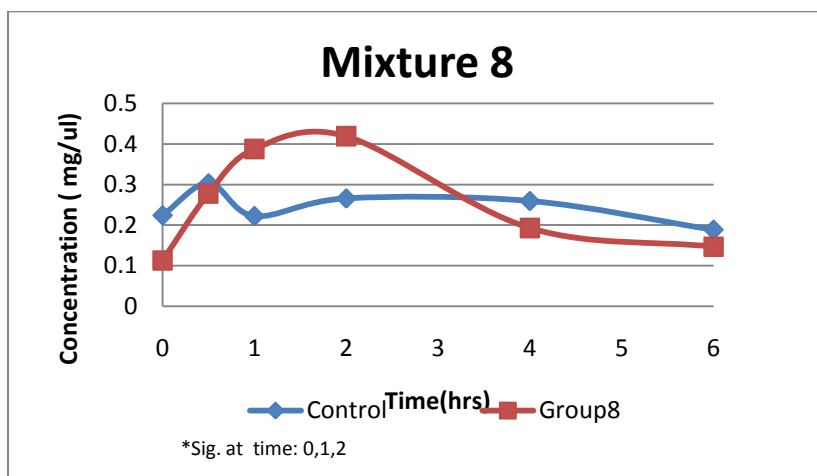


Figure 27. c 0.012 q 0.00025 t 0.0075 h 0.1

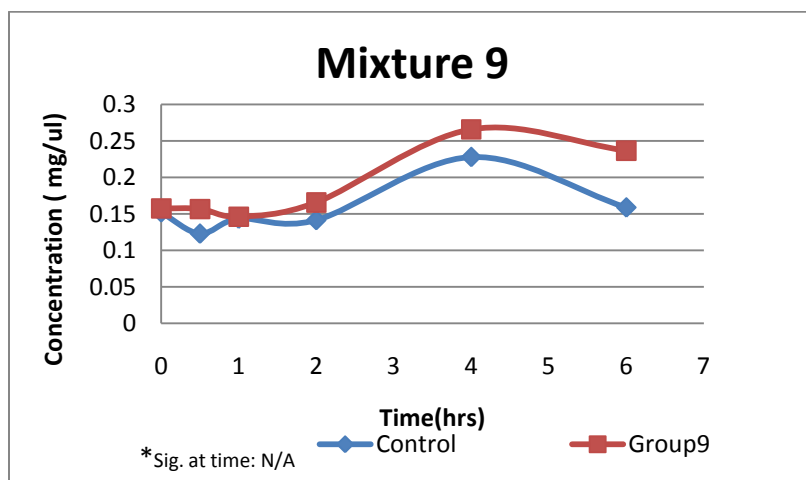


Figure 28. c 0.012 q 0.00025 t 0.0075 h 0.05

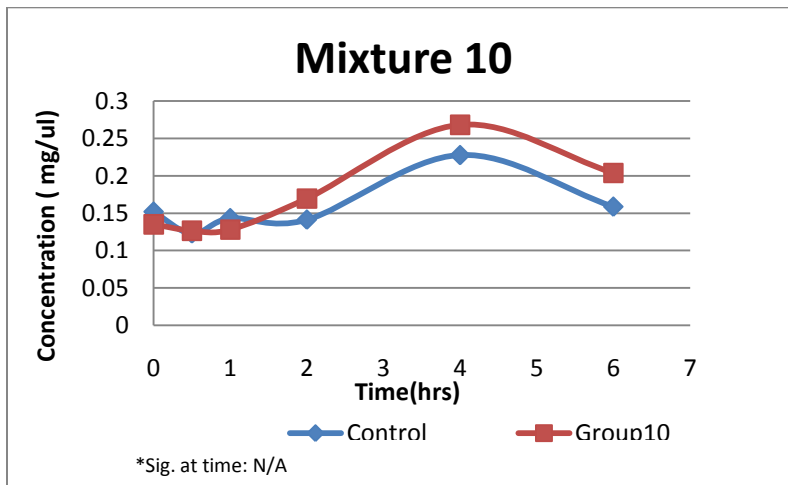


Figure 29. c 0.012 q 0.000125 t 0.03 h 0.2

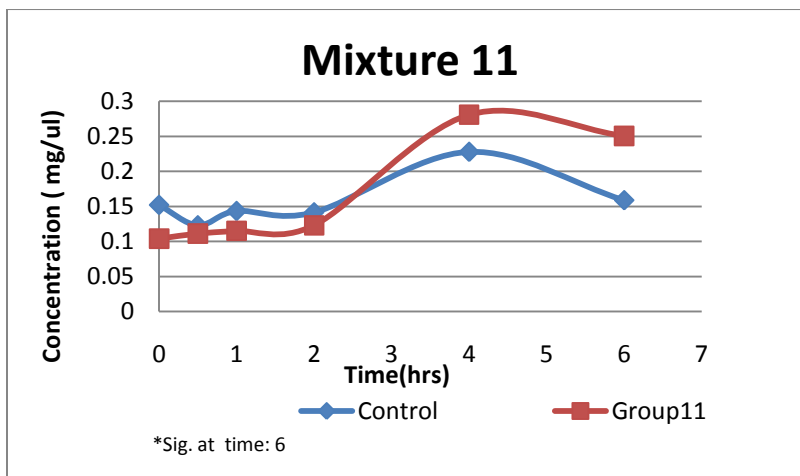


Figure 30. c 0.012 q 0.000125 t 0.03 h 0.1

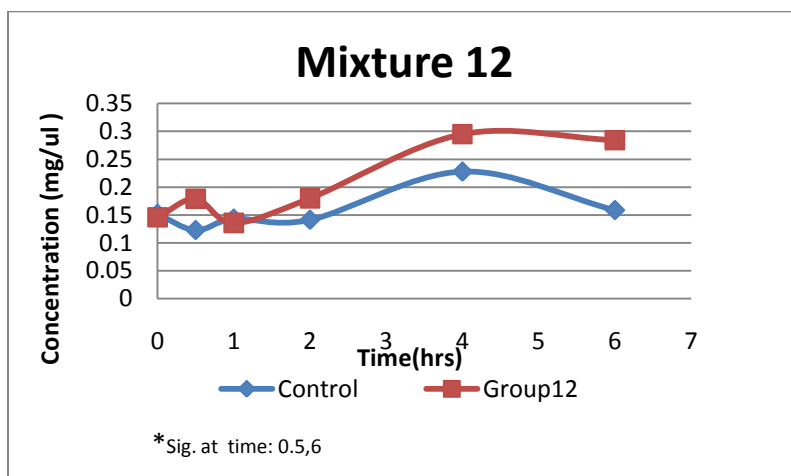


Figure 31. c 0.012 q 0.000125 t 0.03 h 0.05

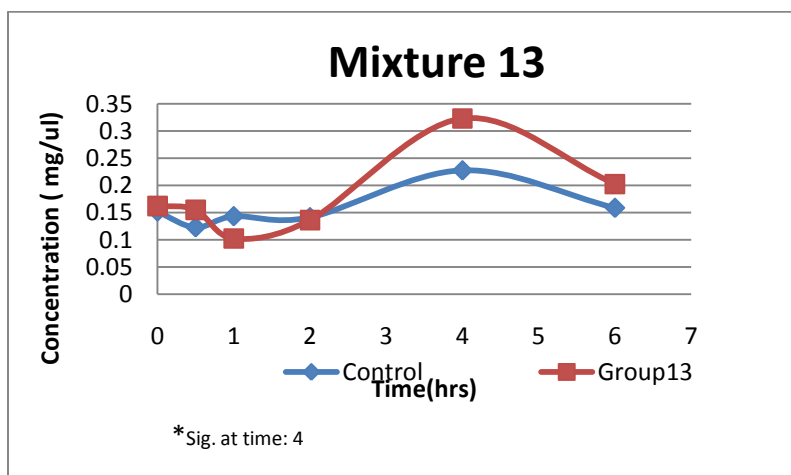


Figure 32. c 0.012 q 0.000125 t 0.015 h 0.2

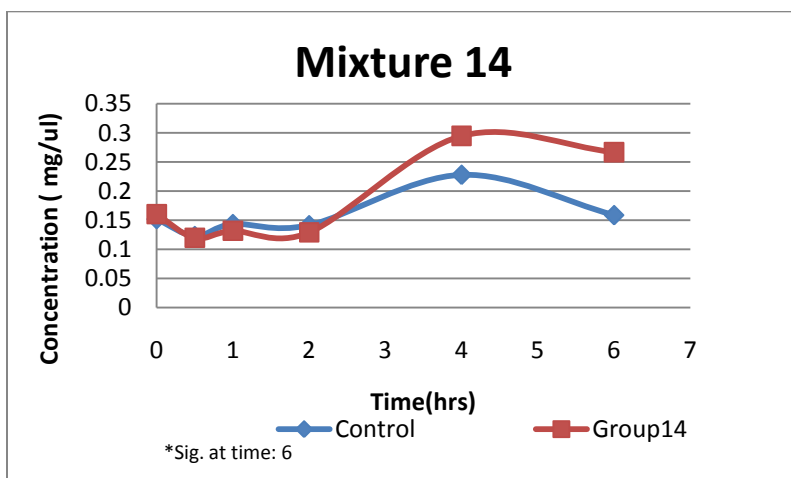


Figure 33. c 0.012 q 0.000125 t 0.015 h 0.1

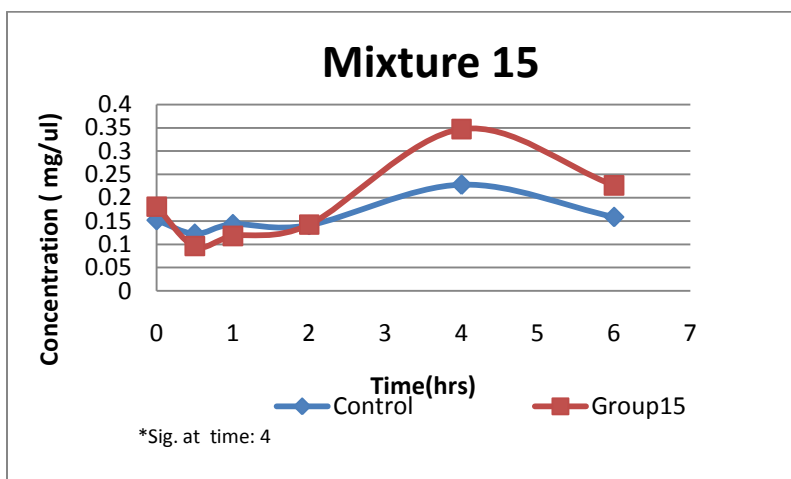


Figure 34. c 0.012 q 0.000125 t 0.015 h 0.05

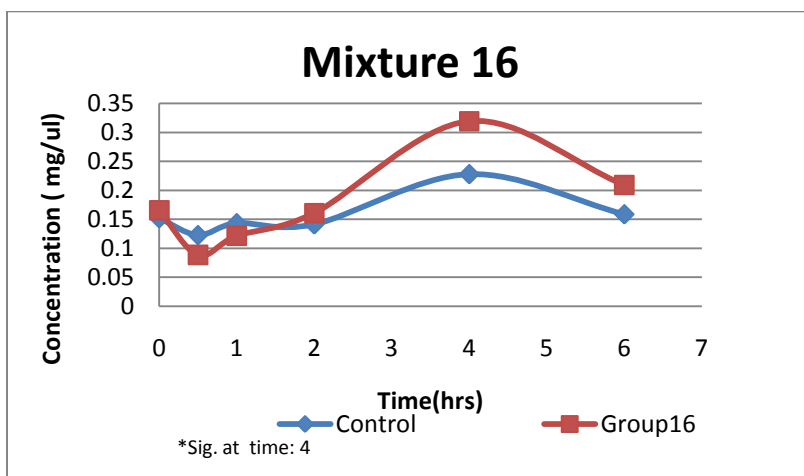


Figure 35. c 0.012 q 0.000125 t 0.0075 h 0.2

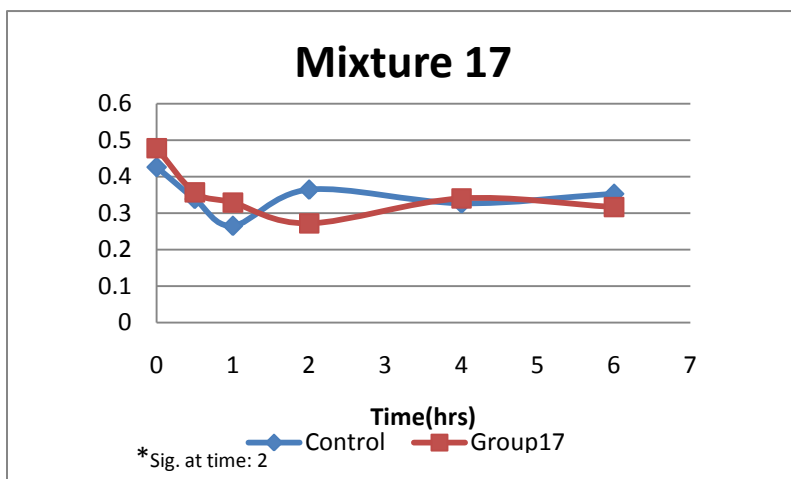


Figure 36. c 0.012 q 0.000125 t 0.0075 h 0.1

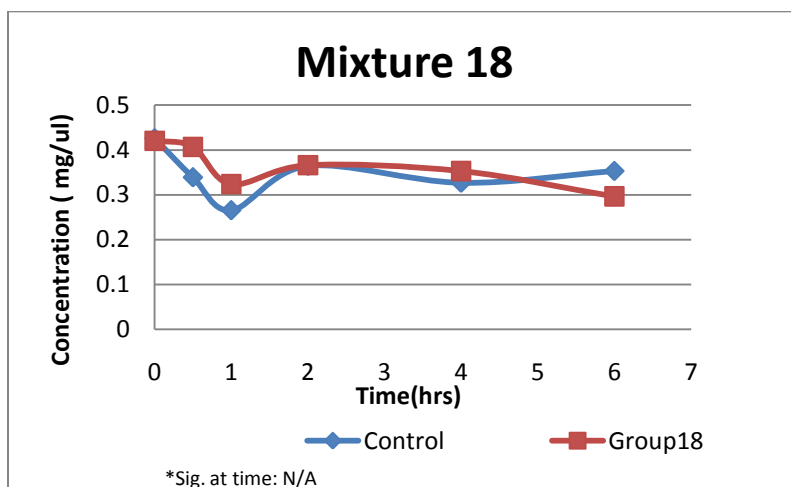


Figure 37. c 0.012 q 0.000125 t 0.0075 h 0.05

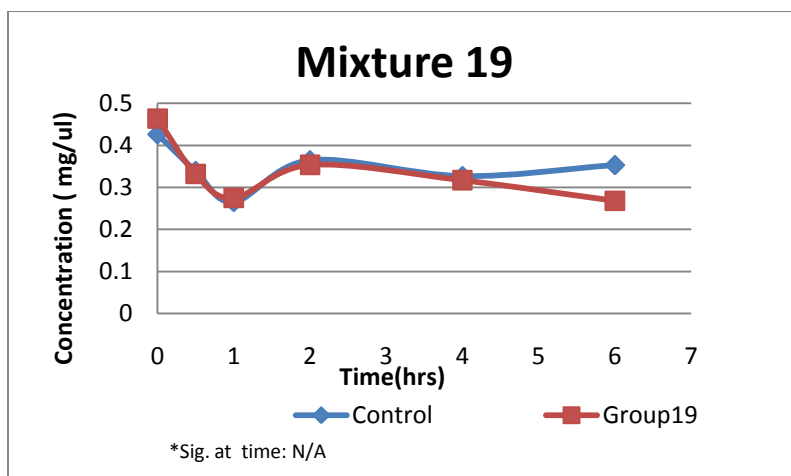


Figure 38. c 0.012 q 0.0000625 t 0.03 h 0.2

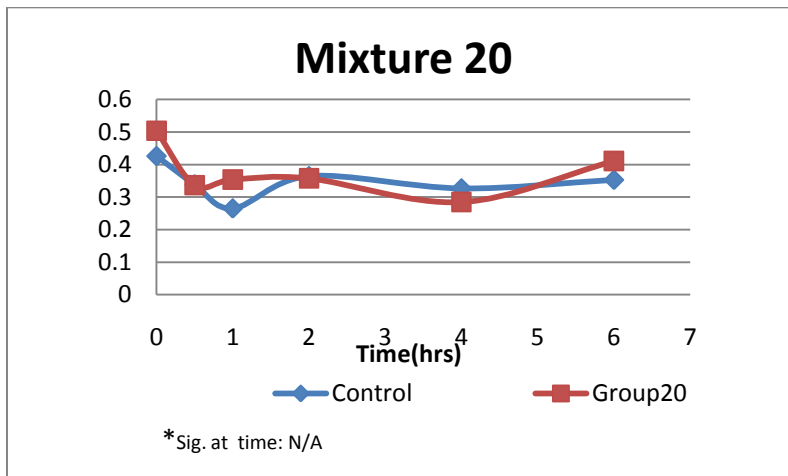


Figure 39. c 0.012 q 0.0000625 t 0.03 h 0.1

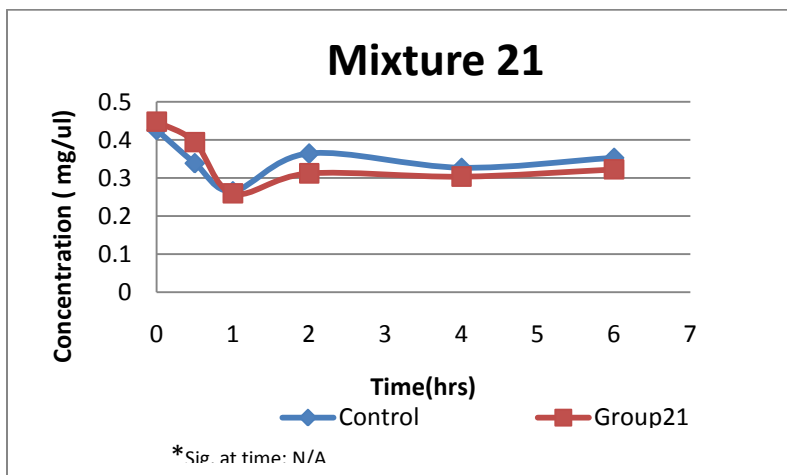


Figure 40. c 0.012 q 0.0000625 t 0.03 h 0.05

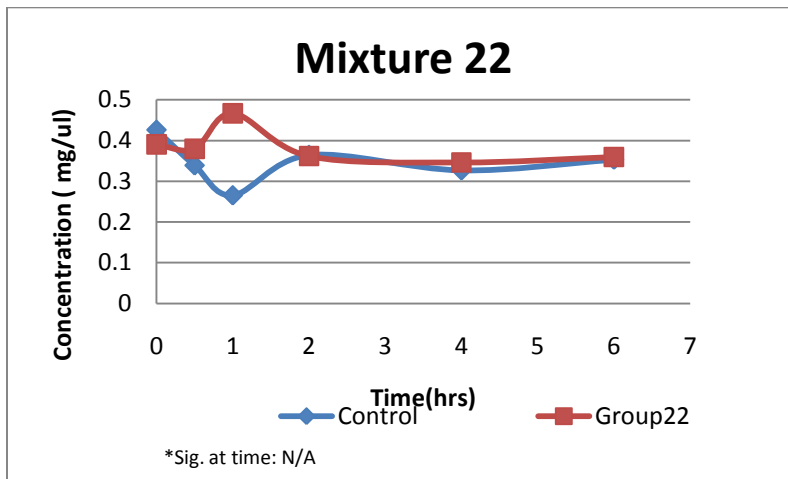


Figure 41. c 0.012 q 0.000625 t 0.015 h 0.2

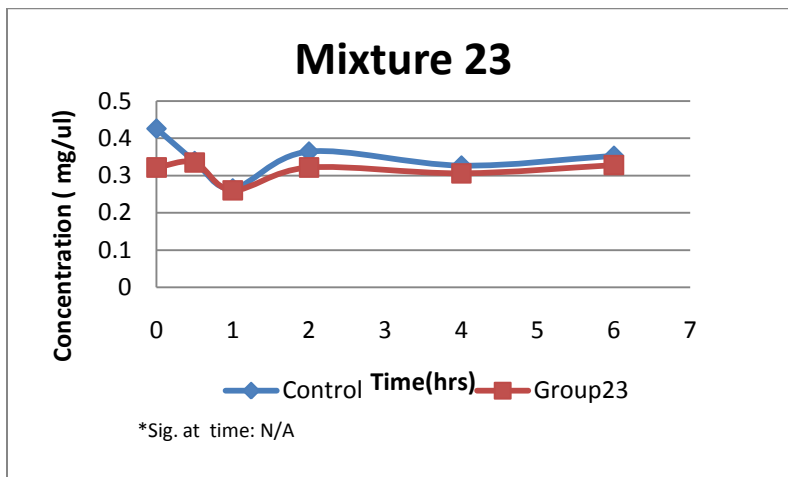


Figure 42. c 0.012 q 0.000625 t 0.015 h 0.1

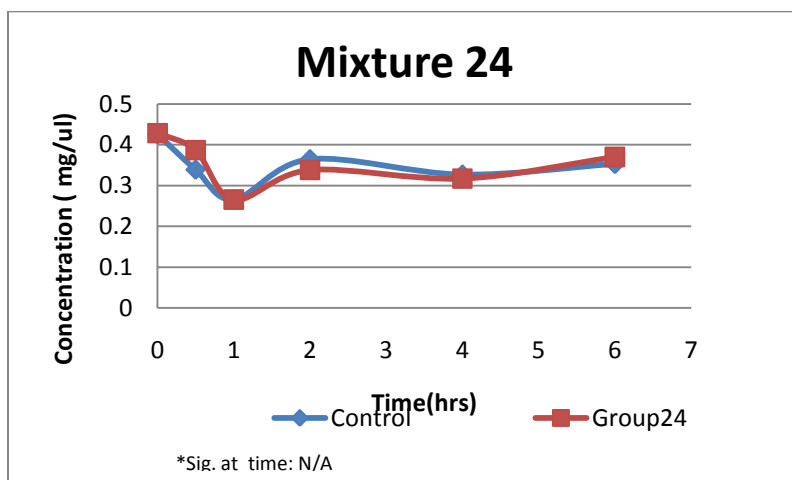


Figure 43. c 0.012 q 0.000625 t 0.015 h 0.05

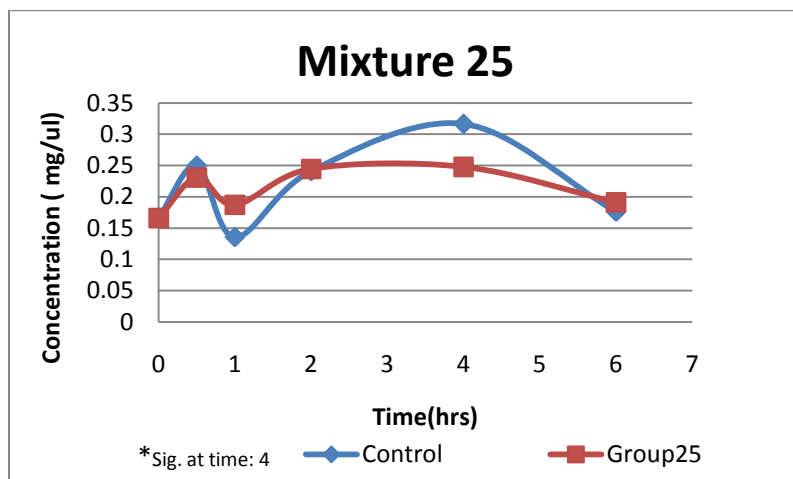


Figure 44. c 0.012 q 0.000625 t 0.0075 h 0.2

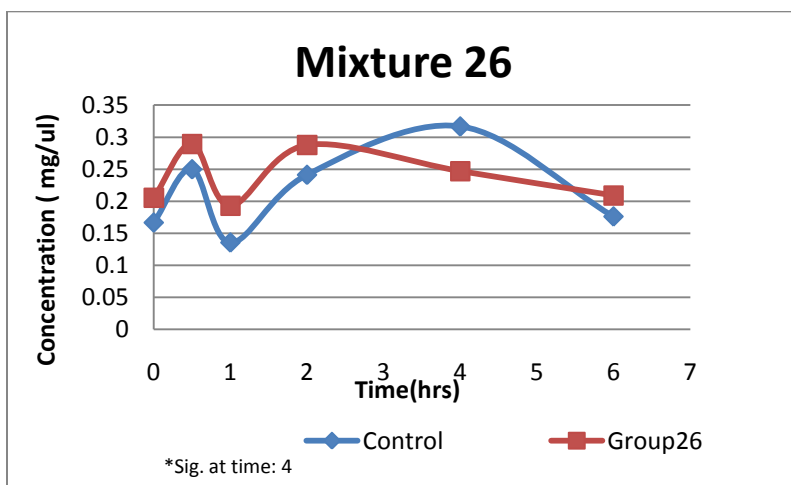


Figure 45. c 0.012 q 0.0000625 t 0.0075 h 0.1

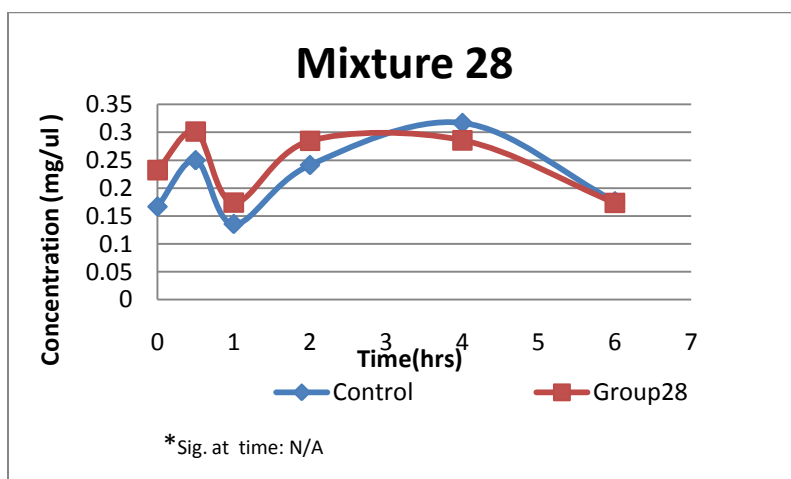


Figure 46. c 0.006 q 0.00025 t 0.03 h 0.2

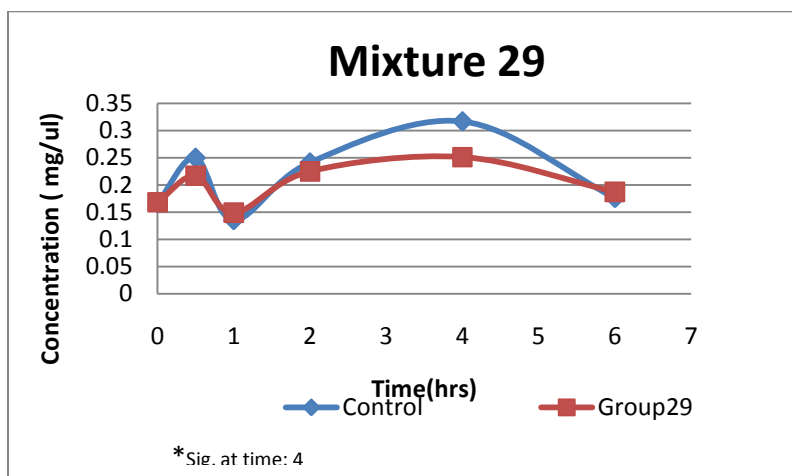


Figure 47. c 0.006 q 0.00025 t 0.03 h 0.1

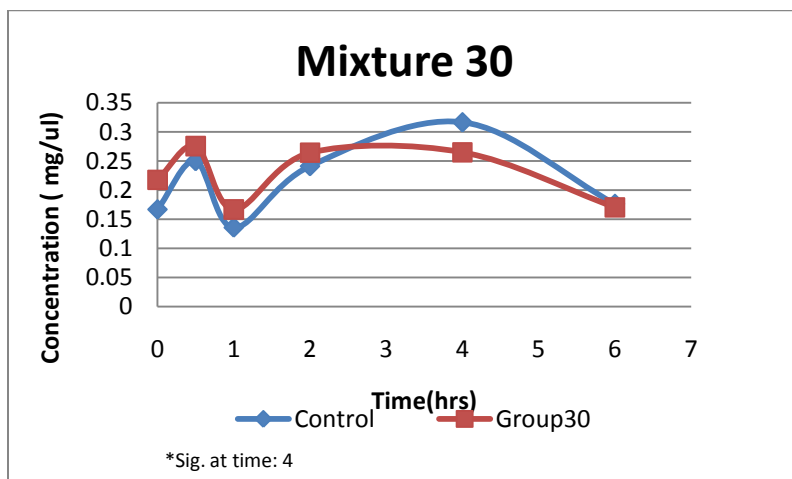


Figure 48. c 0.006 q 0.00025 t 0.03 h 0.05

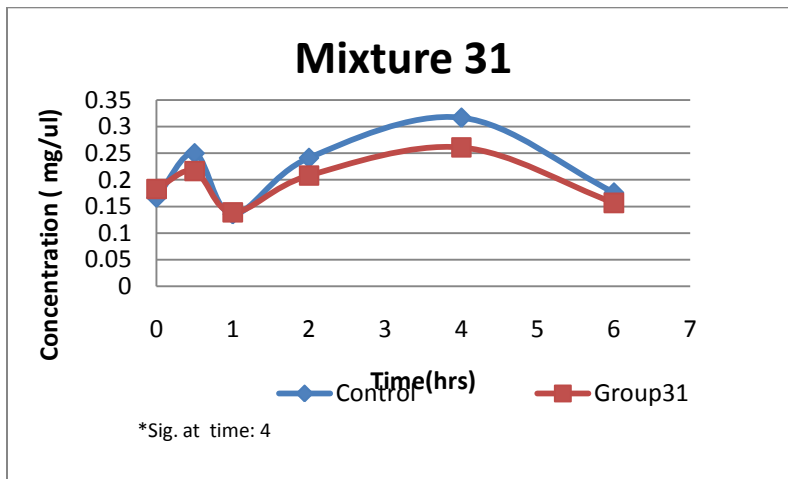


Figure 49. c 0.006 q 0.00025 t 0.015 h 0.2

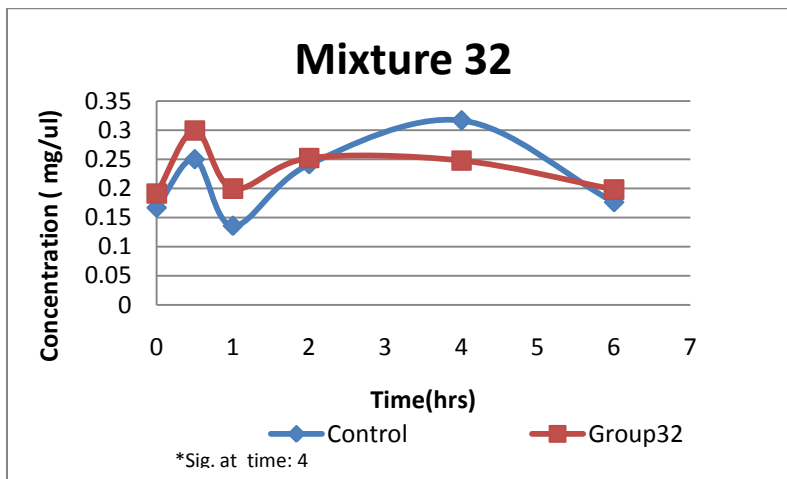


Figure 50. c 0.006 q 0.00025 t 0.015 h 0.1

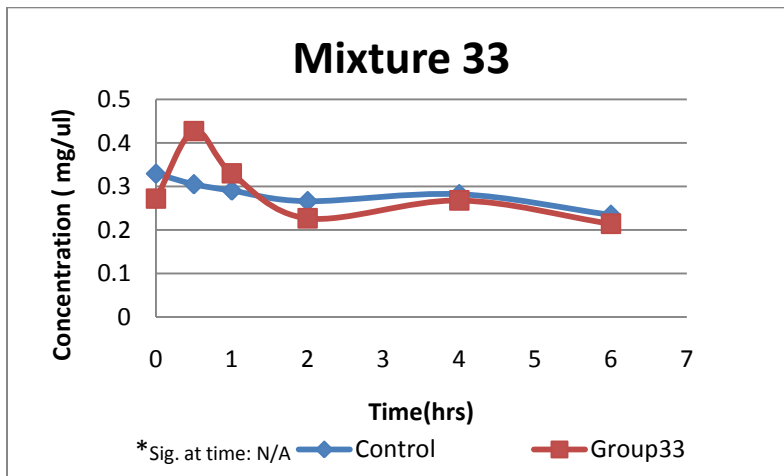


Figure 51. c 0.006 q 0.00025 t 0.015 h 0.05

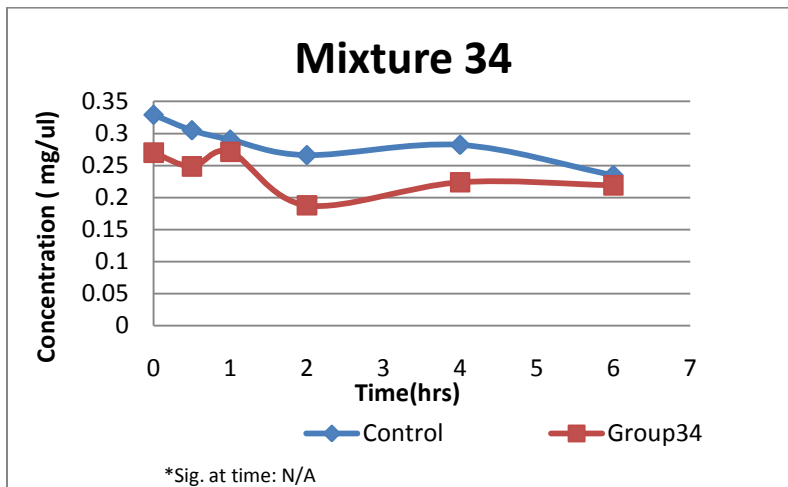


Figure 52. c 0.006 q 0.00025 t 0.0075 h 0.2

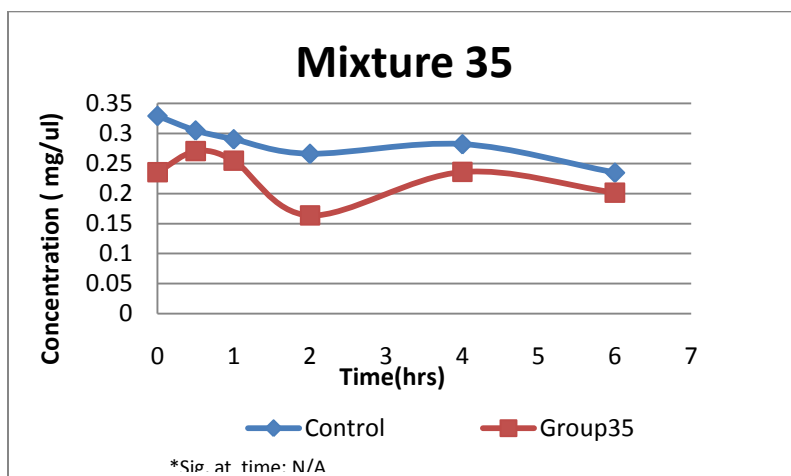


Figure 53. c 0.006 q 0.00025 t 0.0075 h 0.1

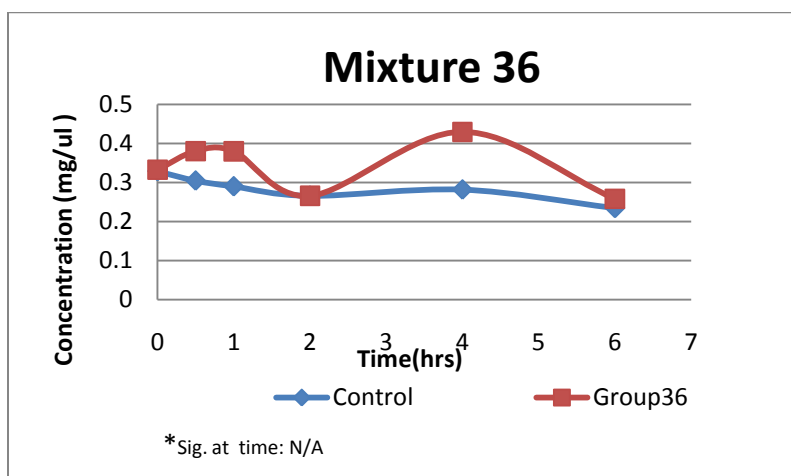


Figure 54. c 0.006 q 0.00025 t 0.0075 h 0.05

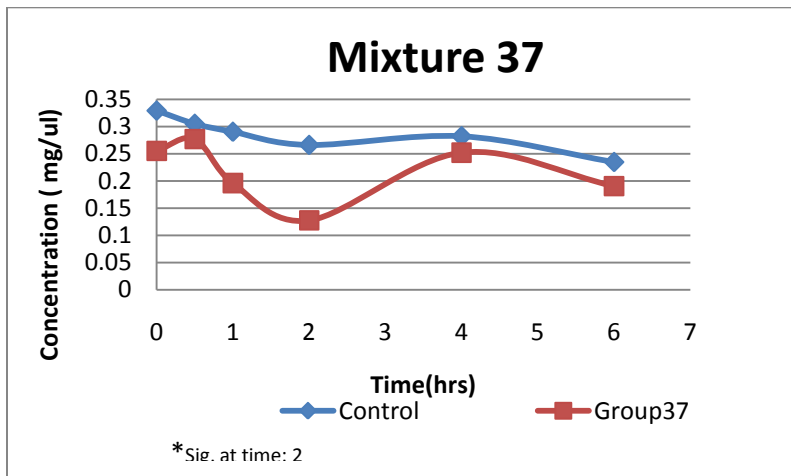


Figure 55. c 0.006 q 0.000125 t 0.03 h 0.2

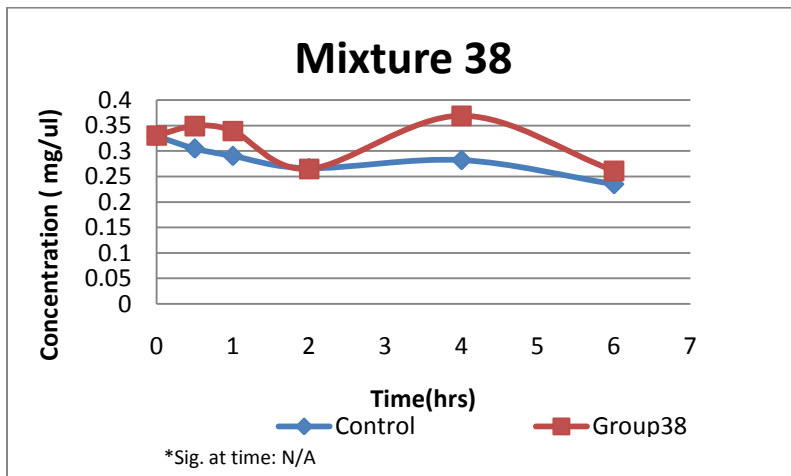


Figure 56. c 0.006 q 0.000125 t 0.03 h 0.1

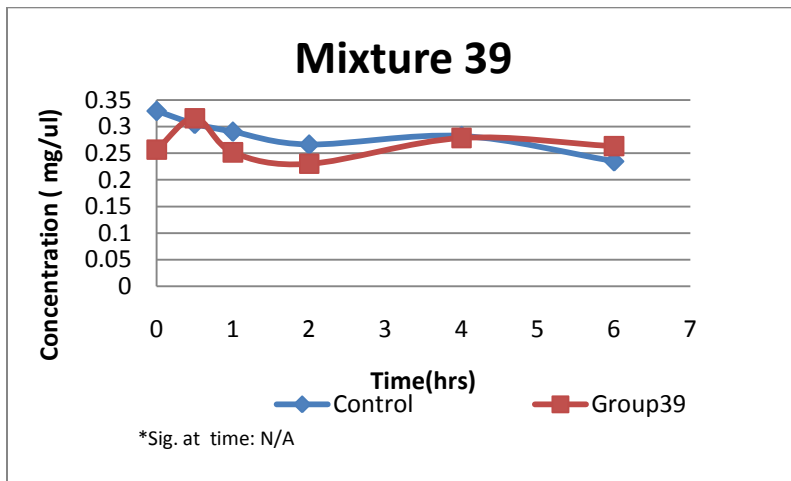


Figure 57. c 0.006q 0.000125 t 0.03 h 0.05

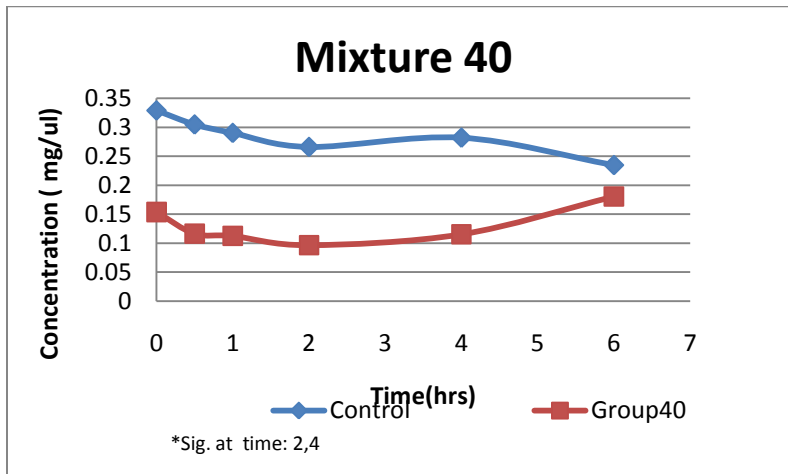


Figure 58. c 0.006 q 0.000125 t 0.015 h 0.2

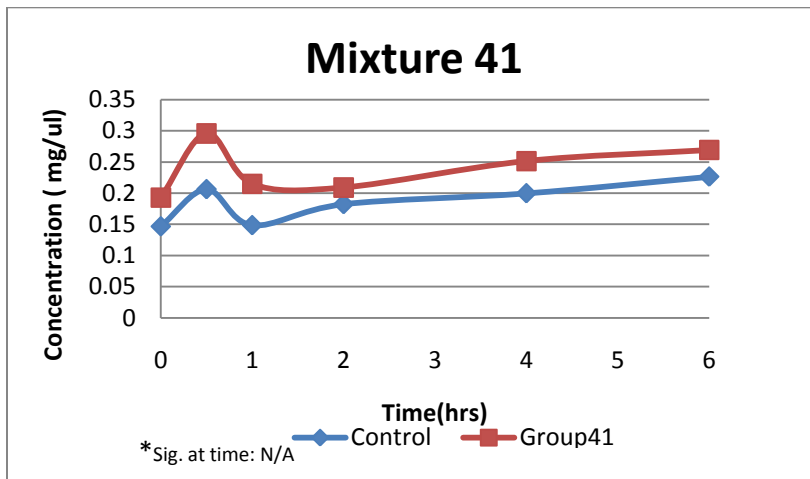


Figure 59. c 0.006 q 0.000125 t 0.015 h 0.1

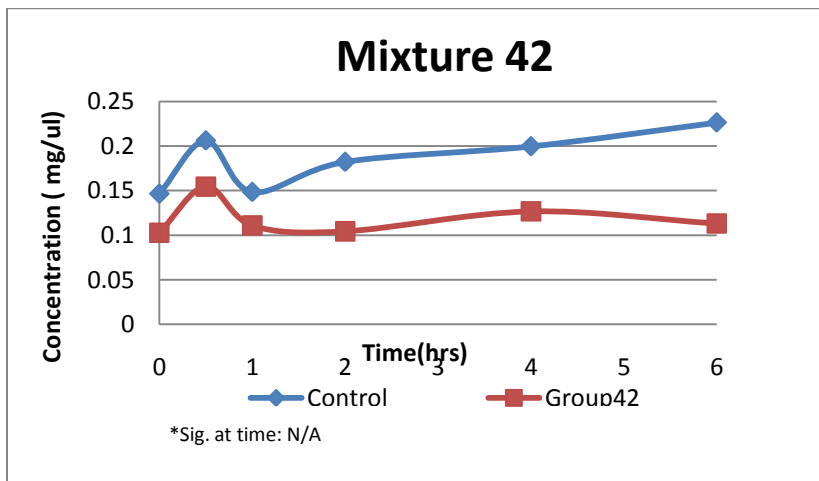


Figure 60. c 0.006 q 0.000125 t 0.015 h 0.05

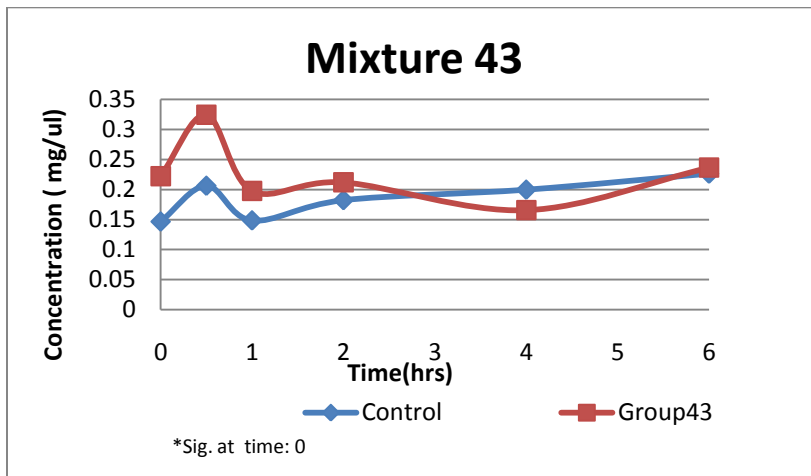


Figure 61. c 0.006 q 0.000125 t 0.0075 h 0.2

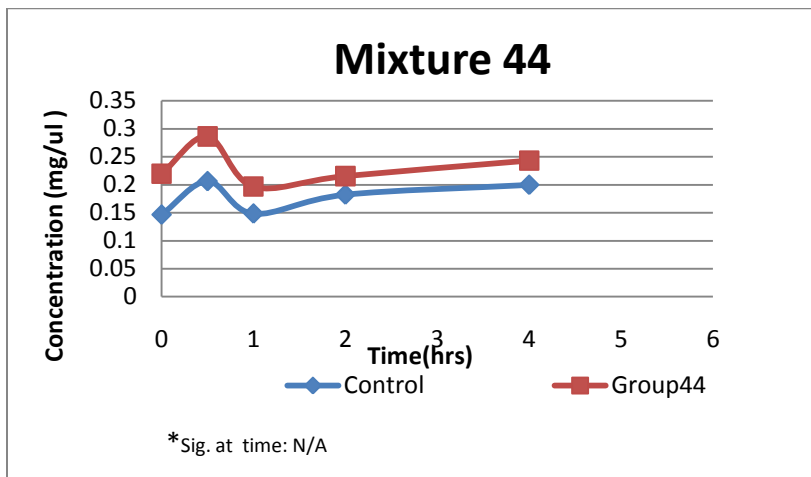


Figure 62. c 0.006 q 0.000125 t 0.0075 h 0.1

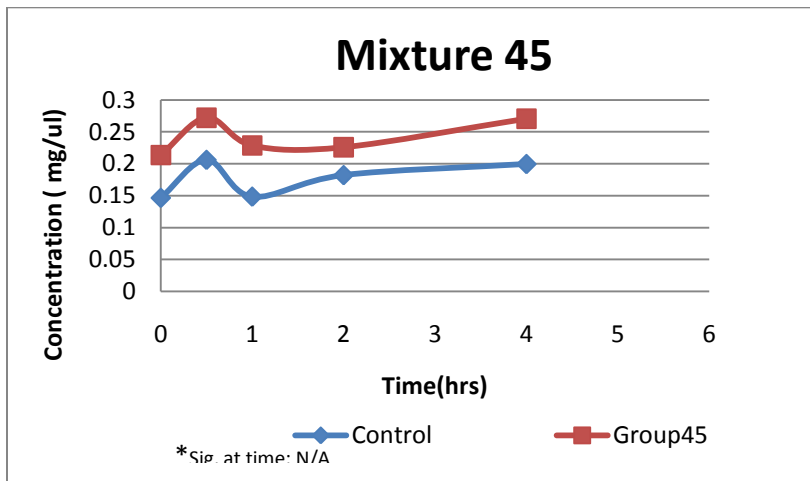


Figure 63. c 0.006 q 0.000125 t 0.0075 h 0.05

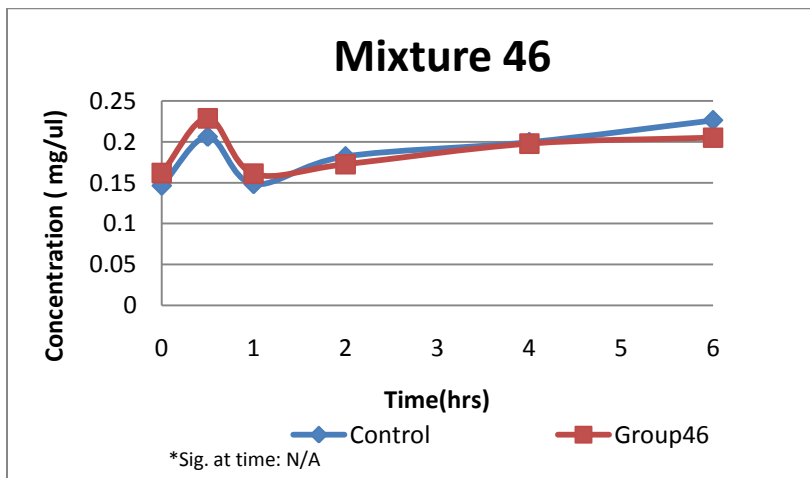


Figure 64. c 0.006 q 0.0000625 t 0.03 h 0.2

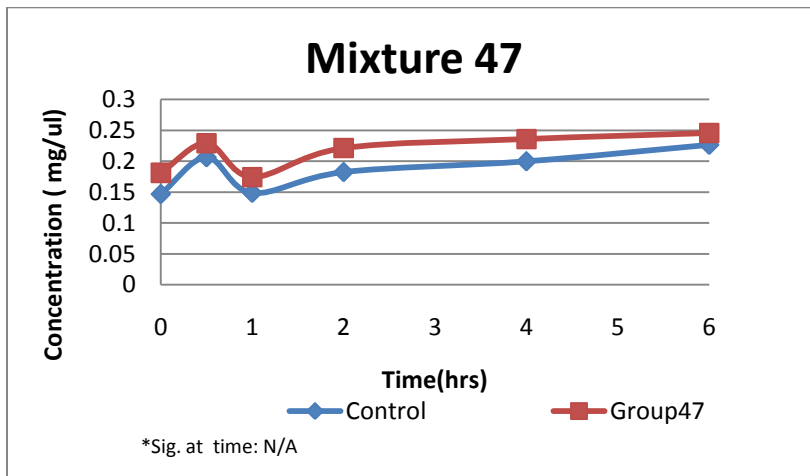


Figure 65. c 0.006q 0.0000625 t 0.03 h 0.1

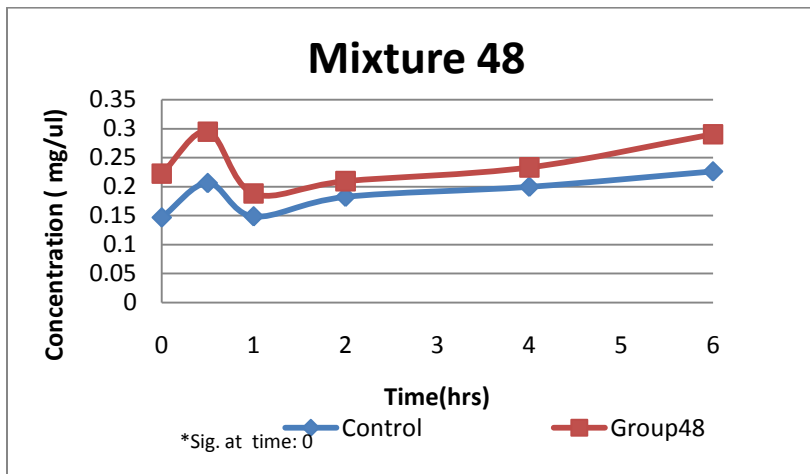


Figure 66. c 0.006q 0.0000625 t 0.03 h 0.05

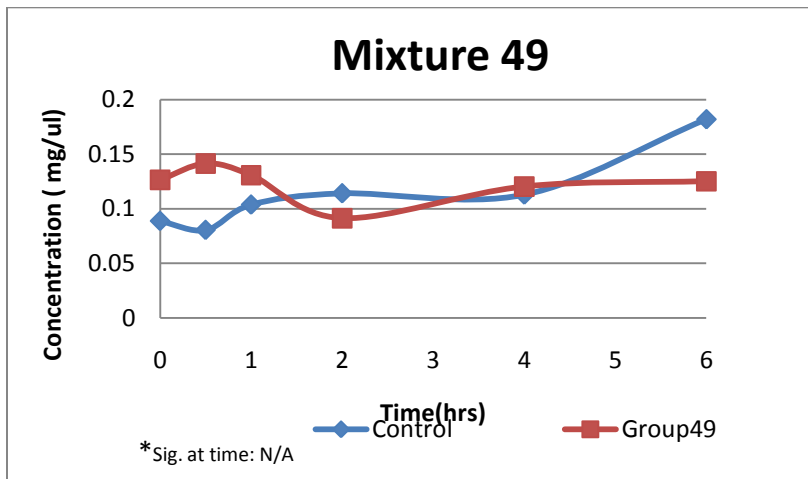


Figure 67. c 0.006 q 0.0000625 t 0.015 h 0.2

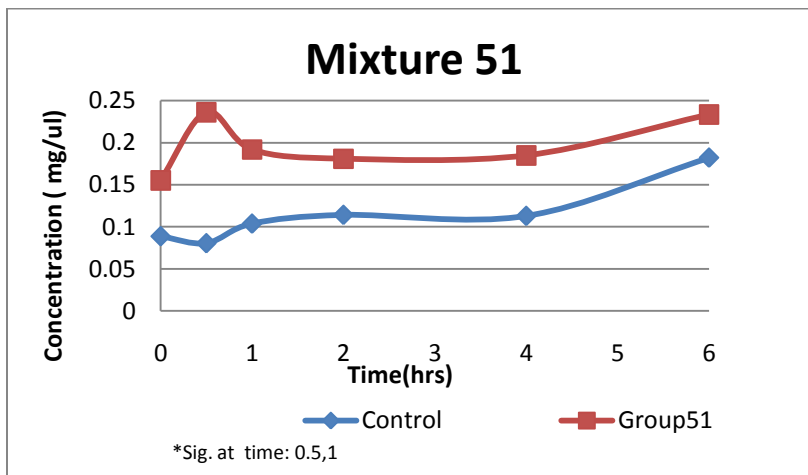


Figure 68. c 0.006 q 0.0000625 t 0.015 h 0.05

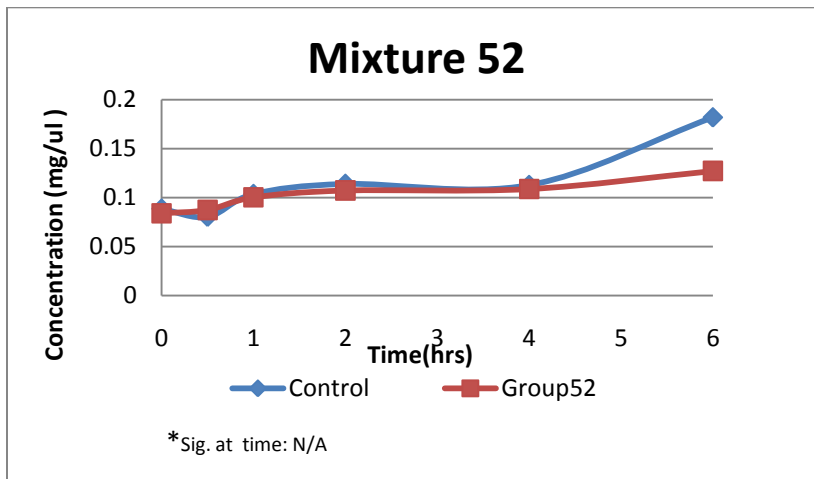


Figure 69. c 0.006 q 0.0000625 t 0.0075 h 0.2

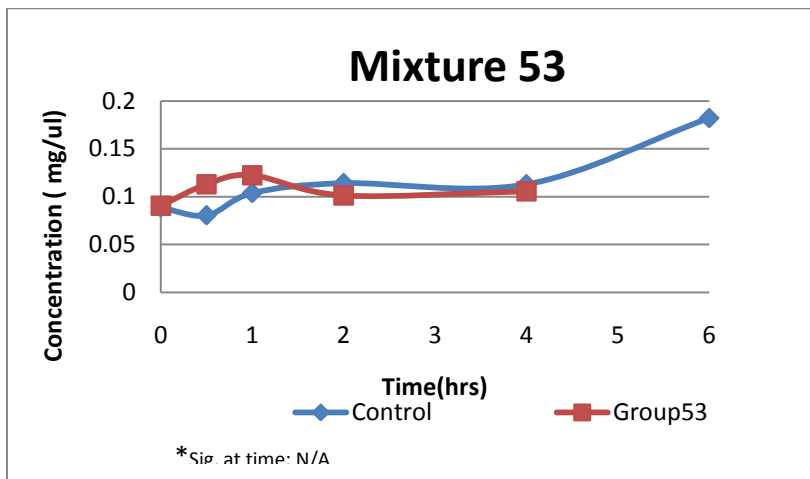


Figure 70. c 0.006 q 0.0000625 t 0.0075 h 0.1

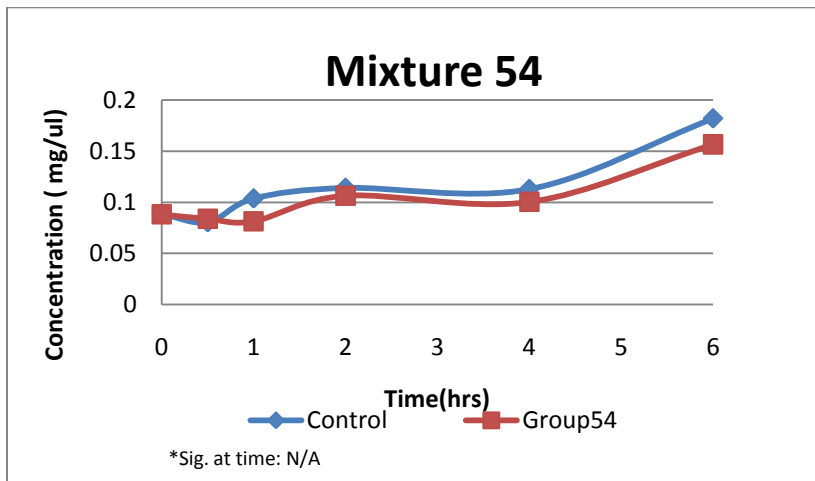


Figure 71. c 0.006 q 0.0000625 t 0.0075 h 0.05

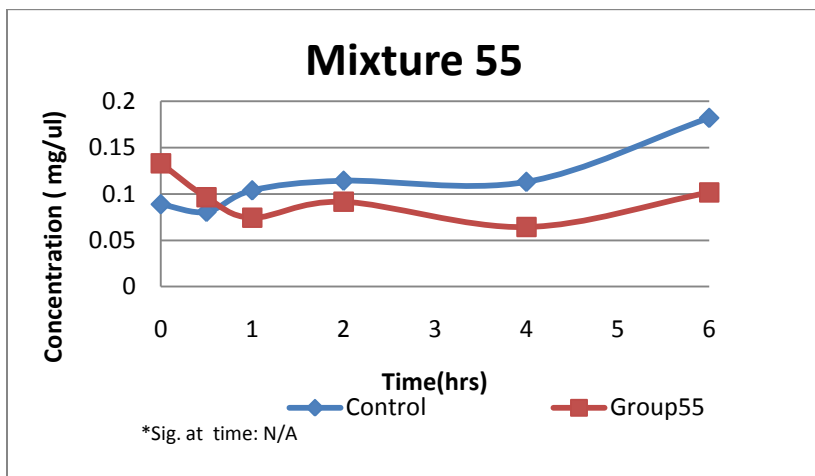


Figure 72. c 0.003 q 0.00025 t 0.03 h 0.2

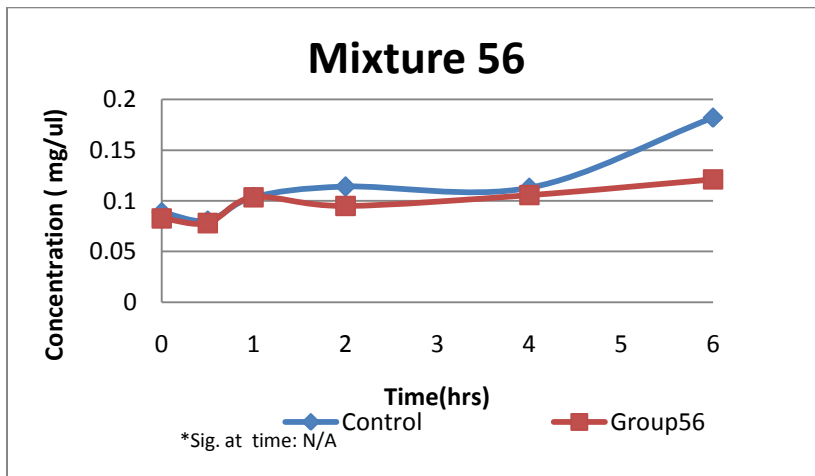


Figure 73. c 0.003 q 0.00025 t 0.03 h 0.1

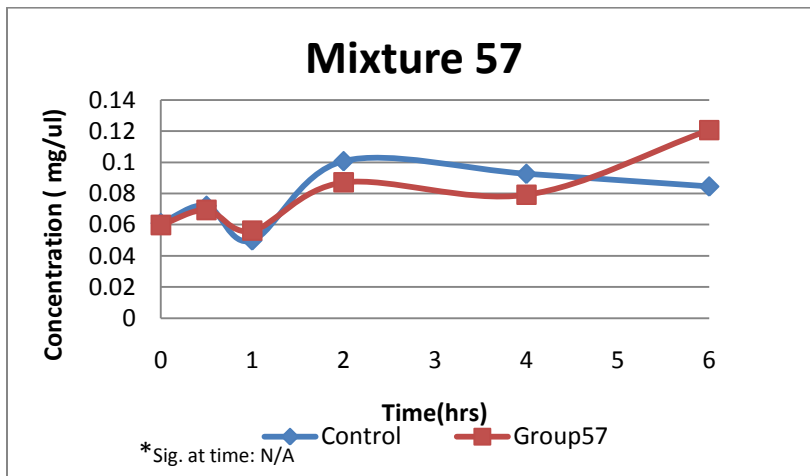


Figure 74. c 0.003 q 0.00025 t 0.03 h 0.05

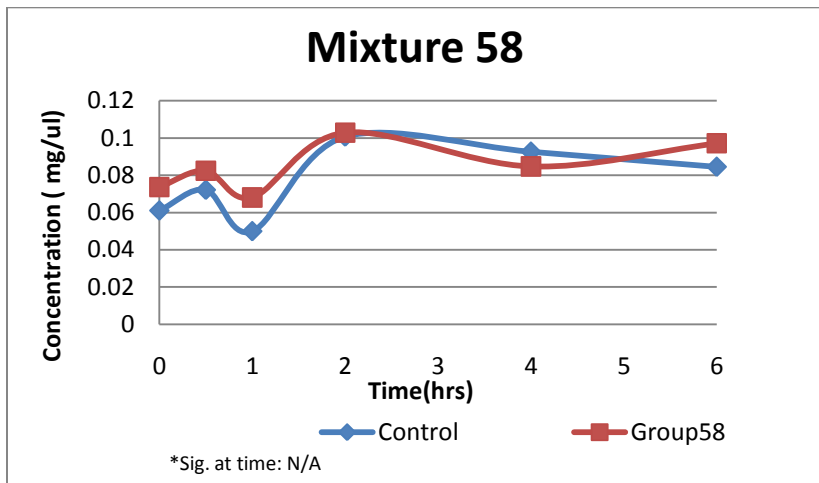


Figure 75. c 0.003 q 0.00025 t 0.015 h 0.2

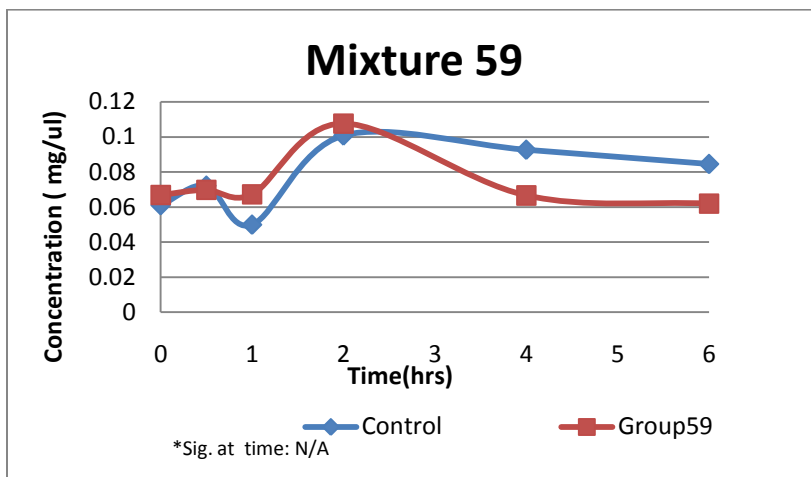


Figure 76. c 0.003 q 0.00025 t 0.015 h 0.1

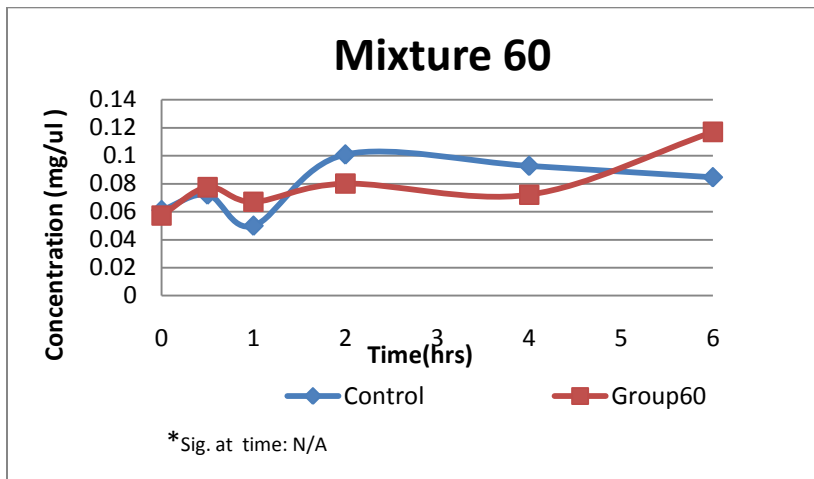


Figure 77. c 0.003 q 0.00025 t 0.015 h 0.05

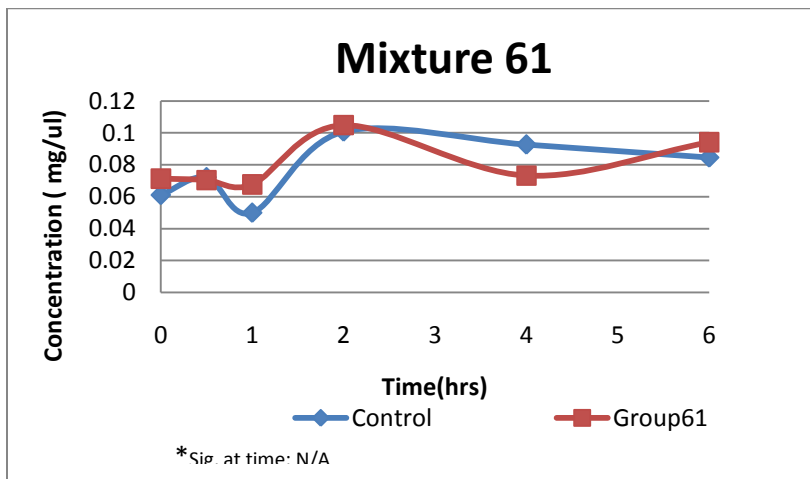


Figure 78. c 0.003 q 0.00025 t 0.0075 h 0.2

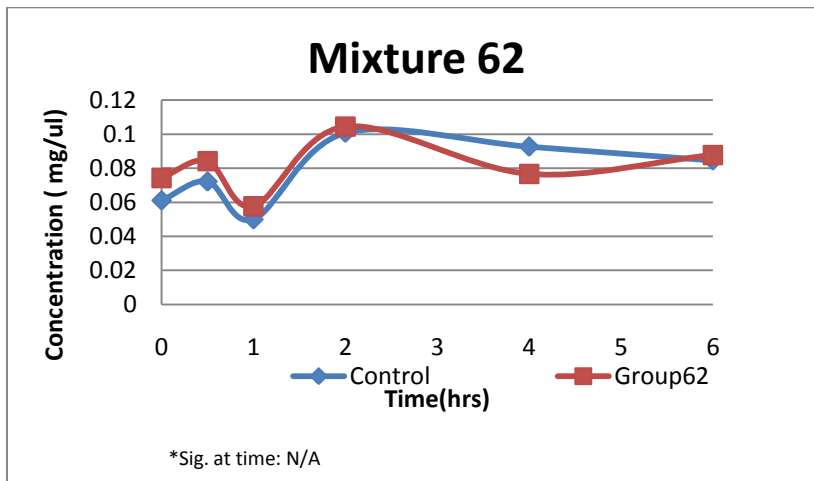


Figure 79. c 0.003 q 0.00025 t 0.0075 h 0.1

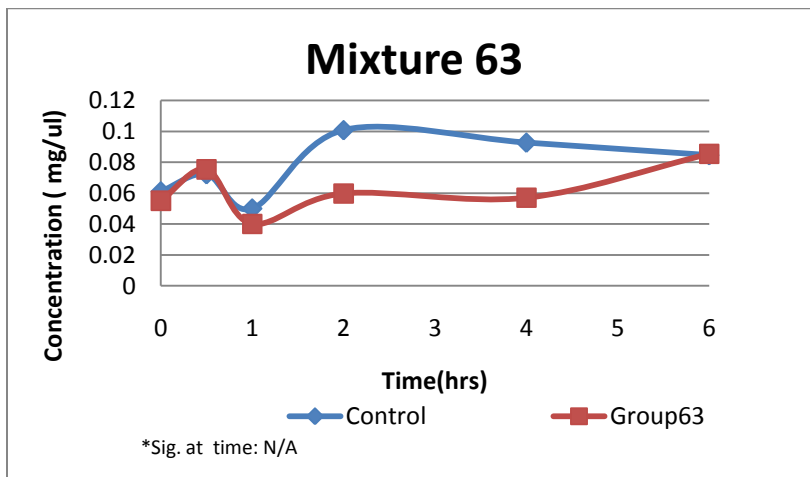


Figure 80. c 0.003 q 0.00025 t 0.0075 h 0.05

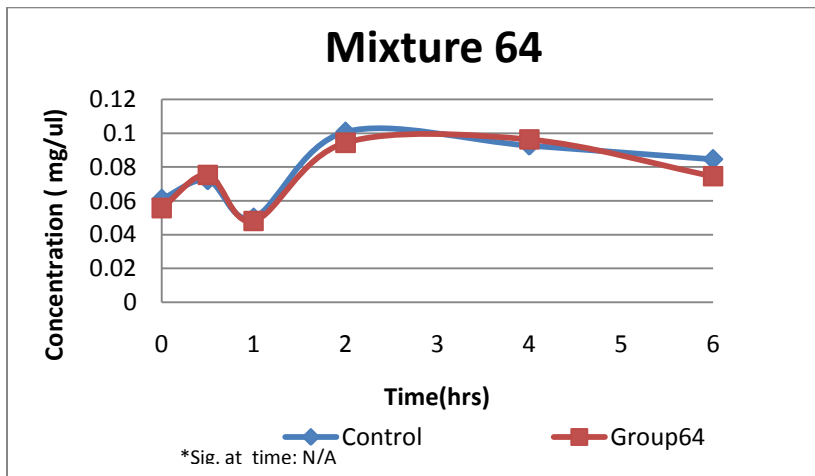


Figure 81. c 0.003 q 0.000125 t 0.03 h 0.2

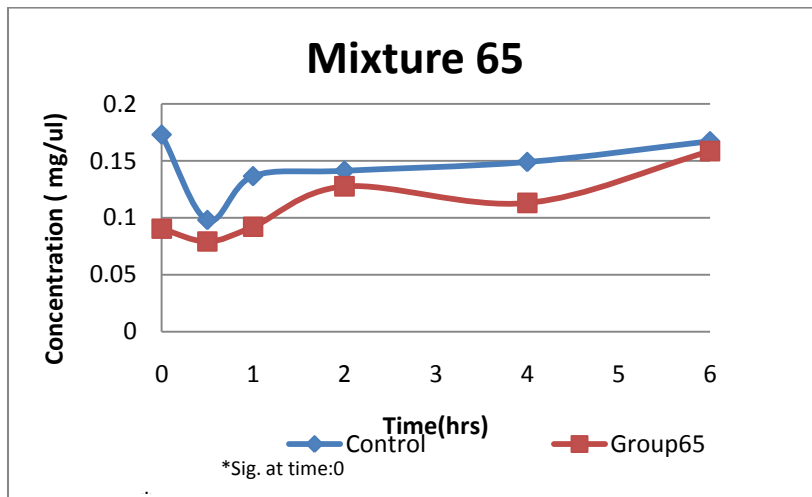


Figure 82. c 0.003 q 0.000125 t 0.03 h 0.1

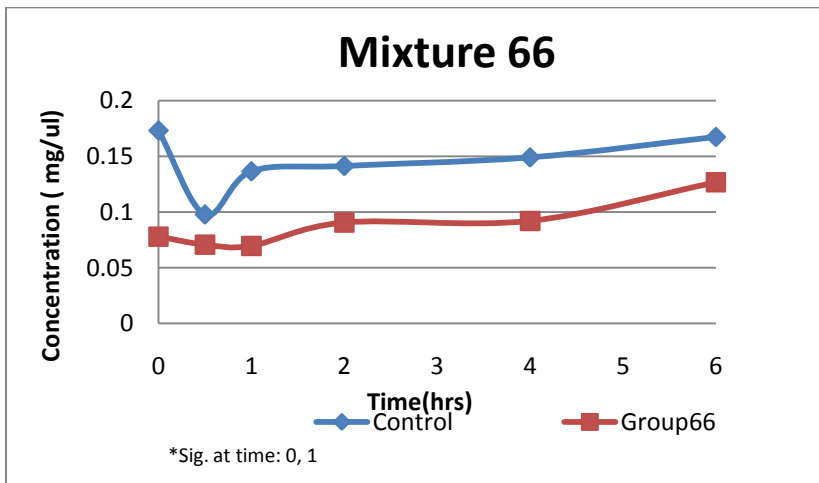


Figure 83. c 0.003 q 0.000125 t 0.03 h 0.05

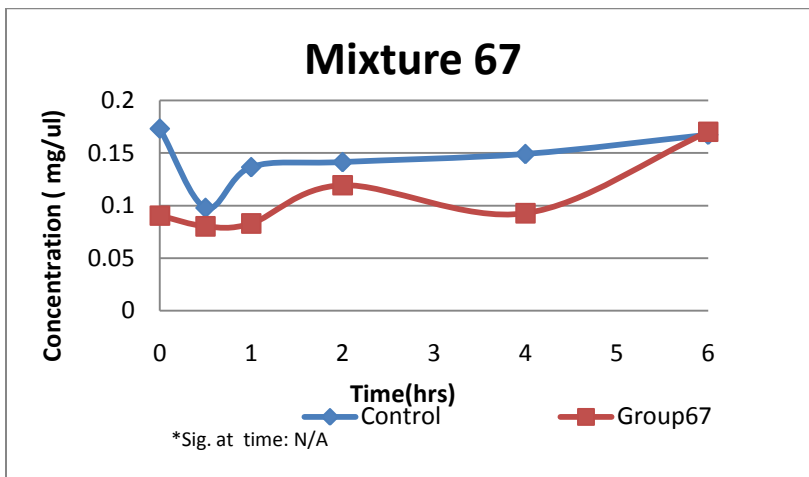


Figure 84. c 0.003 q 0.000125 t 0.015 h 0.2

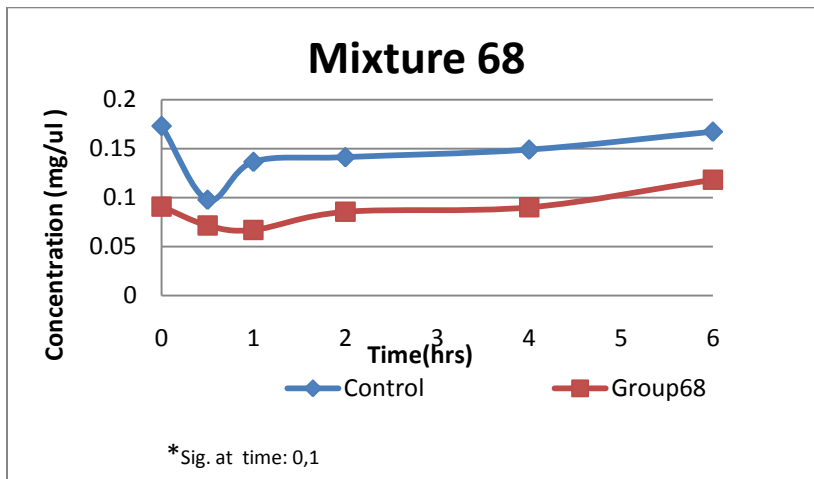


Figure 85. c 0.003q 0.000125 t 0.015 h 0.1

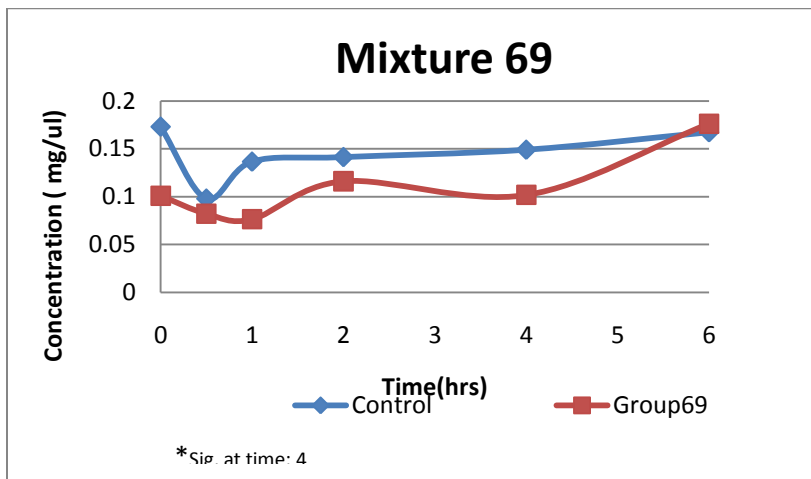


Figure 86. c 0.003 q 0.000125 t 0.015 h 0.05

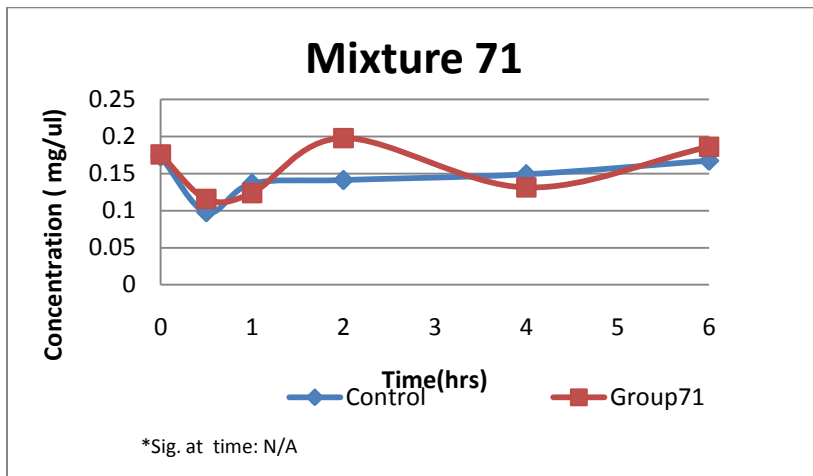


Figure 87. c 0.003 q 0.000125 t 0.0075 h 0.1

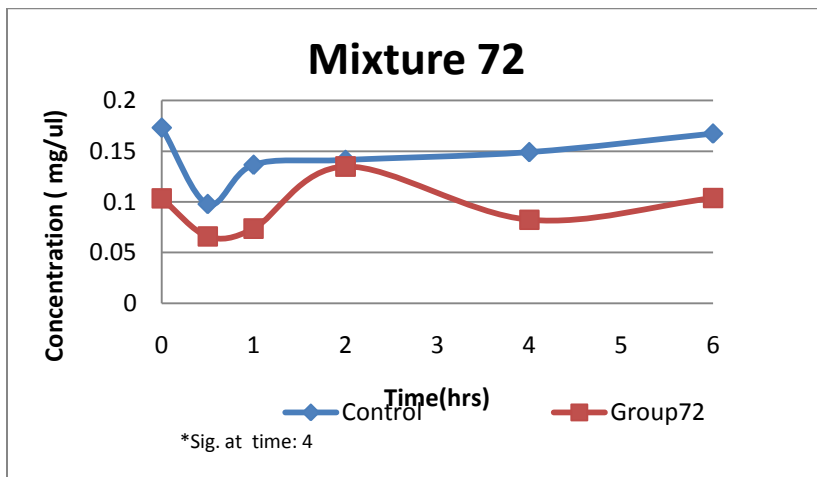


Figure 88. c 0.003 q 0.000125 t 0.0075 h 0.05

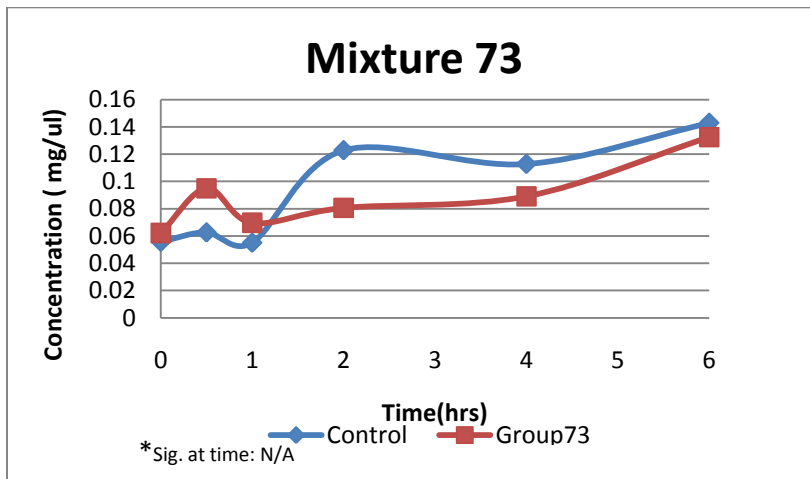


Figure 89. c 0.003 q 0.0000625 t 0.03 h 0.2

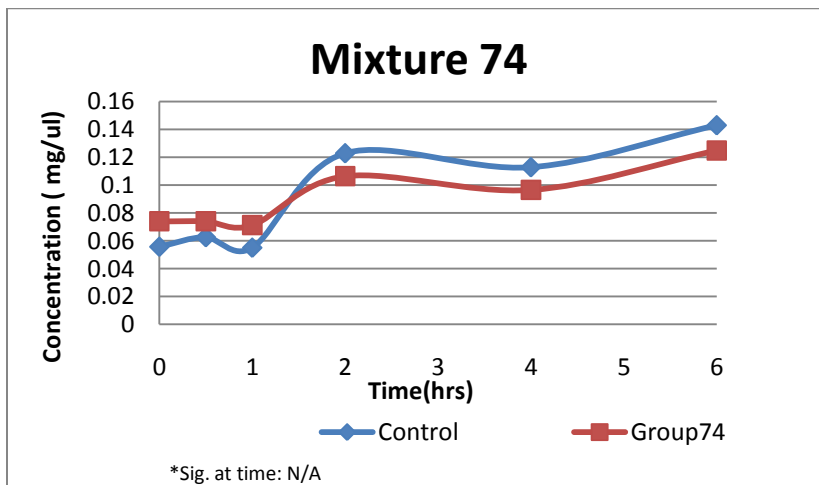


Figure 90. c 0.003 q 0.0000625 t 0.03 h 0.1

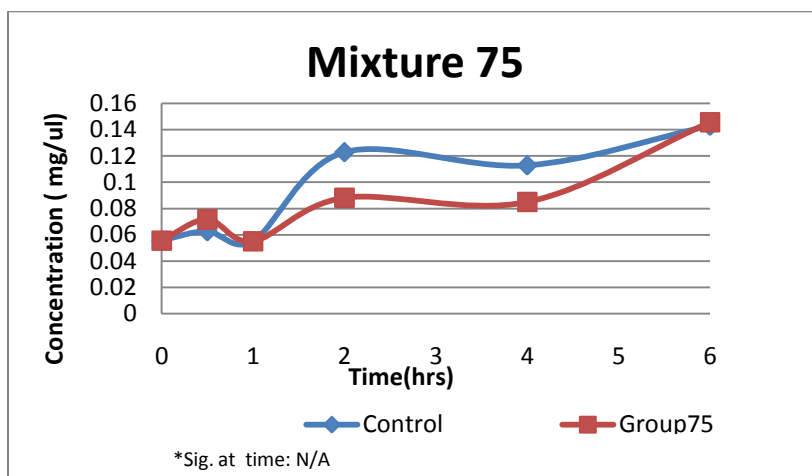


Figure 91. c 0.003 q 0.0000625 t 0.03 h 0.05

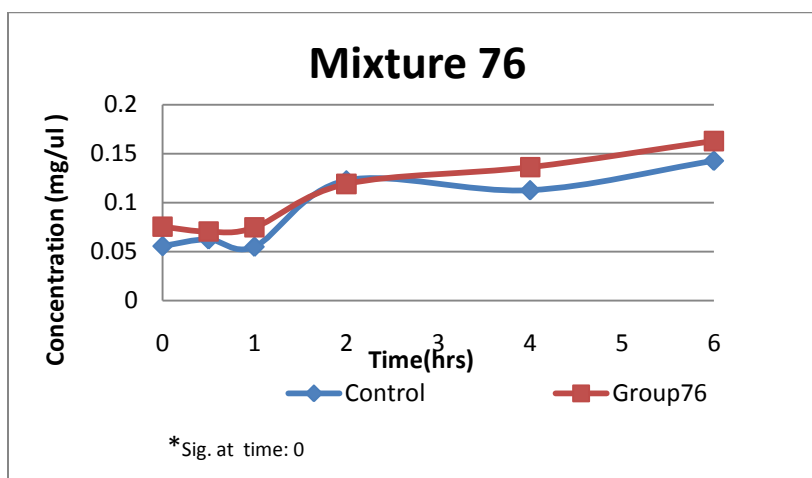


Figure 92. c 0.003 q 0.0000625 t 0.015 h 0.2

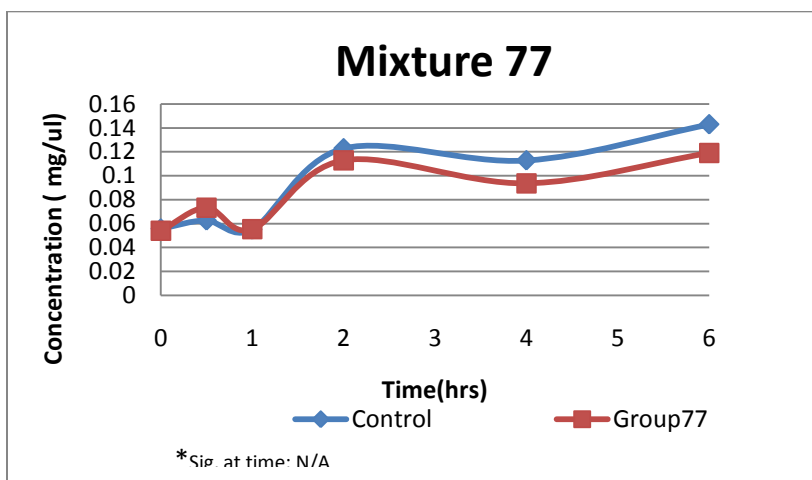


Figure 93. c 0.003 q 0.0000625 t 0.015 h 0.1

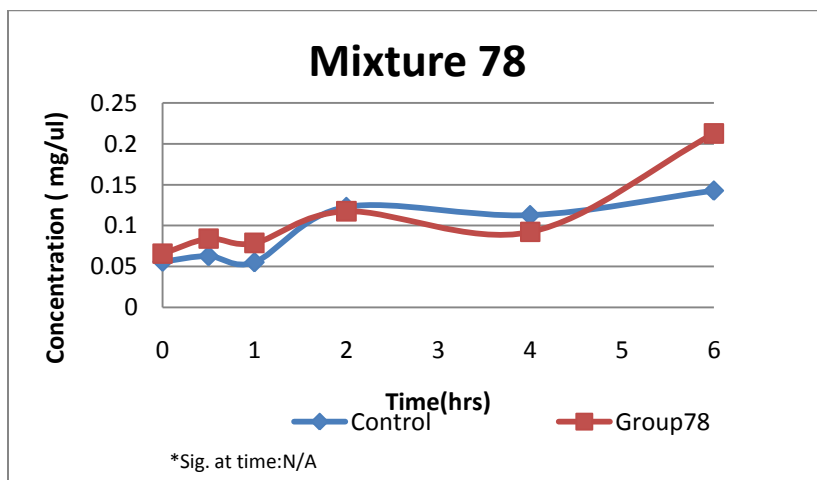


Figure 94. c 0.003 q 0.0000625 t 0.015 h 0.05

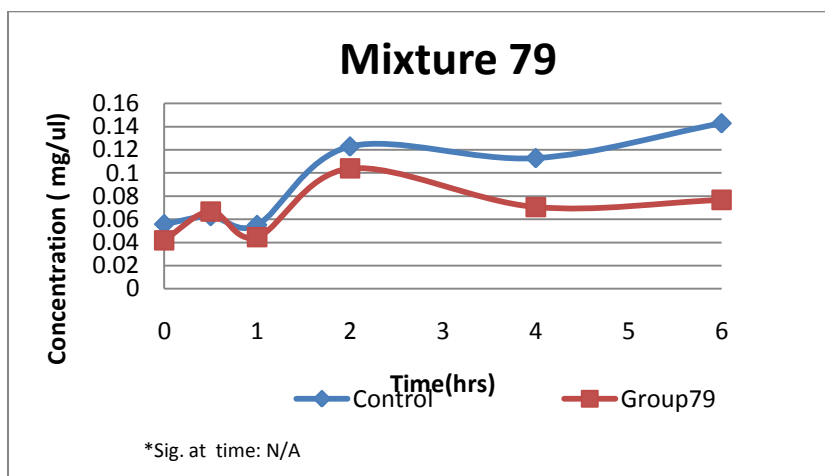


Figure 95. c 0.003 q 0.0000625 t 0.0075 h 0.2

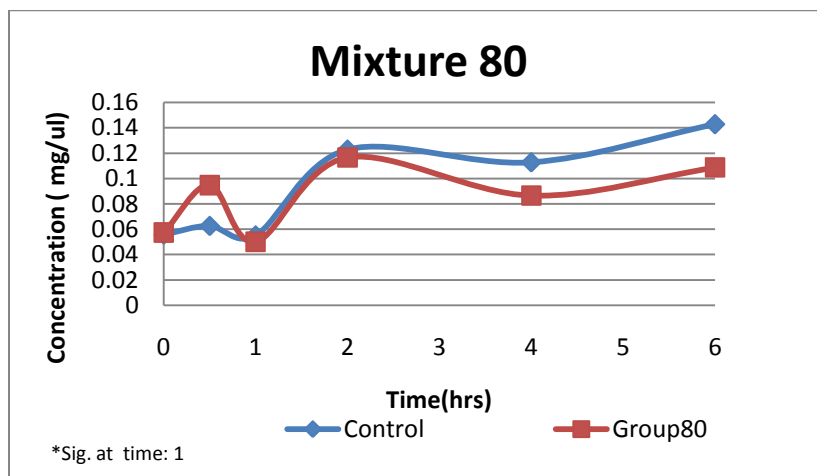


Figure 96. c 0.003 q 0.0000625 t 0.0075 h 0.1

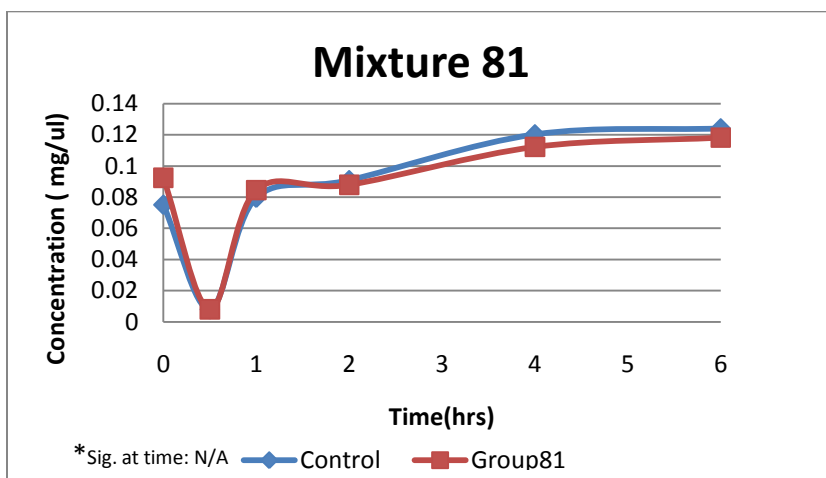


Figure 97. c 0.003 q 0.0000625 t 0.0075 h 0.05