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Adverse Effect of Alcohol Drinking:

The Role of Oxidative Stress

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Resumo

Atualmente, o álcool tem um papel importante na saúde pública e surge como um dos principais problemas sociais no mundo, dado que é a droga mais viciante aceite em encontros sociais. Provavelmente, por essa razão, os riscos do consumo abusivo do álcool são subestimados pelos jovens, mulheres grávidas e idosos. O álcool, quando ingerido em altas proporções, pode afetar todos os órgãos e desencadear inúmeras doenças, tais como a doença cardíaca coronariana, doença neurodegenerativa, as doenças crónicas e câncer. O álcool afeta ainda o estado psicológico, induzindo a violência, o estado antissocial e situações de risco de comportamentos.

Por estas razões, o álcool tornou-se um foco principal da investigação, avaliando os seus efeitos sobre o corpo humano. Nesta pesquisa, foram suscitadas amostras de sangue de um grupo de pacientes em tratamento psicológico e/ou farmacêutico que serão analisadas com quatro métodos: Teste de Radicais Livres do Oxigénio (FORT), Defesa contra Radicais Livres do Oxigénio (FORD), cromatografia gasosa (GC) e cromatografia líquida de alta pressão (HPLC).

Ambos os métodos FORT e FORD avaliam o stress oxidativo pela quantificação de radicais livres e a capacidade de antioxidantes em eliminar esses radicais livres, respetivamente. O stress oxidativo é o efeito do excesso de consumo de álcool, que é reduzido pela capacidade de ação dos antioxidantes. A boa reprodutibilidade, precisão e exatidão de ambos os métodos indicam que estes podem ser aplicados em rápidos diagnósticos.

Para o método **FORT** e considerando o início do tratamento, os **pacientes alcoólicos** apresentaram uma média de **3,59±1.01mmol/LH_2O_2** e o **grupo de controlo** uma média de **1,42±0.53mmol/LH_2O_2**, o que mostra uma diferença significativa entre os dois grupos (**P=0,0006**). Para o método **FORD**, **pacientes alcoólicos** apresentam uma média de **1,07±0.53mmol/LH_2O_2** e o grupo de controlo, uma média de **2,81±0.46mmol/LH_2O_2**, mostrando também uma média significativa (**P=0,0075**).

Após 15 dias de tratamento observou-se que há uma diferença entre os dois grupos de pacientes alcoólicos, mas não há nenhum melhoramento em relação ao grupo de pacientes em tratamento. No método **FORT** os grupos mostram uma diferença significativa (**P=0,0073**), tendo os pacientes sem tratamento farmacêutico melhores resultados (**2.37±0.44mmol/LH_2O_2**) do que os pacientes com tratamento (**3.72±1,04mmol/LH_2O_2**). O oposto ocorre no método **FORD**, os pacientes em tratamento farmacêutico presentam

melhores resultados (**1.16±0.65mmol/LH_2O_2**) do que o outro grupo (**0.75±0.22mmol/LH_2O_2**), não sendo, no entanto, uma diferença significativa entre os dois grupos (**P=0.16**).

Os resultados obtidos para a **concentração de MDA** pelo método de HPLC mostraram que o grupo de controlo tem valores mais baixos do que os pacientes alcoólicos, embora a diferença não seja muito significativa (**P = 0,084**), mas é ainda elevada. Além disso, os dois grupos de pacientes não apresentaram uma diferença significativa entre os seus resultados no início **(P=0,77)** e no fim **(P=0,79)** do tratamento.

De acrescentar ainda que, os resultados da concentração de álcool no sangue determinados pelo método de CG mostraram que só alguns pacientes sem tratamento consumiram álcool durante o período de tratamento, o que influencia negativamente a conclusão sobre o efeito do tratamento.

Contudo, outros fatores externos podem ainda influenciar os resultados finais, tais como o estado nutricional e estado psicológico dos pacientes, se o paciente continua a beber durante o tempo de tratamento ou até mesmo se o paciente é exposto a outros tipos de substâncias nocivas. Existe ainda a possibilidade de o tempo de aplicação do tratamento não ser suficiente para apresentar um efeito positivo em relação ao stress oxidativo e este é um outro fator que contribui para a impossibilidade de confirmar sobre o efeito, quer seja positivo ou negativo, do tratamento antioxidante.

Palavras-Chaves: Álcool, Stress Oxidativo, FORT, FORD, CG, HPLC.

Abstract

Today, alcohol has an important role in heavy health and social problems worldwide because it is the most accepted addictive drug in social gatherings. Probably for this reason, the risks of alcohol abused and its effects are underestimated by young people, pregnant women and elderly people. Alcohol when drunk in high proportions may affect every organ and trigger many diseases such coronary heart disease, neurodegenerative disease, chronic diseases and cancer. It also affects psychologically inducing violence, antisocial and at-risk behaviors.

For these reasons, alcohol has become a major focus of research assessing their effects on the human body. In this research the main point is to evaluate the effectiveness of pharmaceutical treatment in alcoholic patients. A group of alcoholic patients was selected to give four blood samples over 15 days in which seven patients were submitted to a pharmaceutical treatment and the other six patients were submitted only to a psychological treatment. Both groups were compared with control samples. Whole Blood samples and Plasma samples of those patients and control were analyzed in four different methods: Free Oxygen Radicals Testing (FORT), Free Oxygen Radicals Defense (FORD), Gas Chromatography (GC) and High Pressure Liquid Chromatography (HPLC).

Both FORT and FORD methods were useful to evaluate oxidative stress induced by free radicals and the ability of antioxidants to eliminate those free radicals, respectively. The good reproducibility, precision and accuracy of both methods indicate that they can be applied in quickly diagnostics. For **FORT** method and at the beginning of treatment, **alcoholic patients** had an average of **3.59±1.01mmol/LH_2O_2** and **control group** an average of **1.42±0.53mmol/LH_2O_2** showing a significant difference between both groups (**P = 0.0006**). For **FORD** method, alcoholic patients had an average of **1.07±0.53mmol/LH_2O_2** and control group an average of **2.81±0.46mmol/LH_2O_2** showing too a significant difference between both groups (**P= 0.0075**).

After 15 days of treatment it noticed that there is a difference **between two groups of alcoholic patients** but there is no improvement in relation to alcoholic patients under treatment. In **FORT** method the groups show a significant difference (**P=0.0073**) having patients without treatment better results (**2.37±0.44mmol/LH_2O_2**) than patients under treatment (**3.72±1.04mmol/LH_2O_2**). The opposite occurs in **FORD** method, patients under treatment have better results (**1.16±0.65mmol/LH_2O_2**) than the other group (**0.75±0.22mmol/LH_2O_2**), not being however a significant difference (**P=0.16**).

The results obtained for **MDA concentration** by HPLC method showed that control group has lower values than alcoholic patients, although the difference is not very significant (**P=0.084**) but is still high. Also, two groups of alcoholic patients didn`t show a significant difference between their results at the beginning (**P=0.77**) and at the end (**P=0.79**) of treatment.

Furthermore, the results of BAC by GC method showed that only a few patients without treatment have been consuming alcohol during treatment time which may influence the conclusion about the effectiveness of treatment. In patients under treatment was not detected ethanol in their bloodstream.

However, many external factors may influence the final results such the nutritional status and psychological status of the patients, if the patient continues to drink during treatment time or even if the patient is exposed to other types of harmful substances. There is also the possibility that the time of treatment application is not sufficient to show a positive effect in relation to oxidative stress and is another factor that contributes to the impossibility to confirm the positive or negative effectiveness about the antioxidant pharmaceutical treatment.

Keywords: Alcohol, Oxidative Stress, FORT, FORD, GC, HPLC.

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Nomenclature

ADH	Enzyme Alcohol Dehydrogenase
ALDH	Enzyme Aldehyde Dehydrogenase
CYP2E1	Enzyme citochrome
DAD	Diode Array Detector
DGS	Directorate General of Health
FID	Flame Ionization Detector
FORT	Free Oxygen Radicals Testing
FORD	Free Oxygen Radicals Defense
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
I_0	Intensity of radiation lentering the sample cell
I	Intensity of radiation leaving the sample cell
ISS	Institute Superiore di Sanitá
MDA	Malondialdehyde
ROS	Reactive Oxygen Species
ROMs	Reactive Oxidative Metabolites
ROOH	Hydroperoxides

1. Superior Institute of National Health

The Superior Institute of National Health or Institute Superiore di Sanitá (ISS) of Italy is an important and very well known institute in Rome that is the leading technical and scientific public corporation of the Italian National Health Service. ISS activities are related to research, control, training and consultation in relation to public health.

Figure 1.1: Insitute Superiore di Sanitá (ISS), Rome, Italy[1].

The ISS is involved in scientific research in many fields, such as molecular and genetic research, population-based studies of risk factors for disease and disability, in several major clinical trials which are frequently conducted in cooperation with the Scientific Institutes for Research and Care (IRCCS) and Hospitals.

One of the most important activities in ISS is certification of chemical and biological purity of drugs and vaccines, as well as inspection and quality control of medical and diagnostic devices and equipment, food products and packaging. It monitors trends in disease, mortality and other health factors and provides technical support for health-related environmental surveys and for investigations of epidemics and other public health problems at national, regional and local level. It also supervises the laboratories engaged in the testing of prohibited substances in sport and the national veterinary institutes. Finally, it has an important role in monitoring and coordinating blood transfusion services and plasma production in Italy.

The Institute serves as a major source of information relating to public health and biomedicine in Italy through online connections to national and international scientific databases and data banks. It produces too numerous publications, including a quarterly peer-reviewed journal, technical reports, a monthly newsletter describing the Institute's major research activities, and a national epidemiological bulletin.

The Institute plans, implements and evaluates training activities in a wide variety of areas, with courses designed to address the needs of the National Health Service. It develops and tests innovative training methods, including web-based distance learning. Training is provided for various categories of professionals; methods range from short courses and hands-on workshops to more structured and formal courses at national and international level. The topics addressed include health service management and evaluation, epidemiology and biostatistics, training methods, laboratory techniques, diseases control and priority public health issues, and health promotion. The Institute also organizes congresses, conferences, workshops and seminars for national and international participants on fundamental public health issues linked to its institutional activities and research. These events are often organized in cooperation with international organizations such as the European Union, specialized UN Agencies and non-governmental organizations with which the Institute maintains close relations.

The Institute plans, implements and evaluates international health projects, ranging from cooperative research and development projects to humanitarian assistance. It actively promotes cooperation at three different levels of involvement: scientific partnerships with industrialized countries (USA, European partners, Japan); scientific and development projects in partnership with economies in transition (including China, South Africa, the Central Asian Republics and countries in Latin America, the Balkans and the Middle East); development partnerships in Africa and countries in turmoil, where humanitarian and technical assistance is provided in close collaboration with the Italian Ministry of Foreign Affairs, various UN Agencies and the World Bank, as well as the Global Fund for AIDS, Tuberculosis and Malaria. Several members of the Institute serve on panels of experts for the Fund as well as on other international committees and commissions. Current projects include research into the prevention and treatment of HIV/AIDS and poverty-related diseases, capacity-building for central health administrations, technology transfer in health management and technical support for national public health authorities, usually within the context of health care reform. There is growing interest and cooperation in the application of telematics to health issues, in keeping with the priorities recently established by the G8 and the Italian government[1].

2. Alcohol and Some History

Alcohol has been present in human life since the early days. The first traces of alcohol drinking in the humanity appeared in Neolithic period (6000 a.c.) of pre-history, according to the archaeologist records. In this ancient time, alcohol was produced only by fermentation resulting a beverage with low percentage of alcohol and its use was inferior than nowadays as well as its applications in medicinal terms for a very long time.

The primitive peoples prepared their own alcohol with unique characteristics and distinct flavors, such as beer, the oldest alcoholic beverage and the one of the most alcoholic beverages consumed in the world today by humans.

The consumption of alcohol in society always had been connected with celebrations, religious or even in family events[2]. For example, in the Bible there is a citation that reports about the excessive consumption of wine by Noah, who ended up taking his clothes, screaming and fainting (Genesis 9:21). Noah after the flood, planted vines and made wine but he used the drink to the point of getting drunk. Moments later, his son Ham found him “having to show his shame”. It was the first report about drunkenness. Michelangelo, the famous Renaissance painter (1475-1564), inspired about this episode to paint a beautiful fresco by that name, the ceiling of the Sistine Chapel in the Vatican. Note, therefore, that not only the use of alcohol, but its drunkenness, are aspects that accompany humanity since its beginnings.

Alcohol through History had left its mark on many people such Greece and Rome. Their soil and climate were especially rich for grape growing and wine production. These two peoples knew very well fermentation of honey and barley but the wine was the alcoholic beverage most widespread in their empires, having social, religious and medicinal proposes. Although the wine actively participate in social and religious celebrations Greco-Roman, alcohol abuse and alcohol intoxication were already severely censured by the two peoples.

The Egyptian people also left documents which relates about manufacturing, production and marketing of beer and wine. They believed too that fermented beverages eliminated germs and parasites and should be used as drugs in the fight against parasites from the waters of the Nile.

But it was in the Middle Ages that the marketing of wine and beer increased, as well as its regulations. Alcohol intoxication was condemned by the church and considered a sin by itself.

In Modern Ages, during the Renaissance period, cabarets and taverns started to be controlled, as it was stipulated open hours for these places. The cabarets and taverns were the free places where people could express without punishments. In this period, alcohol was also used in political debates that later will trigger the French Revolution.

The end of 18th century was the beginning of Industrial Revolution that was responsible for the changes in demography and social behaviors in Europe. It was this contemporary period that excessive drinking started to be seen as a disease or disorder. It was also in the early 19th century that some scientist begun to make considerations about differences between the distillated alcoholic beverages (spirits) and fermented beverages in particular wine. For example, the famous scientist named Pasteur, in 1865, found no harmful bacteria in wine and so he stated the wine as the most hygienic of all beverages known.

During the 20th century, countries as France created a law that established the age of 18 years for alcohol consumption and in January 1920, States of America decreed the American Prohibition that lasted nearly 12 years. This prohibition outlawed the manufactures, sale, exchange, transport, import, export, distribution, possession and consumption of alcoholic beverages due to the huge disaster for public health and the American Economy.

In 1952, alcoholism started to be treated as a disease and emerged the first edition of the Diagnostic and Statistical Manual of Mental Disorders.

The concept of alcoholism as a disease was incorporated by the World Health Organization, the International Classification of Diseases, from the 8th World Conference on Health. The problems related to alcohol were inserted into a broader category of personality disorders and neuroses. These problems were divided into three categories: dependence, episodes of excessive drinking (abuse) and habitual excessive drinking. Alcohol dependence is characterized by compulsive use of alcohol and the manifestation of withdrawal symptoms after cessation of alcohol use [3].

In conclusion, the presence of alcohol in society today is greater than ever, yet we must make people aware that their excessive consumption is harmful to the health of those who drink as to the health of those who are around them.

3. Consumption of Alcohol: Portugal and Italy

Globally, Europe has a high weight when it comes to alcohol consumption because Europe has an important role in the alcohol global market. Europe is responsible for producing a quarter of alcohol present in the market and more than a half quantity of world wine production. In addition, the trade of alcohol is centered in Europe where they performed 70% of world exports and a little less than half of imports in Europe Union. Besides the production and distribution of alcohol, it is also important to take in account the picture of alcohol consumption in Europe. In the next figure 3.1 is presented a world map which characterizes the global consumption of alcohol per capita of the year 2008.

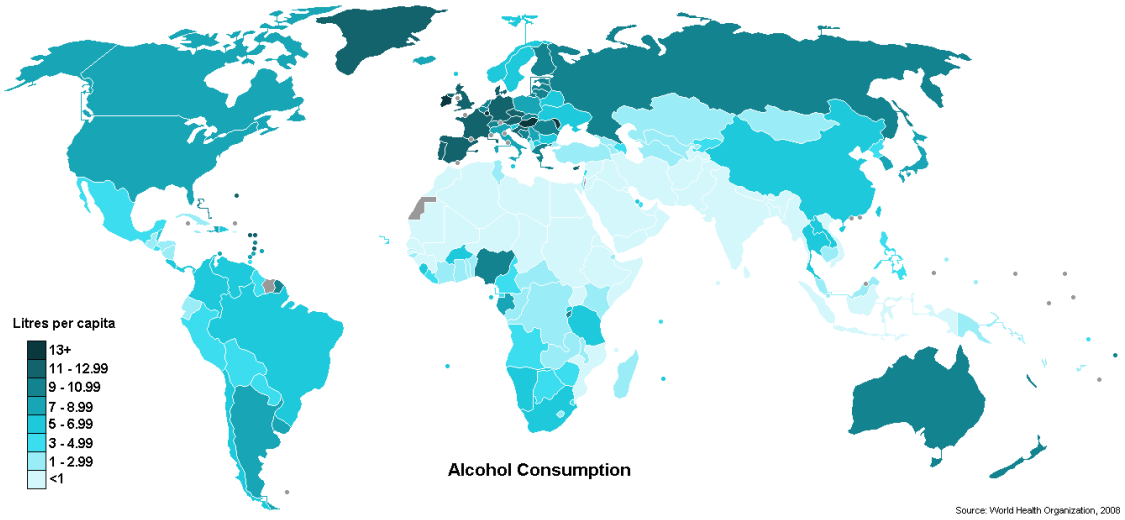

Figure 3.1: Alcohol Consumption per Capita in Adults (2008)[4].

Figure 3.1 shows that countries of Europe are the most consumers of alcohol in the world and this consumption is more frequently in countries that produce alcohol such as wine and beer. Portugal is a good example of a Europe country that produces, distributes and consumes alcohol, as well as the others south countries in Europe such as Spain and Italy where people have a tendency to consume daily. In the North of Europe such as Finland and Sweden, despite their tendency of only to consume occasionally, the quantity of consumption can also be higher in those occasional moments.

In the next figures 3.2 and 3.3 are also presented charts that characterize the number of days that alcohol was consumed on the last month of the year 2006 and the number of excessive consumption on the last month of the year 2006 in Europe countries.

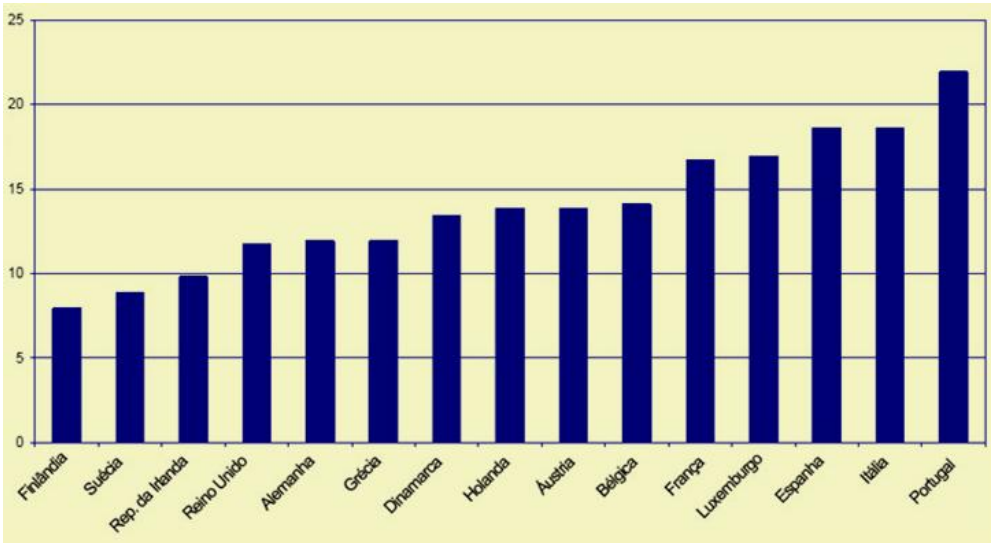

Figure 3.2: Number of days that alcohol was consumed in Europe on the last month of the year 2006[5].

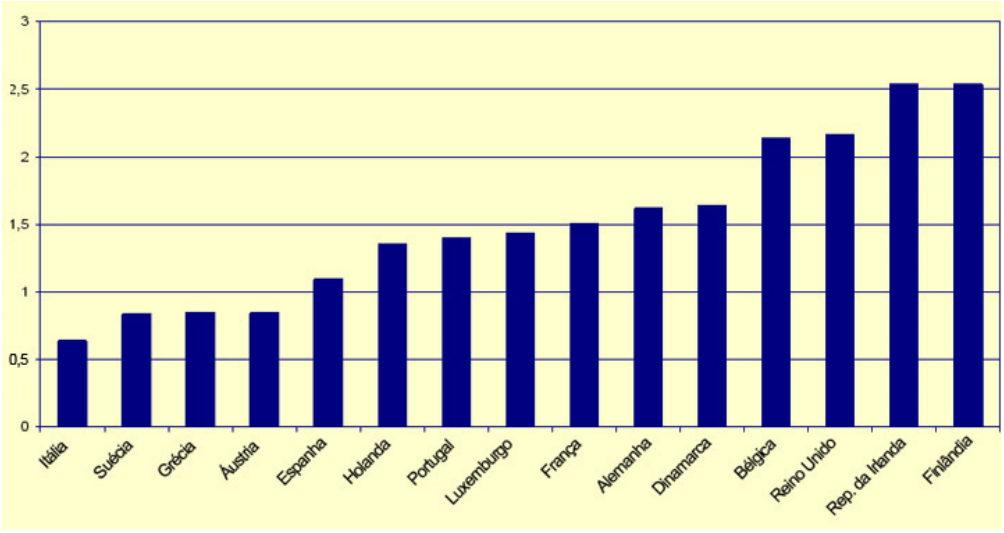

Figure 3.3: Number of excessive consumption in Europe on the last month of the year 2006 [5].

In both figures above, it is possible to observe which countries that consume most and have an excessive consumption of alcohol. In figure 3.2 it is clear that Portugal is the country in Europe with higher consumption of alcohol, followed by Spain and Italy. However, Portugal and Italy are not the countries with the highest excessive consumption of alcohol in Europe (fig.3.3), being Finland and Ireland in this place. Instead, Italy is in the first place as the country with the lowest excessive consumption. Not so great for Portugal which is in seventh place, also ahead of Spain.

To understand better the situation in Portugal in relation to alcohol, it is shown in figure 3.4 the percentage of the amount of alcohol consumption in the continent and both island (Azores and Madeira).

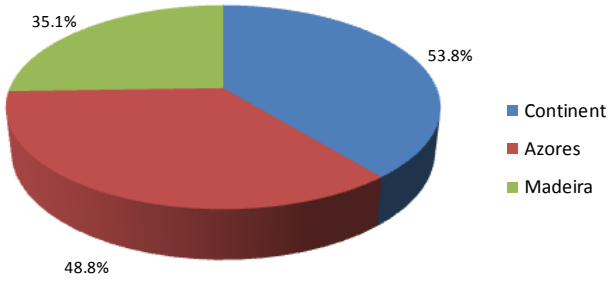

Figure 3.4: Alcohol Consumption in Portugal in 2005/2006[6].

In figure 3.4, it is possible to observe that the highest consumption of alcohol is in the continent with 53.8%, not being Azores too far with 48.8% of alcohol consumption. Madeira is the island of Portugal with lowest consumption of alcohol (35%). These values can be translated also in alcohol consumption in men and women as it is shown in next figure 3.5.

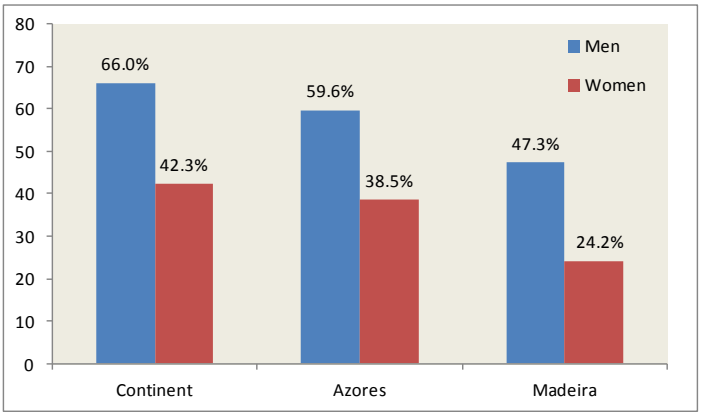

Figure 3.5: Alcohol Consumption of Men and Women in Portugal (2005/2006)[6].

As it is possible to see in figure 3.5, men and women from continent are the highest consumers of alcohol (66% and 42.3%, respectively) in comparison with men and women of Azores and Madeira. It is also clear that men have more tendencies to drink than women in all three cases.

Unfortunately, the tendency to drink alcohol in Portugal and also in other countries begins very early in adolescence and this high consumption increases till it reaches the ages between 45-54 years old, having the consumption the tendency to decrease after these ages but never reaches the lowest values, always higher consumption. This panorama, which can be seen in the chart shown on figure 3.6, happens also in men as in women, being the consumption of men much higher than women during all period of life.

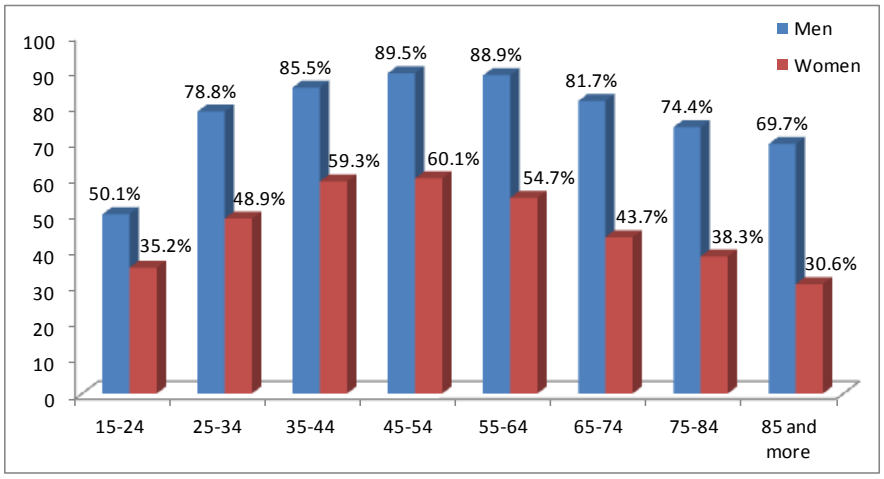

Figure 3.6: Alcohol Consumption in Men and Women during Life (2005/2006)[6]

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The consumption of alcohol in Portugal, by Directorate General of Health (DGS), is a risk factor for road accidents, conflicts and violence. It is also related with many pathologic diseases and physical, psychological and social manners that are the reason why it is considered important for public health.

4. Introduction to Research

Alcohol is a very interesting product from its production and its various production processes up to consumerism and its attendant effects on society today. For these reasons, many studies are being done in many organizations about how alcohol can affect mind and body in a human. The ISS is one of those organizations that conduct studies on alcohol and its effects on humans, from young to adults, women and men.

In this research work, which was held in ISS, will be referred to the effects of alcohol on human body as well as analytical methods that can be applied in this study and types of biomarkers that can help detect the presence of alcohol indirectly.

4.1. Ethanol and Alcohol Drinking: Effects in the Human Organism

Ethanol or ethyl alcohol, as shown in its chemical formula (CH_3CH_2OH or C_2H_6O), is constituted by a hydroxyl group and a short chain of two carbons. It is characterized as an uncolored liquid with a boiling point of 78ºC, volatile, flammable, high solubility in water and a characteristic smell.

This component is an organic substance that can be obtained from the fermentation of sugars, hydration of ethylene or reduction of acetaldehyde. It can be applied as a solvent in the fabric of paints, lacquers, varnishes and perfumes. Ethanol can also be found in beverages such as beer, wine and spirits.

It was after the industry`s revolution and its development that alcohol was produced in high quantities, expanding the market with a variety of beverages. This high availability is associated with personal and social costs due to its abuse with millions of people getting addicted to this type of drug. At the same time, consume of alcohol started to be tolerated, despite of the increase of excessive consume and being convicted in some situations.

The concentration of alcohol in a beverage depends on the type of raw material, fermentation, percentage of additives and the amount of distillation. The typical concentrations of beverages are indicated in table 4.1.

Table 4.1: Alcohol Concentrations (% v/v) in some types of beverages[7].

Beverages	Alcohl Concentration (% v/v)
Beer	6-8
Red wine, Soft wine, Champagne	12
Oporto wine, vertume	14-20
Liqueurs	15-40
Distillates (wisk, rum, ...)	35-45

Nowadays, most of alcoholic beverages have a small quantity of ethanol in their composition because ethanol concentrations above 12% are toxic to yeasts which are responsible for fermentation. Beverages with a concentration higher than 12% are produced by industry processes as distillation of fermented products.

All alcoholic beverages derive from a common process: alcoholic fermentation. This process is reflected by a chemical reaction triggered by the action of yeasts on raw material sugar, producing ethanol and carbonic acid. To these sugars is called wort and it must undergo a pretreatment before brought into contact with the yeast.

Sometimes, the alcohol obtained as a product of wort fermentation is subjected to a distillation process making it a distillated product with a higher concentration of alcohol, such as wine. To separate the alcohol from the remaining components of the wine, whether volatile (water) or solid (yeast and bacterial), is applied multiple distillation operations which are based on different points of the volatile boils present in wine.

Basically, all of the alcohol effects are induced by the presence of ethanol in the organism and its abuse can fortify these effects. After ingestion, ethanol is quickly absorbed in the stomach (20%) and then in the small intestine (80%) passing to the bloodstream. The concentration of ethanol in blood is dependent on quantity of ethanol consumed, concentration of ethanol in beverage, speed of consume and composition of gastric contents, reaching the maximum value after half an hour to one hour and a half after ingestion.

The concentration of ethanol in the organisms decreases during time, even if there is high concentration of ethanol in the stomach. The end of absorption in the stomach and the beginning of absorption in the intestine are the principle factors of why the concentration of ethanol is different in each individual and circumstance. Food delay the absorption of ethanol in the stomach but when it reaches to the intestine the absorption is quickly and complete. Furthermore, as ethanol has high solubility in water, it can easily reach all tissues and organs being distributed uniformly[7].

However, almost total of ethanol is metabolized in the liver due to two enzymatic systems: **alcohol dehydrogenase** and **citochromo P2E1**. These are the systems related to alcohol drinking and, consequently, alcohol drinking is related to oxidative stress, a harmful process to human body which will be explained next.

4.1.1. Oxidative Stress induced by Ethanol

In normal conditions, the oxygen produces continuously reactive oxygen species (ROS) in the cells and these species are called free radicals which are harmful to the organism cells. Frequently, free radical is an often term used to any atom or molecule that exist independently having one or more unpaired electrons. An unpaired electron is the one that singly occupies an atomic orbital or molecule. The presence of one or more unpaired electrons determines an attraction like a magnetic field and sometimes the substance becomes extremely reactive. Most of free radicals have a short period of life because they are able to react very quickly with other compounds or to target cells or membranes.

Between the various free radical species, there are manly oxygen derivatives and transition metals. The oxygen molecule is qualified as a free radical because it has two unpaired electrons and each one is situated in different orbital. As these two electrons have the same type of rotation in the molecule, it provides the most stable state to oxygen.

Understanding oxidative process as a loss of electrons by an atom or molecule and reduction process by a gain of electrons by an atom or molecule, oxygen is considered a good oxidizing agent because it acts as a receptor of electrons from the molecule which oxidizes.

As free radicals, especially oxygen, and others ROS are continuously produced in the organism, the organism itself develops defense systems against this toxic effect that allows the elimination of those free radicals as they are produced by the cell itself. The defense system is composed by enzymes, proteins, vitamins, elements (for example: zinc and copper) and antioxidants. They all neutralize the ROS to prevent the excessive accumulation of harmful substances in the cells that can cause serious injuries.

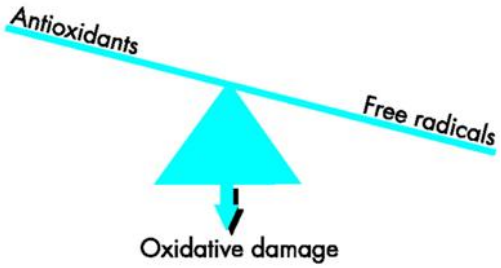

Figure 4.1: Imbalance between antioxidants and free radicals that leads to oxidative stress [8].

Therefore, the oxidative stress is a biological condition in which occurs an imbalance between the production and manifestation of reactive oxygen species and the detoxification by biological systems that remove or repair the damage caused by the oxidative stress (Fig.4.1). This state can be caused by the consumption of alcohol, presence of pollutants, medications, tobacco, stress, toxins and radiations but the main fact is how alcohol induces oxidative stress.

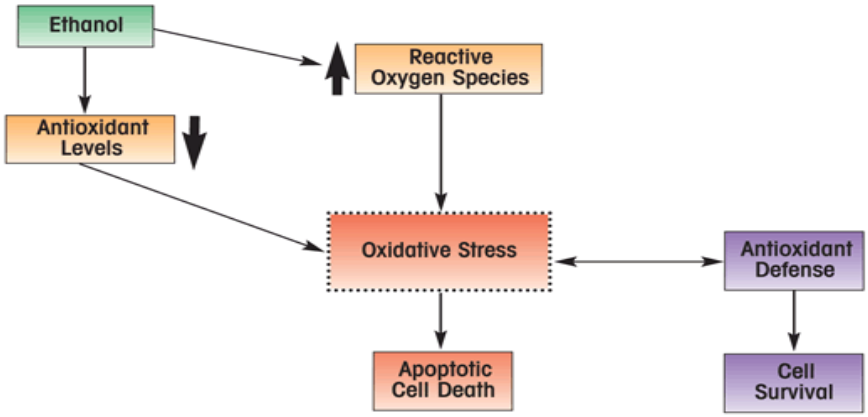

Figure 4.2: Scheme that relates ethanol with oxidative stress status[9].

Chemically, oxidative stress is associated with increased production of oxidizing species (ROS) and/or a significant decrease in the effectiveness of antioxidant defenses. The scheme shown in figure 4.2 helps to understand how the organisms acts when it reaches oxidative stress status caused by alcohol in higher concentrations. If the concentration of antioxidant is higher or sufficient to eliminate ROS, the cell may survive but if the concentration of ROS is superior to antioxidants, it may cause cell death by apoptosis (process of programmed cell death).

All living organisms have an intracellular environment with a reducing nature and there is a balance between oxidized and reduced forms of molecules. This reducing environment is

preserved by enzymes that maintain the reduced state through a constant input of metabolic energy. However, the production of oxidative species must be controlled because disturbances in this balance can cause redox production of peroxides and free radicals that damages all cellular components, including proteins, lipids and DNA.

In humans, the oxidative stress is connected to several diseases, including brain diseases (atherosclerosis, Parkinson's and Alzheimer`s disease), kidney and heart diseases (Fig.4.3). The result of this imbalance can be also injuries in other organs and the appearance of carcinogenic process by the damage of DNA.

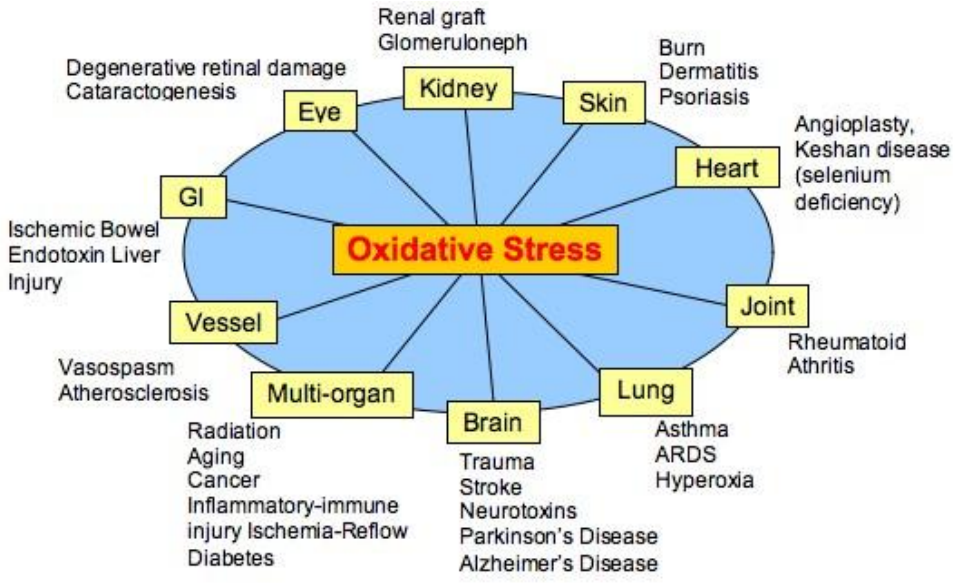

Figure 4.3: Different effects caused by oxidative stress in the human organism[10].

On the other side, reactive oxygen species can also act beneficial to organism when used by immune system to attack and kill pathogenic organisms or molecules when they act as messengers in signaling pathways (also called redox signaling)[11].

4.1.2. Mechanism Generation of Free Radicals

In the human organisms, alcohol can be processed through various metabolic pathways producing toxic biological components which contribute to cell and tissue damage. In each metabolic process there is an enzyme which creates a harmful condition known as oxidative stress and it is associated with an increase in the reaction by which oxygen gains an electron (reduction of oxygen). As explained before, this reaction creates components called ROS that can damage other cellular molecules.

The excessive production of ROS cause oxidative stress by the increased formation of oxygen derived radicals and the decreased antioxidant barrier capacity. Antioxidants have the capacity to eliminate free radicals by combining with them to avoid the dandification of the cell. It can be divided in two different types of antioxidants: enzymatic and non-enzymatic. The enzymatic antioxidants are produced in the organism and there are three: catalase, gluthatione peroxidases (GSH) and superoxide dismutases (SODs)[12].

To understand how the expression of those enzymes can fight oxidative stress is useful first to understand how oxygen functions as an acceptor of electrons in the organisms and how enzymes use oxygen in alcohol metabolism.

The generation mechanisms of free radicals occur in mitochondria, cellular membranes and in the cytoplasm. The mitochondria by means of the electron transport chain is the main source of free radicals.

In certain physiologic conditions, aerobics organisms can metabolize 85-90% of oxygen consumed in mitochondria. The rest 10-15% are used by diverse oxidases and oxygenases enzymes and by oxidation chemical reactions.

When there is an excess of reactive molecules or a lack of molecules that can eliminate free radicals occurs oxidative stress. Free radicals are highly reactive molecules that interact with other cellular structures and they contain unpaired electrons which seek to obtain another electron to produce a stable pair and specie.

Oxygen is the free radical which has two unpaired electrons and so oxygen has the capacity to accept four electrons before becoming a neutral molecule - oxygen can be reduced by four electrons (Fig. 4.4 shows the process and its products).

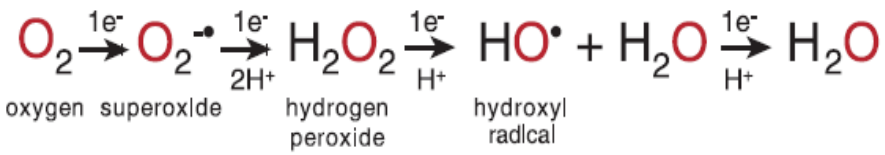

Figure 4.4: Sequential reduction of oxygen in four single electron steps[11].

An increase of free radicals usually is the result of an increased reduction of oxygen to ROS which can react with other cellular molecules. In figure 4.4 is showed the sequential reduction of oxygen starting from the addition of one electron in oxygen that produces superoxide ($O_2^{-\bullet}$), the first reactive specie with a negative sign that shows an electron

reduction. This specie can accept another electron reacting as an oxidant or it can donate the electron to another molecule.

In the cells, there is an enzyme which protects the cells from oxidative stress damages. The first enzyme in the line of defense in the detoxification of products resulting from oxidative stress is called superoxide dismutase and this enzyme converts the superoxide radical to hydrogen peroxide – equation 4.1. Superoxide dismutase protects the cell against high concentrations of this free radical.

$$O_2^- + O_2^- + H^+ \rightarrow H_2O_2 + O_2 \quad \text{(eq. 4.1)}$$

If the concentration of superoxide becomes too high, it can participate in the formation of another free radical called hydroxyl (HO^-) which is very reactive and it will accept another electron from the first molecule that it touches. Superoxide does this by donating the extra electron to metal ions.

Other species can be formed directly by superoxide accepting two hydrogens to produce hydrogen peroxide (H_2O_2). Now, being a neutral molecule, this new species can pass through the cells membranes and cannot be excluded from cells.

At this point, the cell produces certain enzymes such as glutathione peroxidase and catalase that guard against the increased concentration of hydrogen peroxide. Catalase is a hemeprotein cytoplasmic present in major organs and is especially concentrated in the liver. Catalase is able to remove peroxide from the inside of a cell by catalyzing it and its activity depends on the amount of available energy – equation 4.2.

$$2H_2O_2 \rightarrow 2H_2O + O_2 \quad \text{(eq. 4.2)}$$

The enzyme Glutathione pexoridase is able to catalyse the reduction of hydroperoxides reduced glutathione (GSH) leading to formation of oxidized glutathione (GSSG) and water – equation 4.3.

$$SH_2 + H_2O_2 \rightarrow S + 2H_2O \quad \text{(eq. 4.3)}$$

Hydrogen peroxide can also accept another electron and this usually occurs as a result of the transfer from a reduced metal ion. When hydrogen peroxide accepts this electron, it is converted to a hydroxyl radical (HO^-) which combines with hydrogen to produce water.

If no kind of those reactions occurs, then the superoxide accepts two hydrogens which generate two molecules of water, one from each oxygen atom. To better understand the sequential reduction of oxygen, it is possible to observe the scheme of figure 4.5.

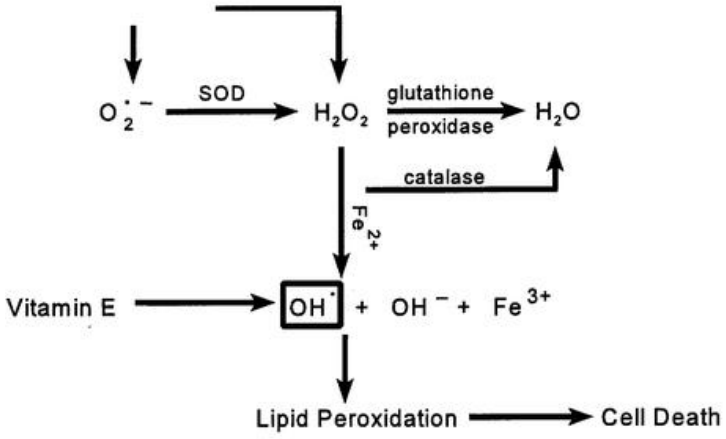

Figure 4.5: Interference of antioxidant enzyms in sequencial reduction of oxygen.

This reaction showed on figures 4.4 and 4.5 generates then a propagation of free radical chains reactions that destroy cell membranes and damage DNA, resulting also in damage to organs.

Besides the cells have enzymes to protect itself from generation of intermediate reduced forms of oxygen, these reactions do occur and they occur more readily when alcohol is consumed[12].

4.1.3. Metabolism and Biochemical process of Oxidative Stress

Individual genetic factors and the environment in which each individual lives have an important role in the development of “alcohol disease”. Each individual also have differences in alcohol metabolism that results in a huge inter-individual variability of toxic effects induced by ethanol.

Alcohol metabolism is dependent of enzymes and its genetic polymorphisms. These enzymes are induced by ethanol. After ingestion, ethanol is absorbed by the organism with a rate varying in timing, dosage and drinking pattern in addition to the nutrition status of the exposed subject. Ethanol is easily distributed to the water space of organism because it has a high solubility in water.

Ethanol suffers a metabolic biotransformation inside the organism (95-98%), while small fractions can be detected in breath (0.7%), sweat (0.1%) and urine (0.3%) but more than

90% of ethanol ingested suffers an oxidative and non oxidative biotransformation in the liver as it said before.

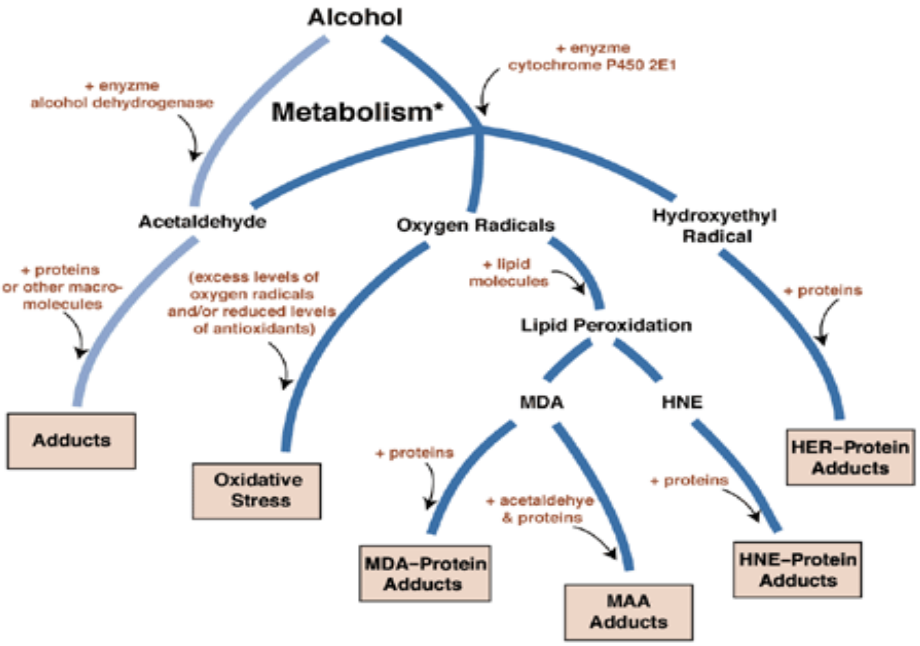

Figure 4.6: Harmful products resulting by alcohol metabolism[13].

In the first way of ethanol metabolism is involved an enzyme named alcohol dehydrogenase (ADH) that produces acetaldehyde which is a very toxic metabolite and it is normally present in the organism at smaller concentrations. This compound is metabolized by the enzyme mitochondrial aldehyde dehydrogenase (ALDH) to acetate and then to dioxide carbonic (CO_2) and water (H_2O) – Figure 4.6 above. Acetaldehyde binds to a specific amino acid residues on the structural and functional proteins to form acetaldehyde-protein adducts, causing a pathological immune response.

The second way of ethanol metabolism involves an enzyme called cytochrome (CYP2E1) that converts ethanol to acetaldehyde in the liver – Figure 4.7. Then, acetaldehyde is oxidized and releases oxygen derived free radicals.

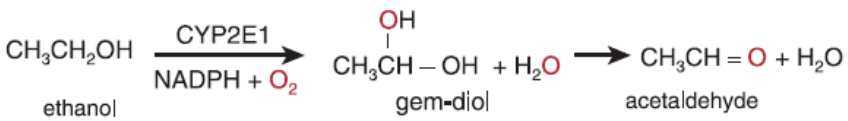

Figure 4.7: Mechanism of ethanol to produce acetaldehyde and water[12].

The enzyme ADH has more affinity to ethanol then cytochrome and that is why cytochrome is just responsible for about 10% of ethanol oxidation to acetaldehyde in cases of moderate alcohol drinking. In presence of an amount of alcohol abuse, ADH gets saturated and CYP2E1 contribution becomes relevant since it is inducible by ethanol itself.

The enzyme CYP2E1 activity occurs mainly in liver and in other organs such as the central nervous and the brain. The increased amount of CYP2E1 oxidative activity leads to the formation of ROS and ethanol-derived free radicals. Thus, CYP2E1 can initiate lipid peroxidation with various breakdown products and promote several damages to DNA. One of those products is malondialdehyde (MDA) which is considered a reliable marker of oxidative stress (it will be mentioned in chapter 4.3).

Alcohol liver disease is associated to mitochondrial damage by oxidative stress and its toxicity is due to ethanol and to its metabolic products like ROS and acetaldehyde. It is now understandable why these compounds have an important role in developing alcohol diseases during ethanol metabolism.

The adverse effects of alcohol and the excessive production of ROS and lack of antioxidants is associated with innumerous consequences for the cell and it has a significant role in the development of alcohol diseases, including pregnancy complications and fetal disorders[14]. However, it is also important to understand that alcohol has different effects in each organism because each organism is different from another.

4.2. Damage due to Alcohol: Differences due to Gender and Ages

Nowadays, alcohol is the most consumed drug among population, including teenagers, pregnant women and elderly people, being considered a social problem. Alcohol problems rises from changes in drinking habits and are generated by some factors such as personal, social and health aspects.

Other factors due to life style (tobacco, drugs, pesticides, pollutants, heavy metals…), together with alcohol consumption, can amplify health damage fortifying the possibilities of damage in various organs like liver and brain.

The effects of ethanol in women and children are different and more dangerous from men because the physiological and metabolism of organism is also different, being more vulnerable to alcohol effects. Blood alcohol concentrations (BAC) depends on body mass index (BMI) and body water, and both are lower in women than men, leading to a lowered

ethanol diffusion in the body and resulting in higher BAC. Besides, studies also support that the amount of enzyme ADH are significantly lower in women and can be close to zero in heavy drinking females, fortifying the alcohol effects.

Even physiologic hormonal patterns can influence the response of the body to alcohol. In different phases of menstrual cycle it is possible to observe different reactions to alcohol and the processes involving hormone estrogens in inflammatory processes explains why damage in liver is quicker in females than males. The use of oral contraceptives may worse the damage because the presence of alcohol actives an endotoxin-induced liver injury. Alcohol may also leads to infertility in women and it increased the risks of breast cancer.

Children can be infected by ethanol during conception, as maternal alcohol drinking during pregnancy may have permanent effects. Alcohol passes easily through placenta and reaches the fetus that has no tolerance to ethanol, being vulnerable to its effects which develop abortion, fetal death, premature birth, low birth weight and abnormalities in mental and physical development. Born children expose to alcohol during pregnancy have fetal alcohol syndrome (FAS) that shows facial anomalies, growth retardation, difficulties in learning and behavior problems. Fetal alcohol damages have no reverse and can be avoided if no alcohol is consumed during pregnancy.

Alcohol can also affects adolescents differently than adults and they show to be more sensitive to its effects such as damage in certain types of memory. The younger a person begins to drink, the more are the possibilities to develop problems later in life. Adolescents drink for various reasons and it can lead to abuse and dependence of alcohol[15]. There is clearly a culture where the encounters between young people must be "watered" with alcohol, its transgressive character that attracts the young and being a disinhibiting that "opens doors" and integrates the individual in the group, like an initiation ritual. The teenager is in a stage of life where alcohol abuse is extremely attractive, especially for its low cost and easy accessibility, even if it is illegal for persons under 16-18 years old.

Adolescence is indeed a period in which the nervous system has not yet completed its development and therefore young people before age 18 cannot properly metabolize the alcohol ingested. Any amount of alcohol, however small it may be, is sufficient to impair the functioning capacity in full development in the young, such as intelligence, reasoning, memory, attention, among others. As the structures and organs of the nervous system is at a growth stage, more sensitive to alcohol will be in the adult stage. On the other hand, alcohol can be evaluated as the true "gateway" to the world of drugs and young people need to be asked to adopt healthy lifestyles[16].

Among elderly people, alcohol may affect memory and brain functions, developing Alzheimer`s disease (AD) which cause dementia. This disease is characterized by progressive changes in cognitive ability, memory and mood.

However, moderate alcohol consumption doesn`t seem to have a significant effect on brain functions and could protect blood vessels in the brain and in the heart. Although, it is important to understand what quantity of alcohol is considered moderate because vulnerability of alcohol depends on individual susceptibility[15].

4.3. Concept of Biomarkers and its importance to evaluate ethanol damage

Biomarkers or biological markers are substances used to indicate a biological state which are objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or treatment responses.

Biomarkers have applications in many scientific fields because they have an important role in understanding the relationship between exposure to environmental chemicals, the development of chronic human diseases and the identification of subgroups that are at increased risk for disease[17].

Biomarkers are used to detect current or past alcohol consumption and they can be defined as trait markers and state markers. Trait markers are biochemical markers which are used to reveal the risks of abusing alcohol and the dependence of alcohol in a person. These markers are connected to brain and ethanol metabolism in terms of polymorphisms of ADH and CYP2E1.

State markers are also biochemical markers but these measures recent drinking patterns of a person. These can reveal information about heavy drinking and/or information about moderate alcohol.

There are many different biomarkers that can be used to evaluate ethanol effects in blood such as:

- **Ethylglucurinide** – minor metabolite of ethanol and a biomarker that is used to determine ethanol up to 80hours consumption. It has a larger field of detection compared with other biomarkers and can be detected also in urine after 1 hour ethanol ingestion. However, incidental exposure to ethanol present in products may cause false results[18];

- **Glutamyl Transferase** (GGT), **Carbohydrate-Deficient Transferrin** (CDT) and **Mean Corpuscular Volume** (MCV) – these biomarkers require several weeks or months of sustained alcohol consumption to be significantly elevated;
- **Blood Alcohol Concentration, Acetaldehyde, 5-hydroxytryptophol** – these biomarkers are able to demonstrated in short-term alcohol use and are measurable soon or only few days after consumption;
- **Vitamine B1 deficiency** – not only considered a biomarkers of alcohol but also a prognostic index of alcohol related to brain disease[19];
- **Malondialdehyde (MDA)**: lipid peroxidation product induced by the presence of free radicals in the organism.

Oxidative stress biomarkers are state markers that are used to measure the progress of alcohol diseases. Lipids are the class of molecules most involved in oxidative stress and lipid oxidation produces aldehydes that exacerbate oxidative damage by interacting with nucleic acids and proteins and damaging cells functions.

In these cases, malondialdehyde (MDA) is the lipid peroxidation biomarker most used to study polyunsaturated fatty acid peroxidation that occurs in the liver. At this point, is easily understandable that in a healthy body, oxidative and reductive processes are in a balance created by antioxidant barriers and its capacity to eliminate free radicals. When this barrier isn`t sufficient free radicals react with cell structures that leads to lipid peroxidation products such as malonialdehyde (MDA) and/or 4-hydroxynonemal. These two secondary products might react with other molecules in the cell. On the other hand, these unwanted reactions might cause DNA modifications in terms of an incorrect transcription of DNA-based guanine and adenine to form several different components that causes mutations and consequently diseases such as cancer. DNA-based are four: adenine, guanine, cytosine and thymine. MDA can also react with proteins which lead to an alteration or loss function. It may show altered physico-chemical behavior and antigenicity.

The participation of lipid peroxidation products have been discussed in several diseases that were mentioned before. Lipid peroxidation is used as an indicator of oxidative stress in cells and tissues and that is why MDA is an useful biological marker.

MDA biomarker has been used in handling several diseases such liver disease, Alzheimer`s disease, Parkinson`s disease and other alcohol symptoms and so it is considered the best in monitoring oxidative stress by alcohol drinking abuse. Reactive oxidative metabolites (ROMs) can also be good markers to evaluate alcohol in blood but

MDA determination in plasma by high performance liquid chromatography (HPLC) is one of the most significant tool to evaluate oxidative stress induced by alcohol[19].

4.4. Biological Samples: Sampling Conditions

Biological samples are fluids, secretions, excretions and fragmented tissues obtained from the human body that can be analyzed.

The most widely used is blood which is considered as a complex system relatively constant consisting of solid elements (blood cells), liquid substance (serum or plasma) and gaseous elements (O_2 e CO_2).

In this research, the biological sample is human blood and its sampling should be adequate to the subject under observation and to the researchers. The adequate collection is dependent on speed, efficiency, quality of care and low suffering as possible to the person. To researchers is important sampling condition as also transport and storage of the biological sample.

For example, in cases where the sample collection is inadequate and may cause some suffer to the person there is a high chance to corrupt the sample because it may occur a phenomenon called hemolysis (Figure 4.8). Hemolysis is the breakdown of red blood cells that releases hemoglobin into the plasma, in other words, is the disruption of red blood cells by disruption of the plasma membrane to release hemoglobin.

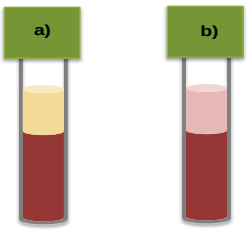

Figure 4.8: Blood Sample after centrifugation where a) is normal blood sample and b) blood sample with hemolysis.

Sampling collection is a fundamental part for determination of an analytical variable because it is the only link between person under observation and laboratory, and a bad sampling collection may lead to bad significant results.

In this work, is important to know the analytical procedures tests and the type of sample required for each test. Blood sample can be divided in three types of samples: whole blood, plasma and serum.

Plasma and serum are both liquid part of blood which main difference between them lies in their clotting factors. When plasma is separated from the blood it still retains a substance named fibrinogen which is a coagulant responsible for blood coagulation. For the plasma is important to avoid blood clotting so it is necessary to add an anticoagulant, for example, heparin, and then goes to centrifuge.

Blood serum is what remains after removing red blood cells, white blood cells and clotting factors. To get serum is necessary to let the blood clot and then centrifuge. The separation is only physiologic.

In this research, it will be used whole blood samples to analyze the oxidative stress caused by free radicals, to analyze the ability of antioxidants to eliminate those free radicals and to determine ethanol concentration in human blood. It will also be used plasma to determine malondialdehyde, a biological marker. Next, it will be developed the methods that will be used to do the mentioned analysis.

5. Analytical Instrumentation

The accuracy in the measurement of any method is crucial because a bad result may lead to a bad consequence and so it is important to use analytical methods that are accurate, fast and efficient to ensure the quality of analysis.

The methods used to analyze human blood should be simple and not demanding in terms of quantity of blood sample. The applied technologies range from the non-instrumental methods such as tritations to more instrumental techniques such as **spectrophotometry** (molecular absorption spectrophotometry, infra-red and atomic absorption), **chromatographic** (gas chromatography and liquid) and **mass spectrometry**.

In this research, it will be used colorimetric methods, which give faster results, to analyze oxidative stress and the ability of antioxidant to reduce free radicals in a blood samples. Chromatography methods, which takes more time but the results have more accuracy than colorimetric methods, will be use to determine the concentration of ethanol in blood samples and a biological marker MDA in plasma. These methods will be described below.

5.1. Colorimetric Methods

Colorimetric methods are quantitative methods of analysis based on the comparison of the color produced by a chemical reaction with an indicator color bound to a specific enzyme. According to the color intensity produced is possible to infer the concentration of a particular analyte. The equipment to determine that concentration is the photometer which measures the absorbance of a sample.

The photometer principle is that monochromatic light is allowed to pass through the cell which contains the solution (or sample) and then reaches a detector that measures the ratio between intensities of final radiation and initial radiation. From the ratio between both light intensities, knowing the capacity of the colored substance to absorb light (absorbance), it is possible to calculate the concentration of the substance using Lambert Beer`s Law.

5.1.1. Lambert Beer`s Law

The Lambert Beer`s law relates the absorption of light to the properties of the material through which the light is travelling. In other words, establishes a relationship between absorbance of a solution and analyte concentration in the solution.

Absorbance is measured when the solution is crossed by a collimated monochromatic light radiation (parallel rays of light). The figure 5.1 below shows a beam of monochromatic radiation with intensity (I_0) across a sample solution.

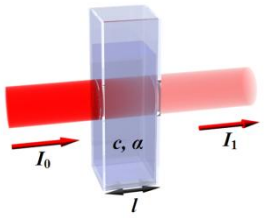

Figure 5.1: Diagram of Lambert Beer absorption of a beam of light as it travels through a cuvette of width l [20].

Throughout the figure 5.1 is easy to understand that, across the sample, the intensity is absorbed and the beam of radiation leaving the sample will have intensity (I) – Equation 5.1.

$$A = \varepsilon l c = -\ln\left(\frac{I}{I_0}\right) \qquad \text{(eq.5.1)}$$

In equation 5.1, ***A*** is absorbance, ***ε*** is the molar absorbtivity (L/mol.cm), l is the path length of the cuvette in which the sample is contained (cm) and ***c*** is the concentration of the compound in solution (mol/L). This equation shows that the absorbance is dependent on the total quantity (concentration) of the absorbing substance which is in the optic way throughout the cuvette[20].

However, not all of colorimetric reactions follow the Lambert Beer`s Law which is valid for strict conditions where the light used is approximately monochromatic, the solutions to be analyzed are diluted to low concentrations and should not be present in the same solution more than one absorbing substance.

The main cause of deviations from the Beer`s Law is the use of concentrated solutions because the increase in concentration is accompanied by the increasing proportional to

absorbance (A) until it reaches a breaking point (B). From this point (concentrated solutions), there is no proportionality between the values as it is possible to observe in the figure 5.2 showed below.

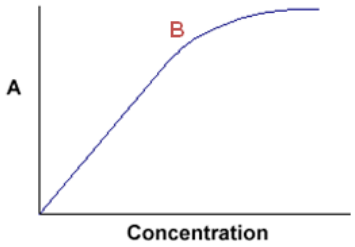

Figure 5.2: Chart which characterizes the absorbance (A) versus concentrations (c) and breakpoint (B)[21].

Linearity limit is the limit of concentration at which Beer-Lambert law is valid. When the concentration is higher than this limit imposed by Lambert Beer`s Law, the linear proportionality between concentration and absorbance ceases to exist[21].

In this research it will be only used two colorimetric methods that are correlated with Lambert Beer`s Law and both will be described below.

5.1.2. Free Oxygen Radicals Testing

Free Oxygen Radicals Testing (FORT) is useful to determine the oxidative stress status and to quantify free radicals on a blood sample. It is a colourimetric test based on the ability of transition metals, such as iron, to catalyze free radicals that are formed in the breakdown of hydroperoxides (ROOH). The free radicals are subsequently trapped by chromogenic substance ($CrNH_2$) which is an amine derivative. Hydroperoxides are easily decomposed in certain conditions as light, heat, reducing agents and metal ions.

In human blood, transport proteins release the transition metal (iron) in acidic medium and, consequently, hydroperoxides are quickly decomposed in alkoxyl (RO•) and peroxyl radicals (ROO•) which can be traduced by equations 5.2 and 5.3. These free radicals are able to convert $CrNH_2$ into the coloured radical cation ($CrNH_2^{+\bullet}$) – equation 5.4.

$$R-OOH + Fe^{2+} \rightarrow RO^{\bullet} + HO^{-} + Fe^{3+} \qquad \text{(Eq.5.2)}$$

$$R-OOH + Fe^{3+} \rightarrow ROO^{\bullet} + H^{+} + Fe^{2+} \quad \text{(Eq.5.3)}$$

$$RO^{\bullet} + ROO^{\bullet} + 2CrNH_2 \rightarrow ROO^{\bullet} + RO^{\bullet} + [CrNH_2^{+\bullet}]_{purple} \quad \text{(Eq.5.4)}$$

The reaction between chromogen and free radicals forms a colored solution (purple) which intensity is dependent on alkoxyl and peroxyl radicals that, in turn, is proportional to the quantity of hydroperoxides present in the blood human sample. Thus, the hydroperoxides concentration and the quantity of free radicals compounds in blood correlate with the intensity of color detected and, consequently, to oxidative status of the sample according to the Lambert Beer`s Law (eq.5.1).

To perform this method is used a photometer in which are carried two readings: one at three minutes and the other after another three minutes. The result is available after six minutes and it traduces automatically in the difference between the two readings and transformed into FORT units based on the calibration curve stores by the microprocessor[22].

5.1.3. Free Oxygen Radicals Defense

Free Oxygen Radicals Defense (FORD) is a colorimetric method to quantify the ability of antioxidants present in plasma to reduce a performed radical cation. In other words, FORD is a test that measures antioxidant ability by the decrease of color intensity before and after adding sample.

The principle of the assay is that in an acidic pH (5.2) and in the presence of a suitable oxidant solution ($FeCl_3$), the chromogen (4-Amino-N,Ndiethylaniline) can form a stable and colored radical cation (purple) – equation 5.5. Antioxidant molecules present in the blood sample have the capacity to transfer a hydrogen atom to the chromogen radical cation reducing the amount of free radicals in the sample. Consequently, there is a reduction of the intensity of the color of the solution which is proportional to concentration of antioxidants in the sample (equation 5.6).

$$Chromogen_{(uncolored)} + oxidant(Fe^{3+}) + H^{+} \rightarrow Chromogen^{+}_{(purple)} \quad \text{(eq.5.5)}$$

$$Chromogen^{+}_{(purple)} + AOH \rightarrow Chromogen^{+}_{(uncolored)} + AO \quad \text{(eq.5.6)}$$

The decrease of intensity of color is determined specially by contribution of antioxidants (proteins, glutathione, vitamins, etc) and these antioxidants are among the most important contributions to antioxidant plasmatic defense[23].

5.2. Gas Chromatography and Liquid Chromatography

Chromatography is a separation method in which components of a sample are separated and distributed between two phases: stationary phase and mobile phase. The stationary bed phase has a large surface area and the mobile phase is a gas or a liquid which percolates through stationary phase.

It is a method in focus between modern analytical methods due to its ease of separation, identification and quantification of different components. This is a physical-chemical method that is based on the displacement of the components of a mixture due to different interactions between stationary phase and mobile phase.

The various chromatographic processes are classified according to physical state of mobile phase, mechanism of separation and by the technique used. The classification can be seen in the figure 5.3.

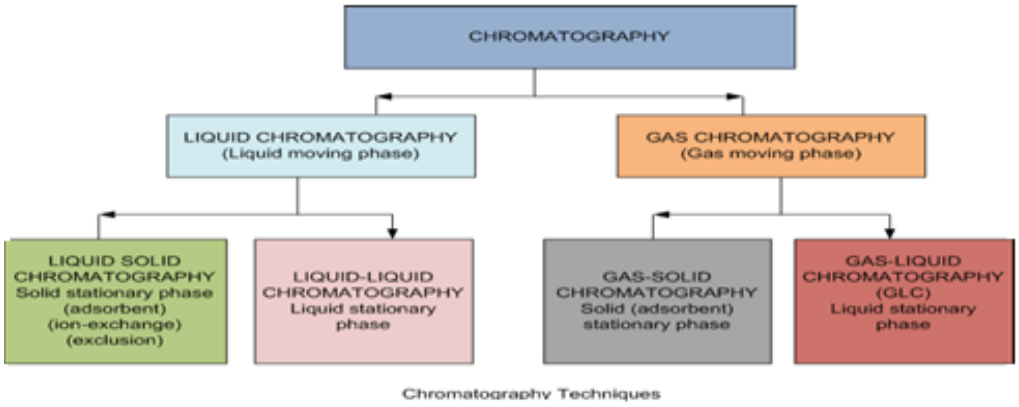

Figure 5.3: Types of Chromatography tecnhiques[24].

According to mobile phase, chromatography can be classified as gas chromatography and as liquid chromatography. In a gas chromatography (GC), the mobile phase is a gas, and in liquid chromatography (LC) the mobile phase is a liquid.

It is also made a subclassification based on stationary phase. If the stationary phase is a solid, the GC technique is called gas-solid chromatography (GSC), and if it is a liquid, gas-

liquid chromatography (GLC). As for the liquid chromatography, if the stationary phase is a liquid, the LC technique is called liquid-liquid-chromatography, and if it is a solid, liquid-solid chromatography (LSC).

Liquid Chromatography can be also dived in classical liquid chromatography (CLC) and high pressure liquid chromatography (HPLC). HPLC is a chromatographic technique that is distinguished among the others by using the mobile phase at high pressure.

Between the various chromatographic processes it will be study in this work gas chromatography coupled with Head Space and FID detector and High Pressure Liquid Chromatography with UV/visible detector. Both methods will be described below.

5.2.1. Gas Chromatography

The Gas Chromatographic (GC) method is a method for separating volatile substances, such as ethanol, of a mixture solution. GC functions is simple, an inert carrier gas (helium, nitrogen or argon) flows continuously from a large gas cylinder through the injection port, the column, and the detector – Figure 5.4. The flow rate of the carrier gas is carefully controlled to ensure the reproducible retention times and to minimize detector drift and noise. The sample is injected into the heat injection port where it is vaporized and carrier into the column (capillary column with 15 to 30m long) coated on the inside with a thin film of high boiling liquid (stationary phase). The sample partitions between the mobile phase and stationary phase and is separated into individual components.

After the column, the carrier gas and the sample pass through a detector. This detector measures the quantity of sample and generates an electric signal that goes to a data system/integrator which generates a chromatogram. The system automatically integrates the peak area, performs calculations and prints out a report with area rate and retention times.

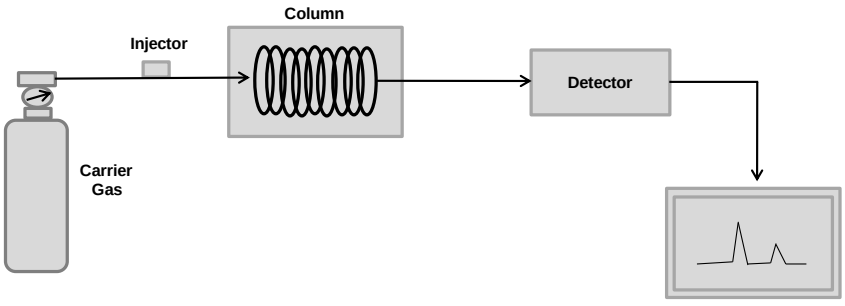

Figure 5.4: Components of Gas Chromatography coupled with a detector.

- **Gas Carrier**

The main purpose of carrier gas is to carry the sample through the column. The carrier gas is the mobile phase which has to be inert and has no interference in the sample. It also provides a suitable matrix for the detector to measure the sample components.

The purity of the carrier gas is very important because the presence of impurities such as oxygen and water can chemically attack the liquid phase in the column and destroy it. The presence of water can also desorbs other column contaminants and produces a high background or even “ghost peaks”.

- **Sample inlets**

The sample inlet should handle a wide variety of samples (gases and liquids) and permit them to be introduced into the carrier gas system after pre-treatment.

Liquid samples expand considerably when they vaporize, only small sample sizes are desirable (microliters). When liquid samples are heated to allow rapidly vaporization, care must be taken to avoid thermal decomposition.

For gas samples is required that the entire sample must be in the gas phase under the conditions in use and for solid samples, they must be dissolved in an appropriate solvent and converted into a liquid solution.

- **Columns**

The column itself can be made of stainless steel or glass. The column that will be used in this chromatographer is made of glass which is more inert and appropriate for biological samples that might react with more active stainless steel tubing.

There are many types of columns but in GC are used capillary columns which are simple, thin and may have fused silica. It also has a thin film of liquid phase inside the wall on its surface. Since this thin tube is open, its resistance to flow is very low and therefore, long lengths up to 100 meters are possible. These columns permit efficient separations of complex samples.

- **Detectors**

A detector senses the effluents from the column and provides a record graphic called chromatogram which express the peaks of each component with the respectively retention time. The detector signals are proportionate to the quantity of each solute making quantitative analysis possible.

The detector is ideally one which has the following characteristics:

- Have high sensitivity, detecting small amounts of sample;
- Be stable, insensitive to temperature and flow, in the case of gradient elutions;
- Linearity: the signal must maintain a linear relationship with the concentration of the sample;
- A continuous reading.

However, to choose a particular detector is important to know the type of analysis that will take place, since each detector is chosen for a given type of analysis.

In GC is used the flame ionization detector (FID) which has high sensivity, linearity, detectable and yet it is relatively simple and inexpensive. There are other popular detectors such as thermal conductivity cell (TCD) and the electron capture detector (ECD), but only FID detector will be mentioned in this work[25].

- **Gas Chromatography coupled with Head Space and FID detector**

In this research, the analytical method for determination of blood alcohol concentration (BAC) is gas chromatography coupled with head space and FID detector.

The technique of head space has a great advantage because it can avoid column contamination and chromatograph injector contamination so can be used different biological samples (blood, urine or saliva). The headspace analysis is generally defined as a vapor extraction involving the partition of a liquid or non-volatile solid phase and vapor phase above the liquid or solid. It is expected that the mixture contains no more vapor components of sample. This technique constitutes evaporation of analytes and sampling vapor above the fluid after reach thermal and gas equilibrium.

The main feature of this head space is the possibility of determining the volatile components in the sample directly. Furthermore, it allows the injection of the sample without prior treatment. The technique headspace has the advantage of protecting the column.

In the FID detector, the effluent from the GC column passes through the flame, which breaks down organic molecules and produces ions. These ions are collected on a negative electrode and produce an electrical signal. The FID is extremely sensitive with a great dynamic range.

Gas chromatography coupled with Head Space is considered the best technique to determine ethanol concentrations in human blood. Its only drawback is that it destroys the sample[7].

5.2.2. High Pressure Liquid Chromatography (HPLC)

The stationary phase can be a solid or a liquid and in mobile phase is used a liquid in which is dissolved the solute. Thus, while mobile phase elutes in stationary phase, the solutes are separated according to their interaction with both phases. The components which have more affinity with the mobile phase are the first components to be separated and then are separated the components which have affinity with stationary phase.

Liquid Chromatography can be subdivided in classical liquid chromatography (CLC) and high pressure liquid chromatography (HPLC). In the first case are used columns with larges diameters, packed with stationery phases finely divided. In these columns the mobile phase passes due to gravity. HPLC uses closed columns that contain very fine particles which provide efficient separations. In this type of liquid chromatography are used high pressures to force the solvent passage and to reduce time of analysis.

It is also important to refer that in HPLC some conditions must be cared such as mobile phase, flow, column and preparations of samples before analysis.

- **Operating Principle**

In HPLC the eluent is fed through one or more pumps to ensure the passing of a controlled flow of eluent through the various components of the chromatographic system – figure 5.5.

The sample is placed in the loop of the injection valve by means of a syringe when this is in the loading position. By turning the valve to the injection position, there is a change in the path of the eluent and within this sample is then drawn into the column. The eluent, which is

continuously pumped into the column will drag the sample components to the first top and then along the column.

If the solute have some affinity for the stationary phase column, along this will be established successive equilibrium distribution of the solute between the stationary and mobile phase. If the set of distribution to various solutes are sufficiently different, they will move along the column at different speeds, leaving the column separated from each other.

Then the sample components pass to a detector. A recorder, which is connected to the detector, allows obtaining automatically the signal sent by detector which is proportional to concentration of the component in solution that passes through the cell at that moment. The graphical representation of the signal sent by detector as a function of time since the injection of sample into the column constitutes what is known as the chromatogram of the mixture.

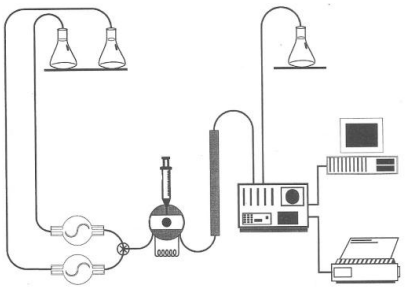

Figure 5.5: Components of liquid chromatography coupled with a detector[24].

This method has high resolution, sensibility and reproducibility but needs expensive equipments with high cost for maintenance and operation and a good expertise of the operator.

- **Mobile Phase**

Mobile phase in HPLC should be a solvent or mix of solvents that respects some characteristics imposed by the method itself. The solvent must be in mobile phase without decomposing the components and chemical interaction. It also should have high purity or being purified to be able to make high sensitivity analyzes, since impurities can strongly interfere with detection of the analyte.

Besides, the mobile phase must be compatible with detector utilized and also having suitable polarity to allow a convenient separation of sample components. Although several solvents, three are most commonly used: water, methanol and acetonitrile.

- **Stationary phase**

In HPLC, the stationary phase must be high resolution components of the sample, easy introduction into the column, have a uniform diameter or particles with pellicle porous.

Stationary phase particles are classified according to their size and the smaller the particle, the greater will be separation efficiency. Thus, the diffusion process of sample molecules is improved because smaller sizes reduce the distance between the analyte and stationary phase. The particles can be spherical or regular and irregular, being the regular more efficient due to its uniform filling.

According to mobile phase and stationary phase, the process can be classified as chromatography with normal phase and chromatography with reversal phase. In the first case, stationary phase used is polar and the mobile phase is nonpolar, referred to elution. The analyte which are more nonpolar are elute in the first place while the analytes more polar are retained by stationary phase and eluted later.

In chromatography with reversal phase, stationary phase is nonpolar while the mobile phase is polar. Polar components are eluted in first place and nonpolar components are eluted in second place.

- **Detectors**

In HPLC, the detectors fall into two broad classes: detectors macroscopic properties, which are those that measure changes in physical properties caused by the solute in mobile phase and detection properties of the solute which are those that respond to a given chemical property or physical solute and are ideally independent of mobile phase.

In HPLC detectors most commonly used are spectrophotometric (UV, DAD or fluorescence), conductometric and refractometric. The detector UV/visible will be used in this research[24].

5.2.3. Differences and Similarities between GC and HPLC

In gas chromatography, the samples must be volatile so they can pass through the column as vapor and thermally stable to not suffer decomposition under separation conditions. This gives some restrictions to GC because only gas and 20% of organic compounds can be analyzed by this method without adding an extra component to increase its volatility. Also in GC the mobile phase must be inert because it can`t interact with sample being its only function to carry the volatilized sample. In this method, the analysis time is reduced and the equipment is less expensive.

In HPLC, as opposite to GC, the samples don`t need to be volatile or thermally stable, they only need to be soluble in mobile phase and that is why HPLC have more applications in many fields than GC. While in GC the sample only interacts with mobile phase, in HPLC the samples interact with both phases, stationary phase and mobile phase, which means that HPLC can have a greater variety of separation mechanisms.

Since the variation may occur in stationary phase and mobile phase, HPLC is more versatile than GC because in GC the mobile phase is always an inert gas.

However, as in GC and HPLC, the results are translated by a chromatogram. This chart shows the different components which constitute the initial sample and are separated during the analysis. This separation occurs at different times, in other words, each component has a separation time called retention time and it is translated as a peak in the chromatogram.

Also in both methods, the identification of various components of a sample is compared with other chromatogram which is obtained by standard solutions at different concentrations. In these standard solutions, the main component is eluted in the same conditions as the sample which will be analyzed, thus components are identified by its retention times. In figure 5.6 is presented a chromatogram which compares standard solutions and components by its retention times.

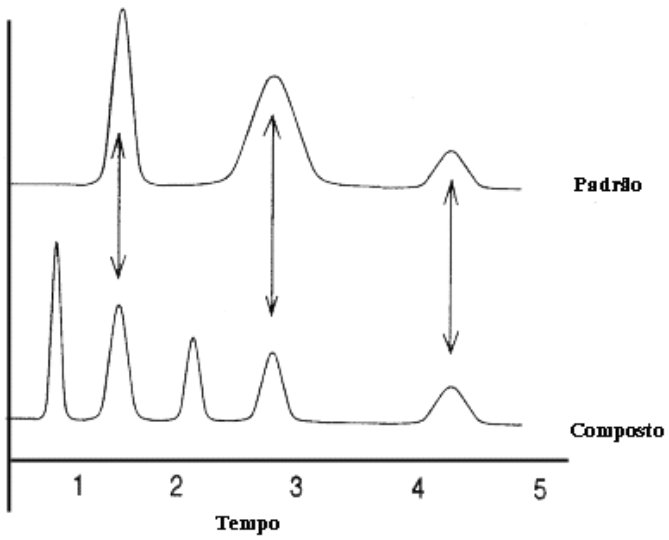

Figure 5.6: Example of a chromatogram which compares components of standard solutions and the same components of a sample by its retention times (RT)[25].

The standard solutions are analyzed in different concentrations to form a calibration curve which is translated in a graph of compound concentration by the peak area obtained. Through this calibration curve it is possible to quantify the different components when it is known the peak area because the area of the chromatogram peak corresponding to a given compound is proportional to the concentration of the detector. The quantification of each compound is done by comparing the peak area of compound present in the sample with the peak area equivalent to a known standard substance[24].

If the temperature of column and if the flow rate of mobile phase are constant, the different components are eluted from the columns at characteristic time (retention time). Retention time is characteristic of compound and type of column which is used. As showed in figure 5.6, the retention time (RT) is the distance on the axes of time from the point of sample injection to peak eluted component. The retention time is useful to do quantification of different compounds[25].

However, retention time depends of some factors such as length, type and temperature of column and mobile phase flow. The type of column is important because different absorbents fillers and oily liquids coating modifies the retention time. Also temperature and length of column can influence retention time. If temperature decreases or length of column increases, retention time will increase. Furthermore, doubling mobile phase flow, retention time is reduced in a half.

5.3. Application of Different Analytical Methods

Today is clearly evident the increasing consumption of alcohol in society and growing number of consumers of alcohol abusers and dependents. Hence, with this negative view of society in relation to alcohol is also evident the importance to create clinical and hospital treatment support for this disease that is addiction to alcohol and any other disease that is triggered by alcohol.

To combat the negative effects of alcohol are carried out investigations that help to clarify how alcohol can affect the body. In many laboratories, biological samples are used in determining blood alcohol concentration (BAC) which results in support of treatment and quality control.

Thus, in this work some alcoholic patients are selected to give blood samples for this research, among which seven are under pharmaceutical treatment and the other six have no pharmaceutical treatment, only psychological treatment. After the selection of patients, it will be set up a protocol for sample collection.

As the next step, blood samples will be used to analyze the status of alcoholic patients in relation to oxidative stress and verify the efficiency of the treatment in relation to oxidative stress and antioxidants capacity. For this are required two colorimetric methods, FORT and FORD, to evaluate the oxidative stress caused by alcohol and the capacity of antioxidants to decrease oxidative stress of each patient during treatment time, respectively. In these tests are used whole blood samples and the tests should be done as soon as after collecting samples. These two methods will be validated and optimized as will be first applied in this type of study which main point is the evaluation of oxidative stress caused by alcohol during treatment time.

To support the results of both methods mentioned above, it will be used also chromatographic methods such as GC and HPLC. GC coupled with Headspace and FID detector is applied to determine blood alcohol concentration (BAC) and this application is important to know if the patient is still drinking during treatment time. This information will help later in the discussion about results and if the pharmacologic treatment is efficient. On the other hand, HPLC is used to determine the concentration of MDA, a biological marker which is produced by lipid peroxidation that is induced by high alcohol consumption, and to be shore about the efficiency of the treatment. For HPLC experimentation, plasma is collected from each sample and placed at a low temperature so it will be possible to analyze

the biological marker. This method will help to monitor the oxidative stress by alcohol drinking abuse.

Blood samples will be assigned by patients who are undergoing treatment and clinical monitoring in the Policlinic Hospital of Rome which has a partnership with the ISS. These analyzes will evaluate status of the patients and will monitor oxidative stress during their treatment. After the experimental phases, there is the results treatment phases in which the results obtained will be traduced to statistical results and these results will be reported to the Policlinic Hospital.

6. Experimentation Part: Procedures

In this chapter will be described the procedure of each method (FORD, FORT, GC and HPLC) used in this research to evaluate oxidative stress, alcohol in human blood and the production of MDA (biomarker).

6.1. Free Oxygen Radical Test Procedure

Free Oxygen Radical Test or FORT method is the colorimetric method applied in quantification of free radicals to measure oxidative stress in a whole blood sample. The description of the procedure can be followed by figure 6.1.

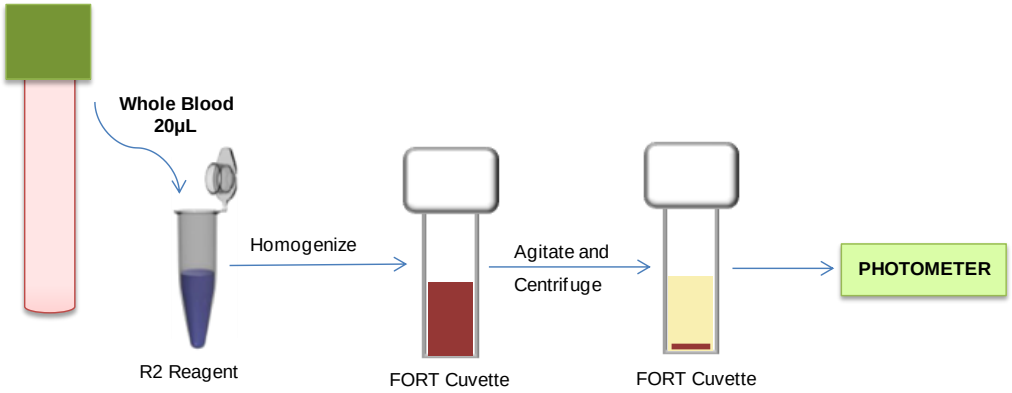

Figure 6.1: Scheme that describes FORT procedure

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The procedure begins by colleting 20µL of whole blood sample with a manual micropipette and placed this volumetric amount inside of an eppendorf. In this eppendorf there is an acid buffer known as R2 reagent with a pH of 5.0 which acts as a stabilizer when in contact with the blood sample. After mixing both liquids, blood and reagent, is important to homogenize the mixture to obtain a clean solution.

The next step is to transfer the clean solution into the FORT cuvette where is a solid mixture called chromogen, an amine derivative. This chromogenic substance is dissolved by a simple agitation with the last clean solution and it has the function of trapping free radicals that were formed in the breakdown of hydroperoxides present in the blood sample. The mixture is the result of the reaction between chromogen and two components derived from

the decomposition of hydroperoxides (free radicals). The intensity of the color is dependent on the concentration of hydroperoxides in the blood sample.

Then, the cuvette with the solution is centrifugated (centrifuge 6000 from Callegari group company) for one minute at 3500rpm, approximately, so the blood cells precipitate and be separated from plasma. It is important that the supernatant is clear after centrifugation so it can be analyzed.

Lastly, put the cuvette with optical path of 1cm into the reading cell (photometer CR3000 series from Callegari group company, fig.6.2) which will detect the chromogenic cation at 505nm and at 37ºC. The result will be available in six minutes.

Figure 6.2: Photometer used in the Laboratory for this experimentation.

In this procedure are also used plasma samples to evaluate oxidative stress status and to compare the results with whole blood samples results. However, instead of using the same aliquots used with whole blood sample must be used half quantity (10µL instead of 20µL) because the volume of blood cells which are removed correspond at almost 50% of whole blood (normal hematocrit).

The parameters for FORT method such as linearity, precision and repeatability, are indicated in table 6.1.

Table 6.1: FORT`s linearity, precision and repeatability.

Linearity	Precision	Repeatability
160 - 600 FORT units	CV < 8.5%	CV < 5%
1.22 - 4.56 mmol//L H_2O_2		

6.2. Free Oxygen Radical Defense Procedure

While FORT has the ability to quantify the presence of free radicals in a blood sample, FORD is applied to determine and quantify the antioxidant capacity to eliminate free radicals in the same blood sample. The description of the procedure can be followed by figure 6.3.

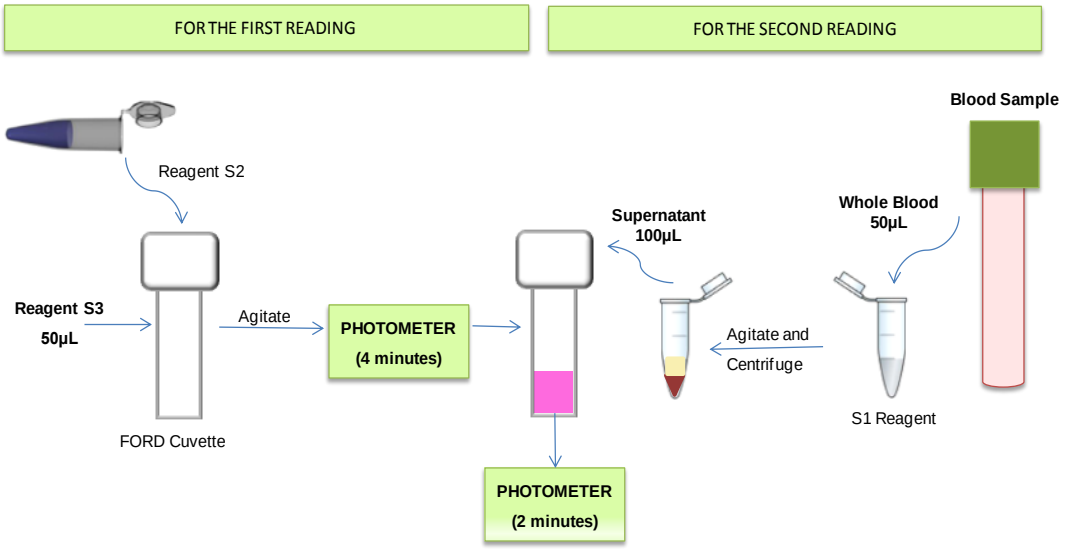


Figure 6.3: Scheme that describes FORD procedure.

To begin, it is transferred all acetate buffer (reagent S2) from the eppendorf with a pH of 5.2 into a FORD cuvette where is also added 50µL of iron solution (reagent S3) using a micropipette. The acetate buffer provides the acid environment for the occurrence of reaction between the oxidizing agent (Fe^{3+}) and chromogen which is present in solid form inside FORD cuvette. The mixture must be homogenized to dissolve the chromogen in the solution and also to obtain a clear pink solution. From the reaction results a chromogen cation that will be reduced by the presence of antioxidant components in the blood sample. Then the solution is ready for the first reading in the photometer that occurs during 4 minutes. The photometer CR3000 Callegari will detect and quantify this chromogen cation at 505nm.

Meanwhile, with a micropipette is placed 50µL of whole blood sample into the eppendorf which contains the reagent S1 (hyperosmolar solution). After homogenization, it is necessary to centrifuge (centrifuge 6000 Callegari company group) the solution during 1 minute to separate the supernatant from the blood cells.

Then, place 100µL of the supernatant with a micropipette into the solution inside the FORD cuvette at the end of the first reading. The FORD cuvette is ready for the second

reading after homogenization. The second reading will evaluate the differences in the intensity of the color from the first reading and after adding the supernatant. If there is a high antioxidant components present in the blood sample, the intensity of the color will decrease because the antioxidants have the ability to eliminate the chromogen cation, a free radical, blocking the coloration of the solution which is proportional to the concentration of antioxidant. The second reading finishes after 2 minutes and the given result is the difference between the two readings.

In this procedure are also used plasma samples to evaluate antioxidant capacity and to compare the results with whole blood samples results. However, instead of using the same aliquots used with whole blood sample must be used half quantity (10µL instead of 20µL) because the volume of blood cells which are removed correspond at almost 50% of whole blood (normal hematocrit).

The parameters for FORD method such as linearity, precision and repeatability, are indicated in table 6.2.

Table 6.2:FORD`s parameters such as linearity, precison and repeatability.

Linearity	Precision	Repeatability
0.25-3.0 mmol/L Trolox equivalents	CV < 5%	CV < 5%

6.3. Gas Chromatography Procedure

Gas chromatography is applied in this research to determine blood alcohol concentration (BAC) in whole blood samples. In this research, it is used a Clarus 600 Gas Chromatography Perkin Elmer and a TurboMatrix 40 Trap HeadSpace Sampler Perkin Elmer (Figure 6.4).

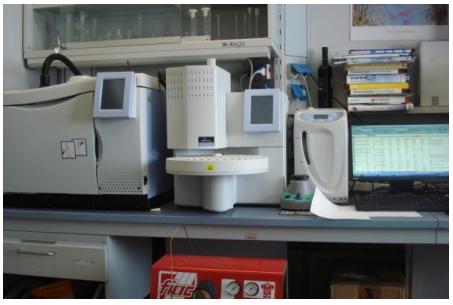


Figure 6.4: Gas Chromatography coupled with HeadSpace and FID detector used in the laboratory for this experimentation.

In this method were used a flame ionization detector at high temperature (200ºC approximately) and pure helium as carrier gas and with a constant flow.

From each blood sample is collected 100µL of whole blood with a micropipette and transferred this volume into a GC vial for headspace analysis. This transference must be quick as possible because ethanol is very volatile. The vial is warmed in headspace sampler at 80°C and after the equilibration period (15 minutes) the gases which are formed inside GC vial will be collected in GC column to be analyzed. If the sample is not analyzed in the same day as its collection, it is important to put paraffin in the top of the vial to prevent the evaporation of ethanol during time and to conserve the vial inside of a refrigerator.

Also, for each analytical run it is necessary to prepare aqueous standard solutions of 100mg%, 50mg% and 25mg% of ethanol and one blank solution and these solutions are obtained by consequents dilutions with distillated water (Figure 6.5). To prepare these solutions it is used a mother solution with a concentration of 200mg% of ethanol (250µL of 99.8% ethanol in 100mL of distillated water) – attached I.

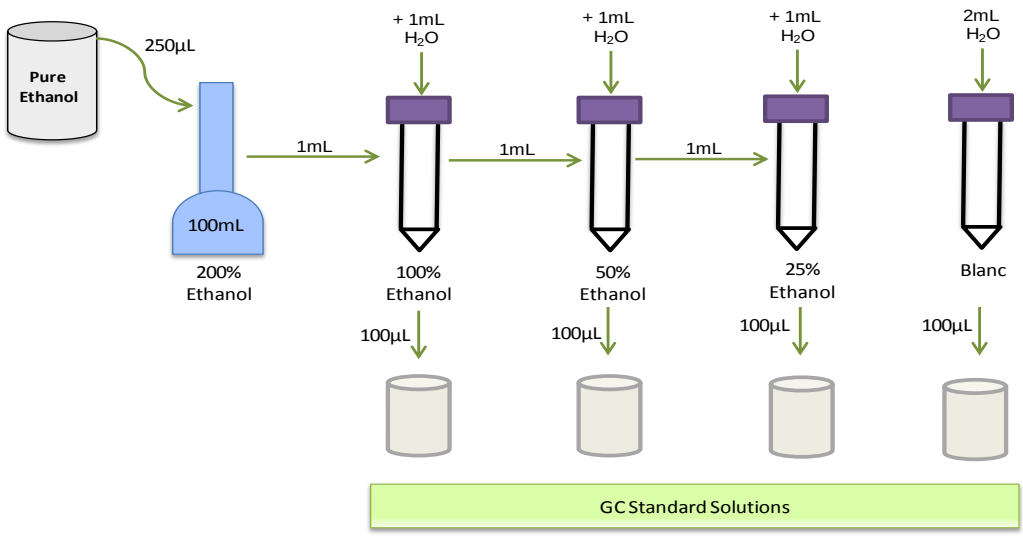


Figure 6.5: Scheme for Gas Chromatography standard solutions.

6.4. High Pressure Liquid Chromatography Procedure

The method of HPLC is used to determine the concentration of MDA, a biological component which is produced by lipid peroxidation induced by alcohol consumption and which is used as a biomarker to evaluate alcohol effect.

In this reaserch it is used a Cromatography Interface 600 series LINK, Series 200 UV/VIS detector, Series 200 pump and series 200 autosampler, in which every equipment belongs to PERKIN ELMER group company (Figure 6.6).

Figure 6.6: High Pressure Liquid Chromatography used in the Laboratory for this experimentation.

To start this method is necessary to prepare standard solutions through serial dilutions to obtained concentrations of 5, 2.5, 1.5 and 0.5µmol from 5mmol MDA concentrated solution that was obtained by diluting 25µl of 1,1,3,3-tetraethoxypropane (TEP) commercial solution (96%) in 20mL of 40% ethanol solution. The preparation of standard solutions can be followed by the scheme shown on figure 6.7.

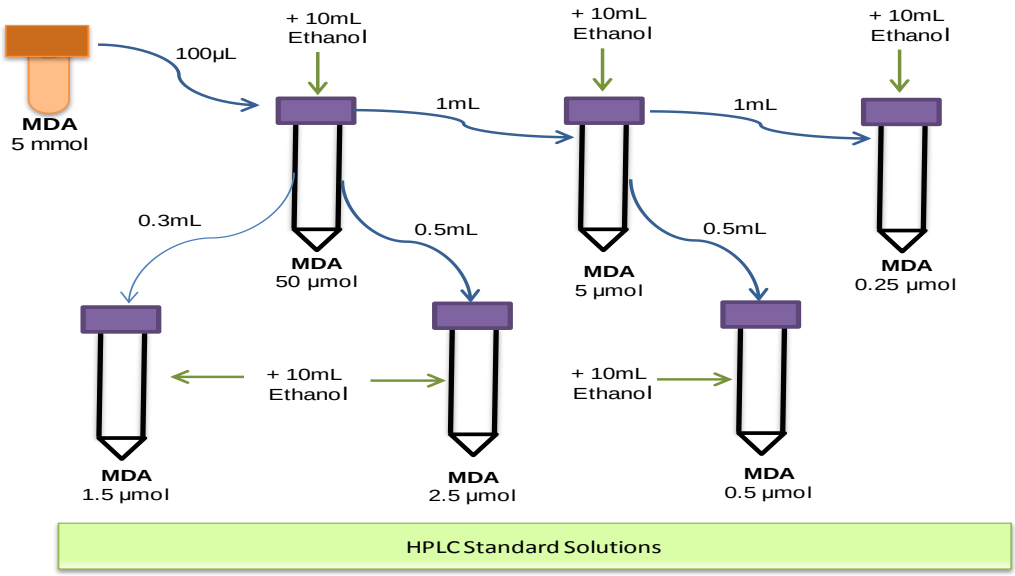


Figure 6.7: Scheme for HPLC Standard Solutions.

Besides preparing standard solutions, is necessary to prepare control samples which are used to compare MDA concentration of healthy subjects with MDA concentration of patients with alcohol abuse.

To perform HPLC it is necessary to prepare the samples of standard solutions, controls and patients. These samples have the same preparation by adding 50µl of plasma or standard solutions in plastic centrifuge tubes. At this volume is also added 50µl of Butylated Hydroxytoluene (BHT) at 0.05%, 400µl of phosphoric acid solution (H_3PO_4) at 0.44M and 100µl of 2-thiobarbituric acid (TBA) at 0.6%.

BHT was obtained by dissolving 0.05 grams of solid BHT in 100mL of distillated water. H_3PO_4 at 0.44M was obtained by diluting 2.56ml of concentrated phosphoric acid to 100ml final volume using distillated water. TBA at 0.8% was obtained by dissolving 0.8 grams of TBA in 100ml of distillated water on a stirring hot-plate at 50-55°C because TBA is only soluble in hot water (solubility increases with temperature).

All of these components are added for the following reasons: BHT is important to precipitate proteins while TBA is used to react with MDA which has no color to form a colored complex. H_3PO_4 solution is important to adjust pH and to provide the acid environment to form that colored complex.

After adding all the components, the samples tubes were capped tightly, vortex mixed, put a hole in the cap and then heated for 1 hour on a 100°C in a hot plate (Accublock Digital Dry Bath Labnet International, Inc. Instruments), so it can occur the reaction between MDA and TBA. Then this is followed by 5 minutes inside the refrigerator to decrease the temperature. When the samples are heating it may occur some evaporation of some components but inside the refrigerator with the decrease temperature the components are again in the solution.

To finalize the preparation of samples, they are transferred into new plastic centrifuge tubes where it is added 250µl of pure n-butanol (BuOH) in each vial. This last component allows the extraction of MDA-TBA colored complex.

Plastic centrifuge tubes were vortex mixed again and put in a ultra-centrifuge for 10 minutes at 10.000 rpm to separate the two phases. Then, aliquots of 100µl of supernatant phase were transferred from the n-butanol layer of each sample and placed in HPLC vials for analysis. The autosampler of HPLC, which is automatic, collects 20µL of each aliquot.

As for the mobile phase used in HPLC, it is a tampon solution of monopotassium fosfate (KH_2PO_4) at 50mM and pure methanol (60:40). The KH_2PO_4 solution is obtained by dissolving 6.8 grams of solid KH_2PO_4 in 1 liter of distillated water. To stabilized the pH to 6.8 is added small quantities of hydroxide potassium (KOH), starting with 3mL. It is used a pH meterMIR 150 pH/temperature bench meter Martini Instruments.

7. Results and Discussion

After the experimental phase, it is important to analyze the results obtained in different methods that were applied in this study to verify the effectiveness of biomarkers to evaluate oxidative stress in alcoholic patients. To recapitulate, in this research a group of alcoholic patients were selected to give blood samples in which seven of them are submitted to a antioxidant pharmaceutical treatment and six of them are only under psychological treatment (without pharmaceutical treatment) during 15 days. The selected patients were two women, one in each group, and eleven men with ages from the range 25 to 50 years old. The patients` list are indicated on table 7.1 where is the information about gender and if they are or not under medicine treatment. The patients were alphabetically classified by order of arrival.

Table 7.1: List of patients that were selected to give blood samples for this research.

Patient	Gender	Pharmacological Treatment
A	M	Yes
B	M	No
C	M	No
D	M	Yes
E	M	Yes
F	M	No
G	F	No
H	M	Yes
I	F	Yes
J	M	No
K	M	Yes
L	M	Yes
M	M	No

Each patient was responsible for giving four blood samples, being the first sampling collection considered as treatment time 0 and the others three samples were collected at treatment time 3, treatment time 7 and treatment time 15 (3, 7 and 15 days after first collection, respectively). From each sample, a small quantity of whole blood was used to analyze presence of free radicals and antioxidant capacity in blood sample applying FORT and FORD methods, respectively. Other small quantity of 100µl of whole blood was quickly transferred to a GC vial to analyze blood alcohol concentration (BAC) using headspace gas chromatography (HS-GC). After collecting aliquots of whole blood to do these analyses, the blood sample with heparin was centrifuged to separate the blood cells from plasma. Plasma samples were also submitted to FORT and FORD tests and used to analyze MDA concentration by HPLC. However, whole blood samples were used only fresh while plasma samples not analyzed were frozen until they were submitted to analysis.

In this research were also used control group samples which were submitted to FORT and FORD methods and used to determine MDA concentration to compare with patient`s results. The samples of control groups correspond to healthy subjects who may be social drinkers and they underwent the same analytical treatment of the patients' samples.

The results obtained in each method will be traduced to statistical results and they will be reported to Policlinic Hospital so they may be utilized for alcoholic patient's treatment.

• **Free Oxygen Radical Test and Free Oxygen Radical Defense`s Results:**

FORT method was applied to quantify free radicals present on a whole blood sample and plasma samples and to evaluate oxidative stress status caused by high alcohol consumption. For this evaluation it was made measurements of aliquots of samples for each treatment time. The free radicals present in this aliquot were catalyzed by iron solution and trapped by chromogenic substance. The concentration of this cation is proportional to the concentration of free radicals in the aliquot, thus the greater the intensity of pink coloration resulting from the reaction, the greater the concentration of free radicals in the solution.

As for FORD method, it is useful to evaluate antioxidant barrier against free radicals that are markedly produced due to excessive alcohol consumption. In this case, the iron solution reacts with chromogen substance to form the colored cation which will be reduced by antioxidants present in an aliquot of whole blood. If the pink color of solution disappears it means that antioxidant capacity in eliminating free radicals is very strong.

Before proceeding to clinical studies, it was crucial to carry out preliminary tests to validate and optimize the method and to evaluate results` stabilization. To achieve this first objective, whole blood samples were analyzed in duplicate or triplicate after 1hour, 3hours and 6hours of sampling collection. These three assays in different times of the day were done as is the first time that photometer used is being applied and also if two methods can be applied as a quick diagnostic procedure, for example in hospitals and pharmacies. The results obtained are presented in table II.4 and II.6 on attachment II.

Evaluating these results obtained by FORT and FORD methods is possible to observe the degree of concordance between the measurements values of the same sample performed under various conditions (for example: lowering blood temperature within six hours) which means the reproducibility between results during 1 day. As results of duplicates and/or triplicates do not show a significant difference between them and between 1, 3 and 6hours, it

can be said that procedure`s reproducibility show a reasonable concordance between results.

To support this observations was calculated the coefficient of variation (CV) that translates to a percentage the procedure`s accuracy. The CV parameter was calculated comparing the results of 1hour with 3hours and 1hour with 6hours and the proximity found between them explains why the coefficient of variation (CV) is low in major cases which statistically mean media dispersion between results. Some high values of CV that can be found may be due to casual errors due to operator on measuring the volume of blood samples aliquots. Besides, it is noticeable that blood temperature influences the results, because temperature influences any measurement that involves a reaction.

The next step was to observe if there is any difference between groups and to start is important to understand the initial situation comparing the results of alcoholic patients at treatment time zero with control group. The results of tables II.1, II.2 and II.3 were traduced to a bar graph presented on next figure 7.1 using a special computer program named MedCalc.

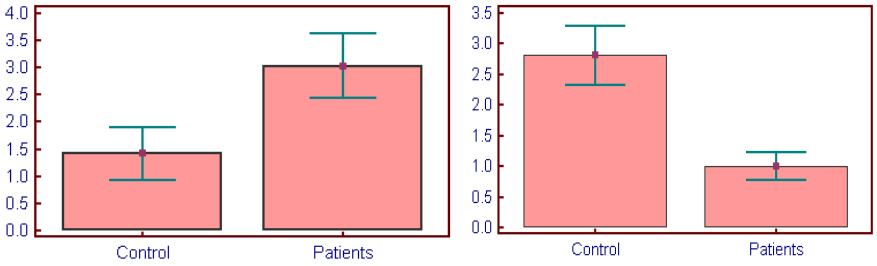


Figure 7.1: Graphs which characterizes FORT results (left) and FORD results (right) between alcoholic patients and control group in the beginning.

Observing the graph on figure 7.1 is noticed that control group values are lower than alcoholic patients. Alcoholic patients have an average of 3.59±1.01mmol/LH_2O_2 and control group have an average of 1.42±0.53mmol/LH_2O_2, showing a significant difference between two groups (P = 0.0006) – attachment II.

Add that it is very important to understand what the referent values are in the human body. For FORT method, the referent values of free radicals present in human body are all values that are under the reference value which is 2.35mmol/LH_2O_2. As for FORD method, the reference values of antioxidants are between the range 1.07 and 1.53mmol/LH_2O_2.

As for FORD results happens the opposite, values of alcoholic patients are lower having an average of 1.07±0.53mmol/LH_2O_2 while control group have an average of 2.81±0.46mmol/LH_2O_2, showing too a significant difference between two groups (P = 0.0075) – attachment II.

To help to evaluate effectiveness of treatment is necessary to analyze if there is any difference after 15 days of treatment between two groups of alcoholic patients. In tables II.1 and table II.2 (attachment II) are presented the results obtained for patients under pharmacological treatment and the results obtained for patients that have only psychological treatment, respectively. These results were traduced to a graph in figure 7.2.

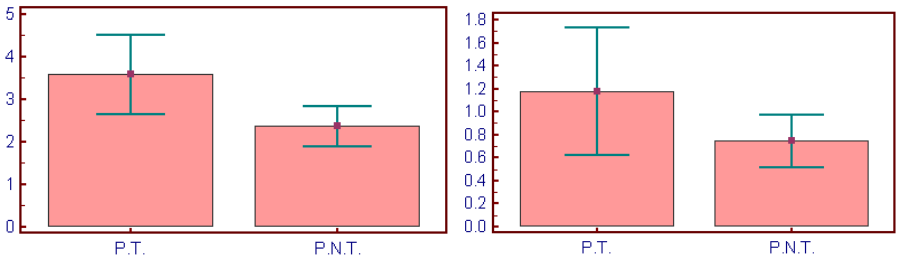


Figure 7.2: Graphs which characterize the difference of FORT (left) and FORD (right) results between patients under pharmaceutical treatment (PT) and patients with no treatment (PNT) after 15days.

Group of alcoholic patients under treatment have an average of 3.72±1.04mmol/LH_2O_2 and group of patients with no treatment have an average of 2.37±0.44mmol/LH_2O_2 in relation to FORT results, showing a significant difference (P=0.0073), although ideally the bar of patients under treatment should not be the highest. For FORD method, group of alcoholic patients under treatment have an average of 1.16±0.65mmol/LH_2O_2 and group of patients with no treatment have an average of 0.75±0.22mmol/LH_2O_2. In this case, the difference is not significant but is still high being values only 16% compatible - attachment II.

Starting from patients that are under pharmacological treatment, observes that major patients have their values above 2.35mmol/LH_2O_2. Between treatments times 0 to 15 observes that there is no marked difference noticing a simple small oscillation. This fact is confirmed by the graph shown on figure 7.3.

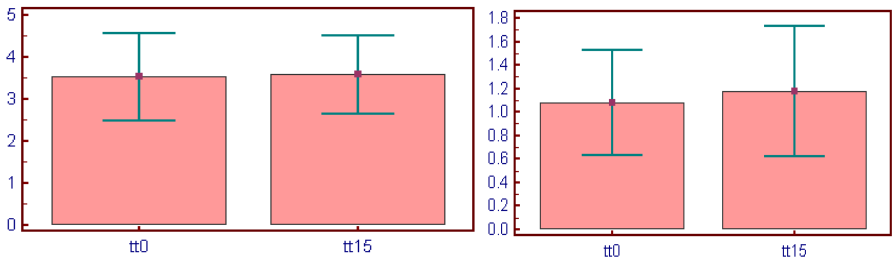


Figure 7.3: Graph which characterizes the difference between the beginning of treatment (tt0) and after 15days (tt15) for patients under pharmaceutical treatment.

In relation to FORD results, is evident that major values are not in reference range mentioned before, indicating a low capacity of antioxidant in eliminating free radicals and normalizing oxidative stress status. It is also found that the antioxidant barrier is constant during 15 days of treatment time and there is a small oscillation in a globally way, being the values practically the same.

The same small oscillation is noticed for patients that are only under psychological treatment, being the values of different treatment times close to each other and not marking too the difference between them.

Another parameter can be mentioned here, a Receiving Operating Characteristic (ROC) curve was determined using MedCalc program and using FORT and FORD methods´ results. ROC curve is a fundamental tool for diagnostic test evaluation and describes in a graph a complete sensitivity/specificity report. In figure 7.4 are presented two ROC curves, one for each method.

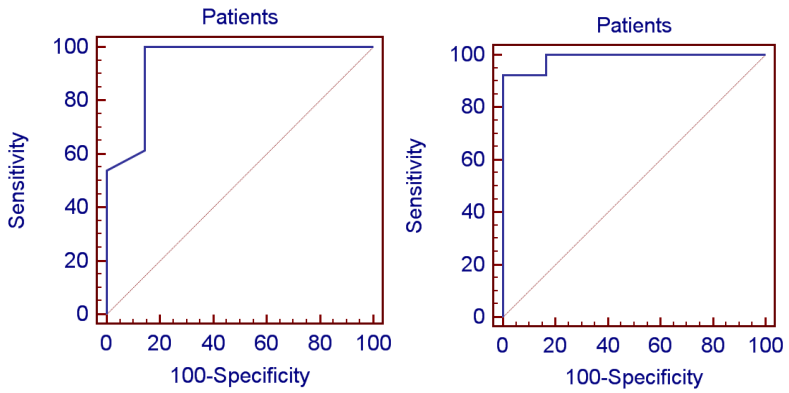


Figure 7.4: ROC curves resulting from FORT (on the right) and FORD (on the left) methods.

In a ROC curve the true positive rate (sensitivity) is plotted in function of false positive rate (100-specificity) for different cut-off points and each point represents a sensitivity/specificity

pair corresponding to a particular decision threshold. The area under ROC curve is a measure how well a parameter can distinguish between two diagnostic groups (disease/normal) which in this case are alcoholic patients and control group. Therefore, both curves show a perfect discrimination because ROC curve passes through the upper left corner (100% sensitivity/100% specificity) and the closer ROC curve is to the upper left corner, the higher the overall accuracy of the test.

• **Malondialdehyde Concentration`s Results:**

Another important point of this work was the determination of MDA concentration which is a lipid peroxidation product used as biological marker. MDA concentration was measured with HPLC method and evaluated as an indicator of oxidative stress status and antioxidant pharmaceutical treatment response.

In analytical terms, chromatograms obtained with HPLC show a well defined and separated peaks and with good resolution. In figures 7.5, 7.6 and 7.7 are showed three examples of chromatograms resulting from a blank, a patient and control samples, respectively.

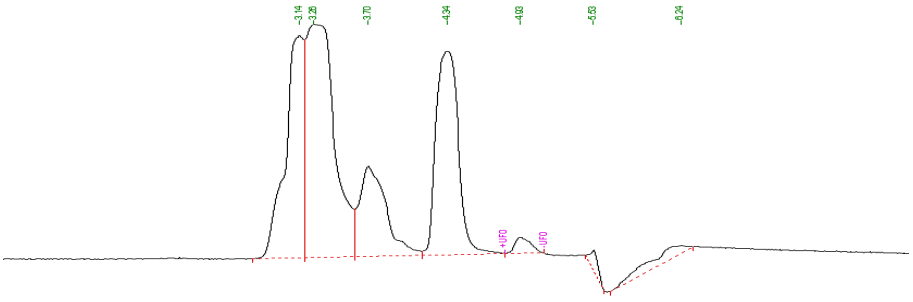


Figure 7.5: HPLC`s chromatogram resulting from a Blank.

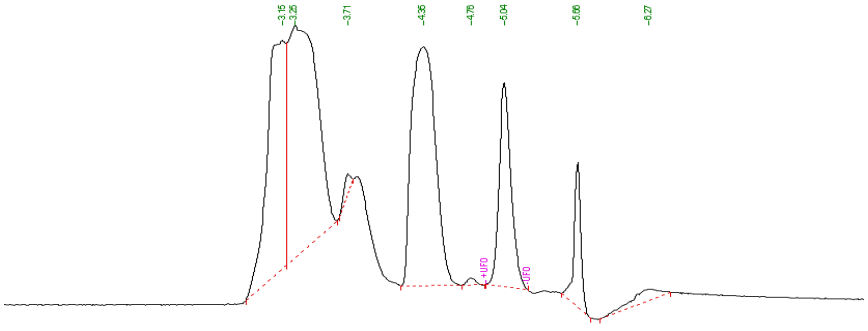


Figure 7.6: HPLC`s chromatogram resulting from one patient sample.

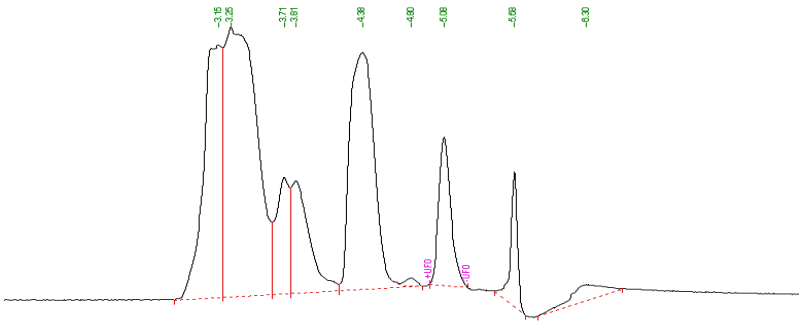


Figure 7.7: HPLC`s chromatogram resulting from sample of one Control sample.

In figure 7.5 which represents blank`s chromatogram, the peak of MDA component doesn`t appear since blank does not contain the major component. Nevertheless, the other components peaks are represented in this order: TBA, H_3PO_4, BHT and butanol. Each component is separated at different retention times which are indicated in table 7.2.

Table 7.2: Retention times of each component.

Component	TBA	H_3PO_4	BHT	MDA	Butanol
Retention Time (min)	3.2	4.6	4.8	5.0	5.7

In the other two chromatograms is noticed the presence of MDA`s peak which has a retention time of 5.0 minutes, approximately. The order of elution is the same as in blank`s chromatogram, although MDA elutes first than butanol. It is also visible a difference in peaks of butanol between these chromatograms. Contrary to what happens in blank`s chromatogram and in chromatograms obtained with standard solutions, chromatograms resulting from patients samples and control group samples show a large peak together with butanol perhaps due to some interference of the sample.

On attachments IV to VII are presented the chromatograms resulting from standard solutions, patients samples and control group samples and in attach III are presented the results for MDA retention times, MDA peaks area and MDA concentration. These results show that both retention times and peaks areas are reproducible because they show a good concordance between duplicates (table III.4). The good reproducibility between duplicates indicates a high precision of the procedure. The little variation that can be found in duplicates may be due to constant use of column.

If there is a small variation in peaks area, the same variation will be noticed in MDA concentration of duplicates because peaks area is directly proportional to MDA

concentration, thus the greater the peak area, the greater MDA concentration. That`s why the values presented in table III.1 and III.2 are the media of duplicates as is also control group values presented on table III.3 in same attach.

It is equally important to add that the concentration values obtained for standard curves of MDA are within limits of concentration 0.5 to 5µmol/L and linearly proportional to analyte concentration. Mention that the correlation factor is always much closed to 1 which proves a good linearity of the method. The accuracy and precision of this method are determined by measuring MDA standard solutions in that concentration range. These results can be seen on table 7.3 and they show a good reproducibility and precision of the method because the highest coefficient of variation is 9.5% and the lowest is 0.82%. Globally, the absolute error is under 4% showing a good concordance between theoretical values and real values obtained in different days (5 days) which means a high accuracy of the method.

Table 7.3: Parameters of variability for HPLC standard solutions.

Theoretical Value	Real Value					Parameters of Variability			
Concentration (µmol/L)	1	2	3	4	5	Media	σ	C.V. (%)	ξ (%)
0.50	0.58	0.55	0.46	0.53	0.48	0.52	0.0497	9.56	3.89
1.5	1.55	1.57	1.54	1.58	1.56	1.56	0.0128	0.823	4.05
2.5	2.74	2.63	2.48	2.42	2.41	2.54	0.145	5.71	1.46
5.0	4.86	4.91	5.00	5.02	5.03	4.96	0.0761	1.53	3.89

For HPLC method the limit of detection (LOD) which is defined as lowest MDA concentration that can be detected with an adequate accuracy is 0.03µmol/L and the limit of quantification (LOQ) which is define as lowest MDA concentration that can be quantified with an adequate accuracy and precision is 0.15µmol/L.

In general, this HPLC procedure is an excellent method to determine MDA concentration in plasma samples and it is also a rapid method because it can give fast results (10 minutes per sample) which give the possibility to analyze many samples per day.

Turning now to the mean values of MDA concentration is possible to check whether there is any difference between results of alcoholic patients and control group at beginning of treatment (treatment time 0). These results were translated to bar graph shown on figure 7.8.

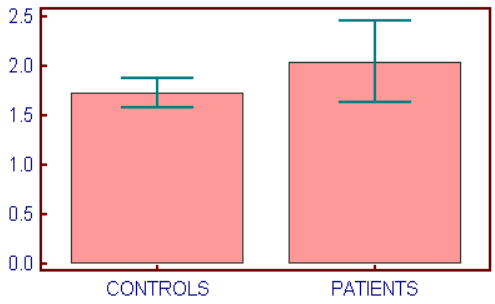


Figure 7.8: Difference between alcoholic patients and control group in relation to MDA concentration.

MDA concentration of both groups doesn't show a significant difference with an average of 2.04±0.71µmol/L for alcoholic patients and 1.73±0.26µmol/L for control group, being a difference between both groups, although it is close to significance (P=0.084) – table III.5. The reference values for MDA concentration are 1.38±0.8µmol/L and they were obtained by determination of MDA in 11 healthy subjects.

It is also important to compare patients under antioxidant pharmaceutical treatment and patients under no treatment to understand the effectiveness of treatment in reducing oxidative stress status. These results were traduced to a bar graph (figure 7.9) calculating the total area under curve obtained with different treatment times and to statistical results (attach III).

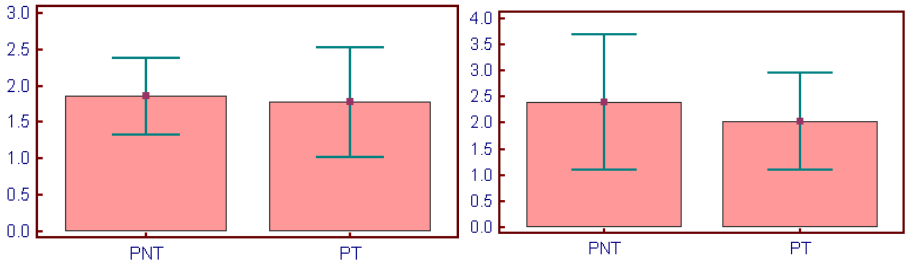


Figure 7.9: Difference between alcoholic patients under pharmaceutical treatment and alcoholic patients with no treatment at the beginning (left) and at the end (right) of treatment.

Looking to the graph on figure 7.9 there is no difference between two groups of alcoholic patients at the beginning of treatment and after 15 days of treatment. At treatment time 0 (tt_0), patients under treatment have an average of 1.72±0.88µmol/L and patients without treatment have an average of 1.86±0.50µmol/L, being the difference not significant (P=0.77) – attach III. Now, at the end of the 15 days, patients under treatment have an average of 2.26±0.85µmol/L and patients without treatment have an average of 2.40±1.23µmol/L, being

the difference also not significant (P=0.79) and practically the same as it was at beginning of treatment.

As the results show no difference between three groups, it is important to consider an external factor that can influence the final results as for example patients keep drinking during treatment time and this is why it is very important the determination of BAC by GC method.

• **Blood Alcohol Concentration`s Results:**

The determination of Blood Alcohol Concentration (BAC) is fundamental to verify whether or not the alcoholic patient continues to drink during 15 days of treatment because it is a fact that influences the outcome about effectiveness of antioxidant treatment.

The goal of HS-GC is to quantify the presence of ethanol in bloodstream. From each whole blood sample were transferred 100µL to GC vial which were tightly closed and heated up to 80°C. Volatile compounds in the vials are analyzed by GC. This method analyze one sample in 5 minutes which gives the possibility to analyze many samples per day and even to finish the experiment in a couple of days. HS-GC is independent from biological matrix since only volatile phase is analyzed.

In the attachment VIII are presented BAC results and in attach IX are presented the chromatograms resulting from standard solutions and alcoholic patients samples. In the figures 7.10 and 7.11 that appear below are represented a standard solution`s chromatogram and a alcoholic patient`s chromatogram.

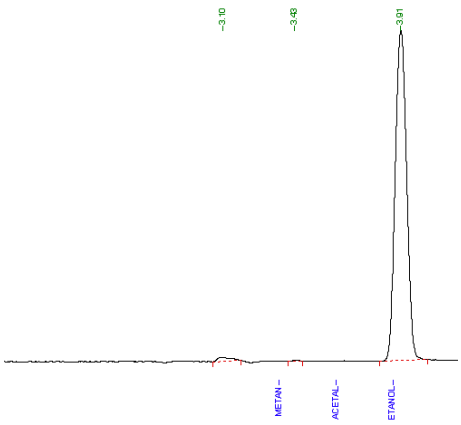


Figure 7.10: 25mg% Standard Solution`s Chromatogram resulting from GC method.

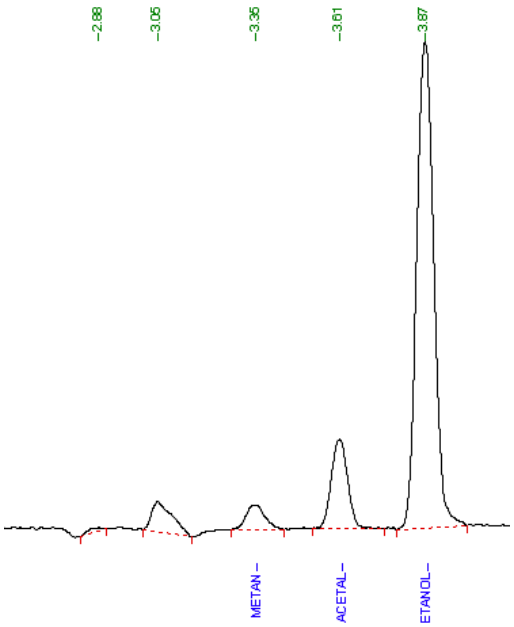


Figure 7.11: Chromatograms of an alcoholic patient resulting from GC method.

In standard solution`s chromatogram on figure 7.10 there is only ethanol to be separated and detected because standard solution is composed only by water and ethanol so in the chromatogram only appears ethanol`s peak with a retention time of 3.9 minutes.

When the alcoholic patient drinks alcohol, it is possible to separate and quantify the presence of three components in a whole blood sample such as methanol, acetaldehyde and ethanol. In the chromatogram of figure 7.11 are represented the three components where methanol is eluted first in carrier gas following acetaldehyde and ethanol in this order. The retention times of each component which indicates the different times of separation are presented on table 7.4. As it is also possible to see, the three component`s peaks are well define and with good resolution.

Table 7.4: Retention time of methanol, acetaldehyde and ethanol, approximately.

Component	Methanol	Acetaldehyde	Ethanol
Retention Time (min)	3.4	3.8	3.9

Besides ethanol, acetaldehyde can be detected in bloodstream because acetaldehyde is a product resulting from ethanol metabolism where is involved the two enzymes alcohol dehydrogenase and cytochrome. Also methanol can be detected in bloodstream even because some wines have methanol in their composition due to degradation of peptic

substances present in grape by the action of its enzymes which in high proportions is harmful to the human body.

Analyzing now BAC results (attachment VIII) it was found high quantity of ethanol in some alcoholic patients who are not under antioxidant treatment. In next table 7.5 are presented BAC (%) that was found in some alcoholic patients without treatment. All patients under treatment gave negative results for BAC. The results of BAC in this table were determined from calibration curve and from the sum of peak areas corresponding to acetaldehyde and ethanol.

Table 7.5: Positive Blood Alcohol Concentration (%) of some alcoholic patients without treatment.

Patient	Treatment Time (days)	BAC (%)
B	0	75
	15	92
F	3	60.4
	15	92
M	0	77

In general, it is possible to make some considerations concerning the results presented before which were obtained by four different methods.

Starting from FORT and FORD methods, as both showed having a good procedure`s reproducibility, both methods can be applied for this type of study and/or used in pharmacies or hospitals to give a quickly result.

Between control group and alcoholic patients group, alcoholic patients have a higher quantity of free radicals in their blood than controls because the higher the consumption, the higher the quantity of free radicals. In relation to antioxidant barrier capacity in eliminating these free radicals, alcoholic patients have a lower capacity than controls because excessive alcohol consumption decreases that capacity. Although controls may be social drinkers, the quantity of alcohol consumed is not sufficient to cause an imbalance between free radicals and antioxidants.

Regarding the results obtained for the two groups of alcoholic patients, the group under antioxidant pharmaceutical treatment has the highest quantity of free radicals showing a significant difference in comparison with patients without treatment, although ideally it should be the opposite. However, the difference in relation to antioxidant barrier capacity is not significant between both groups. It should also add that there is no difference in quantity of free radicals and antioxidant capacity between the beginning and at the end of treatment for patients under treatment.

In case of MDA concentration, control group and alcoholic patients group show a high difference but not significant between the values at the beginning of treatment. In some cases of control group samples the concentration of MDA is a little higher than normal perhaps due to the fact that they may be in contact with other type of harmful substance such as tobacco. Add also that MDA concentration obtained for the two groups of alcoholic patients is practically the same at the beginning and at the end of treatment, showing no improvement in MDA concentration and no difference between them. However, in literature was found that TBA component which reacts with MDA can also react with other components of plasma increasing artefactually the concentration of MDA.

Although the results show no difference between three groups, it is not completely correct to affirm that the antioxidant pharmaceutical treatment has no effect in reducing oxidative stress by reducing MDA because MDA concentration is not directly proportional to alcohol consumption.

About results of alcohol concentration in blood (BAC), it was not detected recent use of alcohol in patients who are under antioxidant treatment but it was found a high alcohol concentration in some patients without treatment. However, in cases where ethanol wasn't detected in alcoholic patients samples it doesn`t mean surely that alcoholic patients didn`t drink during sample collections because alcohol is metabolized and eliminated quickly in the organism and can only be detected a few hours after consumption. It is also important to add that alcoholic patients know when they have to give blood sample and they may drink a few days before or after sampling collection so it won`t be detected when the sample is analyzed.

Furthermore, other factors can influence the final results as for example: patients' organism tolerance for the treatment, psychological instability and low nutritional status of the patient, patient continues to forget to take the medicine, appearance of other vicious such as tobacco or even the consumption of cannabis, etc.

There is also other possibility that a treatment time of only 15 days is not sufficient to stabilize the oxidative stress in alcoholic patients whose body is accustomed with presence of alcohol.

For all of these reasons, it is not correct to confirm if antioxidant treatment applied for only 15 days has a negative or a positive effect in relation to oxidative stress.

8. Conclusion and Suggestions for the Future

Since the time of industrial revolution alcohol has become one of the products most marked and consumed all over the world. However, the easily acceptance by society turn to a certain additive drug and a drug that, when consumed in excess, causes psychological problems, violence and diseases such as cancer and liver disease.

Because the number of people affected by alcohol is increasingly high, some investigations are being conducted to combat the excessive alcohol consumption and its effects. In this research the mains purpose was to study the effect of alcohol in oxidative stress in alcoholic patients that were submitted to an antioxidant pharmaceutical treatment which purpose was to reduce the oxidative stress status caused by excessive alcohol consumption. Thus, a group of alcoholic patients were selected for giving blood samples during 15 days of treatment. In this group some have undergone treatment and some were only subjected to psychological treatment.

From each blood sample, whole blood aliquots and plasma aliquots were analyzed in four different methods: FORT, FORD, GC and HPLC. In FORT and FORD methods, the group of alcoholic patients showed to have a higher quantity of free radicals in their whole blood samples (average of 3.59±1.01mmol/H_2O_2) and a lower antioxidant barrier in eliminating those free radicals (1.07±0.53mmol/H_2O_2) than group of control (1.42±0.53mmol/H_2O_2 and 2.81±0.46mmol/H_2O_2, respectively). The difference between these two groups is very significant because the probability is lower than 5%. Now the difference between two groups of alcoholic patients in relation to free radicals quantity is very significant (P=0.0073) but in relation to antioxidant capacity is practically the same (P=0.16), being the group under pharmaceutical treatment with highest values.

In case of MDA concentration, it is lower in quality control groups than alcoholic patients, although the difference is not very significant (P=0.084), being the average of 2.04±0.71µmol/L for alcoholic patients and 1.73±0.26µmol/L for quality control. Between two groups of alcoholic patients, there is no difference at the beginning and at the end of treatment (P=0.77 and P=0.79, respectively).

Furthermore, the results obtained for BAC by GC method showed that only a few patients of alcoholic group without pharmaceutical treatment have been drinking during treatment time. Alcoholic patients under treatment showed no ethanol concentration in their blood.

It also adds that these results may have been influenced by other external factors such as nutritional status of patients, patients who are smokers, patients who continue to drink during treatment time, patients that consumes other type of drugs (cannabis), etc.

For all of these reasons, no confirmation about the effectiveness of pharmaceutical treatment can be made because the behavior of the results it is not very clear and the fact that maybe the application period of treatment be to short to have a clear and positive effect.

In the following investigations it may be important to study the influence of low temperature on plasma and MDA concentration and the fact that plasma is kept conserved for a long period of time. It is also important to ensure that the group of quality control is not in contact with other harmful substances such as tobacco which may influence the final results and conclusion about the effectiveness of treatment. Another fact that influenced the confirmation about the effectiveness is the impossibility in determining blood alcohol concentration with GC method due to short period of work time.

It will be interesting in the future to conduct a study on the influence of alcohol in different age groups, including young teens and its effect in school and social life. Another study which may be accomplished is the effect of mixing alcohol with energy drinks in young people and how can accelerate the probability of a heart attack. Or even study the influence of alcohol during cancer induction in different genders.

Another good possibility is to perform a partnership between several European countries to conduct studies on the influence of alcohol and to combat the excessive consumption of this drug which is camouflaged in society.

Bibliography

[1] Institute Superiore di Sanitá (ISS); "What we do"; URL: www.iss.it (Accessed on March 11, 2012);

[2] Tratamento do Alcoolismo; "*Os Riscos do Consumo de Álcool"* ; July, 2010; URL: http://alcoolismotratamento.net/historia-alcool-sociedade/ (Accessed on March 10, 2012);

[3] Centro de Informações Sobre Saúde e Álcool (CISA); "*História do Álcool";* 2012. URL:http://www.cisa.org.br/categoria.html?FhIdTexto=25ff28cda5f109c71bb2387dd75df853&ret=& (Accessed on March 10, 2012);

[4] World Helth Organization (WHO); "*Consumption of Alcohol per capita in adults*"; Report EURO/10/2008 of September 12th, 2005. URL: www.who.int (Accessed on March 10, 2012);

[5] Milhano, A. I; Álcool e Segurança Rodoviária; "Álcohol"; December, 16th 2009; URL: http://www.zona-s.pt/default.aspx?Page=3053&articleId=543&mId=8518&SNPrintMode=true&articleType=ArticleView&SkinSrc=[G]Skins/Common/PrintModule&ContainerSrc=[G]Containers/Common/None (Accessed on March 10, 2012);

[6] Direcção-Geral de Saúde; "*Consumo de bebidas álcoólicas – Estado de saúde e seus determinantes*"; Elementos Estatísticos: Informação geral; Saúde 2008; Report ISSN 0872-1114; p.41; December, 2012.

[7] Rego, Teresa Cristina; "Avaliação de um método de Cromatografia em fase gasosa – Head Space e Estudo da estabilidade do Etanol em Amostras de Sangue"; Pós-Graduação em Ciências Farmacêuticas; Centro de Ciências da Saúde; Universidade Federal do Rio Grande do Norte; Natal 2008. 81pages.

[8] Keely, J. Frank; "*Oxidative Stress: its role in air pollution and adverse health effects*"; Occupation and Environmental Medicine; Environmental Research Group, School of Health & Life Science; London 2003.

URL: http://oem.bmj.com/content/60/8/612.full (Accessed on April 17, 2012);

[9] Nirelia M. Idrus, Ph.D., and Jennifer D. Thomas, Ph.D; "*Fetal Alcohol Spectrum Disorders: Experimental Treatments and Strategies for Intervention*"; National Institute of Alcohol Abuse and Alcoholism; Alcohol Research & Health, Volume 34, Issue Number 1. URL: http://pubs.niaaa.nih.gov/publications/arh341/76-85.htm (Accessed on April 22, 2012);

[10] Technology, N. I. (s.d.); From Online Resources for Disorders Caused by Oxidative Stress; URL: http://www.oxidativestressresource.org/ (Accessed on March 2, 2012);

[11] *Oxidative Stress: what is it?* (2009); Medical Aesthetic Clinic; Accessed on March, 2, 2012. URL: http://www.thalassa-lisboa.com/Stress-Oxidativo-O-que-e

[12] ph.D R. Koop, D. (2006). Alcohol Metabolisms Damagin Effects on the Cell. *Reactive Oxygen Generation by the Enzyme Cytochrome P450 E1* , p. 2.

[13] "*Harmful Byproducts Resulting from Alcohol Metabolism*"; National Institute of Alcohol Abuse and Alcoholism; Source: Tuma, D.J., and Casey, C.A. Dangerous byproducts of alcohol breakdown—Focus on adducts. *Alcohol Research & Health* 27(4):285–290, 2003. Accessed on April 22, 2012.

URL:
http://www.niaaa.nih.gov/Resources/GraphicsGallery/Liver/Pages/metabolismp286.aspx

[14] Mancinelli, Rosanna; Barlocci, Eleonora; Palminiello, Sonia; Saso, Luciano; "Oxidative stress and brain diseases: Biomarkers and analytical methodologies"; National Centre for Chemical Substances, Istituto Superiore di Sanità; Department of Physiology and Pharmacology "Vittorio Erspamer", "Sapienza" University of Rome, Rome, Italy; Indian Jornal of Biotechonology; Vol.10; October 2011; pages 395-403.

[15] Mancinelli, Rosanna; Binetti, Roberto; Ceccanti, Mauro; "Female drinking, environment and biological markers"; Dipartimento di Ambiente e Connessa Prevenzione Primaria, Institute Superiore di Sanitá; Centro Riferimento Alcologico Regione Lazio, Dipartimento di Medicina Clinica; Unviersitá degli Studi "La Sapienza", Rome, Italy.

[16] Enfermeira Mineiro; Cândida; Enfermeiro Pinto, Carlos; "*Adolescentes e o Álcool"*; Jornal das Caldas; April, 8th 2009; Acessed on March 2, 2012;

URL: http://www.jornaldascaldas.com/index.php/2009/04/08/adolescencia-e-o-alcool/

[17] "Biomarkers"; Health and Education; National Institute of Environmental Health Sciences – National Institute of health; Acessed on March 2, 2012;

URL: http://www.niehs.nih.gov/health/topics/science/biomarkers/index.cfm

[18] Kissack, JC; Bishop, J; Roper, AL; "Ethylglucuronide as a biomarker for ethanol detection." College of Pharmacy, Harding University, Searcy, Arkansas 72149-2230, USA.

[19] Mancinelli, Rosanna; Binetti, Roberto; Ceccanti, Mauro; “Female drinking, environment and biological markers”; Dipartimento di Ambiente e Connessa Prevenzione Primaria, Institute Superiore di Sanitá; Centro Riferimento Alcologico Regione Lazio, Dipartimento di Medicina Clinica; Unviersitá degli Studi “La Sapienza”, Rome, Italy.

[20] Lambert Beer`s Law; Bioscience and chemestry; Sheffield Hallam University; Acessed on March 12, 2012;

URL: http://teaching.shu.ac.uk/hwb/chemistry/tutorials/molspec/beers1.htm

[21] Almeida Mendes, Marcus Fabiano; Benfato, Mara Silvera; “Lambert Beer`s Law”; Práticas de Laboratório de Bioquímica e Biofísica, Guanabara Koogan, Compri-Nardy M., Stella M.B., Oliveira C. (2009), 200 págs. Acessed on March 14, 2012;

URL: http://www.ufrgs.br/leo/site_espec/bibliografia.html

[22] “FORT: Free Oxygen Radicals Testing”; The form system: AN EPR Analysis; Callegari S.p.A.; Catellani Group; 16/07/2009.

[23] Palmieri, B.; Sblendorio, V.; “Oxidative Stress Tests: overview on reliability and use”; European Review for Medical and Pharmacological Sciences 2007; 11: 383-399; Part II; Department of General Surgery and Surgical Specialities, University of Modena and Reggio Emilia; Medical School, Surgical Clinic, Modena, Italy.

[24] Rutz, Josiane Kuhn; “Avanços na Cromatografia Líquida”; Bacharelado em Química de Alimentos; Departamento de Ciência dos Alimentos; UNIVERSIDADE FEDERAL DE PELOTAS; Seminários em Alimentos: Pelotas, 2009.

[25] Gouveia, Jorge Luiz Nobre; Tadeu, Marcos; “*Monitoramento Ambiental*”; Curso de Auto-Instrução em Prevenção, Preparação e Resposta para desastres envolvendo produtos químicos; Acessed on Aprile 25, 2012.
URL: http://www.bvsde.paho.org/tutorial1/p/monimedi/

ATTACHMENTS

Attachment I: Preparation of solutions needed in GC and HPLC procedures

In this attached it is presented how are prepared the solutions which are needed to GC and HPLC trials.

• Preparation of 200% ethanol solution from a solution of ethanol at 99.8%: this solution is useful to prepare standard solutions of 100%, 50% and 25% ethanol.

$$\rho = \frac{m}{V} \Leftrightarrow V = \frac{mg\,\%}{\rho_{ethanol}} = \frac{200\,\%}{0.8} = 250\,\mu L \qquad \text{(Equation I.I)}$$

Adding 250µL of 99.8% ethanol in 100mL of water, it is obtained a solution of 200% ethanol.

• Preparation of BHT at 0.05mM: this solution is important to use in HPLC samples preparation.

BHT is provided in solid form so it is necessary to measured 0.05g of BHT in an analytical balance and dissolving this quantity in 100ml of H2O using a volumetric flask to obtained a BHT solution of 0.05%.

• Preparation of phosphoric acid (H_3PO_4) solution at 0.44M: this acid solution is important to use in HPLC samples preparation.

MM (H_3PO_4) = 98 g/mol

P (H_3PO_4) = 1.68 g/L

$$C = \frac{n}{V} \Leftrightarrow C = \frac{m}{MV} \Leftrightarrow m = CMV \Leftrightarrow m = 0.44 \times 98 \times 1 \Leftrightarrow m = 43.12g \quad \text{(Eq. I.2)}$$

$$\rho = \frac{m}{V} \Leftrightarrow V = \frac{m}{\rho} \Leftrightarrow V = \frac{43.12}{1.68} \Leftrightarrow V = 25.67L \qquad \text{(Eq. I.3)}$$

Transferring 2.56mL of at 85% H_3PO_4 into a total of 100mL of water, it is obtained a solution of H_3PO_4 at 0.44M.

• Preparation of acid tiobarbituric (TBA) from 98%TBA: this component is provided in solid form. Dissolving 0.8gr of TBA in 100ml of distillated water obtains a TBA solution at 0.8%. This dissolution must occur at high temperatures (50-55ºC) because TBA is only soluble at these temperatures.

• Preparation of mobile phase: it is composed by tampon solution of monopotassium fosfate (KH_2PO_4) at 50mM and pure methanol (60:40) using hydroxide potassium to control pH at 6.8.

Preparation of hydroxide potassium (KOH) at 5M

MM (KOH) = 56.11 g/mol

$$C = \frac{n}{V} \Leftrightarrow C = \frac{m}{MV} \Leftrightarrow 5 = \frac{m}{56.11 \times 1} \Leftrightarrow m = 280.55g$$ in 1L of water

Dissolving 28.55g of KOH in 100mL of water, it is obtained a solution of KOH at 5M.

Preparation of monopotassium fosfate (KH_2PO_4) at 50mM

This component is provided in solid form. Dissolving 6.8 grams of KH2PO4 in 1L of distillated water obtains a solution of KH2PO4 at 50mM.

Attachment II: Results obtained by FORT and FORD methods

In this attached it will be present the results obtained with FORT method and FORD method in which are the results of alcoholic patients, quality control, reproducibility of both methods between duplicates of first hour after sampling collection and between 1hr, 3hrs and 6hrs. It is also included the results obtained with plasma samples and the statistical results to compare the different groups.

Table II.1: FORT and FORD results for Patients under antioxidant pharmaceutical treatment obtained with Whole Blood and Plasma samples.

Patient	Type of Sample	FORT (mmol/L H_2O_2)				FORD (mmol/L H_2O_2)			
		Time Treatment (days)				Time Treatment (days)			
		0	3	7	15	0	3	7	15
A	Blood	5.55	4.38	3.20	4.77	2.14	1.89	3.43	2.38
D	Plasma	3.44	3.42	2.56	2.47	0.900	0.855	0.788	0.595
E	Plasma	2.63	2.56	2.83	3.33	0.885	1.13	0.985	0.665
H	Blood	3.00	3.40	2.98	2.65	0.670	1.20	0.860	0.970
I	Blood	4.34	4.12	3.79	4.56	0.925	0.945	1.13	1.06
K	Blood	3.60	3.35	-	4.56	0.920	1.00	-	1.31
L	Blood	2.20	1.45	1.92	2.78	1.14	1.05	1.45	1.27

Table II.2: FORT and FORD results for alcoholic patients with no antioxidant pharmaceutical treatment obtained with Whole Blood and Plasma Samples.

Patient	Type of Sample	FORT (mmol/L H_2O_2)				FORD (mmol/L H_2O_2)			
		Time Treatment (days)				Time Treatment (days)			
		0	3	7	15	0	3	7	15
B	Plasma	2.46	2.04	1.94	2.30	0.875	1.15	1.10	0.805
C	Plasma	1.85	1.91	1.87	2.20	0.865	0.785	0.940	0.525
F	Plasma	2.11	2.56	2.37	2.12	0.730	0.635	0.860	0.695
G	Plasma	2.12	1.87	1.92	1.80	0.875	0.770	0.585	0.505
J	Blood	2.61	2.62	2.68	2.98	0.97	1.27	1.23	1.07
M	Blood	3.20	4.09	4.32	2.79	1.14	1.12	1.19	0.885

Table II.3: FORT and FORD results for control group obtained with plasma.

Control	Age	Gender	FORT (mmol/H_2O_2)	FORD (mmol/H_2O_2)
1	58	M	2.61	1.88
5	53	F	<1.22	>3.00
7	50	M	<1.22	>3.00
8	26	F	<1.22	>3.00
9	27	F	<1.22	>3.00
10	49	M	<1.22	>3.00

Table II.4: Reproducibility of FORT method using Whole Blood samples between duplicates of first hour after sampling collection.

FORT (mmol/L H_2O_2)						
Sample	Measurements at 1hr			Average	σ	C.V. (%)
	1	2	3			
1	4.08	4.68	-	4.38	0.42	9.69
2	5.08	4.46	-	4.77	0.44	9.19
3	4.56	4.52	-	4.54	0.03	0.62
4	1.70	1.50	-	1.60	0.14	8.84
5	4.42	4.56	-	4.49	0.10	2.20
6	4.56	4.55	-	4.56	0.01	0.16
7	4.28	3.94	-	4.11	0.24	5.85
8	2.71	3.01	2.84	2.85	0.15	5.27
9	3.95	3.51	3.31	3.59	0.33	9.12
10	2.80	2.87	2.71	2.79	0.08	2.87
11	2.64	2.74	2.87	2.75	0.12	4.19
12	2.91	2.61	-	2.76	0.21	7.69
13	3.01	2.87	3.11	3.00	0.12	4.02
14	3.38	3.48	3.34	3.40	0.07	2.12
15	2.91	3.04	-	2.98	0.09	3.09
16	2.58	2.71	-	2.65	0.09	3.48
17	4.56	4.12	-	4.34	0.31	7.17
18	4.48	3.75	-	4.12	0.52	12.5
19	3.75	3.82	-	3.79	0.05	1.31
20	4.56	4.56	-	4.56	0.00	0.00
21	2.8	2.44	-	2.62	0.25	9.72
22	2.77	2.58	-	2.68	0.13	5.02
23	3.18	2.77	-	2.98	0.29	9.75
24	3.58	3.61	-	3.60	0.02	0.59
25	3.18	3.51	-	3.35	0.23	6.98
26	4.56	4.56	-	4.56	0.00	0.00
27	1.47	1.43	-	1.45	0.03	1.95
28	1.97	1.87	-	1.92	0.07	3.68
29	2.71	2.84	-	2.78	0.09	3.31
30	3.25	3.15	-	3.20	0.07	2.21
31	4.35	3.83	-	4.09	0.37	8.99
32	4.29	4.35	-	4.32	0.04	0.98
33	2.84	2.74	-	2.79	0.07	2.53

Table II.5: Reproducibility of FORT method using Whole Blood Samples between 1hr, 3hrs and 6hrs after sampling collection.

Sample	FORT (mmol/L H_2O_2)									
	Assay`s time after Sampling Collection			Average			σ		C.V. (%)	
	1hr	3hrs	6hrs	1hr	3hrs	6hrs	(1hr-3hrs)	(1hr-6hrs)	(1hr-3hrs)	(1hr-6hrs)
1	4.94	5.60	5.88	5.55	5.51	5.62	0.0141	0.0495	0.257	0.881
	6.16	5.42	5.36							
2	4.08	5.82	5.22	4.38	5.49	4.98	0.392	0.424	7.15	8.52
	4.68	5.16	4.74							
3	3.20	4.30	-	3.20	4.15	-	0.336	-	8.09	-
	-	4.00	-							
4	1.70	1.45	-	1.6	1.48	-	0.0424	-	2.87	-
	1.50	1.51	-							
5	4.42	3.72	4.56	4.49	3.415	4.56	0.760	0.0495	22.3	1.09
	4.56	3.11	-							
6	2.71	4.56	3.15	2.85	4.43	2.69	1.12	0.118	25.2	4.39
	3.01	4.35	2.51							
	2.84	4.39	2.40							
7	2.64	2.37	2.17	2.75	2.27	2.505	0.339	0.173	15.0	6.92
	2.74	2.17	2.84							
	2.87	-	-							
8	3.01	3.25	2.58	3.00	3.52	2.61	0.367	0.273	10.4	10.5
	2.87	3.78	2.64							
	3.11	-	-							
9	3.38	4.56	3.41	3.40	4.51	2.67	0.781	0.520	17.3	19.5
	3.48	4.45	1.92							
	3.34	-	-							
10	2.91	4.01	2.64	2.98	4.22	2.81	0.877	0.117	20.8	4.15
	3.04	4.42	2.98							

Table II.6: Reproducibility of FORD method using Whole Blood between duplicates of first hour after sampling collection.

FORD (mmol/L H_2O_2)						
Sample	Measurements at 1hr			Average	σ	C.V. (%)
	1	2	3			
1	2.22	2.06	-	2.14	0.1131	5.29
2	1.80	1.98	-	1.89	0.1273	6.73
3	2.93	2.87	-	2.90	0.0424	1.46
4	2.74	2.80	-	2.77	0.0424	1.53
5	2.18	2.53	-	2.36	0.2475	10.5
6	1.08	1.13	-	1.11	0.0354	3.20
7	0.88	0.83	-	0.86	0.0354	4.14
8	1.06	1.20	1.06	1.11	0.0808	7.30
9	1.03	1.03	-	1.03	0.0000	0.00
10	0.84	0.87	-	0.86	0.0212	2.48
11	0.62	0.75	0.67	0.68	0.0656	9.64
12	0.70	0.77	0.73	0.73	0.0351	4.79
13	0.95	0.96	1.00	0.97	0.0265	2.73
14	0.92	0.93	-	0.93	0.0071	0.76
15	0.92	0.97	-	0.95	0.0354	3.74
16	1.11	1.15	-	1.13	0.0283	2.50
17	1.04	1.07	-	1.06	0.0212	2.01
18	0.99	0.95	-	0.97	0.0283	2.92
19	1.33	1.21	-	1.27	0.0849	6.68
20	1.2	1.25	-	1.23	0.0354	2.89
21	1.06	1.07	-	1.07	0.0071	0.66
22	0.93	0.91	-	0.92	0.0141	1.54
23	0.99	1.01	-	1.00	0.0141	1.41
24	1.21	1.4	-	1.31	0.1344	10.3
25	1.19	1.08	-	1.14	0.0778	6.85
26	0.99	1.1	-	1.05	0.0778	7.44
27	1.47	1.43	-	1.45	0.0283	1.95
28	1.24	1.23	-	1.24	0.0071	0.57
29	1.11	1.17	-	1.14	0.0424	3.72
30	1.19	1.05	-	1.12	0.0990	8.84
31	1.18	1.19	-	1.19	0.0071	0.60
32	0.89	0.88	-	0.89	0.0071	0.80

Table II.7: Reproducibility of FORD method using Whole Blood between 1hr, 3hrs and 6hrs after sampling collection.

Sample	FORD (mmol/L H2O2)									
	Assay`s time after Sampling Collection			Average			σ		C.V. (%)	
	1hrs	3hrs	6hrs	1hrs	3hrs	6hrs	(1h-3h)	(1h-6h)	(1h-3h)	(1h-6h)
1	2.22	2.26	1.96	2.14	2.1	1.92	0.0283	0.156	1.35	8.10
	2.06	1.94	1.88							
2	1.80	1.84	1.76	1.89	1.95	1.98	0.0424	0.064	2.18	3.21
	1.98	2.06	2.20							
3	2.56	5.50	-	3.43	4.29	-	0.608	-	14.2	-
	4.30	3.08	-							
4	1.29	1.62	1.62	1.47	1.495	1.495	0.0177	0.018	1.18	1.18
	1.65	1.37	1.37							
5	1.08	1.16	1.2	1.105	1.11	1.155	0.00354	0.0354	0.319	3.06
	1.13	1.06	1.11							
6	1.03	0.86	0.89	1.03	0.905	0.915	0.0884	0.0813	9.77	8.89
	1.03	0.95	0.94							
7	0.62	0.72	0.62	0.68	0.675	0.57	0.0035	0.0778	0.524	13.6
	0.75	0.63	0.52							
	0.67	-	-							
8	0.95	1.05	1.13	0.97	1.02	1.13	0.0354	0.113	3.47	10.0
	0.96	0.99	-							
	1.00	-	-							
9	1.00	0.96	1.1	0.90	1.03	0.97	0.0860	0.0436	8.39	4.52
	0.87	1.09	0.83							
	0.84	-	-							
10	0.69	0.69	0.51	0.67	0.81	0.585	0.0990	0.0601	12.2	10.3
	0.63	0.93	0.66							
	0.69	-	-							
11	0.77	0.92	0.83	0.86	0.935	0.775	0.0530	0.0601	5.67	7.76
	0.95	0.95	0.72							

Table II.8: Plasma results obtained with FORT and FORD methods.

Patient	Treatment time (days)	FORT (mmol/L H_2O_2)			FORD (mmol/L H_2O_2)		
		1	2	Average	1	2	Average
A	0	2.74	2.94	2.84	1.13	1.08	1.11
	3	2.80	2.61	2.71	0.87	0.73	0.800
	7	3.08	3.61	3.35	0.88	0.79	0.835
	15	2.8	3.11	2.96	0.86	0.8	0.830
B	0	2.54	2.37	2.46	0.96	0.79	0.875
	3	2.07	2.00	2.04	1.14	1.15	1.15
	7	2.04	1.83	1.94	1.1	1.09	1.10
	15	2.23	2.37	2.30	0.77	0.84	0.805
C	0	1.83	1.87	1.85	0.90	0.83	0.865
	3	1.87	1.94	1.91	0.80	0.77	0.785
	7	1.87	1.87	1.87	0.96	0.92	0.940
	15	2.40	2.00	2.20	0.62	0.43	0.525
D	0	3.65	3.31	3.48	0.37	0.38	0.375
	3	3.55	3.28	3.42	0.79	0.92	0.855
	7	2.54	2.58	2.56	0.72	0.84	0.780
	15	2.54	2.40	2.47	0.64	0.55	0.595
E	0	2.64	2.61	2.63	0.85	0.92	0.885
	3	2.58	2.54	2.56	1.10	1.15	1.13
	7	3.01	2.64	2.83	0.95	1.02	0.985
	15	3.41	3.25	3.33	0.79	0.54	0.665
F	0	2.14	2.07	2.11	0.73	0.73	0.730
	3	2.51	2.61	2.56	0.74	0.53	0.635
	7	2.37	2.37	2.37	0.91	0.81	0.860
	15	2.20	2.04	2.12	0.74	0.65	0.695
G	0	2.20	2.04	2.12	0.85	0.9	0.875
	3	2.11	1.63	1.87	0.68	0.86	0.770
	7	2.04	1.80	1.92	0.63	0.54	0.585
	15	1.80	1.80	1.80	0.49	0.52	0.505
H	0	2.77	2.34	2.56	1.01	0.95	0.980
	3	2.77	2.61	2.69	0.83	0.77	0.800
	7	3.04	2.47	2.76	0.83	0.59	0.710
	15	2.30	2.40	2.35	0.79	0.54	0.665
I	0	2.94	2.77	2.86	0.74	0.62	0.680
	3	2.71	2.77	2.74	0.66	0.65	0.655
	7	2.58	2.64	2.61	0.90	0.82	0.860
	15	2.44	2.37	2.41	0.69	0.65	0.670
J	0	1.73	1.4	1.57	0.66	0.85	0.755
	3	1.76	1.76	1.76	0.86	0.89	0.875
	7	2.14	2.14	2.14	0.76	0.81	0.785
	15	2.34	2.04	2.19	0.96	0.66	0.810
K	0	2.14	2.07	2.11	0.72	0.59	0.655
	3	2.64	2.47	2.56	0.67	0.75	0.710
	7	-	-	-	-	-	-
	15	2.51	2.37	2.44	0.99	0.98	0.985
L	0	1.40	1.30	1.35	0.99	1.07	1.030
	3	1.57	1.22	1.40	0.82	0.92	0.870
	7	1.63	1.5	1.57	1.21	1.71	1.460
	15	1.97	2.11	2.04	0.93	0.70	0.815
M	0	2.37	2.44	2.41	0.75	0.63	0.690
	3	2.27	2.14	2.21	0.65	0.77	0.710
	7	2.51	2.37	2.44	0.80	0.75	0.775
	15	2.40	2.58	2.49	0.78	0.69	0.735

Table II.9: Statistical results obtained for Whole Blood samples and Plasma samples results by FORT and FORD methods.

Method	Sample	Average ± σ (mmol/L H_2O_2)	Variance	Probability test-t (P)
FORT	Whole Blood	3.40 ± 0.96	0.914	0.0001
	Plasma	2.46 ± 0.48	0.232	
FORD	Whole Blood	1.39 ± 0.68	0.461	0.0001
	Plasma	0.793 ± 0.18	0.0313	

Table II.10: Variability of FORT and FORD results between alcoholic patients and control group at treatment time 0.

Method	Sample	Average ± σ (mmol/L H_2O_2)	Variance	Probability test-t (P)
FORT	Alcoholic Patients	3.59 ± 1.01	1.02	0.0006
	Control Quality	1.42 ± 0.53	0.276	
FORD	Alcoholic Patients	1.07 ± 0.53	0.209	0.0075
	Control Quality	2.81 ± 0.46	0.282	

Table II.11: Variability of FORT and FORD results between two groups of alcoholic patients.

Method	Alcoholic Patients	Average ± σ (mmol/L H_2O_2)	Variance	Probability test-t (P)
FORT	Pharmaceutical treatment	3.72 ± 1.04	1.07	0.0073
	No Pharmaceutical treatment	2.37 ± 0.44	0.194	
FORD	Pharmaceutical treatment	1.16 ± 0.65	0.424	0.16
	No Pharmaceutical treatment	0.75 ± 0.22	0.0474	

Table II.12: Statistical results of ROC curve obtained for FORT method.

Variable	Patients
Classification variable	diagnosis

Sample size		20
Positive group :	diagnosis = 1	13
Negative group :	diagnosis = 0	7

Disease prevalence (%)	unknown

Area under the ROC curve (AUC)	0.940
Standard Error [a]	0.0635
95% Confidence Interval [b]	0.736 to 0.997
z statistic	6.920
Significance level P (Area=0.5)	<0.0001

[a] DeLong et al., 1988
[b] Binomial exact

Criterion values and coordinates of the ROC curve [Hide]

Criterion	Sensitivity	95% CI	Specificity	95% CI	+LR	-LR
>=1.22	100.00	75.3 - 100.0	0.00	0.0 - 41.0	1.00	
>1.22 *	100.00	75.3 - 100.0	85.71	42.1 - 99.6	7.00	0.00
>2.47	61.54	31.6 - 86.1	85.71	42.1 - 99.6	4.31	0.45
>2.61	53.85	25.1 - 80.8	100.00	59.0 - 100.0		0.46
>4.77	0.00	0.0 - 24.7	100.00	59.0 - 100.0		1.00

Table II.13: Statistical results of ROC curve obtained for FORD method.

Variable	Patients
Classification variable	Diagnosis

Sample size		19
Positive group :	Diagnosis = 1	13
Negative group :	Diagnosis = 0	6

Disease prevalence (%)	unknown

Area under the ROC curve (AUC)	0.987
Standard Error [a]	0.0181
95% Confidence Interval [b]	0.801 to 1.000
z statistic	26.870
Significance level P (Area=0.5)	<0.0001

[a] DeLong et al., 1988
[b] Binomial exact

Criterion values and coordinates of the ROC curve [Hide]

Criterion	Sensitivity	95% CI	Specificity	95% CI	+LR	-LR
< 0.67	0.00	0.0 - 24.7	100.00	54.1 - 100.0		1.00
<=1.14 *	92.31	64.0 - 99.8	100.00	54.1 - 100.0		0.077
<=1.88	92.31	64.0 - 99.8	83.33	35.9 - 99.6	5.54	0.092
<=2.14	100.00	75.3 - 100.0	83.33	35.9 - 99.6	6.00	0.00
<=3	100.00	75.3 - 100.0	0.00	0.0 - 45.9	1.00	

Attachment III: Results of MDA concentration resulting from HPLC

In this attached it will be present the results of MDA concentration, a biological marker used as an indicator of oxidative stress, obtained by HPLC for patients under pharmaceutical treatment, patients with no pharmaceutical treatment and quality control samples, as well the reproducibility of results and the variability found between three groups in terms of MDA concentration.

Table III.1: Media of MDA concentration results for Patients under antioxidant pharmaceutical treatment.

Patient	Age	MDA (µmol) Time Treatment (days)			
		0	3	7	15
A	27	1.08	1.41	1,56	1.25
D	?	2.21	1.38	2.33	2.58
E	?	1.62	2.02	1.67	2.40
H	?	3.22	3.61	3.04	3.71
I	29	1.44	1.61	1.67	1.83
K	44	0.760	1.22	-	1.80
L	?	2.12	2.80	3.68	0.595

Table III.2: Media of MDA concentration results for patients with no pharmaceutical treatment.

Patient	Age	MDA (µmol) Time Treatment (days)			
		0	3	7	15
B	42	2.13	2.50	2.56	1,87
C	31	1.73	1.55	3.17	4.78
F	?	1.62	1.55	1.42	2.47
G	36	1.59	1.71	3.34	2.27
J	50	2.75	2.07	1.40	1.63
M	?	1.35	0.945	2.02	1.38

Table III.3: Results of retention times, peaks areas and MDA concentration for control group samples.

Control	Gender	Age	Retention Time (min)		Peak Area		MDA Concentration (µmol/L)		MDA Concentration media (µmol)
			1	2	1	2	1	2	
1	M	58	5.06	5.04	8095	7973	1.64	1.61	1.63
2	F	54	4.81	4.78	5753	5673	1.75	1.72	1.74
3	F	?	5.02	4.97	7997	12496	1.51	2.30	1.51
4	F	32	5.69	5.64	8572	9624	1.86	2.10	1.98
5	F	53	5.58	5.47	10351	10024	2.18	2.12	2.15
6	F	24	5.25	5.27	7453	6652	1.62	1.46	1.54
7	M	50	5.85	5.87	4892	4599	1.38	1.30	1.34
8	F	26	5.52	5.50	6512	7652	1.71	2.01	1.71
9	F	27	5.40	5.38	5119	5011	1.33	1.30	1.32
10	M	49	5.19	5.17	8965	10848	1.93	2.31	2.12
11	M	39	5.17	5.16	9004	9173	1.78	1.82	1.80
12	M	53	5.08	5.05	9785	9754	1.91	1.90	1.90
13	F	44	5.08	5.05	10454	9800	2.02	1.88	1.95
14	F	38	5.15	5.13	10638	11003	2.12	2.20	2.16

Table III.4: Reproducibility between retention time and peaks area resulting from HPLC.

Patient	Treatment Time (days)	Retention Time (min)					Peak Area					MDA Concentration (µmol/L)	
		1	2	Average	σ	CV (%)	1	2	Average	σ	CV (%)	1	2
A	0	5.52	5.49	5.51	0.0212	0.385	4481	5124	4803	455	9.47	1.01	1.14
	3	5.44	5.43	5.44	0.0071	0.130	6015	6757	6386	525	8.22	1.33	1.48
	7	5.80	5.76	5.78	0.0283	0.489	6355	6391	6373	25	0.40	1.55	1.56
	15	5.66	5.68	5.67	0.0141	0.249	4772	5452	5112	481	9.41	1.17	1.33
B	0	4.72	4.70	4.71	0.0141	0.300	11038	12535	11787	1059	8.98	1.99	2.28
	3	4.68	-	4.68	-	-	8002	-	8002	-	-	2.50	-
	7	4.66	4.64	4.65	0.0141	0.304	8097	8225	8161	91	1.11	2.54	2.58
	15	4.63	4.63	4.63	0.0000	0.000	5670	6572	6121	638	10.4	1.72	2.02
C	0	4.93	4.94	4.94	0.0071	0.143	8501	9948	9225	1023	11.1	1.60	1.85
	3	4.87	4.87	4.87	0.0000	0.000	7765	8623	8194	607	7.40	1.47	1.62
	7	5.59	5.60	5.60	0.0071	0.126	13676	11560	12618	1496	11.9	3.45	2.90
	15	5.51	5.47	5.49	0.0283	0.515	18742	18617	18680	88	0.47	4.79	4.76
D	0	5.63	5.63	5.63	0.0000	0.000	8482	9726	9104	880	9.66	2.06	2.37
	3	5.87	5.75	5.81	0.0849	1.460	4541	6271	5406	1223	22.6	1.15	1.61
	7	5.73	5.70	5.72	0.0212	0.371	7745	10123	8934	1681	18.8	2.01	2.65
	15	5.64	5.61	5.63	0.0212	0.377	8444	11267	9856	1996	20.3	2.20	2.96
E	0	4.95	5.00	4.98	0.0354	0.711	8022	8034	8028	8	0.11	1.62	1.62
	3	4.92	4.91	4.92	0.0071	0.144	10902	10981	10942	56	0.51	2.01	2.03
	7	4.83	4.90	4.87	0.0495	1.017	9339	9391	9365	37	0.39	1.67	1.68
	15	4.77	4.75	4.76	0.0141	0.297	12192	13165	12679	688	5.43	2.30	2.51
F	0	5.39	5.36	5.38	0.0212	0.395	6386	7539	6963	815	11.71	1.59	1.65
	3	5.34	5.31	5.33	0.0212	0.398	6755	7210	6983	322	4.6	1.51	1.59
	7	5.37	5.34	5.36	0.0212	0.396	6275	6638	6457	257	3.98	1.38	1.46
	15	5.31	5.31	5.31	0.0000	0.000	11759	11429	11594	233	2.01	2.50	2.43
G	0	5.87	5.87	5.87	0.0000	0.000	4856	6308	5582	1027	18.4	1.37	1.80
	3	5.88	5.87	5.88	0.0071	0.120	5847	6141	5994	208	3.47	1.66	1.75
	7	5.76	5.68	5.72	0.0566	0.989	14303	10902	12603	2405	19.1	3.79	2.88
	15	5.61	5.57	5.59	0.0283	0.506	7129	10121	8625	2116	24.5	1.87	2.67
H	0	5.44	5.44	5.44	0.0000	0.000	12028	12274	12151	174	1.43	3.18	3.25
	3	5.63	5.68	5.66	0.0354	0.625	10943	17110	14027	4361	31.1	2.82	4.40
	7	5.49	5.45	5.47	0.0283	0.517	13801	9782	11792	2842	24.1	3.55	2.52
	15	5.44	5.30	5.37	0.0990	1.843	12589	16262	14426	2597	18.0	3.24	4.18
I	0	4.83	-	4.83	-	-	7010	-	7010	-	-	1.440	-
	3	4.87	4.86	4.87	0.0071	0.145	6586	7735	7161	812	11.3	1.46	1.75
	7	4.87	4.82	4.85	0.0354	0.730	9593	9169	9381	300	3.20	1.71	1.63
	15	4.89	4.83	4.86	0.0424	0.873	5403	5160	5282	172	3.25	1.06	1.00
J	0	5.04	5.02	5.03	0.0141	0.281	13816	14075	13946	183	1.31	2.72	2.77
	3	5.02	5.01	5.02	0.0071	0.141	10752	10668	10710	59	0.55	2.08	2.06
	7	5.31	5.25	5.28	0.0424	0.804	6995	7417	7206	298	4.14	1.35	1.44
	15	5.10	5.07	5.09	0.0212	0.417	8297	8276	8287	15	0.18	1.63	1.62
K	0	4.89	4.87	4.88	0.0141	0.290	4042	4293	4168	177	4.26	0.733	0.793
	3	4.78	4.77	4.78	0.0071	0.148	5872	5362	5617	361	6.4	1.28	1.15
	7	-	-	-	-	-	-	-	-	-	-	-	-
	15	4.80	4.75	4.78	0.0354	0.740	8880	8692	8786	133	1.51	1.82	1.77
L	0	5.12	5.11	5.12	0.0071	0.138	10624	10636	10630	8	0.08	2.11	2.12
	3	5.25	5.17	5.21	0.0566	1.086	13975	15051	14513	761	5.24	2.70	2.90
	7	5.03	5.01	5.02	0.0141	0.282	21540	16713	19127	3413	17.8	4.13	3.22
	15	5.18	5.18	5.18	0.0000	0.000	3602	3717	3660	81	2.22	0.58	0.61
M	0	5.47	5.39	5.43	0.0566	1.042	6870	5152	6011	1215	20.2	1.52	1.18
	3	5.32	5.27	5.30	0.0354	0.668	3837	4110	3974	193	4.86	0.92	0.97
	7	5.42	5.41	5.42	0.0071	0.131	10304	10008	10156	209	2.06	2.05	1.99
	15	5.31	5.25	5.28	0.0424	0.804	6850	7122	6986	192	2.75	1.35	1.41

Table III.5: Statistical results for MDA concentration between alcoholic patients and control group.

Sample	Average ± σ (µmol/L)	Variance	Probability test-t (P)
Alcoholic Patients	2.04 ± 0.71	0.51	0.084
Control Quality	1.73 ± 0.26	0.07	

Table III.6: Statistical results for MDA concentration between alcoholic patients under pharmaceutical treatment and alcoholic patients with no pharmaceutical treatment at treatment time 0.

Alcoholic Patients	Average ± σ (Area Under Curve)	Variance	Probability test-t (P)
Pharmaceutical treatment	1.72 ± 0.88	0.78	0.77
No Pharmaceutical treatment	1.86 ±0.50	0.25	

Table III.7: Statistical results for MDA concentration between alcoholic patients under treatment and alcoholic patients without treatment at the end of 15 days.

Alcoholic Patients	Average ± σ (Area Under Curve)	Variance	Probability test-t (P)
Pharmaceutical treatment	2.26 ± 0.85	0.73	0.79
No Pharmaceutical treatment	2.40 ±1.23	1.52	

Attachment IV: HPLC Chromatograms Resulting From Standard Solutions

In this attached it will be presented the chromatograms obtained by HPLC method for standard solutions with a limit of concentration of 0.5 to 5µmol/L**.**

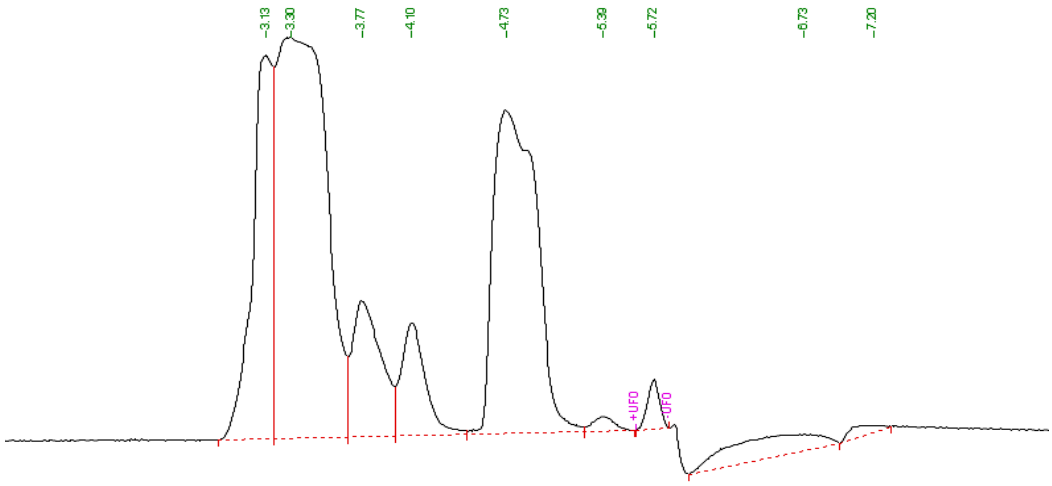


Figure IV.1: Chromatogram resulting from standard solution of 0.5umol/L with a retention time for MDA of 5.7 minutes.

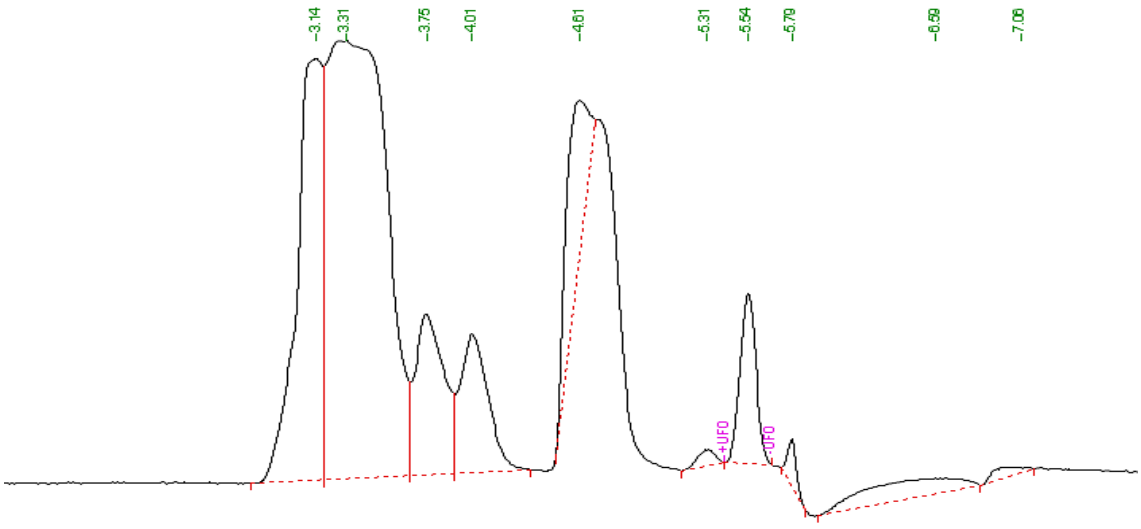


Figure IV.2: Chromatogram resulting from a standard solution of 1.5umol/l with a retention time for MDA of 5.5 minutes.

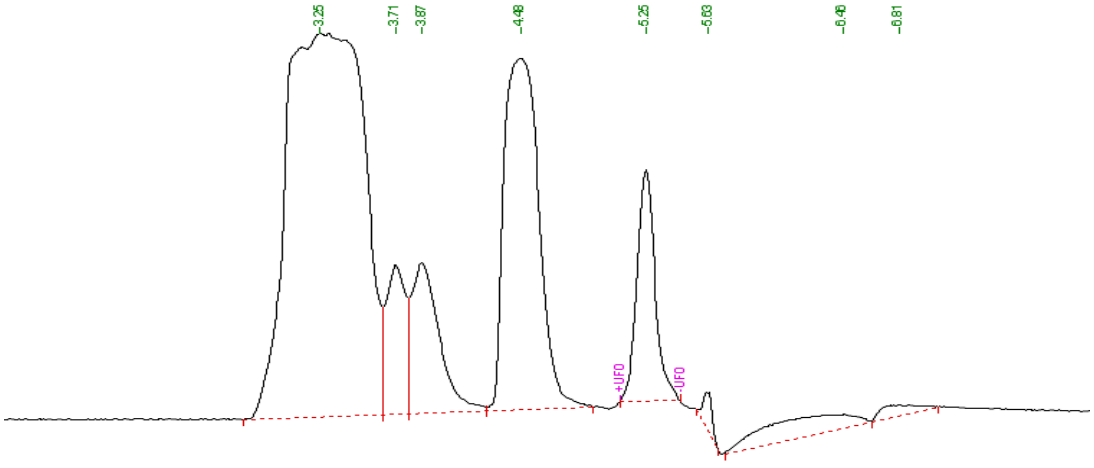


Figure IV.3: Chromatogram resulting from standard solution of 2.5umol/L with a retention time for MDA of 5.3minutes.

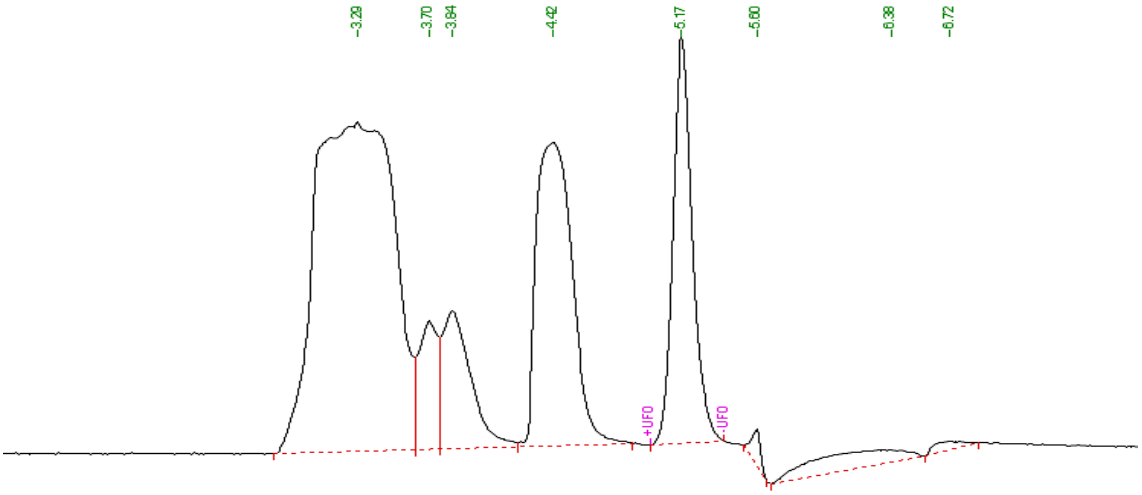


Figure IV.4: Chromatogram resulting from a standard solution of 5umol/L with a retention time for MDA of 5.2minutes.

Attachment V: HPLC Chromatograms Resulting From Patients under pharmaceutical treatment

In this attached it will be presented HPLC chromatograms resulting from patients under pharmaceutical treatment for different treatment times (0, 3, 7 and 15 days).

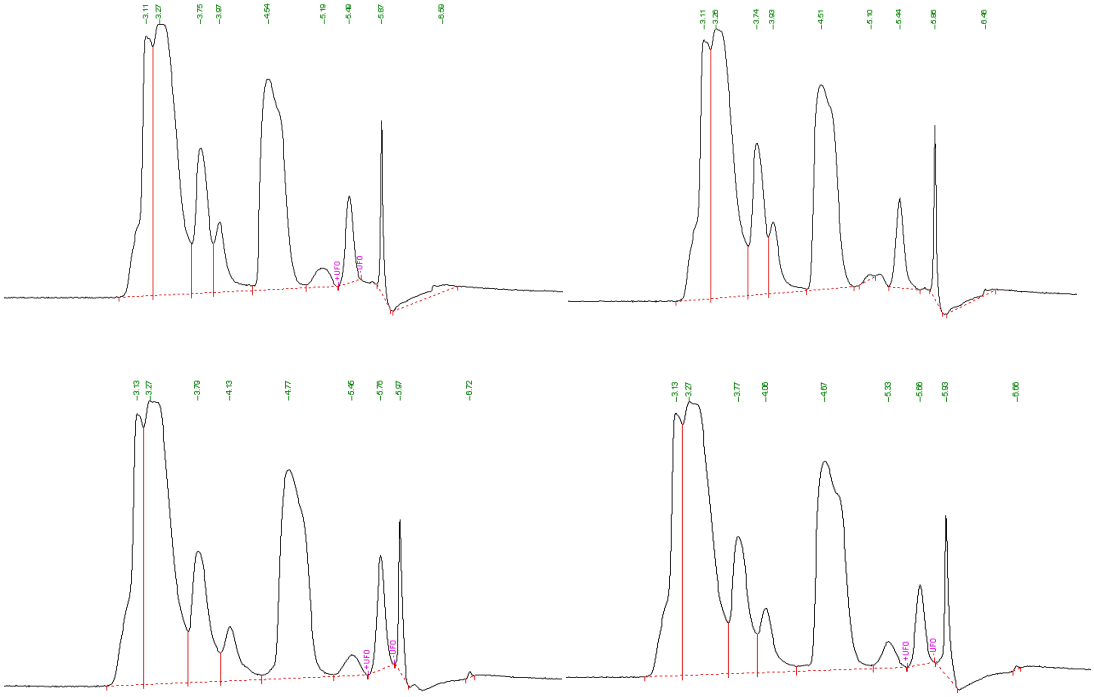


Figure V.1: HPLC Chromatograms resulting from Patient A for treatment times (tt) 0, 3, 7 and 15.

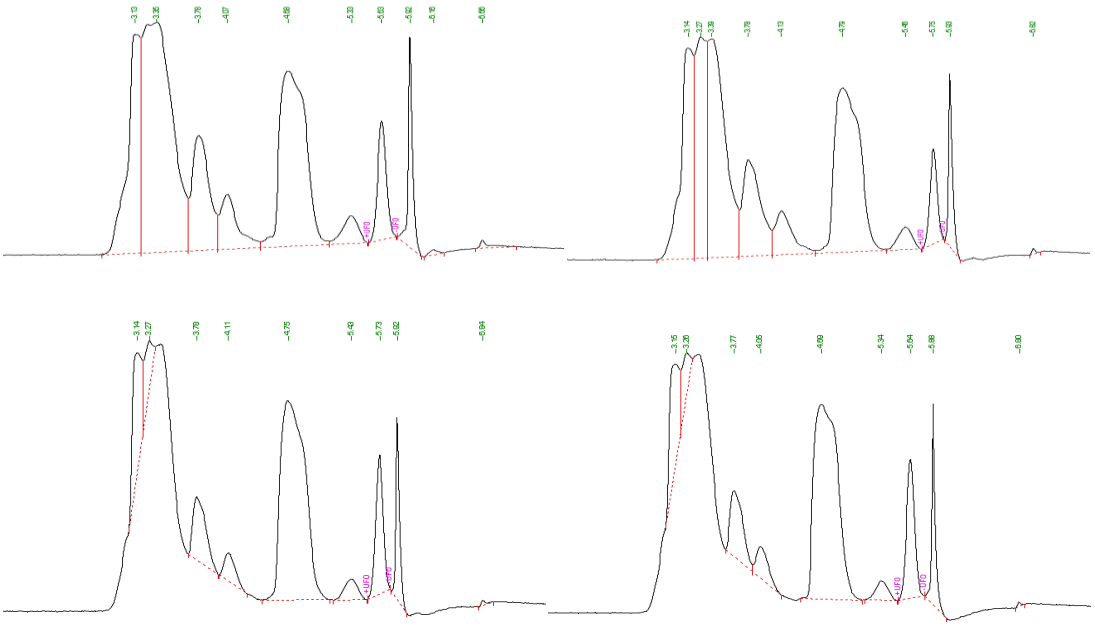


Figure V.2: HPLC Chromatogram resulting from Patient D for treatment time (tt) 0, 3, 7 and 15.

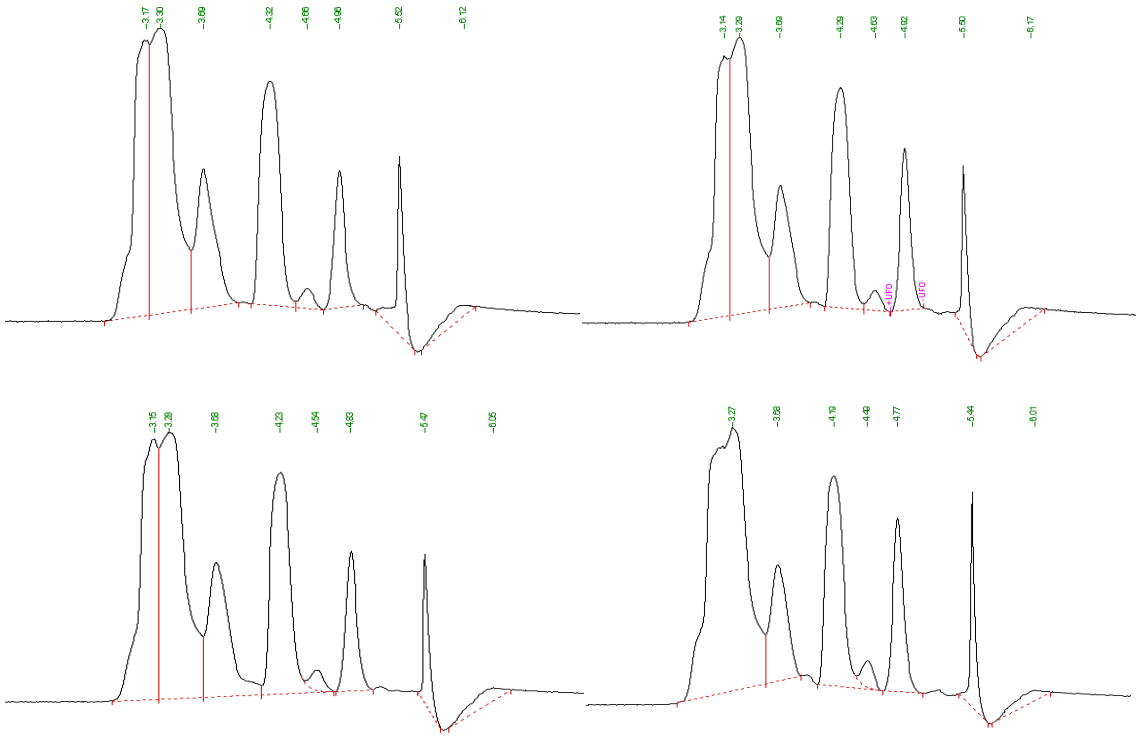


Figure V.3: HPLC Chromatograms resulting from Patient E for treatment times (tt) 0, 3, 7 and 15.

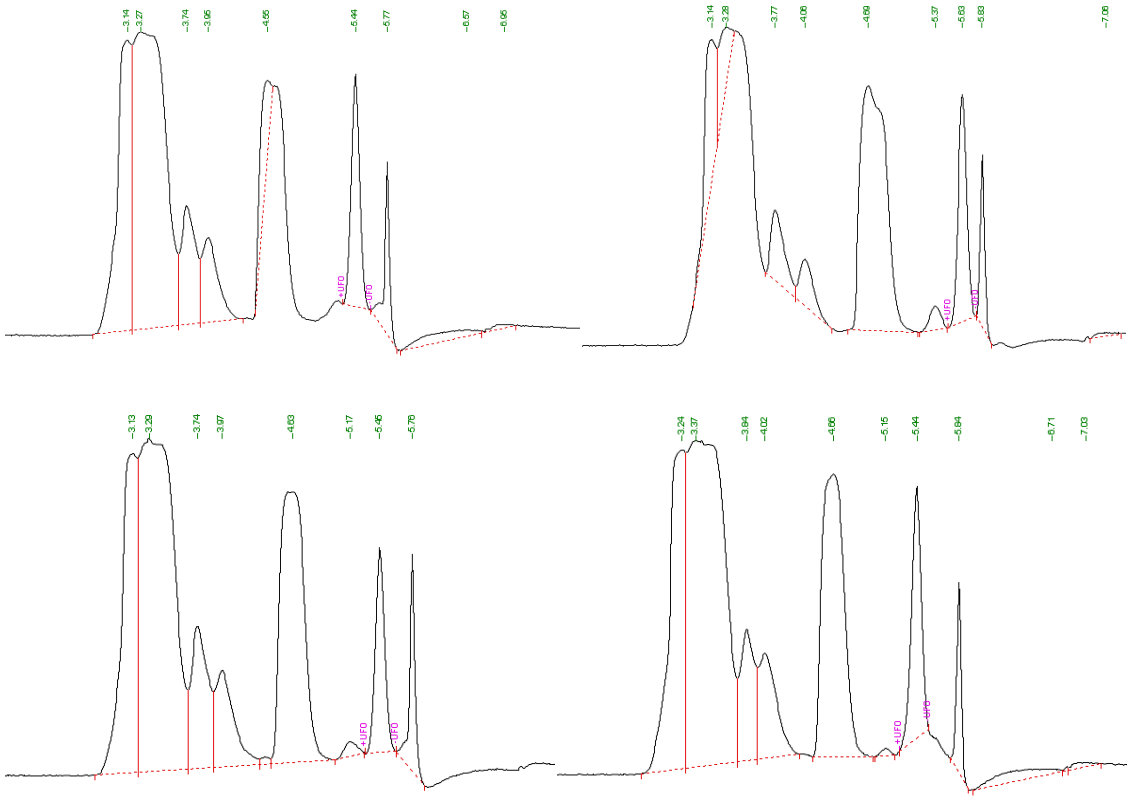


Figure V.4: HPLC Chromatograms resulting from Patient H for treatment time (tt) 0, 3, 7 and 15.

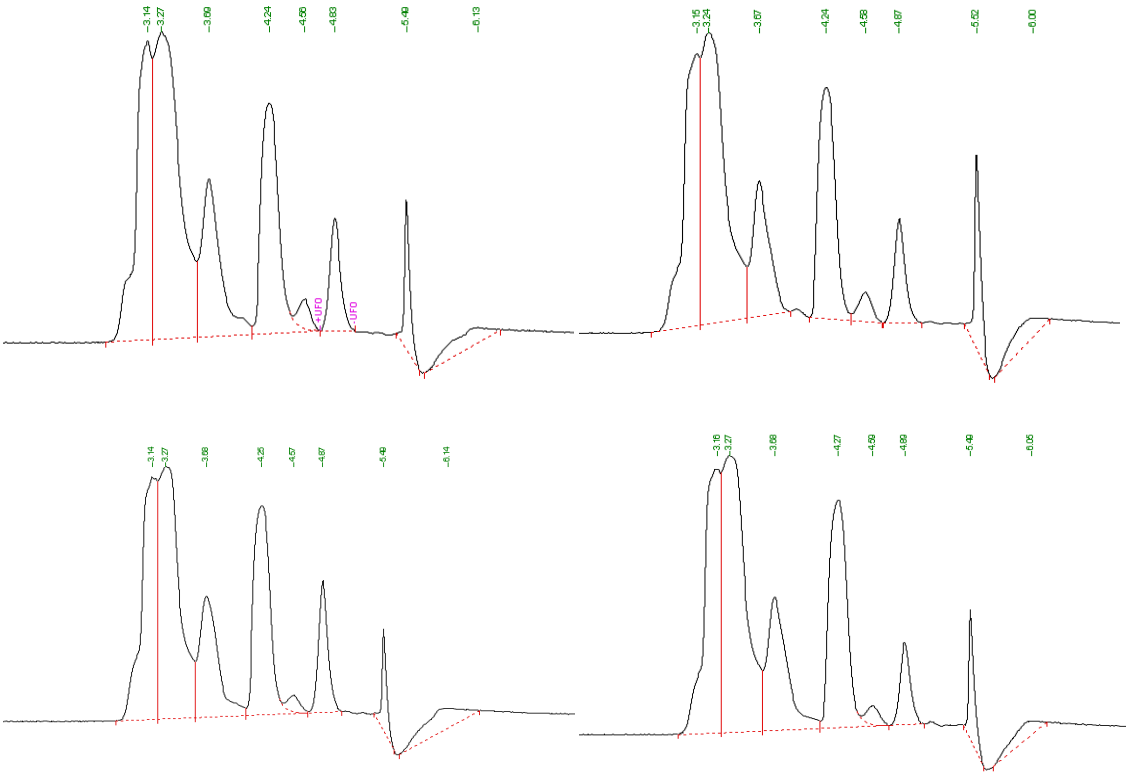


Figure V.5: HPLC Chromatograms resulting from Patient I for treatment time (tt) 0, 3, 7 and 15.

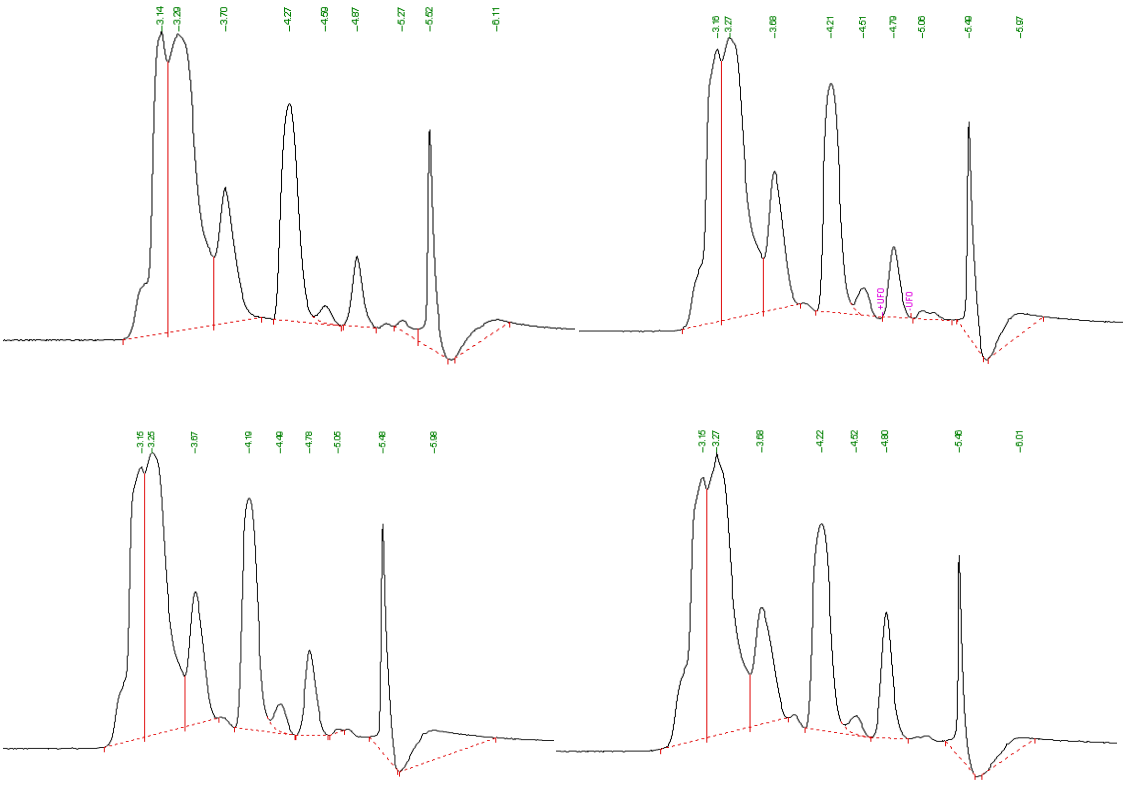


Figure V.6: HPLC Chromatograms resulting from Patient K for treatment time (tt) 0, 3, 7 and 15.

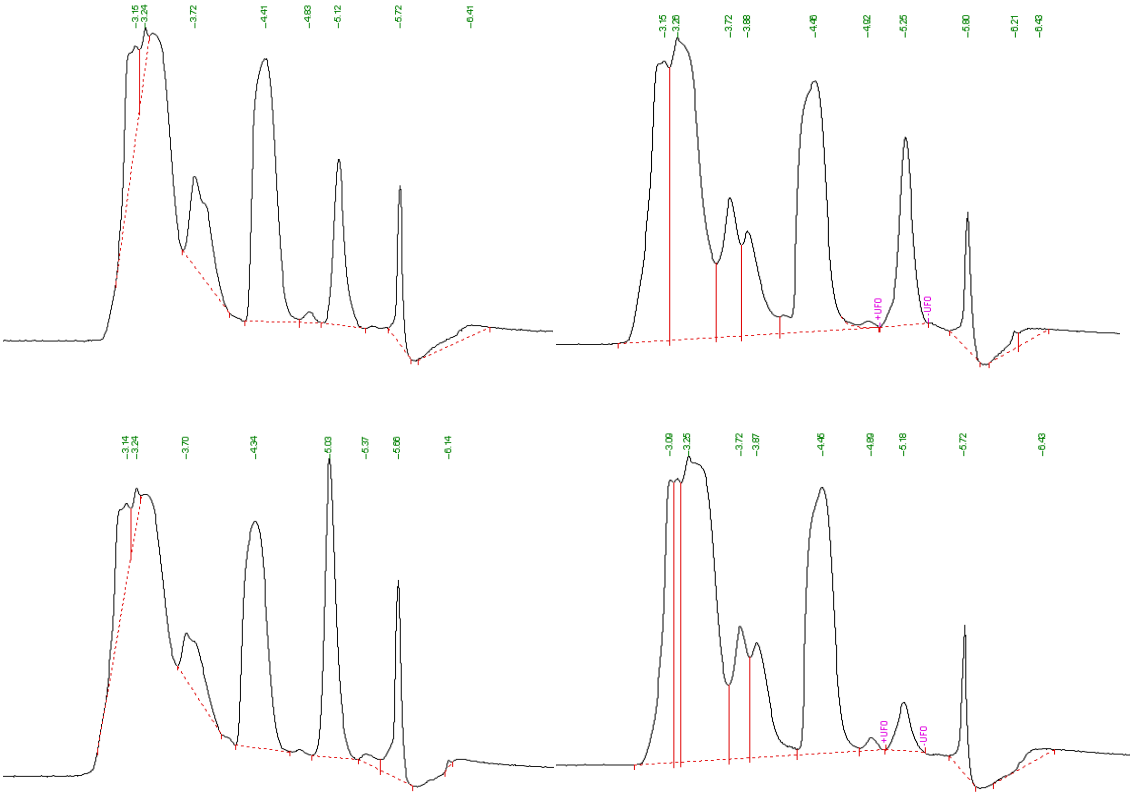


Figure V.7: HPLC Chromatograms resulting from Patient L for treatment times (tt) 0, 3, 7 and 15.

Attachment VI: HPLC Chromatograms Resulting From Patients under no pharmaceutical treatment

In this attached it will be presented HPLC chromatograms resulting from patients under no pharmaceutical treatment for different treatment times (0, 3, 7 and 15 days).

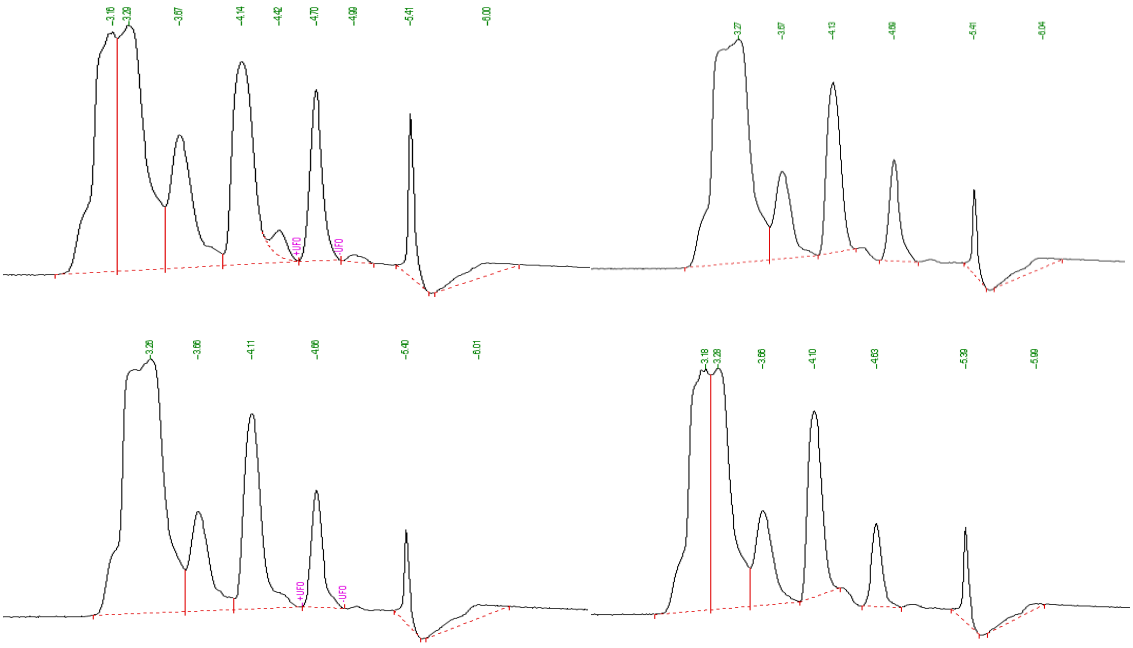


Figure VI.1: HPLC Chromatograms resulting for patient B for treatment times (tt) 0, 3, 7 and 15.

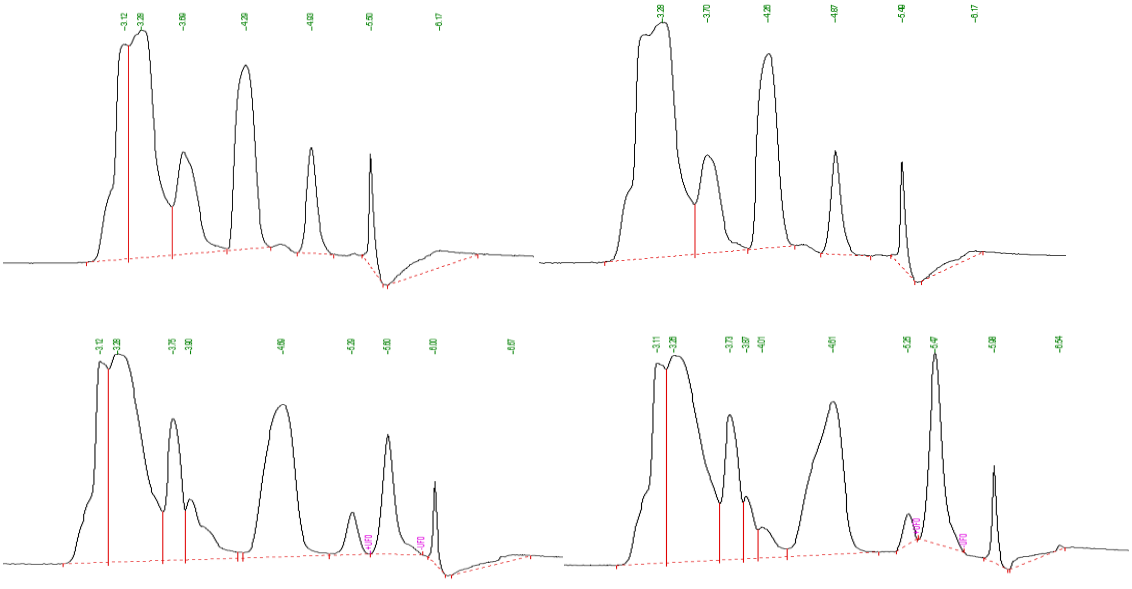


Figure VI.2: HPLC Chromatograms resulting from Patient C for treatment times (tt) 0, 3, 7 and 15.

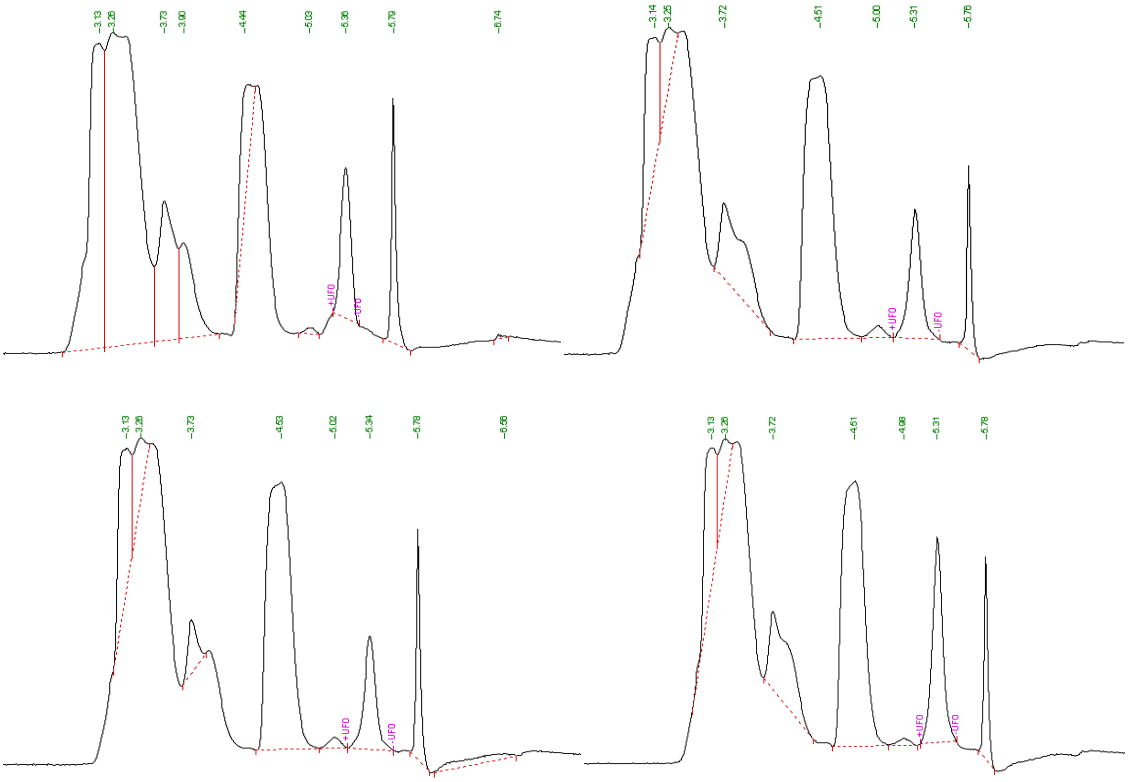


Figure VI.3: HPLC Chromatograms resulting from Patient F for treatment times (tt) 0, 3, 7 and 15.

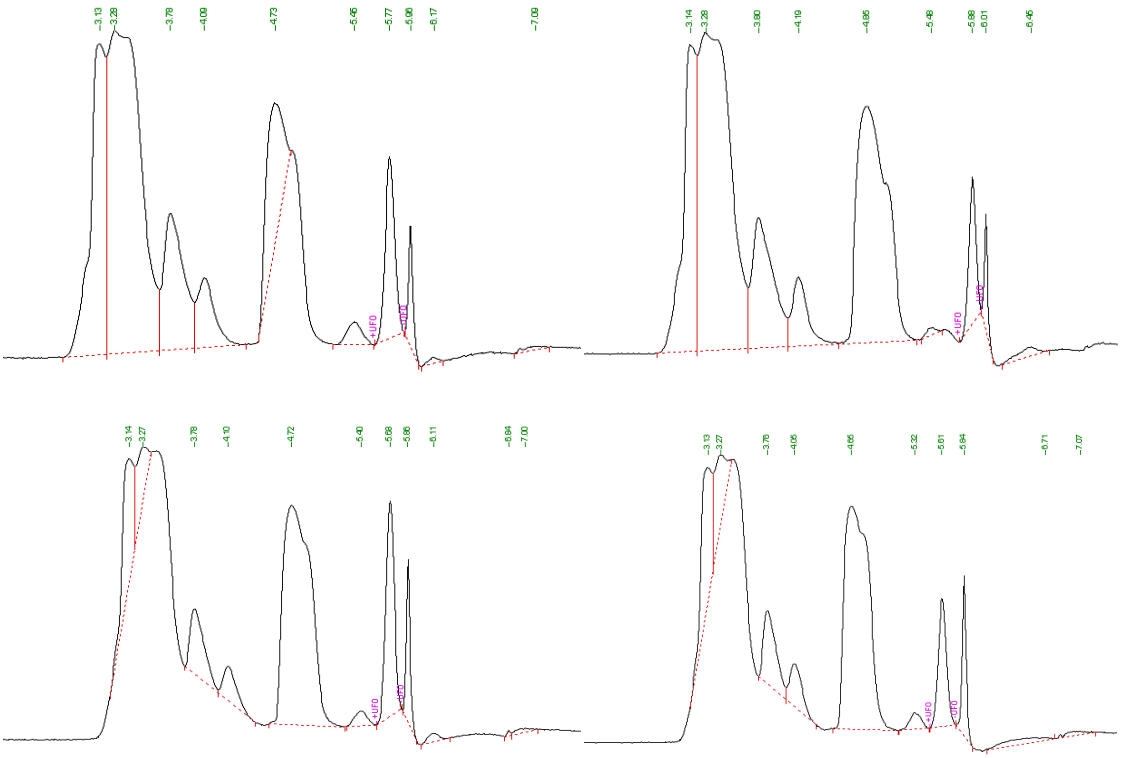


Figure VI.4: HPLC Chromatograms resulting from Patient G for treatment times (tt) 0, 3, 7 and 15.

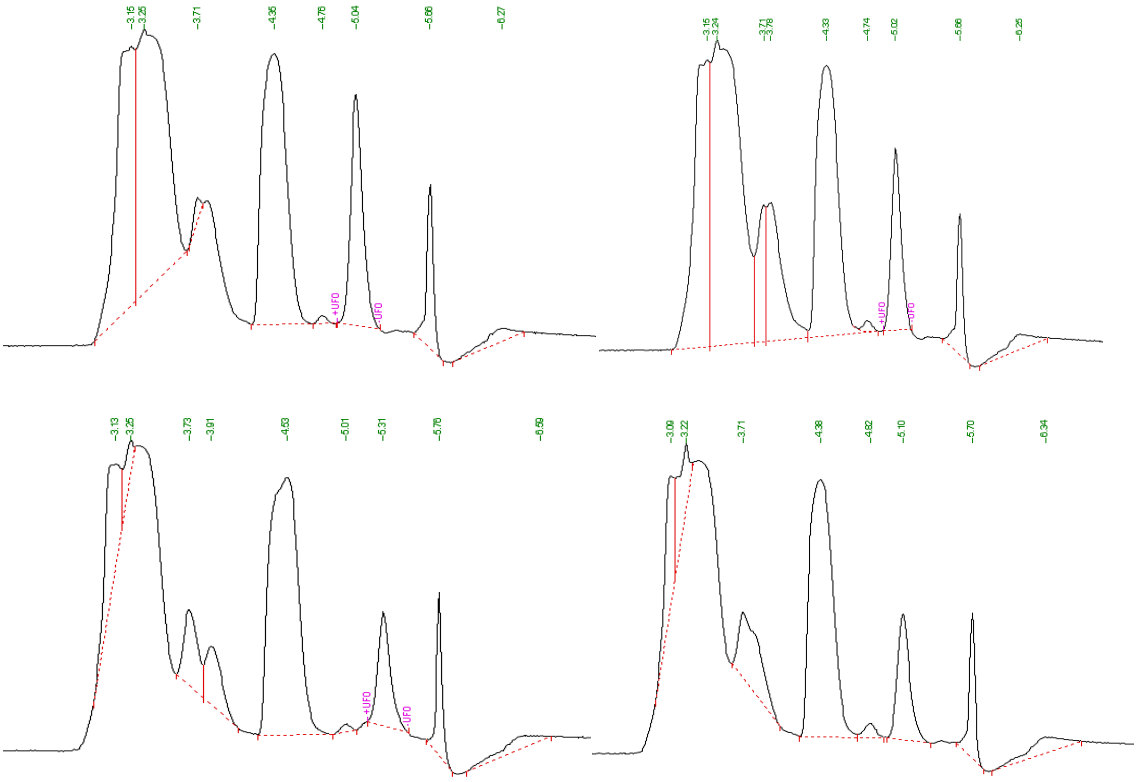


Figure VI.5: HPLC Chromatograms resulting from Patient J for treatment times (tt) 0, 3, 7 and 15.

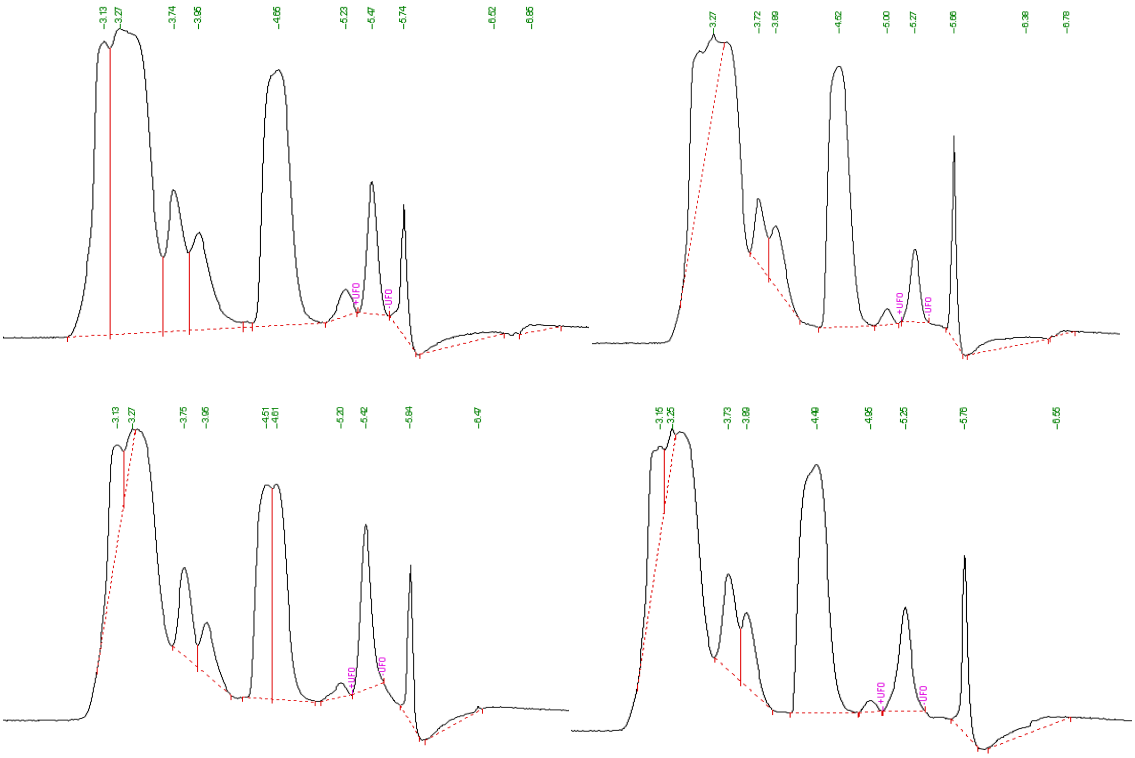


Figure VI.6: HPLC Chromatograms resulting from treatment times (tt) 0, 3, 7 and 15.

Attachment VII: HPLC Chromatograms Resulting From Control Group

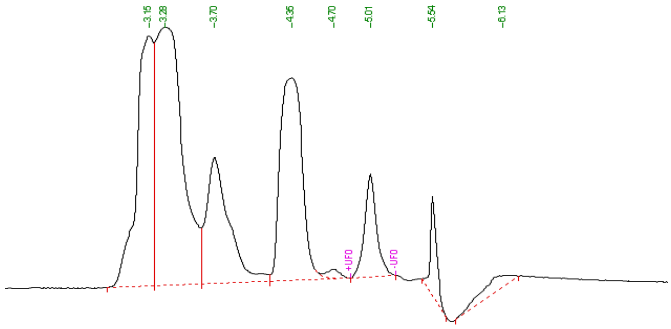


Figure VII.1: HPLC Chromatogram resulting from Control number 1.

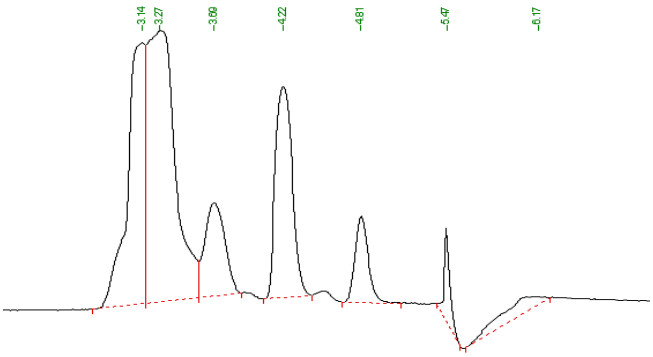


Figure VII.2: HPLC Chromatogram resulting from Control number 2.

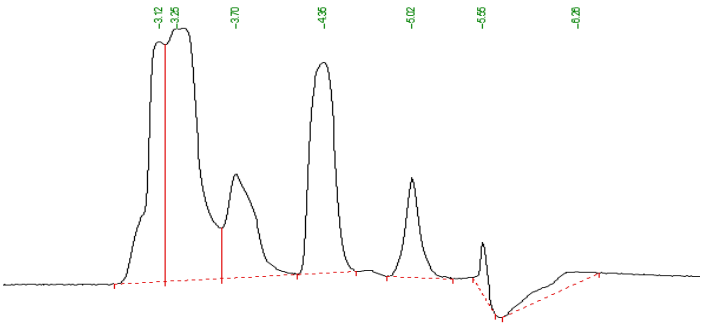


Figure VII.3: HPLC Chromatogram resulting from Control number 3.

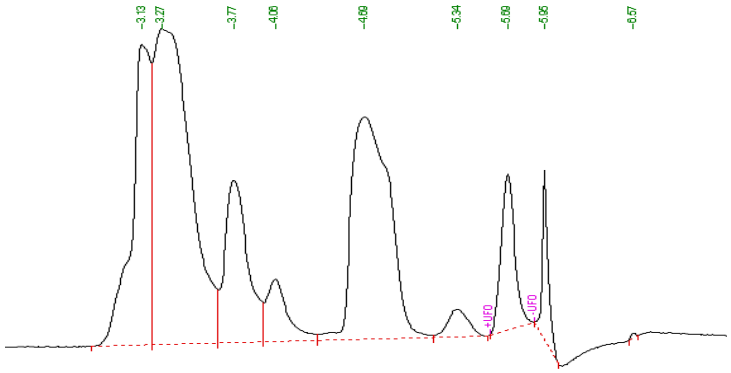


Figure VII.4: HPLC Chromatogram resulting from Control number 4.

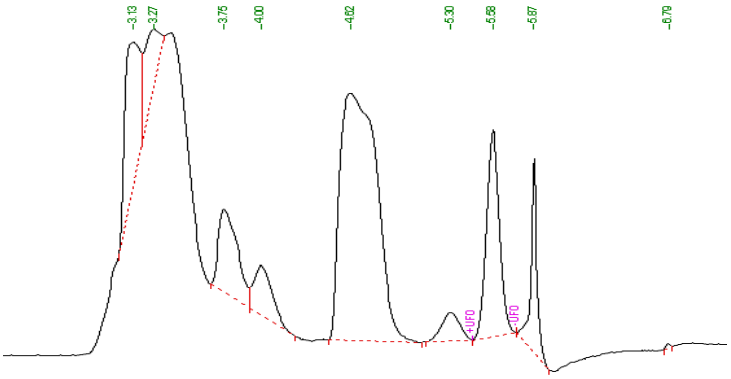


Figure VII.5: HPLC Chromatogram resulting from Control number 5.

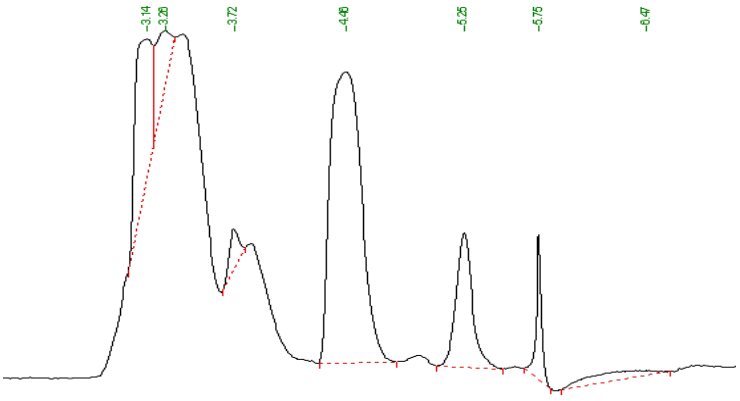


Figure VII.6: HPLC Chromatogram resulting from Control number 6.

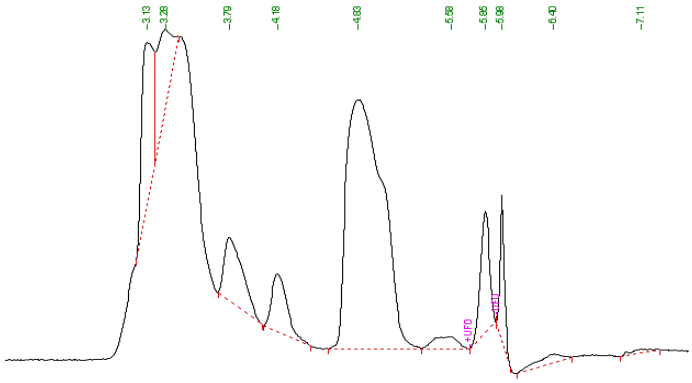


Figure VII.7: HPLC Chromatogram resulting from Control number 7.

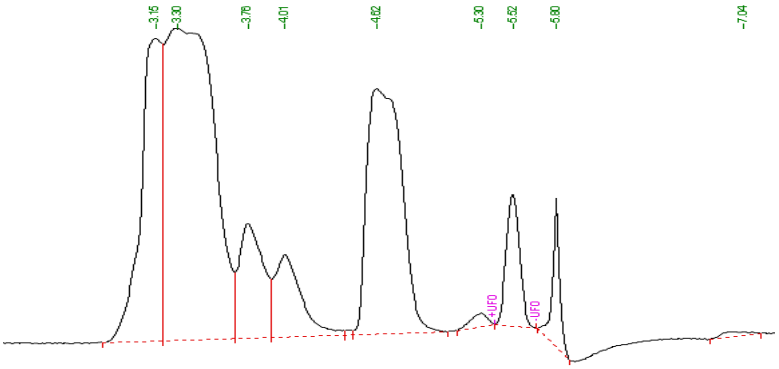


Figure VII.8: HPLC Chromatogram resulting from Control number 8.

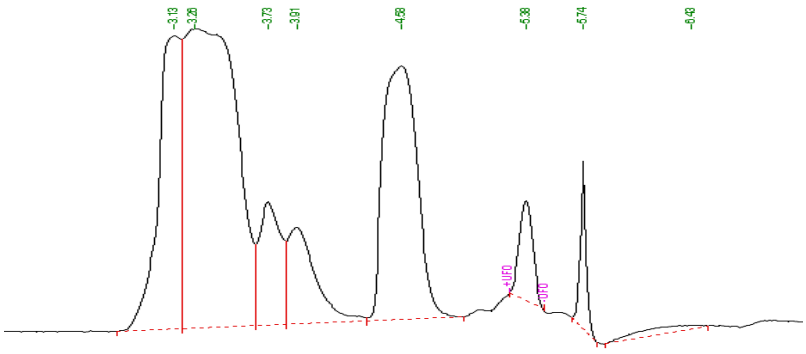


Figure VII.9: HPLC Chromatogram resulting from Control number 9.

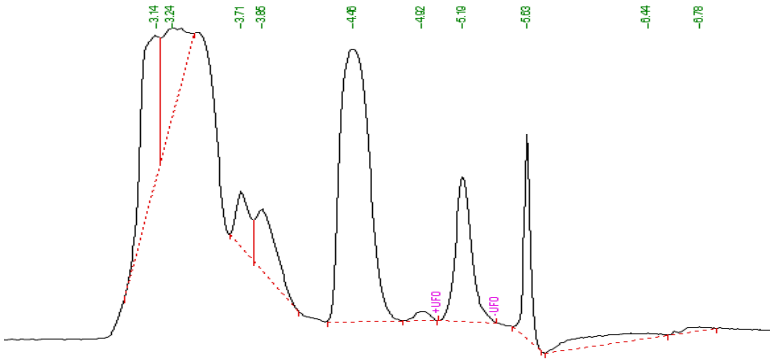


Figure VII.10: HPLC Chromatogram resulting from Control number 10.

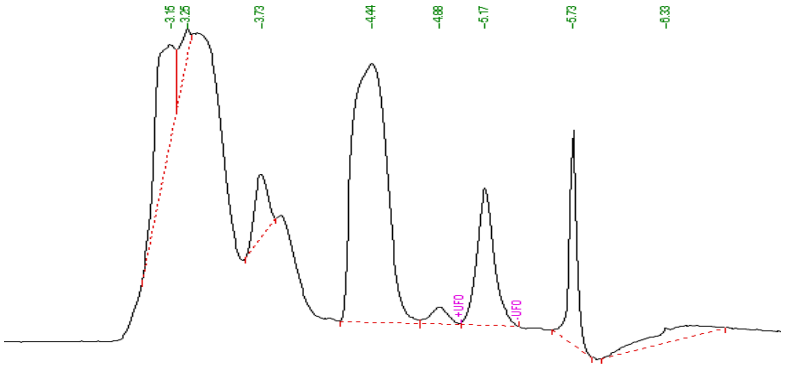


Figure VII.11: HPLC Chromatogram resulting from Control number 11.

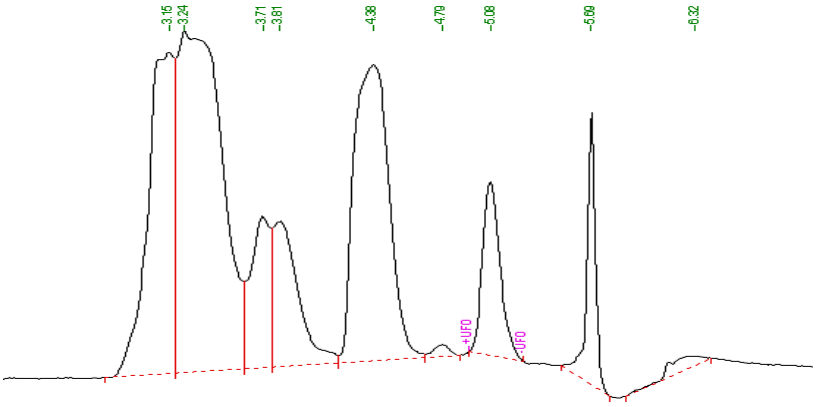


Figure VII.12: HPLC Chromatogram resulting from Control number 12.

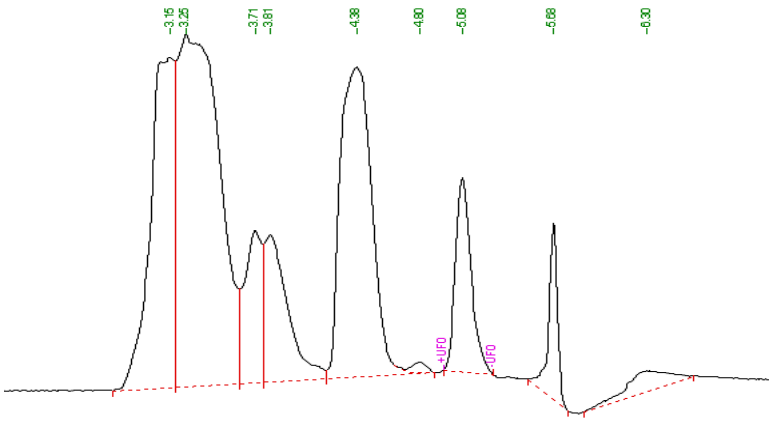
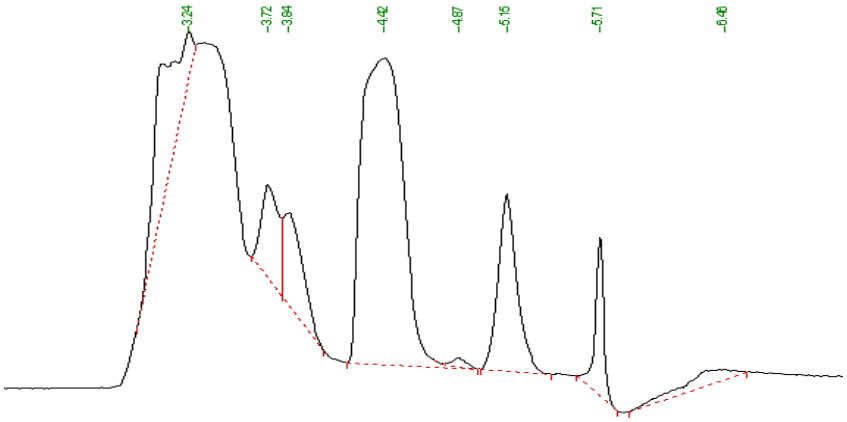


Figure VII.13: HPLC Chromatogram resulting from control number 13.



VII.14: HPLC Chromatogram resulting from Control number 14.

Attachment VIII: Results of Blood Alcohol Concentration resulting from GC

In this attachment are presented the results of Blood Alcohol Concentration (BAC) of alcoholic patients.

Table VIII.1: Results of Blood Alcohol Concentration of Alcoholic Patients.

Patient	Treatment Time (Days)	Component Found	Retention Time (min)	Area	BAC (%)
A	0	No Component	-	-	0
	3				
	7				
	15				
B	0	Ethanol	3.92	434922	75
	3	No component	-	-	0
	7	No component	-	-	0
	15	Methanol	3.53	2579	3.73
		Acetaldehyde	3.61	10200	6.35
		Ethanol	3.87	249027	88.6
C	0	Methanol	3.41	1070	0.99
	3	No component	-	-	0
	7	No component	-	-	0
	15	No component	-	-	0
D	0	No Component	-	-	0
	3				
	7				
	15				
E	0	No Component	-	-	0
	3				
	7				
	15				
F	0	No component	-	-	0
	3	Methanol	3.41	260	0.35
		Acetaldehyde	3.67	3626	1.01
		Ethanol	3.93	302897	59.73
	7	No component	-	-	0
	15	Methanol	3.35	2540	3.71
		Acetaldehyde	3.61	8048	5.61
		Ethanol	3.87	45530	18.52
G	0	No Component	-	-	0
	3				
	7				
	15				
H	0	No Component	-	-	0
	3				
	7				
	15				
I	0	No Component	-	-	0
	3				
	7				
	15				
J	0	No Component	-	-	0
	3				
	7				
	15				
K	0	No Component	-	-	0
	3				
	7				
	15				
L	0	No Component	-	-	0
	3				
	7				
	15				
M	0	Methanol	3.63	2133	3.57
		Acetaldehyde	3.61	522	3.02
		Ethanol	3.87	214400	76.8
	3	No component	-	-	0
	7	No component	-	-	0

Attachment IX: Chromatograms resulting from GC method.

In this attachment are presented the chromatograms obtained by gas chromatography method for standard solutions and for alcoholic patients that show some recently alcohol consumption.

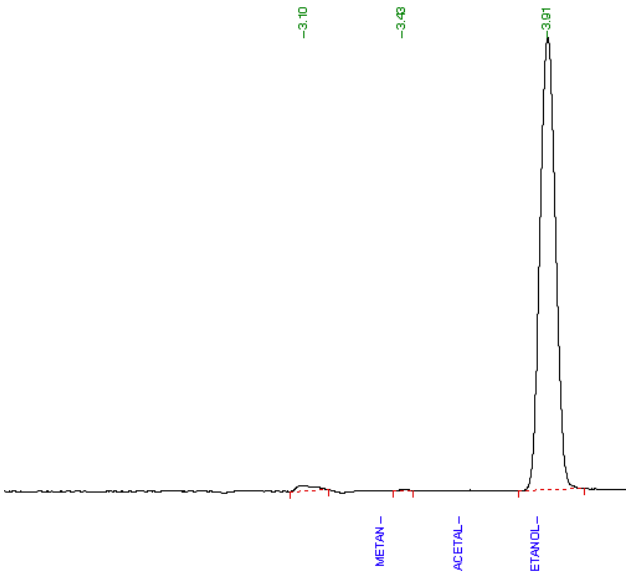


Figure IX.1: Chromatogram resulting from a standard solution of 25% with a retention time of 3.9 minutes for ethanol.

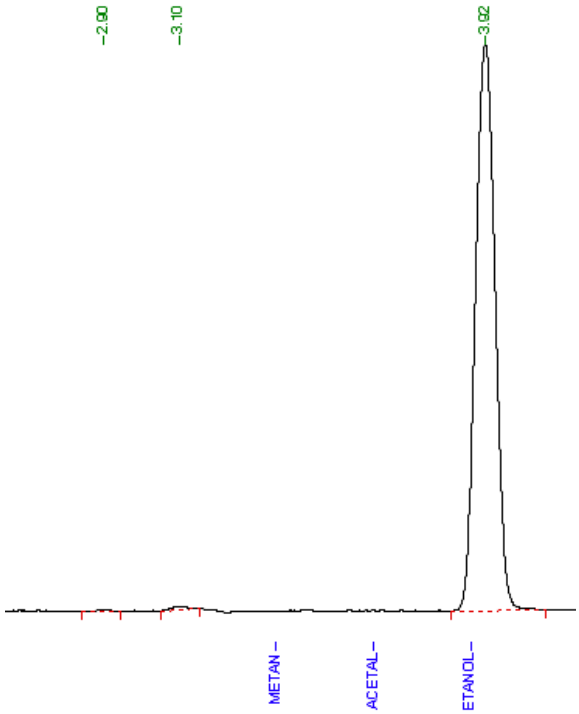


Figure IX.2: Chromatogram resulting from a standard Solution of 50% with a retention time of 3.9 minutes for ethanol.

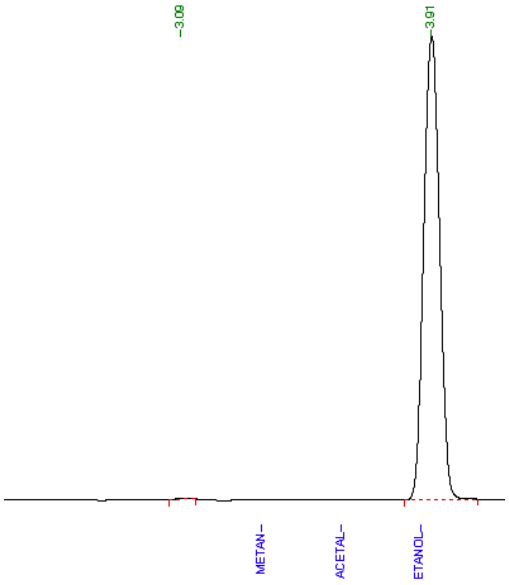


Figure IX.3: Chromatogram resulting from a 100% standard solution with a retention time of 3.9 minutes for ethanol.

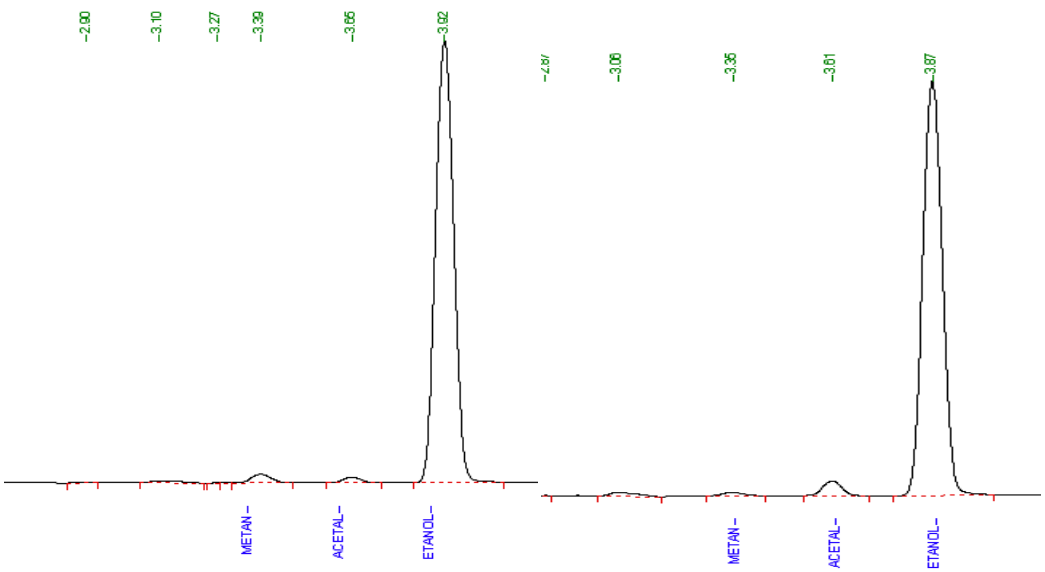


Figure IX.4: Chromatogram resulting from a whole blood samples of patient B at treatment time 0 (on the right) and at treatment time 15 (on the left).

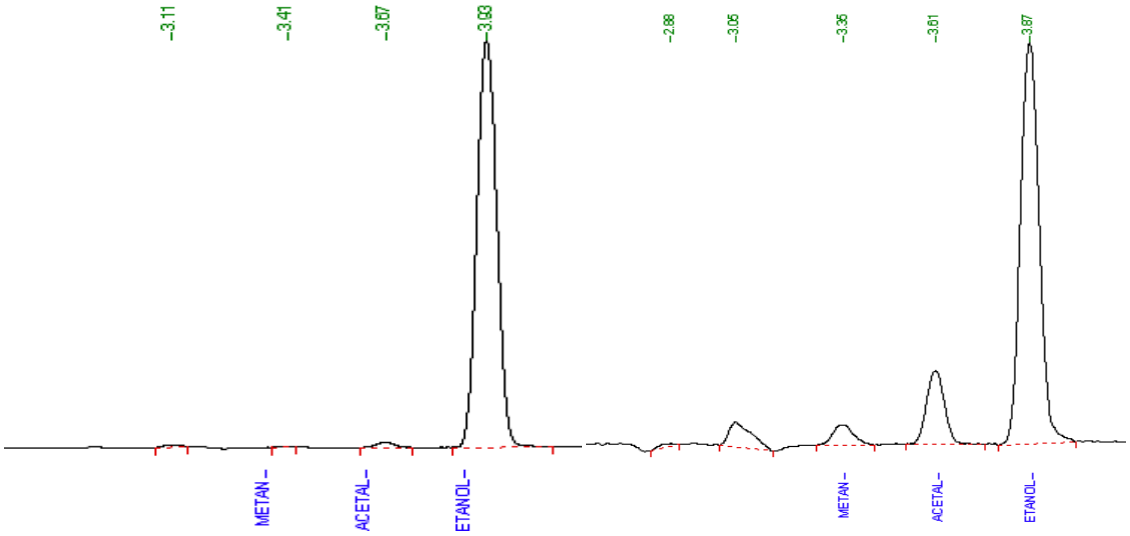


Figure IX.5: Chromatogram resulting from a whole blood samples of patient F at treatment time 3 (on the left) and at treatment time 15 (on the right).

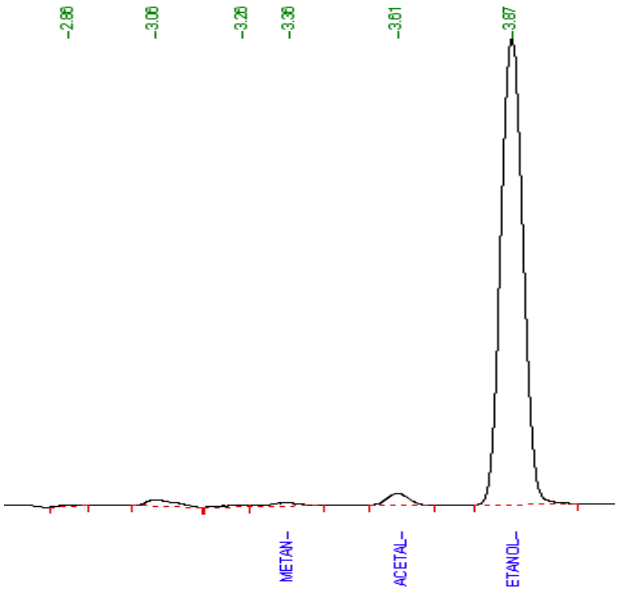


Figure IX.6: Chromatogram resulting from a whole blood sample of patient M at treatment time 0.

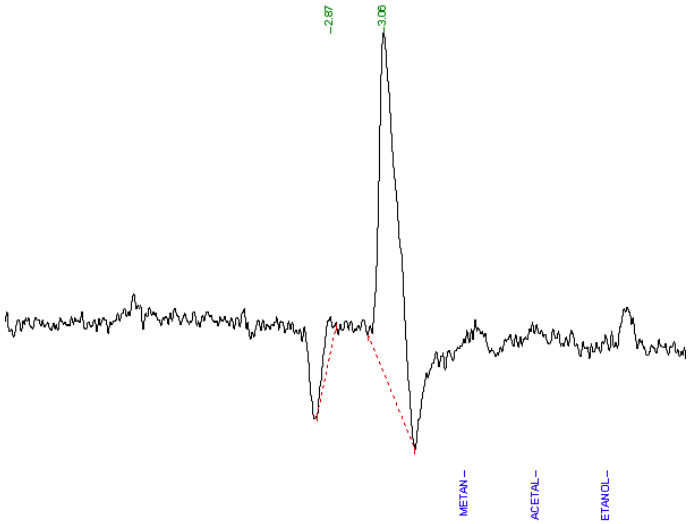


Figure IX.7: Chromatogram resulting from a whole blood sample of an alcoholic patient in which ethanol was not detected.