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# Determination of Cholesterol in Foods by Flow Injection Analysis With Perroxyoxalate Chemiluminescence

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A thesis

presented to

the faculty of the Department of Chemistry

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Master of Science in Chemistry

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by

Abisake Feleke

August 2006

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Keywords: Cholesterol, Hydrogen Peroxide, Cholesterol Oxidase, Cholesterol Esterase, Chemiluminescence's and Peroxyoxalate, and TCPO

ABSTRACT

Determination of Cholesterol in Foods by Flow Injection Analysis with Perroxyoxalate Chemiluminescence

by

Abisake Feleke

Cholesterol is an important biological molecule with many important functions. However, high serum cholesterol is a health hazard. Thus analysis of cholesterol is important and many analytical techniques have been developed. The objectives of the proposed research are to develop an economical, rapid method for the determination of total cholesterol with good selectivity and enhanced sensitivity. For evaluation of proposed flow injection analysis with peroxyoxalate chemiluminescence (FIA-POCL) method, figures of merit such as accuracy, precision, and linear dynamic range will be assessed. The proposed procedure was then applied to the determination of total cholesterol in foods. The procedure was linear for cholesterol from 0.01 to 0.120 mg/mL. The relative standard deviation was 2.57 %. The recoveries were 97.5 – 103.3 % for commercial standard cholesterol sample, and 101.5 – 108.0 % for butter. The proposed method was applied to analysis of cholesterol in food and the results were consistent with expected values.

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# CHAPTER 1

## INTRODUCTION

Cholesterol (cholest-5-en-3ß-ol), a fat like substance, with a polar secondary hydroxyl head group at the $C_3$ position in the non-polar hydrocarbon body, is the major steroid in animal tissue. Cholesterol is an important steroid and it is a common component of the membranes of animal cells. It is synthesized in many types of tissue but particularly in the liver and intestinal wall. Cholesterol is formed from squalene via lanosterol. [1, 2] Its structure is shown in Figure 1.

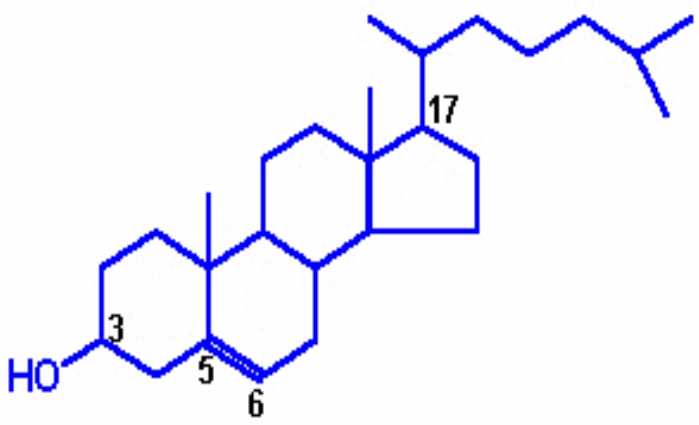



Figure 1. Chemical Structure of Cholesterol ($C_{27}H_{46}O$)

Cholesterol is present in the free and esterified cholesterol forms. However, there is no significant difference between the free and the esterified form of cholesterol other than the fact that in the case of the esterified form the cholesterol has a fatty acid attached to the hydroxyl group through the ester linkage. [1, 2]

Cholesterol is an essential molecule in many animals, including humans. It is not required in the mammalian diet however, because all cells can synthesize it from simple precursors. Moreover, cholesterol is important for the metabolism and transport of fatty acids and in the production of hormones and Vitamin D. [2, 3] Cholesterol gives our cells the strength they need to maintain their shape. Furthermore, it is essential for the formation and maintenance of cell membranes, which help the cells to resist changes in temperature, to protect and insulate nerve fibers. In addition to its roles in the structure and function of membrane, cholesterol has another extremely important function. Cholesterol is the basic building block of the steroid hormones that are stored in the adrenal glands, testes, and ovaries where they are available whenever there is a need. The major groups of the steroid hormones include the male and female sex hormones. [2]

Cholesterol is found in the blood stream and body cells. It comes from two main sources. Cholesterol is ingested whenever animal product such as egg yolks, meat, fish, and dairy products are consumed by us. Cholesterol helps to develop cellular and tissue membrane and is stored in organs and tissues of the body. Cholesterol is also obtained from foods having saturated fatty acids or animal fat. The liver is the secondary source of cholesterol. Livers produce various amounts of cholesterol up to about 1,000 mg a day. [3-4] Our bodies produce three to four times more cholesterol than what we get from our diet; therefore, about 80 % of the cholesterol is made by the liver. Normally, the total amount of cholesterol from these two sources remains constant because the rate of cholesterol synthesis in the liver is under feedback control. When the dietary intake is high, liver synthesis of cholesterol is low; and when intake is low, synthesis of cholesterol increases. Naturally, cholesterol is manufactured by the liver and located in

cell walls, but it is also created by cells lining the small intestine and by individual cells in the body. Lipoproteins, a combination of lipids (fats) and proteins, are made in the liver and carry cholesterol, fats, fat-soluble vitamins, and other lipids, to different parts of the body that requires energy production and any repair. [3, 4, 5]

Bile salts such as taurocholic acid are also synthesized from cholesterol in the liver. The synthesis of bile acid is one of the predominant mechanisms for the extraction of excess cholesterol from the body. Bile acid is stored in the gallbladder and released into the small intestine after ingestion of fatty foods. It acts as a biological detergent with four physiological functions, elimination of excess cholesterol, facilitating the digestion of dietary triacylglycerols by acting as emulsifying agents rendering access to pancreatic lipases, and solubilizing the cholesterol as well as facilitating the intestinal absorption of fat-soluble vitamins. However, high cholesterol concentration in the bile may lead to gallstone formation. [2, 3]

Excessive cholesterol in the blood contributes to the development of plaques in the inner wall of the arteries, a condition known as atherosclerosis. Plaques can restrict the blood flow to the heart and if they burst, they can form clots. Blood clots can cause the blockage of the small blood vessels in the heart that leads to heart attack. Furthermore, if clots block blood vessels in the brain it can cause brain stroke. [4] The risk of developing heart disease or atherosclerosis increases with the increase of the level of cholesterol in blood. The normal range for total blood cholesterol is between 140 and 200 mg per decilitre (mg/dL). The levels between 200 and 240 mg/dL indicate moderate risk, and levels above 240 mg/dL indicate high risk for coronary heart disease. Triglycerides are another form of fat in the blood stream, and high levels can increase the

risk for heart disease. If triglyceride level is at the border line or more than normal then medical treatment is necessary. [4] Table 1 shows the guideline developed by the National Cholesterol Education Program (NCEP) of the National Heart, Lung and Blood Institute, National Institutes of Hearth (NIH) and the National Cholesterol Education Program (NCEP). [4, 7]

Table 1. Serum Cholesterol and Triglyceride Levels (mg/dL)

| Total Cholesterol | |
|---|---|
| Less than 200 | Desirable |
| 200-239 | Borderline High |
| 240 and above | High |
| LDL Cholesterol | |
| Less than 100 | Optimal |
| 100-129 | Near optimal/above optimal |
| 130-159 | Borderline High |
| 160-189 | High |
| 190 and above | Very High |
| HDL Cholesterol | |
| Above 60 - 90 | Desirable |
| 35 - 40 | High |
| Less than 35 | Very High |
| Triglycerides | |
| Less than 100 | Normal |
| 100 - 149 | Borderline High |
| 200 - 399 or above 400 | Very High Risk |

Cholesterol, like fat, is insoluble in water; however, it is transported in the blood by water-soluble proteins, which have been classified on the basis of their densities. Very low-density lipoproteins (VLDL) serve mainly to transport triglycerides. Low densities lipoproteins (LDL), known as bad cholesterol, carry cholesterol to different parts of the body. In the LDL form, cholesterol is transported and supplied to different body tissues. LDL carries about 60-70 percent of the cholesterol around the body. Studies show that excess blood cholesterol leads to a much higher risk of heart attack and stroke. The LDL- cholesterol level should be less than 160 mg/dL. Although, there are other factors that can cause an increase in the blood cholesterol level such as age, gender, smoking, family history of heart disease, and diabetes mellitus, high dietary cholesterol is a major contributing factor that should not be ignored. [3-5]

High density lipoprotein (HDL) is involved in the transportation of cholesterol in the opposite direction, that is, from the tissues and organs back to the liver for recycling. They are either reused, converted to bile acids, or be disposed. HDL is called good cholesterol and high HDL levels are a good indicator for a healthy heart because less cholesterol is present in the blood. HDL also helps to reduce the risk of heart attack and stroke because they consist of more protein than that of triglycerides or cholesterol and they work hard to remove LDL from the walls of arteries. The HDL level should be more than 60 mg/dL, the higher the HDL the lower the risk for heart disease. However, if the HDL level is less than 40 mg/dL then we are in a higher risk for coronary heart disease. Numerous studies show that women have a higher HDL level than men and this is caused by estrogen, which tends to elevate HDL level in both men and women. [8] Regular exercise is associated with increasing the HDL level and alcohol consummation

between one or three ounce a day may also help increase the HDL concentration. [7, 10]

Biosynthetic Pathway of Cholesterol

Cholesterol, containing 27 carbons, suggests a complex biosynthetic pathway from a single pre-cursor of acetic acid. [2] Cholesterol, like a long chain fatty acid is made from the two-carbon acetate group of acetyl-CoA. This process occurs in four stages. In the first stage, the three acetate units condense to form a six carbon intermediate, mevolonate, where the two molecules of acetyl-CoA condense to form acetoacetyl-CoA. Stage two involves the conversion of mevalonate into activated isoprene units, when three phosphate groups are transported from ATP molecules to mevolonate. In the third stage, the isopentenly pyrophosphate and dimethylallyl pyrophosphate undergo a head-to-tail condensation where one pyrophosphate group is displaced and the 10-carbons chain becomes geranyl pyrophosphate. In the final stage, the cyclization of squalene forms the four rings of the steroid nucleus. Furthermore, series of changes, oxidations, removal or migration of methyl groups, lead to the production of cholesterol. Figure 2 illustrates the biosynthetic pathways of cholesterol.

The acetyl-CoA used for cholesterol biosynthesis is derived from an oxidation reaction (fatty acids or pyruvate) in the mitochondria and is transported to the cytoplasm by the same process as that described for fatty acid synthesis. Acetyl-CoA can also be derived from cytoplasmic oxidation of ethanol by *acetyl-CoA synthetase*. All the reduction reactions of cholesterol biosynthesis use nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor. [2, 5]

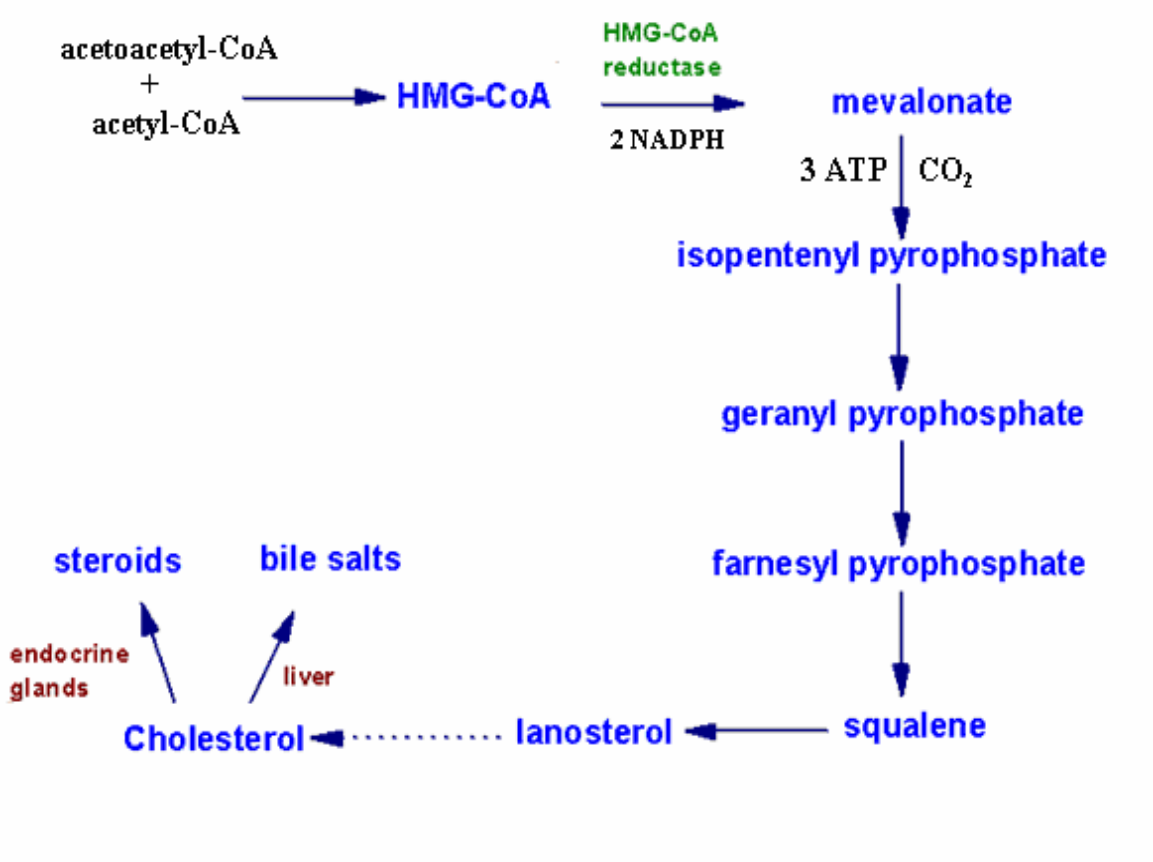



Figure 2. Overview of Cholesterol Biosynthesis

The isoprenoid intermediates of cholesterol biosynthesis can be diverted to other synthetic reactions, such as those for an isoprenoid compound similar to cholesterol that is used in the synthesis of N-linked glycoproteins, coenzyme Q (of the oxidative phosphorylation) pathway or the side chain of heme. Additionally, these intermediates are used in the lipid modification of some proteins. Acetyl-CoA units are converted to mevalonate by a series of reactions that begins with the formation of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). [2, 3]

HMG-CoA is converted to mevalonate by 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR); this enzyme is bound in the endoplasmic reticulum (ER). HMGR

absolutely requires NADPH as a cofactor and two moles of NADPH are consumed during the conversion of HMG-CoA to mevalonate. [2]

The synthesis and use of cholesterol must be tightly regulated in order to prevent over-accumulation and abnormal deposition within the body. The abnormal deposition of cholesterol and cholesterol-rich lipoproteins in the coronary arteries eventually leads to atherosclerosis and is considered as the leading contributory factor in diseases of the coronary arteries. Cholesterol synthesis is a very complex and energy expensive process; therefore, it is advantageous to an organism to be able to regulate the synthesis of cholesterol to complement dietary intake. Unregulated cholesterol production can lead to serious health problems. [7, 10]

Cardiovascular Disease

Cholesterol has a profound effect on our health and it is very important to understand this fact about cholesterol as well as finding a way to manage blood cholesterol level. Cardiovascular disease (CVD) is one of the leading causes of morbidity both man and women of any ethnicity. Each year, almost half a million deaths in the United State are caused by cardiovascular disease and 160,000 of those deaths are in individuals who are 35 to 64 years of age. According to studies conducted by the Center for Disease Control (CDC), lifestyle behaviors and environmental pollution can be factors for causing a heart disease as well. [4, 7]

Cholesterol is the most publicized lipid because of the strong correlation between high level of cholesterol in blood and incidences of human cardiovascular disease. Most often, the final blow from cardiovascular disease is either a heart attack or a stroke. These disasters are often associated with thrombus, a blood clot that blocks a key artery.

If the thrombus blocks one of the coronary arteries that supply blood to the cardiac muscle, a heart attack occurs. A thrombus that causes a heart attack may form in a coronary artery itself or it may develop elsewhere in the circulatory system and reaches a coronary artery via the bloodstream. Such a moving clot is called an embolus. Similarly, many strokes are associated with a thrombus or an embolus that clogs an artery in the brain, and, therefore, the brain tissue that is supplied by that artery dies. [7, 8, 10]

The suddenness of a heart attack or stroke belies the fact that the arteries of most victims had become gradually impaired by arteriosclerosis. Arteriosclerosis is associated with high level of cholesterol in the blood and particularly a higher LDL level and can greatly increases the risk of a blood clot plugging an artery. The narrowing of blood vessels does not occur suddenly but builds up over several years when cholesterol and fat transform the smooth muscle cells lining the blood vessels into thickened plaques that can become hardened by calcium deposits, resulting in a form of arteriosclerosis or hardening of the arteries. This causes the arteries to become constricted, diminishing their elasticity and reducing the volume of blood able to travel through them at any time. Healthy arteries have smooth linings, but the rougher lining of an artery affected by atherosclerosis encourages the adhesion of platelets that triggers the clotting process. Arteriosclerosis causes more deaths from heart disease than any other single condition. [6-10]

Hypertension (high blood pressure) promotes atherosclerosis and increases the risk of heart attack and strokes as well. Hypertension is sometimes referred to as the silent killer because a person with this disease may experience no symptoms until the tragedy occurs. Fortunately, hypertension is simple to diagnose and can usually be

controlled by drugs, diet, and exercise. To some extent, the tendency for hypertension and atherosclerosis is inherited, making certain people more predisposed than other to cardiovascular disease. Smoking, lack of exercise, and a diet rich in animal fats and cholesterol are among the factors that have been correlated with increased risks of cardiovascular disease. [7]

Besides diet, the increased serum cholesterol level can also caused by underlying diseases such as diabetes mellitus, kidney disease, liver disease, or hypothyroidism. It also can be caused by an inherited disorder in which cholesterol is not metabolized properly by the body. Obesity, which generally results from eating a diet high in fat, also can lead to elevated blood cholesterol levels. This is because obesity itself leads the body to produce excessive amounts of cholesterol. Hypercholesterolemia is a condition of high level of cholesterol in the blood that can lead to atherosclerosis that increases the risk for heart attack, stroke, circulation problems, and death. [8]

Familial hypercholesterolemia is also a syndrome that is due to a high level of low-density lipoprotein (LDL). Approximately 85% of men with this disorder have experienced a heart attack by the time they reach 60 years old. Among the affected males, a first heart attack typically occurs in their 40s to 50s. The incidence of heart attacks among women with this disorder is also increased. However, it is delayed by 10 years as compared to men. In contrast, the HDL level is nearly the same in both men and women for the first 15-20 years of life and then it declined by about 20-25% in men but by a lower percentage in women. [8, 10]

Symptoms of high cholesterol usually are rare. High cholesterol levels are generally identified from a blood test. The primary symptom associated with coronary

heart disease is called angina (chest pain). When a person experiences this, he or she describes a feeling of pressure, squeezing, or a general feeling of someone sitting on their chest. These symptoms can spread to the jaw, neck, or arm regions of the body. Initially the symptom will start with pressure in the chest region; however, some individuals experience that feeling of pressure in other areas and not the chest. In addition to the reported pressure, other symptoms include nausea, shortness of breath, sweating, lightheadedness or dizziness, and heart palpitations. [6, 7] With blood flow impeded, the heart becomes starved for oxygen, causing angina. Angina can be directly correlated to coronary heart disease and should be taken seriously. However, there are other stressors such as over-exertion, extreme emotion, or even after eating a huge meal. In these circumstances, a short period of rest, five minutes or so, will reduce or eliminate the symptoms. Again, if one has any of these symptoms, it is better to be checked out by a physician than to assume everything is fine.

Stroke is also one of the leading causes of deaths in the U.S. There are four primary symptoms pertaining to a stroke, although most of the time this is a sudden event with little or no warning. The sudden onset of numbness or weakness of face, arm, or leg, especially on one side of the body, confusion, difficulty speaking or understanding, sudden trouble with vision in one or both eyes and dizziness, loss of balance, and lack of coordination are of the symptoms. Other common signs include nausea and vomiting, fever and fainting, convulsions. [7-10]

Peripheral vascular disease is caused by high cholesterol in which the arteries that carry blood to our extremities become narrow or clogged. The outcome is that the blood flow is slowed down or stopped completely. The symptom includes numbness or tingling

in the lower extremities, a cold sensation in the lower extremities and open sores or ulcers on the lower extremities that do not heal normally.

Lipid-Lowering Treatments

Lifestyle and eating excessively "rich" diets and obesity have contributed to a dramatic increase in the occurrence of cardiovascular disease. Many studies have shown that a high serum concentration of low density lipoproteins cholesterol (LDL-C) is a major risk factor for coronary heart disease (CHD). Lowering of LDL-C level will reduces the risk of major coronary event. Drug therapy also can be considered for lowering the blood cholesterol level. Additionally, dietary therapy, regular physical activity, and weight loss can be prescribed treatment for elevated blood cholesterol levels. However, the treatment of high cholesterol is aimed at lowering the LDL-C, lowering triglyceride levels, and increasing the HDL or good cholesterol. The most effective lipid lowering agents are the 3-hydrox-3-methylglutaryl-coenzyme, a reductase inhibitor, more commonly known as statins. [6, 8]

Currently, a number of lipid lowering drugs available in the United State includes bile-acid sequestrants, cholesterol-absorption inhibitors, fibric-acid derivatives, statins, and niacin/nicotinic acid as shown Table 2. Statin is the primary treatment for patients who are at high risk of CHD. There are six types of statins such as lovastain (Mevacor), Atorvastatin (Lipotor), Cerivastatin (Baycol), fluvastatin (Lescol), Pravastatin (Pravachol), and Simvastatin (Zocor). These six types of statins differ in their effectiveness in reducing serum cholesterol. [8, 9]

Table 2. Available Lipid-Lowering Drugs

| Statins | |
|---|---|
| Avorvastatin (Lipotor) | Lovastain (Mevacor) |
| Fluvastatin (Lescol) | Cerivastatin (Baycol) |
| Pravastatin (Pravachol) | Simvastatin (Zocor) |
| Nacin/nicotinic Acid | |
| Extended Release (Naspan) | Immediate Release |
| Fibric-acid Derivatives | |
| Fenofibrate | Gemfibrozil |
| Bile-acid Sequestrannts | |
| Cholestyramine | Colesevelam |

Statins can block the conversion of HMG-CoA to mevalonate reductase that controls the rate of cholesterol production in the body. This results in an increase in hepatic LDL receptor expression to enhance the removal of apo B lipoprotein containing particles from the circulation and lower the total cholesterol, LDL-C, and triglycerides. Statins can lower the blood cholesterol level by slowing down the production of cholesterol and by increasing the liver's ability to remove the LDL-cholesterol already in the blood. Additional benefits of statins include atherosclerotic plaque stabilization, positive effects on endothelial dysfunction, and reduced level of C-reactive proteins all of which has been associated with CHD. Statins do show low side effects including hepatic or muscle toxicity and it is advisable to avoid using higher dose. [6, 9, 10]

Lovastatin is a natural product, while simvastatin and prevastatin are semi-synthetic products. [6] Atorvastatin is the most effective statin and has the ability to reduce the LDL-C level by about 52 % to 55 % at its highest dose of 80 mg.[6] Pravastatin and fluvastatin show nearly 34 % LDL-C reduction at their higher doses. However, lovastatin and simvastatin reduce cholesterol by about 6 % and 12 % at their higher dose.

Another class of drugs for lowering LDL is the bile acid sequestrants (BAS) such as cholestyramine, colestipol, and, nicotinic acid (niacin). [8-9] These have been shown to reduce the risk for coronary heart disease in controlled clinical trials. Bile acid sequestrants interrupt the enterohepatic circulation of bile acids by binding with them in the bowel to form an insoluble complex that is excreted in the stool. Bile acid depletion leads to an increase in hepatic LDL cholesterol receptor expression and consequently a fall in total and LDL cholesterol levels. The BAS drugs have been used for more than three decades and are moderately effective in lowering LDL-C levels from 15 % to 25%. [6] However, there are some side effects including gas bloating and constipation.

Niacin (nicotinic acid) is a water-soluble B-Complex vitamin, inexpensive and widely accessible to patients since the 1950s. [6] It is also another type of lipid lowering drug and can increase the concentration of HDL while decreasing the concentration of LDL level. Niacin undergoes two major metabolic pathways, one involves conjugation with glycine to form nicotinuric acid and it is associated with the flushing of niacin. The conjugate pathway is a low affinity, high-capacity pathways that are used when the non-conjugative pathway is saturated. However, they have significant problems for patients with higher LDL level because they are associated with flushing or hot flashes, abnormal

liver function, and other problems. [8] Dietary supplement niacin must not be used as a substitute for prescription niacin. If physician approves, over-the-counter niacin can be used as a substitute. But it must not be used for cholesterol lowering without being monitored by a physician because it is associated with flushing and skin irritation of the face and neck. [6-8] All patients taking niacin to lower blood cholesterol should have their doctor closely monitor them to get the best effect and to avoid complications. Self-medication with niacin should definitely be avoided.

The large reductions in total blood cholesterol level and LDL-C produced by these drugs resulted in large reductions in heart attacks and heart disease deaths. The main goal of cholesterol-lowering treatment is to lower LDL level enough to reduce the risk of having a heart attack or other diseases caused by hardening of the arteries. [6-9, 10]

Cholesterol is essential for normal functioning of the immune system, particularly for the body's response to the millions of cancer cells that every person makes in the body each day. For all the health problems associated with cholesterol, this important substance is not something we should try to eliminate from our bodies. Cholesterol does far more good than harm. The good news is that all these can be brought under control either by changes in lifestyle such as diet, losing weight, an exercise program, or quitting a tobacco habit. Drugs also may be necessary in some people. Sometimes one change can help bring several risk factors under control. For example, weight loss can reduce blood cholesterol levels, help control diabetes, and lower high blood pressure.

# CHAPTER 2

# ANALYSIS OF CHOLESTEROL

The determination of cholesterol content in either serum or foods has been the subject of extensive research. The need for suitable analytical methods for the determination of total cholesterol in biological blood and food product is highly significant for both our health and nutrition. Cholesterol determination is a valuable aid to diagnosis and treatment in a variety of clinical states, such as arteriosclerosis, thyroid metabolism, diabetes, and other disorders. [10-12] However, clinical laboratories are often unable to keep pace with the growing demand for these determinations because of the challenging and unique requirements placed on the techniques. The methods that are developed for the analysis of cholesterol present in the blood and for any of its fractions have proceeded through several pathways. Many different analytical approaches have been described for the analysis of cholesterol and its esters.

In late nineteenth century, Salkowski [13] described the first color reaction, where his analyte was isolated from gallstones. The combination and digitonide precipitation for purification of color reaction for quantification led to the first colorimetric procedure for serum reaction based on the gravimetric method of Windaus. [13] Digitonin was one of the specific reagents commonly employed for precipitating the cholesterol for its determination.

Methodologies for determinations of total cholesterol are subdivided into two main groups, extraction and direct methods. In the extraction methods the cholesterol is extracted from the serum into a suitable solvent, and after purification the cholesterol is subjected to a color reaction. [14] In the direct reaction methods, the reagents are added

to the sample without fractionation of the sample because the proteins are removed before measurement is allowed to take place. Additionally, there is no phase separation in direct reactions. Furthermore, the analyses of cholesterol are divided in two categories, non-enzymatic and enzymatic reactions. Non-enzymatic reactions involve the formation of color compounds. In contrast, the enzymatic reactions involve reaction between free cholesterol with cholesterol oxidase to generate hydrogen peroxide. The hydrogen peroxide can then be determined by various techniques. There are some significant differences between the non-enzymatic and enzymatic methodologies. The non-enzymatic method is more time consuming and requires the use of corrosive chemicals, whereas enzymatic reaction is fast and uses a direct assay and can be an automated procedure. [14, 15]

Lieberman-Burchard Reagents

The colorimetric methods for the determination of cholesterol are based upon the original reaction of Liebermann and Burchard, but often they differ in the extraction procedure. Liebermann-Burchard (L-B) reagent is one of the most significant and widely used reagents for non-enzymatic method of production of color. The reactions were described initially by Lieberman in 1885 [16] in which the reaction of cholesterol in acetic anhydride with sulfuric acid produced color that went from red through violet to blue-green. Burchard [16] applied the reaction to determine cholesterol in $CHCl_3$ instead. It was believed that the acetic anhydride served only as a diluents for the sulfuric acid because acetic anhydride can be replaced by acetic acid, ethyl acetate, or butanol.

The Lieberman-Burchard color reaction is complex and can be affected by many variables including concentrated sulfuric acid, glacial acetic acid, length of reaction,

light, and temperature. Since the L-B reagent is unstable, it is prepared fresh daily before performing the reaction and it is to be stored at low temperature. The reaction produces stable color between 430 to 630 nm. However, the green color at the maximum peak of 620 nm was more stable than the blue L-B color. [17]

In the early nineteenth century, Kim and Goldberg [18] developed a technique for serum cholesterol assay using a stable reagent. The reagents were prepared just as Liebermann and Burchard prepared them, using glacial acetic acid, acetic anhydride, and sulfuric acid. They pipetted 6.0 mL of the reagent and mixed it with 0.1 mL of the unknown sample or with 0.1 mL of cholesterol standard (200 mg/d). The mixtures were incubated at 37 $^{0}$C for about 18 min and absorbance measured at 625 nm. However, Burke and his coworker [19] indicated that the L-B and Zak color reactions had similar oxidative mechanisms with the active oxidants of $SO_3$ and $Fe^{3+}$. Further evidence showed their similarity where the L-B reaction was performed in acetic acid, sulfuric acid, and acetic anhydride medium, except for Zak [15] reaction which was performed using iron (III) for color production instead of acetic anhydride. The color study also indicated that the color species observed in the two systems are enylic carbonium ions formed during the protonation of the parent polyenes. The red product from Zak reaction was a cholestateraenylic cation, but the blue color from L-B reaction was the pentaenylic cation.

The L-B reagent has been modified in many different ways for the determination of cholesterol in serum; however, the color production per molecule differs for pure cholesterol and for total cholesterol in serum. The reason for this difference is because the cholesterol in serum is present as esters with long-chain fatty acid instead of free

cholesterol. It also can be because the L-B reagent interacts differently with the matrix of serum. However, L-B reagent seems to give a better result when it is used in a direct method compared to the other methods mentioned above.

Non-Enzymatic Method of Cholesterol Analysis

Non-enzymatic procedures involve the reaction of cholesterol with certain reagents that result in the production of color products obeying Beer's Law over a wide analytical range.

Non-enzymatic methods used for the determination of total serum cholesterol were very tedious and errors may result from incomplete cholesterol extraction. These sources of error were eliminated in a new rapid and accurate method of Pearson, Stern, and McGavack. [20] In 1952, they introduced a method in which cholesterol in serum is reacted directly with p-toluenesulfonic acid. In this method, glacial acetic acid, p-toluenesulfonic acid, and acetic anhydride were added directly to serum followed by addition of concentrated sulfuric acid. The determination was carried out in test tubes in a short time. They found that the color was stable for 10 to 15 minutes. The color stability was measured in the range of 550 to 650 nm and the absorption maximum was at 650 nm. However, the color density seems to differ with time.

Cholesterol, as well as other steroids, give intense colors when treated with Lewis acids, and the actual color produced depends on the specific acid used, its concentration, as well as other variables. In 1953, Zlatkis [13, 14] presented the first automated, sensitive, and stable color reaction for the simple determination of serum cholesterol by direct treatment of the serum with a reagent consisting of ferric chloride dissolved in glacial acetic acid. Full development of the purple color appeared in one minute. The

rates of color formation between cholesterol and cholesterol ester were identical. Additionally, precision and reproducibility were demonstrated with the absorption characteristic of color product through the spectral ranges of 400 to 700 nm. [17] The result followed Beer's Law and the color remained stable for several hours. Zak's direct reaction method was highly satisfactory because color formation was stable and it had good sensitivity.

In 1964, Jamieson [21] modified Pearson's technique for an automated direct cholesterol determination without preliminary solvent extraction. The reagents used in Pearson's method were also used in Jamieson's procedures but with slightly different concentration, volumes, and temperatures. The time for the color production was increased to 50 minutes rather than 30 minutes. The procedure did not require high concentration of toluene-p-sulphonic acid and it was found that keeping the concentration of sulfuric acid close to 15 % was very important. Lower concentration of sulfuric acid resulted in incomplete dissolution of the protein and led to instrumental fluctuations. More importantly, the automated method was fast, requiring only a very small amount of serum, and was very economical in terms of reagents. Furthermore, there were fewer sources of error compared with the more elaborate extraction method. Jamieson's automatic method, using a variable temperature heating bath, required about 0.2 mL of serum, standard, and reagents, showed satisfactory results for measuring serum and standard cholesterol. Compared with manual methods, there was no significant difference in the result. Jamieson's method was fast, stable, and more accurate than Pearson's method.

Wybenga and co-workers [22] described a simple, direct, and specific manual

method for determination of total cholesterol in serum. Their method did not require the extraction of cholesterol from the serum and was relatively free of interference from hemoglobin. Like the method of Zlatkis [14] the reactions were performed using a mixture of sulfuric acid, acetic acid, and ferric chloride. The only difference was the addition of a 50 μL of sample of cholesterol standard in Wybenga's method. The maximum color was achieved within 30 minutes at room temperature similar to Pearson's method. The results obtained by Wybenga et al. did not differ statistically from those obtained by the method of Abell and coworkers. [18]

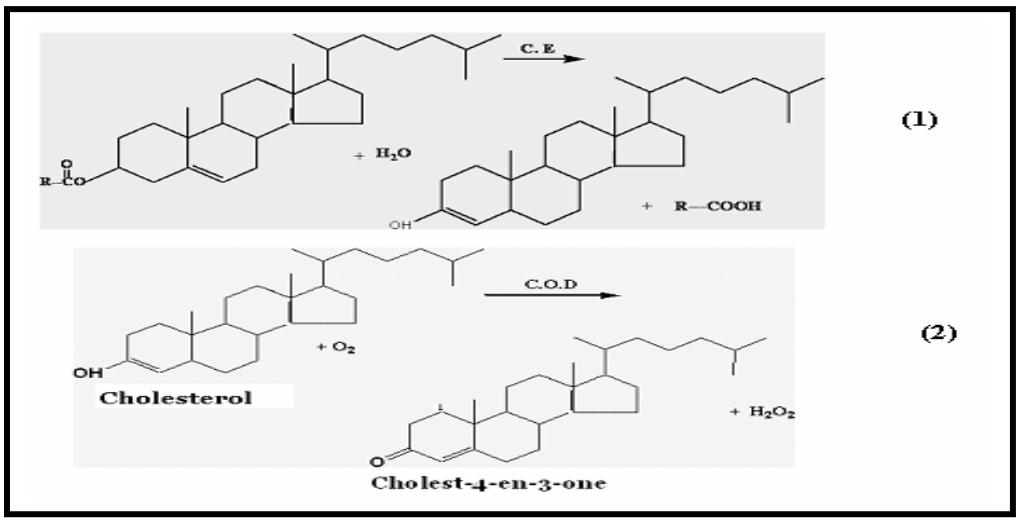
In 1982, Abell's reaction [16, 18] was performed for direct addition of Liebermann-Burchard reagent to cholesterol sample. Their results were calibrated with human serum pools assayed. Accuracy and precision were monitored for six years by analysis of internal-control pools and blind external-control pools. For the various internal controls pool the imprecision of the long-term averages ranged from 0.5 to 0.9 %. It was concluded that the use of serum calibrator eliminated the bias inherent in direct L-B methods because the color yield per molecules differs for cholesterol in serum and pure cholesterol in calibration solutions.

The determination of serum cholesterol has changed from a comparatively time-consuming procedure to simpler and more rapid technique. Bowmen and Wolf [23] had examined the absorbance spectrum of Zlatkis's cholesterol reaction. The reaction showed not only the expected peak at 560 nm but also an additional higher peak at 490 nm. Under the optimum condition of time and color reagent, the 480-nm peak approximately double the sensitivity for cholesterol determination as compared to the 560-nm peak. The observed interference of iodide in Zlatkis reaction resulted from an

enhancement effect of the color by the cholesterol itself. However, adding an extra 100 μg NaBr per milliliter of glacial acetic acid produced 50 % greater color and eliminated interference at 560 nm.

Enzymatic Method of Cholesterol Analysis

Enzymatic methods of analysis are becoming very important in food. Clinical chemistry also has benefited from the advantages in the use of enzymes for analytical purposes. Enzymes are biological catalysts that enable many complex chemical reactions to proceed at a reasonable rate. Several enzymatic methods for free cholesterol determination and total cholesterol had been published. [26-29] Enzymatic determination of total cholesterol is based on the hydrolysis of esterified cholesterol catalyzed by cholesterol esterase (CE) to free cholesterol. The free cholesterol is then oxidized in the presence of cholesterol oxidase (COD) to yield the corresponding ketone (cholest-4-en-3-one) and hydrogen peroxide, as shown in equations 1 and 2.



Cholesterol ester (CE) and free cholesterol can be measured after enzymatic reaction using either colorimetric or fluorometric methods. Furthermore, CE content in human plasma, rat liver, and culture cell can be measured using the above methodology and its usefulness evaluated.

Huang and his associates [24] had described a fluorometric enzymatic method for determining total serum cholesterol based on the hydrolysis of cholesterol esters to free cholesterol. They described the procedure in which free cholesterol was oxidized by oxygen gas in the presence of cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide. A calibration curve was constructed form the collected data on a series of standard cholesterol solutions versus the fluorescence change ($\Delta f/5$ min). The curve was linear up to 4.0 g of total serum cholesterol per liter of serum with correlation coefficients of about 0.98. This method was specific, precise, accurate, rapid, and simple. Moreover, the results correlated well with both Liebermann-Burchard procedure and the colorimetric enzymatic method.

Amundson et al, [25] described a simple and sensitive fluorometic assay for the enzymatic determination of cholesterol in serum and food samples. The complete reaction mixture of the Amplex red-based cholesterol assay contain Amplex Red, cholesterol oxidase, cholesterol esterase, HRP (horseradish peroxidase), and cholesterol. The assay was performed in one step using a 96-well microplate. The assay allowed the detection of 5 pmol (2 ng) cholesterol per well, which is 100-fold more sensitive than the fluorometic and colorimetric methods. In addition to its high sensitivity, the Amplex Red-based cholesterol assay showed excellent linearity for cholesterol concentration from

10 nM to 10 μM. This method was superior as it used longer wavelength spectra that are less susceptible to interference from biological compounds.

Allain and his associates [26] described the enzymatic hydrolysis of cholesterol esters and the colorimetric measurement of peroxide by a peroxidase coupled reaction with 4-aminoantipyrine and phenol. This procedure was adapted to continuous-flow automated procedure for serum cholesterol using the AutoAnalyzer II. The calibration curve showed linearity at 600 mg/dL. This method is reproducible and the result correlated with automated Liebermann-Burchard procedure and the method of Abell et al.

Enzymatic Flow Injection Analysis (FIA)

Flow injection analysis has been used for the determination of total cholesterol level in the presence of various enzymes such as cholesterol oxidase, cholesterol esterase, and peroxidase.

Fernandez-Romero and co-workers [27] had proposed four different flow injection methods for enzymatic determination of total cholesterol in serum. Using the merging-zones or merging zone/stopped flow procedure with a photometric or fluorimetic detection, they developed four different precise methods for cholesterol analysis. The first two steps are the enzymatic reactions involving cholesterol esterase and cholesterol oxidase. However, the third step depended on characteristic of the detection systems. The first step was the catalyzed hydrolysis of cholesterol ester by cholesterol esterase (CE) to generate free cholesterol and fatty acid. The second step was the oxidation of free cholesterol by atmospheric oxygen in the presence of cholesterol oxidase (COD) to produced cholest-4-en-3-one and hydrogen peroxide. The horseradish peroxidase (HPOD) catalyzed the reaction of hydrogen peroxide with 4-aminoantipyrene

and phenol to generate highly colored quinoneimine dye with maximum absorbance at 520 nm. Both free and esterified cholesterol, are determined in the absence and presence, respectively, of cholesterol oxidase. Their reaction time was 10 min at 37 $^{o}$C. The calibration curves were linear for ranges of 0.020 – 0.20 and 0.005 – 0.050 g/L and the method showed excellent results.

Richmond [28] described an assay for total and free cholesterol determination in serum with the use of the AutoAnalyzer II (Technicon). An alkaline ethanolic hydrolyzing reagent at pH 7.5 was used to release free cholesterol from its lipoprotein complexes and hydrolyzed the ester. The hydrolysis was kept at pH 7.5 to prevent formation of a precipitate with the addition of $Hg^{2+}$. Furthermore, the free cholesterol was maintained in miceller solution for enzymatic oxidation with surfactant Triton X-100 and hydrogen peroxide was measured by reaction with $Ti^{4+}$ and xylenol orange.

The use of immobilized enzymes for analysis has been considered to be one of the most important advancements in enzyme technology. It improves enzyme stability, activity, re-usability, and prevents its deactivation due to environmental perturbations. In 1985, Masson and Townshend [29] proposed a method based on immobilization of an enzyme for the determination of cholesterol using flow injection analysis (FIA). In this method 50 U of cholesterol oxidase were immobilized on 200 mg of controlled porosity glass (CPG) by cross linking with glutaraldehyde. The cholesterol oxidase immobilized on CPG showed very high stability. The calibration curves gave a linear response for 0 – 80 mg $dL^{-1}$ cholesterol with relative standard deviation of 1.0- 3.0 %. The sample throughput was 80 $h^{-1}$.

Later, optimization of the co-immobilized cholesterol esterase and cholesterol oxidase on to cellulose acetate membrane was introduced by Bariyah and co-worker. [30] Cholesterol esterase and cholesterol oxidase which were co-immobilized by the adsorption method on to cellulose acetate membrane coated on glass beads. A maximum adsorption of 75% for cholesterol oxidase and 63% for cholesterol esterase were obtained under the condition consisting of an agitation rate of 70 oscillations per min for 18 h using cholesterol esterase and 0.08 U/ $cm^2$ of cholesterol oxidase at 37 $^oC$. A satisfactory stability of 8 repeated uses of the co-immobilized preparation was obtained with a $t_{1/2}$ of 15 consecutives uses. When compared to the commercially available Sera-Pak and CHOD-PAP, the co-immobilized enzyme preparation showed a linearity with correlation coefficient of 0.95 and 0.99.

Moody and co-workers [31] developed an amperometric enzyme electrode in a flow analysis system for determination of cholesterol. The electrode consisted of cholesterol oxidase immobilized on nylon mesh and held over platinum. The electrode was run under +600 mV vs a silver–silver chloride reference electrode, with a carrier stream of 2.3 $cm^3$ $min^{-1}$ at pH 7.0 phosphate buffer containing Triton X-100 (1%). The electrode exhibited linearity between 10 μL and 1.03 mM of cholesterol and it was also concluded that the enzyme electrode lifetime were about 6 weeks and it was governed by the deleterious effect of cholesterol on the surfactant of electrode. The respective linear ranges were $5.17 \times 10^{-5} – 5.17 \times 10^{-4}$ and $1.30 \times 10^{-5} – 1.30 \times 10^{-4}$ mol $dm^{-3}$ of cholesterol.

Potentiometric detection was an interesting method because of its inherently wide linear range. However few studies have been done in its use for analysis of cholesterol. Situmorang and co-workers [32] reported a study using flow injection potentiometry

(FIP) for this purpose. They developed a method that used a tungsten wire electrode as a sensor vs. Ag/AgCl in conjunction with a redox mediator ferrocyanide. Ferrocyanide is converted to ferricyanide by hydrogen peroxide catalyzed by peroxidase, and the tungsten electrode responds to the ratio of ferricyanide to ferrocyanide. Their assays were done with a combination of three enzymes, cholesterol oxidase, cholesterol esterase, and peroxidase (POD). Flow injection potentiometry gave well defined peaks and had very fast response (30 s). Calibration curves were obtained from 0.05 to 3.0 mM cholesterol with a slope of 60.2 mV/decade and the detection limit was 0.01 mM cholesterol (S/N = 3.0).

All of these works illustrated enzymatic or non-enzymatic reactions that resulted in the formation of hydrogen peroxide detected either in color form or as end-point signal. Generally, the determinations of serum cholesterol and cholesterol ester have included both enzymatic and non-enzymatic reaction with four separation steps namely: extraction, saponification, isolation, and end-point. Cholesterol determinations have become simpler and it is generally feasible for most clinical laboratories.

# CHAPTER 3

# LUMINESCENCE AND TECHNIQUES

## Principle of Luminescence

In 1568, Cellini described the first recognized nature of luminescent diamond and it was about the same times that fluorescence was discovered. Luminescence phenomena have been known since ancient times and related mainly to living organisms that emit light including fireflies, luminous bacteria, fungi, fishes, and insects. In 1888 Wiedemann [33] was the first person that defined the term luminescence to distinguish between the light emission from thermally excited substance and light emission from molecules excited by other mechanism. He then classified luminescence phenomena into six different categories according to their excitation methods. The list of the present recognized types of luminescence includes photoluminescence, thermo-luminescence, electroluminescence, crystalloluminescence, triboluminescence, and chemiluminescence. Photoluminescence is caused by absorption of light can be subdivided into fluorescence and phosphorescence. Electroluminescence is produced in gases by an electric discharge, thermoluminescence is produced by slight heating, triboluminescence is a result of friction, crystalloluminescence a result of crystallization, and chemiluminescence is caused by chemical reaction. [34-36]

Luminescence applied to the reemission of previously absorbed radiation. When a photon of electromagnetic radiation is absorbed by a molecule, the molecule is raised to the excited state and when it returns to the ground state, the molecule emits radiation. [33] Some of the most important features of luminescent method is their inherent

sensitivity, low detection limit compared to absorption methods. Additionally, it has large linear dynamic range and selectivity.

Fluorescence, phosphorescence, and chemiluminescence provide some of the most sensitive and selective methods of analysis of many compounds. Fluorescence and phosphorescence are alike in their excitation method; however, chemiluinescence was based on an excited species that is produced from a chemical reaction. Generally, fluorescence is a phenomenon in which absorption of light of a given wavelength by a fluorescent molecule is followed by the emission of light at longer wavelengths. [37, 38] Fluorescence can occur in simple, complex gaseous, liquid, and solid chemical phase. In 1935, Jablonski [39] proposed the scheme of electronic energy level that becomes the basis of interpretation of all luminescence phenomena. The Jablonski diagram shown in Figure 3 shows the activation and deactivation processes of a molecule.

A Jablonski diagram is a partial energy diagram for a typical photo-luminescent molecule. The lowest heavy horizontal line represents the ground-state energy of the molecules, indicating singlet state and is labeled as $S_0$. At room temperature this state represents the energy of essentially all of the molecules in a solution. The three lines above $S_0$ represent the energy levels of the ground vibrational states.

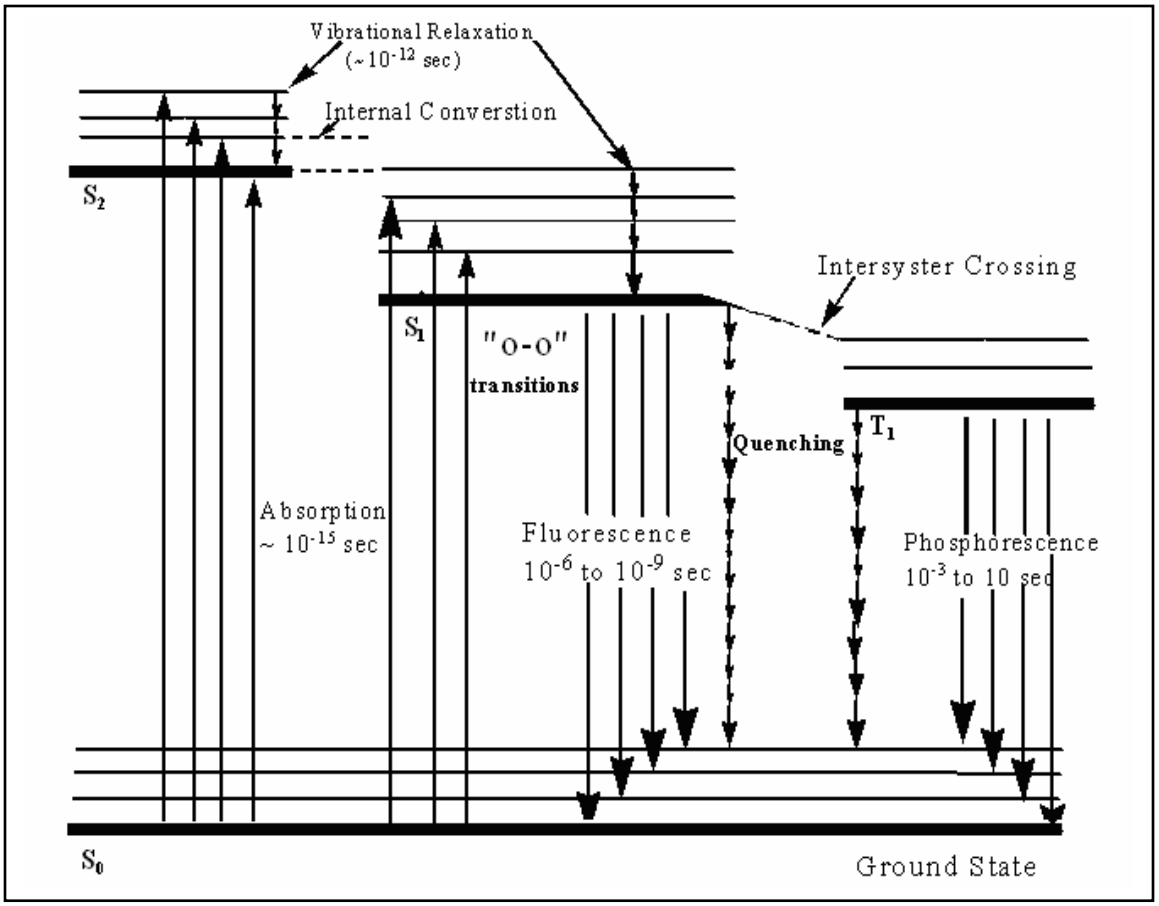



Figure 3. Jablonski Energy Diagram

The upper heavy horizontals lines represent the first electronic singlet state ($S_1$) and second electronic singlet state ($S_2$). A singlet state is a molecular electronic state in which all electron spins are paired, and no splitting of electronic energy level will occur when the molecules are expose to a magnetic field. The energy level located on the right side, $T_1$ represents triplet state. The energy of the first excited triplet state is lower than the energy of the excited singlet state. In the triple state the two electrons become unpaired and parallel. [37, 39]

The difference between the singlet and triplet state are the electronic spins, which are equal to + ½ and – ½. The expressions of multiplicity are related to the spin of the

orbital and are used to express the orbital angular momentum of a given state. [38] The term multiplicity is used to express the orbital angular momentum of a given state that is related to the spin by the equation 3.

$$M = 2S + 1 \tag{3}$$

When all the electrons are paired, S = 0, (+ ½ – ½ = 0) and the multiplicity will be a singlet electronic state. However, when the spin of singlet state is reversed, the molecules find itself with unpaired electrons and resulted a multiplicity of 3 or called a triplet state.

At room temperature, the ground singlet state $S_0$ represents the energy of all the molecules in the solution. The excitation of the molecules takes place by absorption of quanta of radiation of appropriate wavelength. If the molecules absorbed energy equal to the energy difference between the ground state and one of the excited single states, then the molecules are raised to that excited single state.

Deactivation Process

There are several mechanisms as illustrated by the Jablonski diagram that an excited molecule, can return to its ground state. Fluorescence and phosphorescence involve the release of a photon returning to $S_0$ from the lowest singlet excited state $S_1$ or from the triplet excited state $T_1$, respectively. The different processes responsible for the dissipation of the excess energy of an excited species can be classified as in Figure 4.

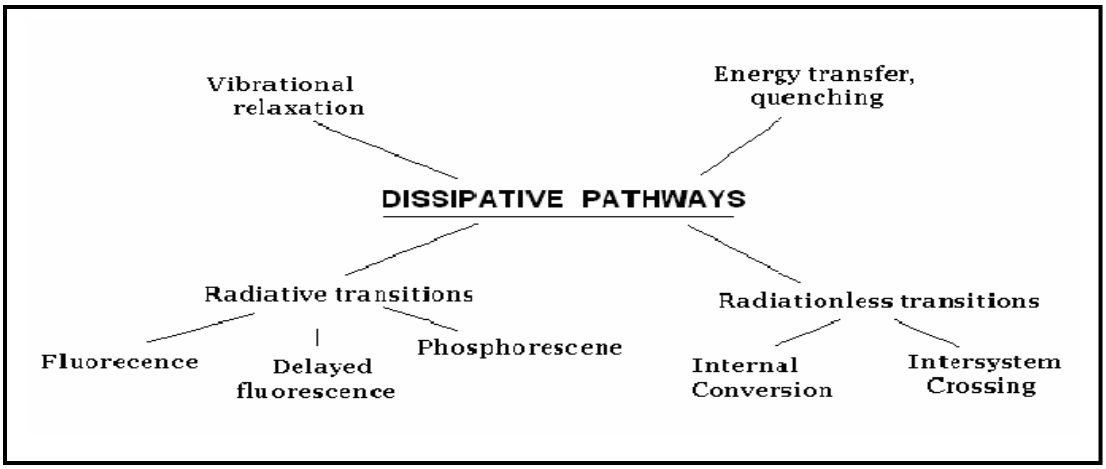



Figure 4. Pathways for the Dissipation of Electronic Energy

The non-radiative relaxation processes are so efficient that the average lifetime of a vibrationally excited molecule is about $10^{-12}$ sec. In solution, the excess vibrational energy is immediately lost as a consequence of collisions between the molecules of the excited species and those in the solvent, resulting in energy transfer and an increase in the temperature of the solvent. This means that most excited state molecules never emit energy from higher electronic states. This process is known as vibrational relaxation. [39]

Internal conversion (IC) occurs when a molecule is de-excited from a lower vibrational level of the upper excited state ($S_2$) to the higher vibronic levels of the lower excited state ($S_1$). [39] Internal conversion appears to be very efficient when two electronic energy levels are sufficiently close for there to be an overlap in their vibrational energies. IC through the overlapping vibrational level is usually more than the loss of energy by fluorescence from a higher excited state. IC may also results in

phenomenon of pre-dissociation, where the electron moves from the higher electronic state to the upper vibrational level of the lower electronic singlet state. [38-39]

In molecules where the singlet $S_1$ and the triplet $T_1$ energy level are closely spaced the molecules can drop into the lowest energy $T_1$ state through a process known as intersystem crossing (ISC). [39] Sometimes under the right conditions the molecules may flip its spin and get to the triplet state. This process is normally a forbidden transition. However, due to spin orbital coupling ISC may take place between $10^{-3}$ to $10^{-4}$ s, a relatively slow process. When energy is applied some molecules will reverse the intersystem crossing process to bring molecules back to the singlet state. ISC is the most common for molecules containing heavy atoms. ISC can decrease the occurrence of fluorescence and increase the probability of populating the triple state. Transitions from the triplet excited state to the singlet ground state are forbidden. However, when the molecules returned to the ground state $S_0$ by emission of radiation from the $T_1$ to $S_0$ phosphorescence occurs. Phosphorescence is observed in transition times of $10^{-4}$ to 10 s. An after glow is one of the characteristic features of phosphorescence because emission tends to continue after the exciting source is removed. Because of the relatively long lifetime of the triplet state, molecules in this state are much more susceptible to radiationless deactivation processes. [37-39] Fluorescence is statistically much more likely than phosphorescence.

Deactivation of an excited molecule involved with interaction and energy transfer between with other molecules or solvent molecules are known as external conversion or collisional quenching. Quenching results from a specific interaction between a fluorophor and another substance present in the system. [37-38]

Fluorescence quenching is a process which decreases the intensity of the fluorescence emission. The general mechanisms for quenching are given below. In these equations, M represent the fluorophor, Q is the quencher, and M* is the excited fluorophor.

$$M + h\upsilon \longrightarrow M^{*} \text{ (light absorption)} \quad (4)$$

$$M^{*} \longrightarrow M + h\upsilon \text{ (fluorescence emission)} \quad (5)$$

$$M^{*} + Q \longrightarrow Q^{*} + M \text{ (quenching )} \quad (6)$$

$$Q^{*} \longrightarrow Q + \text{energy} \quad (7)$$

Quenching can occur via collision (dynamic quenching), charge transfer reaction, and energy transfer. Dissolved oxygen [37, 38] is the most common luminescence quencher and removal of oxygen from the sample before luminescence analysis is advisable.

<u>The Principle of Chemiluminescence</u>

The phenomenon of chemiluminescence (CL) was first discovered in the mid-nineteen century by Radziszewski. In 1877, Radziszewski [42] reported the green light emitted by reaction of organic compounds. The emitted green light was observed from lophine (2, 4, 5 –triphenylimidazole) reacting with oxygen in the presence of a strong base.

Visible radiation that is observed when chemical reaction yields an electronically excited intermediate or products that either luminesce or donate its energy to another molecule that can luminesce is called chemiluminescence. If radiation is emitted by

energy transfer, it is known as chemi-excitation. When the chemical reaction is enzymatic or occurs in living organisms, the phenomenon is named as bioluminescence (BL). [41]

Many more CL reactions were discovered during the early 20th century. In 1905, Trauzt [42] described and published a review of known CL and BL reactions and he attributed the reaction to active oxygen. In his study, he described more extensively the CL and the luminescent properties of the reaction from the organic compounds with oxidants. In 1927, Mallet [42] discussed energy transport to fluorophores and in 1928, Albrecht [43] reported the intense luminescence associated with alkaline oxidation of the most widely used CL reagent known as luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione).

Generally, a CL reaction can be generated by two basic mechanisms. In the direct reaction involving two reagents, a substrate and an oxidant react in the presence of a co-factor to form a product which is chemiluminescent. The product is formed in the electronically excited state that can subsequently relax to the ground state with the emission of a photon as shown by the equations below:

$$A + B \longrightarrow C^{*} + D \qquad (8)$$

$$C^{*} \longrightarrow C + h\upsilon \qquad (9)$$

A and B represent the reactants and C* is the excited stats of the product. The indirect or sensitized CL reaction is based on energy transfer of the excited species to a fluorophore. The following reactions summarize the process.

$$A + B \longrightarrow P^* + D \quad (10)$$

$$P^* + F \longrightarrow P + F^* \quad (11)$$

$$F^* \longrightarrow F + h\upsilon \quad (12)$$

In step one, a key excited intermediate P* is formed by a reaction. In the second step the chemical energy of the intermediate is transferred to excite the fluorphore F. Finally, the CL occurs when the excited fluorophore F* returns to the ground state with the emission of light.

Three conditions are required for CL reaction. First the chemical reaction must be highly exothermic to release sufficient energy to populate the excited state molecules. Secondly, the reaction pathway must favor the formation of the excited state product. Finally, the excited state product must be capable of emitting a photon itself or transferring its energy to another molecule that can emit. [44, 45]

In all luminescent processes, intensity of CL ($I_{CL}$) depends on the efficiency of the formation of excited state molecules to luminesce, which is represented by quantum efficiency ($\Phi_{CL}$). Thus $I_{CL}$ is given by the following.

$$I_{CL}\,(\#\text{ of photons/s}) = \Phi_{CL}(\#\text{ of photons}/\#\text{ of molecules reacted}) \times -dC/dt\,(\#\text{ of molecules/s}) \quad (13)$$

The CL quantum yield is actually express as a product of two efficiencies: [37-38]

$$\Phi_{CL} = \Phi_{ex} * \Phi_{L} \quad (14)$$

where $\Phi_{ex}$ equals the ratio of the number of electronically excited molecules formed divided by the number of the molecules reacted. The $\Phi_L$ is the luminescence efficiency of the luminescent species and it equals the fraction of the number of photons emitted divided by the number of electronically excited molecules formed. Therefore, CL signal decreases over a period of time after the reagents are consumed.

There are many inorganic and organic chemical reactions that produce CL in the liquid phase. However, there are only a few systems that have been used for analytical purposes, including luminol ( 5-amnio-2,3-dihydrophathalazine-1, 4-dione), lucigenin (N,N-dimethyl-9, 9-biacridinium nitrate), lophine (2,4,5-triphenylimidazole), bis(2,4,5-tricholorophenyl oxalate), and luciferin. Luminol, followed by lucigenin, are the two most commonly employed CL reaction. [38, 46, 47] Because many of the solution phase systems use hydrogen peroxide or organic peroxides as oxidants, these can be generated in many ways in liquid systems to extend the applicability of CL for analysis.

## Types of Chemiluminescence

### Lminol Chemiluminescence

Luiminol (5-amino-2, 3 dihydrophthalazine-1, 4-dione) is one of the most popular, commonly used, and functional chemiluminescent reagents. It can produce its blue fluorescent under basic condition with an oxidizing agent. Specifically, lumniol is used for the detection of horseradish peroxidase (HPR) using hydrogen peroxide ($H_2O_2$) as a substrate. [47] Lumniol is a relatively simple compound and was often used in biochemistry for determination of enzymatic reaction as well as in blood detection in crime scene.

The chemiluminescent emitter is a "direct descendent" of the oxidation of luminol or an isomer like isoluminol by an oxidant in basic aqueous solution. Light is produced when luminol reacts with an oxidizing agent to yield an excited singlet state of aminonaphthalate anion and emits an intense blue color. [39] The oxidation of luminol is one of the more commonly known non-enzymatic chemiluminescent reactions. Probably the most useful oxidant is hydrogen peroxide. However, other oxidants have also been used such as perborate and permanganate. It is very stable in the absent of oxygen.

Figure 5 shows how luminol reacts with hydrogen peroxide with the formation of dinegative ion of luminol, which then reacts with oxygen or an oxidizing agent. Nitrogen gas is discharged during the reaction, leaving the luminol in an excited state in the form of 3-aminophthalate ion which emits light at a maximum wavelength of 425 nm. [37, 38]

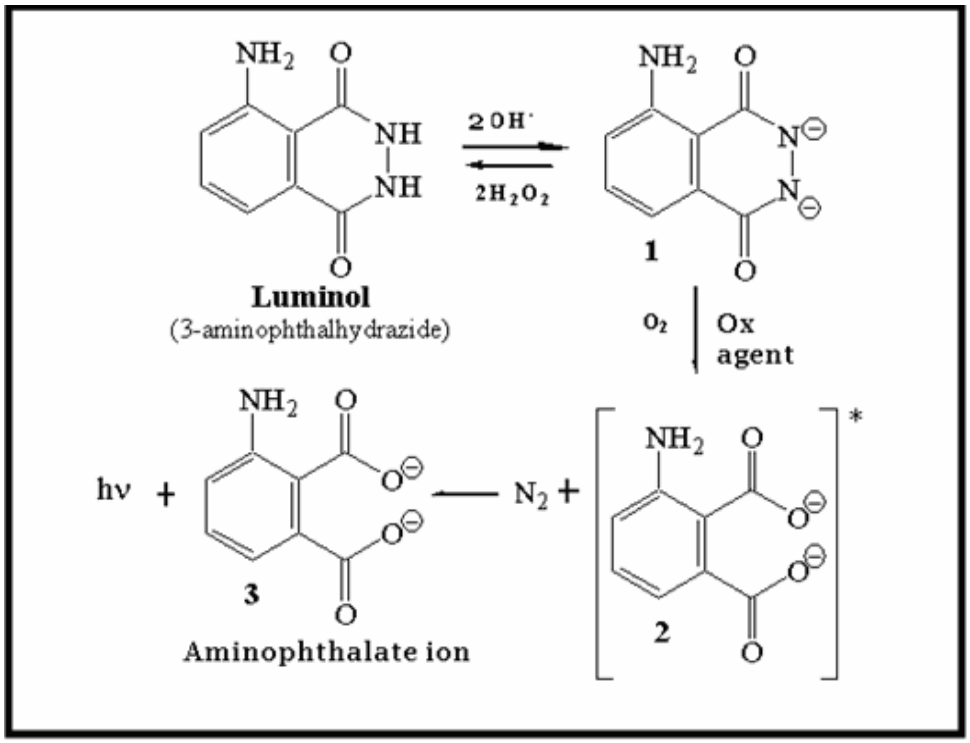



Figure 5. Reaction of Luminol with Hydrogen Peroxide

Luminol CL has been shown to be a very sensitive method for the determination of metal ions such as $Cu^{2+}$, $Co^{2+}$, and $Fe^{2+}$. Some of the catalysts that have been used are Cu(II), Cr(II), $VO^{2-}$, Mn(II), Ni(II), and Co(II) and the co-oxidants are $S_2O_8^{2-}$, $Fe(CN)_6^{3-}$, heamine and peroxidase and permangnate are required for CL.[39, 40, 49]

When luminol is placed in a basic solution with perborate, permanganate, hypochlorite, iodine, or hydrogen peroxide, and a catalyst such as iron, manganese, copper, nickel, or cobalt is added, the luminol is oxidized. A catalyst is the most important ingredient to the reaction because the more effective the catalyst the longer and brighter the light will glow. Hydrogen peroxide works best in base, and cobalt has proven to be the best metal catalyst. Some metals, however, repress CL at different concentrations and within certain limits. The CL intensity of luminol is directly proportional to the concentration of the oxidant, the catalyst, or the luminol itself. [46, 47]

Olsson [48] studied the determination of hydrogen peroxide using CL with a reagent consisted of 100 μM of luminol and 3 μM of the peroxidase at pH 10 of carbonate buffer. In his method an automated flow injection system with throughput of two samples per minute was used and the linear log-log calibration curve resulted in a slope of 1.3 from the detection limits of $3 \times 10^{-9}$ up to $10^{-5}$ M of $H_2O_2$.

The chemiluminescent properties of luminol are remarkable in fact that instead of heat, reactions involving luminol produce a cool light that has become a useful tool for both scientific community and average individuals. It has aided research in blood, forensic, and chemical analyses.

Peroxyoxalate Chemiluminescence (PO-CL)

Proxyoxalate Chemiluminescene was introduced by Chandross [50] in 1963. He found a very bright CL resulted when he added acid chloride to hydrogen peroxide containing a fluorophore such as anthracence and 9, 10-diphenylanthracene or N-methylacridone. Later, Rauhut et al. [51] proposed 1, 2-dioxetanedione as a possible high energy-rich intermediate and capable of transferring energy to fluorophores in POCL reactions.

POCL reaction is one of the most frequently used CL analytical applications. It has unique properties, which permits many applications that cannot be served by electrically powered devices. For example, in areas where the possibility of explosion exists, chemical light can be employed safely; being a cold luminescent source it cannot be a source of ignition. An amazing variety of useful techniques of PO-CL has been developed in recent years such as emergency lights for fuse in power failures; small portable lights for camping, backpacking, jogging; marking lights for life jackets; lures for commercial fishing; and a wide spectrum of novelty uses. [50, 51] Whereas most CL reactions involve emission from a reaction intermediate derived from one of the reagents, the peroxyoxalate reaction transfers energy to a variety of fluorescent molecules that in turn emit light during relaxation from the first singlet excited state.

POCL is a chemi-excitation process involving an oxalic acid derivative, usually an activated diaryl oxalate. It is based upon the reaction between hydrogen peroxide with an activated oxalate, leading to the formation of one or more energy-rich intermediates,

and capable of exciting a large number of fluorphores through the chemically initiated electron exchange luminescence mechanisms. The process is shows in the following equations.

$$\text{TCPO} + H_2O_2 \longrightarrow [C_2O_4] + \text{ArOH} \quad (15)$$

$$[C2O4] + \text{Fluorophore} \longrightarrow 2CO_2 + \text{Fluorophore}^* \quad (16)$$

$$\text{Fluorophore}^* \longrightarrow \text{Fluorophore} + h\upsilon \quad (17)$$

PO-CL involves three steps. In the first step, an aryl oxalate ester like bis(2,4,6-tricholorphenly)oxalate (TCPO) reacts with hydrogen peroxide to produce an intermediate 1, 4-dioxetanedione ($C_2O_4$). In the next reaction, the intermediate decomposes into carbon dioxide giving its energy to an available fluorophore. The final step is the emission of light energy when the excited fluorophore returns to the ground state. It is evident that $H_2O_2$ is essential for CL generation and, therefore, the removal of hydrogen peroxide would lead to CL inhibition. Acidic and basic moderators or other catalyst affect the reaction rate of $H_2O_2$ with oxalate. [50- 51]

The oxalate, Bis(2, 4, 6-tricholophenly) oxalate (TCPO) is one of the most common aryl oxalate esters used in the chemiluminescent analysis. Its structure is shown in Figure 6.

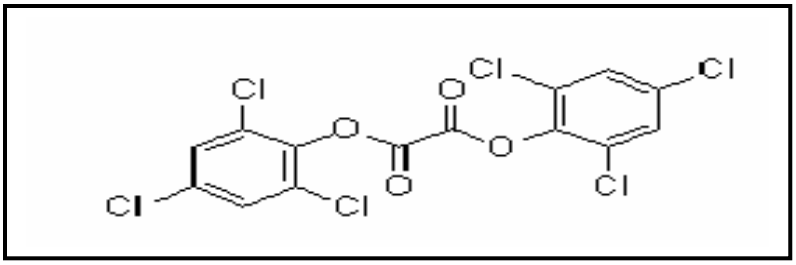



Figure 6. Bis (2, 4, 6-tricholorphenly) oxalate (TCPO)

Oxalate esters such as TCPO allow production of light from fluorophores when reacting with hydrogen peroxide. To increase the sensitivity, a suitable fluorophore such as perylene is used. [51] This reaction has been successfully used as highly sensitive detection in procedures developed for low-level determination of a wide variety of analytes. The reaction can be used for the detection of fluorophore or hydrogen peroxide. Additionally, PO-CL can also be coupled with a various instrumentation including flow injection analysis (FIA), high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE) for the detection of flurophore or fluorescent derivatives. It has been also used to detect analytes that can produce hydrogen peroxides in enzymatic reactions. [52-55]

PO-CL has many advantages. It has greater than four orders of magnitude of linear dynamic range. TCPO is a very stable organic compound and is easy to prepare. The reaction can occur very stable over a wide range of pH. But there is one important difference between the peroxyoxalate reaction and other CL reactions which involve emission from the intermediate itself. PO-CL involves an energy transfer to a fluorophore. This feature allows peroxyoxalate reaction to be applied to a wide variety of analytes. TCPO has one major disadvantage, it is very insoluble in water, which requires

the use of organic solvent such as acetonitrile, dioxane, tert-butanol, and ethyl acetate. [54]

Application of Peroxyoxalate Chemiluminescence

Over the years, there have been numerous studies on PO-CL that involve the hydrogen peroxide oxidation in the presence of a catalyst to analyze various trace amounts of fluorphores.

Sigvardson et al., [55] used PO-CL detection for many amino-substitution polycyclic aromatic hydrocarbon (amino-PAH) in high-performance liquid chromatography (HPLC). They observed the detection limits to be typically one to two orders of magnitude lower than those achieved in the same chromatographic system using fluorescence detection. The amino-PAH were selectively detected with high sensitivity. Sigvadson and his coworkers believed that the high selectivity of the amino-PAH over other fluorescent compounds arose from differences in the excitation step. The energy transfer from the key intermediate to an aromatic amine appeared to be very efficient. For most aromatic compounds with no functioned group, the ability to undergo charge transfer is most important in the excitation step. It also had been shown that a linear relationship existed between the log of the corrected CL response and the oxidation potential when electron transfer mechanism was Nernstian.

Arnous and his associates [56] developed a simple, rapid, sensitive, and enzyme-free analytical method for estimating the scavenging of hydrogen peroxide using 9, 10-diphenylanthracene as fluorophore. Aliquot of 1.8 mL of 9, 10-diphenylanthracene was mixed with 0.2 mL of imidazole solution (4.5 mM) and 0.025 mL of $H_2O_2$ (2.25 mM).

The entire sample was mixed with 0.2 mL of TCPO solution (0.45 mM) and 0.05 mL of test compound for about 5 s. The reactions were performed in organic solvents of acetonitrile and ethyl acetate (9:1). The CL signal was found to be linear for $H_2O_2$ in the concentration range of 9.0 to 72.0 μM. However, when the reaction was performed in acetonitrile only, no satisfactory result was obtained and it was highly unstable. Using a mixture of ethyl acetate and acetonitrile gave excellent linear calibration curve with 0.999 correlation coefficient.

Substitutions of the imidazole and other leaving groups have been tested for catalytic efficiency in PO-CL reaction using TCPO as reagent. Catalytic activity was discovered for 1,2,4-triazole, 1-methlimidazole, 2-methylimidazole, 4-methylimidazole, and 4,5-dichloroimidazoe. [56-57] Derivatives with constituent having only moderate influence on the electronegativitiy of the imidazole ring were capable of catalyzing PO-CL reaction. However, none of the compounds mentioned above was more efficient than imidazole. The spectrophotometric measurements of the catalytic breakdown of TCPO showed that the intermediate was formed with all catalysts, and the stable intermediate led to a reduced catalytic efficiency in the PO-CL reaction with TCPO. Evidently, the ability of the imidazole to catalyze the PO-CL reaction of TCPO is largely dependent on the ability to destabilize the TCPO reagent. This is most efficiently done by forming an intermediate that is less stable than TCPO, which is then more susceptible to nucleophlic attack by hydrogen peroxide. [56-58]

Advantages and Limitations of Luminescence Technique

Some of the advantages of molecular emission such as fluorescence, phosphorescence, and chemluminescence are their extreme sensitivity, good selectivity,

and wide linear range of analysis. Their extreme sensitivity is the most important aspect of luminescence techniques. The minimal detectable concentration for an assay ultimately depends on how sensitively light can be detected and on the quantum efficiency (Φ) of the fluorophor. This sensitivity is because fluorescence signal is directly proportional to the concentration of the substance; and the emitted radiation can be measured by a photomultiplier tube extremely senstitively. [39] Absorbance measurements can reliably determine concentrations only as low as several tenths of a micromolar. Fluorescence techniques can accurately measure concentrations one million times smaller, at pico and even femtomolar level. Quantities less than an attomole ($<10^{-18}$ mole) can be detected.

However, there has been a very little improvement in the selectivity of fluorescent measurements by instrumental means. These limitations include an inherent broad-band structure of fluorescenct spectra of many molecules at room temperature. This limits the ability of conventional measurement system to resolve the overlapping bands which are often observed in mixtures of fluorescent compounds. For some molecules, low temperature measurement helps to sharpen bands and improve spectral selectivity. [37-40]

The specificity of the BL and CL is possible by using luminescence as an indicator reaction coupled to the intermediates such as ATP, NADH, and $H_2O_2$ which are generated during enzymatic reactions. Because of the enzymatic process, BL reactions are very specific. However, CL reactions are not as specific. For instance, luminol will undergo CL reaction with various oxidants such as oxygen, peroxide, superoxide, and iodine, and the reaction is subject to interference by reducing agents. [41]

Since CL is an emission process as opposed to absorption, response is usually linearly proportional to the concentration from the minimal detectable up to the point where it is no longer possible to maintain an excess of other reactants relative to the analyte.

The distinct advantage of CL analysis is the absence of external light source, the emission can be measured against a completely dark background. The only background signal is that of the PMT's dark current. Therefore, light source warm-up drift and interference from light scattering are absent. In systems where red and near infrared light are observed in analytical detection, red sensitive PMT's dark current can be minimized by cooling; with blue light emission detection, cooling is not required. Thus methods are highly sensitive because of low light level monitored in the absence of noise. Detection limits is orders of magnitude lower than fluorescence methods. [39-40] The detection limits can be the range of femtomole. For instance, in liquid phase down to 1 fmole of the fluorophore may be detected using PO-CL system and 0.10 fmole peroxidese when using luminol. [39] However, the detection limits of the CL are determined by the reagents purity rather than by the transducer sensitivity. CL is $10^5$ times more sensitive than absorption spectrometry and at least $10^3$ times more sensitive than fluorimetry.

Flow Injection Analysis (FIA)

In the mid-70s, flow injection analysis (FIA) became a popular powerful analytical technique for analysis. It was introduced and developed by Ruzicka and Hansen [60] from Denmark and Stew from United States. It is based on the injection of a liquid sample into a moving, non-segmented continuous carrier stream of a suitable liquid. The injection sample forms a zone that is transported toward a detector that

records the absorbance, electrode potential, or other physical parameter as it changes due to the passage of the sample through the flow cell.

The flow injection analyzer consists of a pump that is used to propel the carrier stream through a narrow tube; an injection port that introduces reproducibly a well-defined volume of a sample solution into the carrier stream; and a micro-reactor in which the sample zone disperse and reacts with the component of the carrier stream forming a species that is sensed by a detector and recorded.

FIA [61] is based on the combination of three principles: sample injection, controlled dispersion of the injection sample zone, and reproducible timing of its movement from the injection point toward and into the detector. The chemical analyses are taking place while the sample material is dispersing within the reagent, that is, while the concentration gradient of the sample zone is being formed by the dispersion process. FIA also is potentially a useful technique for field operation due to its portability, flexibility, and low cost. Photomultiplier tubes (PMT) requiring a high voltage power supply have traditionally been used for detecting the CL emission. However, low voltage (12V), low power PMT and solid state detector like photodiodes array detector have also been used for monitoring total CL emission.

FIA has the advantages of very low sample reagent consumption. The small volume of the flow cell prevents excessive dilution. The signal is highly reproducible because the signal is acquired as a narrow peak and corresponds to the portion of analyte passing through the cell. FIA is now well established as an excellent technique for rapid, automated, quantitative analysis that can perform on-line chemical and physical sample treatment with flow through detection in an enclosed and continuous flow environment.

It is particularly well-suited to monitoring transient light emission from liquid phase CL reaction due to the rapid and reproducible mixing of sample and reagents in close proximity to the detector.

# CHAPTER 4

# METHODOLOGY AND EXPERIMENTAL PROCEDURE

Cholesterol determination is very important in clinical diagnosis because high levels of cholesterol are associated with the risk factor of heart disease, arteriosclerosis, and hypertension. [3, 4] As a result, intensive research efforts have been made to understand the properties of cholesterol and to find ways for lowering cholesterol levels. Normally, cholesterol is also partly present in an esterified form and the proportion [4] by weight of esterified to free cholesterol in the body is about 60:40. This means analytical determination of cholesterol in food products is important.

As shown before, various analytical methods have been used for the determination of free cholesterol. Most methods now involve enzymatic procedures because of their rapidity, selectivity, sensitivity, and greater accuracy. They are based on the hydrolysis of cholesterol ester to free cholesterol that is then oxidized in the presence of cholesterol oxidase (COD) to produce cholest -4-ene-3-one and hydrogen peroxide. After this, various methods can be devised to measure the amount of hydrogen peroxide.

## Proposed Research

The aim of this research project is to develop a flow injection analysis procedure based on the peroxyalate chemiluminescent detection, or FIA-POCL, of the hydrogen peroxide produced by enzymatic reaction of esterified and free cholesterol in the presence of cholesterol esterase and cholesterol oxidase to determine the total cholesterol level in foods. The proposed reaction scheme is shown Figure 8

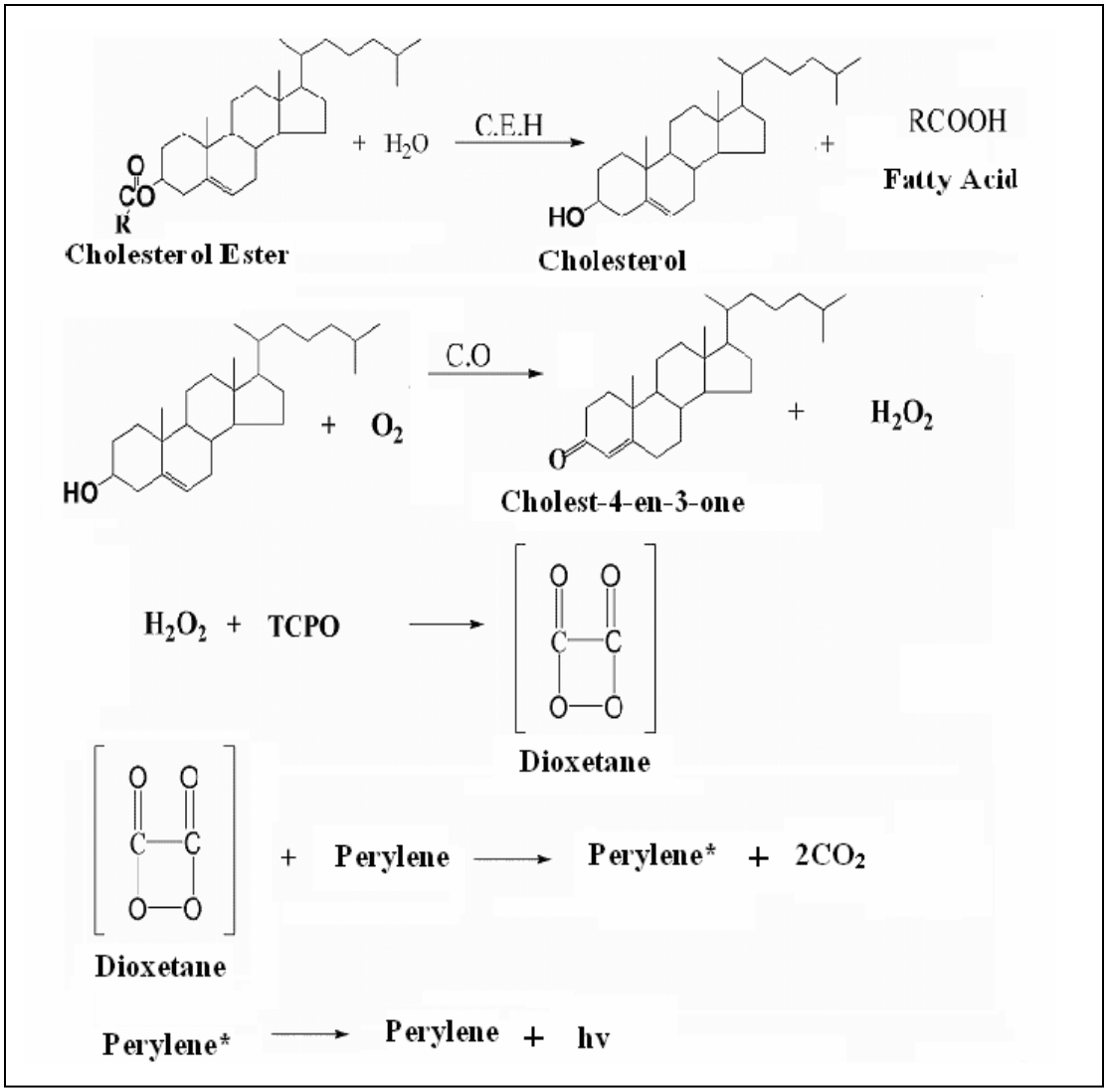



Figure 7. Reaction Scheme for FIA-POCL Analysis of Cholesterol based on Enzymatic Cholesterol esterase (CE) and cholesterol Oxidase (COX) and CL Procedures

Objectives of the Proposed Research

The objectives of the proposed research are as follow:

1. To develop a sensitive and economical method for the analysis of total cholesterol in foods.

2. To use enzymatic methods to confer selectivity and chemiluminescence detection to

enhance sensitivity of analysis.

3. To designed a flow injection system for rapid and reproducible measurements.
4. To evaluate the validity of the method, figures of merits such as precision, accuracy, and linearity will be assessed.
5. To apply the proposed procedure to the analysis of total cholesterol in commercial products. These samples including commercially available butter, cheeses, lard, chicken fat, and beef fat.

## Experimental Procedure

### Reagents

Cholesterol standard (200 mg/dL) in a buffered aqueous solution of cholesterol with stabilizers, surfactants, and preservative was obtained from Stanbio Laboratory (Fort Worth, TX).

Cholesterol oxidase, 250 U, cholesterol esterase 100 U, Sigma (St. Louis, MO).

Cholesterol (98%), Imidazole, 99 %, Aldrich Chemical Company (Milwaukee, WI)

Triton-X-100, Eastman Kodak Company (Rochester, NY).

Hydrogen peroxide 30%, ethanol 95%, methanol, ethanol, ethyl ether, petroleum ether, isoproprly alcohol, acetonitrile, potassium hydroxide, glacial acetic acid, concentrated sulfuric acid, acetic anhydride, di-sodium phosphate, potassium dihydrogen phosphate, methylene chloride, and sodium salt of cholic acid, all ACS reagent grade, Fisher Scientific (Fair Lawn, NJ) were used as received.

Bis (2,4,6 tricholorophenyl)oxalate (TCPO) was synthesized in the laboratory by the method of Mohan and Turro. [62]

Stock Solutions

The following stock solutions are prepared for long-term storage from which working solutions are made.

1. Cholesterol stock solution A (9.99 mg/mL). Prepared by dissolving 0.2489 g of 98 % cholesterol with 2-propanol in 25-mL volumetric flask.
2. Cholesterol oxidase (COX) (2.06 U/mL), dissolved 250 units of cholesterol oxidase with pH 7.0 phosphate buffer with 50 µL of Triton X-100 added, and the solution diluted to the mark in a 100-mL volumetric flask.
3. Cholesterol esterase (CE) (1 U/mL), dissolved 100 unit of cholesterol esterase with phosphate buffer with 50 µL of Triton X-100 added, and the solution diluted to the mark in a 100-mL volumetric flask.
4. Perylene ($1.86 \times 10^{-4}$ M), dissolved 0.0047 g in acetonitrile and diluted to 100 mL in a volumetric flask.
5. Imidazole solution (100 mg/mL): prepared by dissolving 1 g of imidazole in a 10-mL volumetric flask and diluted to the mark with pH 7.0 phosphate buffer.
6. Phosphate buffer, pH 7.0 (0.20 M), dissolved 0.9422 gram of disodium phosphate, and 1.57 gram of potassium di-hydrogen phosphate in 200 mL of deionized water.
7. Sodium of cholic acid (129.6 mg/mL), prepared by dissolving 1.296 g of sodium cholate salt in 10 mL of phosphate buffer. This solution was made fresh daily.

8. 30 % Hydrogen peroxide was used as provided and was stored in its original plastic bottle in the refrigerator.

Working Solutions

Cholesterol working solution, 0.9992 mg/mL was prepared from the stock solution B by dissolving 1.0 mL of stock standard cholesterol and diluted with 2-propanol to the mark in a 10-mL volumetric flask.

Hydrogen peroxide, 3%, was freshly prepared by diluting 1 mL of the 30 % $H_2O_2$ in deionized water in 10-mL volumetric flask.

TCPO, (1.20 mg/mL), was prepared by dissolving 0.120 g of TCPO in 100 mL of acetonitrile with sonication for 45 minutes. Any unused solution was stored in brown glass bottle in the refrigerator.

The color reagent for spectrophotometric measurements was prepared by dissolving 50 mg of 4-aminophenazone, 100 mg of phenol and 1 mg of peroxidase per 100 mL of 0.4 M sodium phosphate pH 7.0 buffer. It was stored in amber colored bottle before use at 4 $^{o}$C and prepared freshly every week. [64]

Instrumentation

The CL signals were monitored by an American Instrument Company microphotomer (Silver Spring, MD), with a Hamamatsu R928 photomultiplier tube mounted in front of the home made flow cell of about 90 µL in volume. The CL signals were recorded with the Model 680 Hewlett-Packard, Strip chart recorder. Cole Parmer Master Flex peristaltic pump (Barrington, IL) was used to pump reagent solution at 1 mL/min.

Rheodyne 7125 injector with 20-µL sample loop (Cotati, CA) was used to introduce the sample into the flowing stream.

Figure 9 is the schematic diagram of the FIA-POCL system for cholesterol determination. The TCPO / perylene solution is pumped at the rate of 1.0 mL/min by the Cole-Palmer Master Flex peristaltic pump. Aliquots of samples were injected into the Rheodyne 7125 injector 20-µL sample loop using a 100-µL syringe. The mixtures pass through the home-made flow cell where the photomultiplier tube measured the CL intensity emitted. The signals were processed by the American Instruments micro-photometer and also recoded on the Hewlett Packard strip chart recorder.

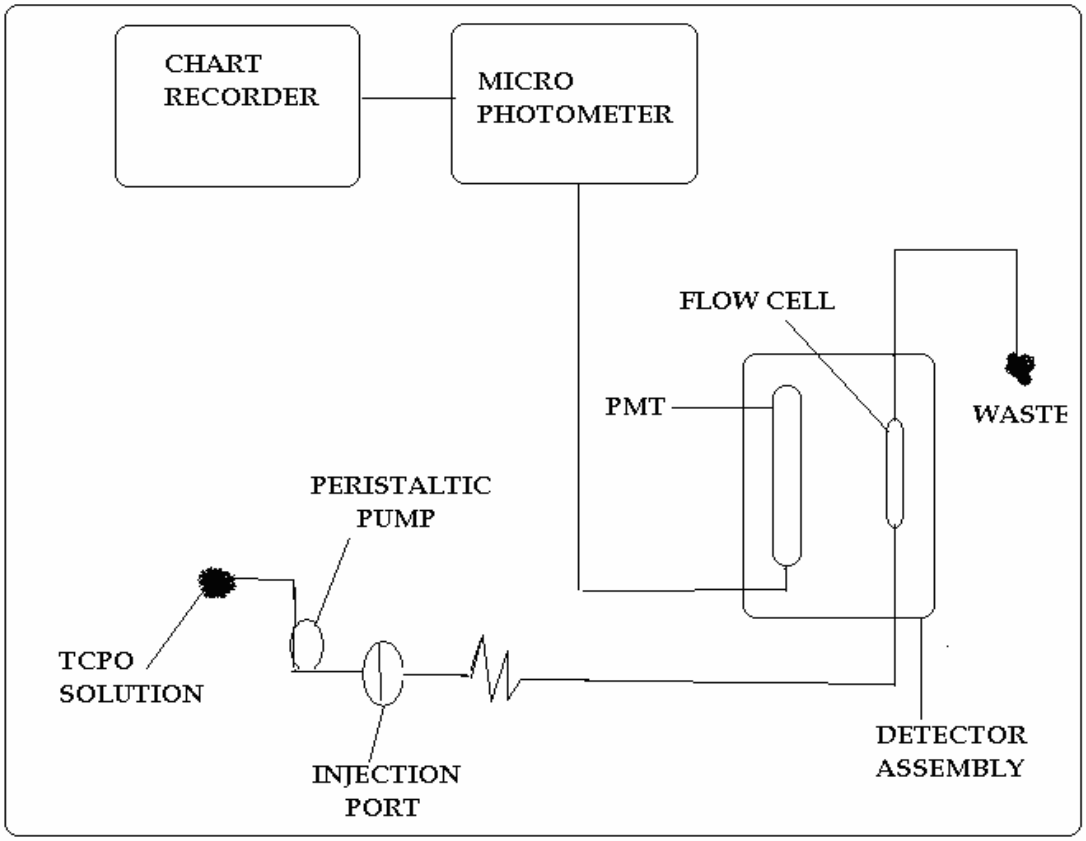



Figure 8. Instrumentation of FIA-POCL system for CL intensity measurements

Preparation of Cholesterol Calibration Standard Solutions

From the cholesterol standard working solution (9.9992 mg/mL), a more dilute working solution of 0.9992 mg/mL was prepared by pipetting 1-mL of 9.9992 mg/mL solution into a 10-mL volumetric flask and diluting to the mark with 2-propanol. Calibration working solutions were then prepared by pipetting 50, 100, 200, 400, and 600 μL into 5-mL volumetric flasks. Into each flask 250 μL cholesterol esterase, 500 μL cholesterol oxidase and 500 μL sodium cholate were added. After mixing, the samples were incubated for 30 min at 37–40 $^{o}$C in a water bath. Addition of 100 μL of imadazole solution followed the incubation period and the mixtures were finally diluted to the mark with pH 7.0 of phosphate buffer. These samples were then ready for CL measurements.

Preparation of Solutions for Optimization of Imidazole

To determine the optimum amount of imidazole used, the following solutions were made. Into each 10- mL volumetric flask 100 μL of cholesterol working solution (0.9992 mg/mL), 500 μL cholesterol oxidase (2.06 U/mL), and 250 μL cholesterol eastrase (1.0 U/mL) were mixed in the flask. The mixtures were incubated for 30 minutes in a water bath of 37-40 $^{0}$C. The addition of imidazole was made right after the incubation period. The different amounts of imidazole added were 1, 5, 40, 100, 250, and 500 μL respectively. Finally, each flasks were diluted to the mark with pH 7.0 phosphate buffers and were ready for CL measurement.

Preparation of Hydrogen Peroxide Solutions for Linearity Studies

To determine the linearity of CL with hydrogen peroxide, five different concentrations of hydrogen peroxide were prepared in 10-mL volumetric flasks. Into five different flasks 50, 100, 150, 200, and 250 μL of 3% hydrogen peroxide working solution were pipeted, respectively. To all the flasks 100 μL imidazole solution was added and finally the solutions were diluted to the mark with phosphate buffer. These samples were then ready for CL measurement.

Preparation of Solutions for Reproducibility Studies

The solutions for these experiments were prepared in two ways. The first 8 solutions were made using the standard working cholesterol solution (0.9992 mg/mL). Into each 5-mL volumetric flasks, 100-μL aliquots of working cholesterol were pipeted. Then into all the flasks, 250 μL cholesterol esterase, 500 μL cholesterol oxidase, and 500 μL of sodium cholate were added. The mixtures were incubated at 37-40 $^{o}$C in a water bath for 30 min. After incubation, 100 μL of imidazole solution were added as a catalyst and the mixtures diluted to the mark with pH 7 phosphate buffer. The samples were now ready for CL measurements.

The second set of experiment was performed using a butter sample to determine the reproducibility of the proposed procedure. Eight aliquots of 100 μL of working butter sample were pipeted into 5-mL volumetric flasks. Then 250 μL cholesterol esterase, 500 μL cholesterol oxidase, and 500 μL of sodium cholate were added. The mixtures were incubated at a 37-40 $^{o}$C in a water bath for 30 minutes. After incubation 100 μL of imidazole solution were added to the mixture and diluted to the mark with the phosphate

buffer. The samples were then ready for CL measurement.

Preparation of Solutions for the Recovery Studies

To determine the accuracy of the proposed method, recovery studies were performed. A calibration curve and a series of spiked samples were prepared. Five standard cholesterol working solution ranging from 0.0100 to 0.1199 mg/mL were prepared as described for use in constructing a calibration curve. Aliquots of 500 μL standard commercial cholesterol (2 mg/mL) were pipetted into nine 5-ml volumetric flasks. Into all flasks, 250 μL cholesterol esterase, 500 μL cholesterol oxidase, and 500 μL sodium cholate were added. The mixtures were then divided into three group triplicates. For the first triplicate, no standard cholesterol working solution was added, while 100 μL and 200 μL of standard cholesterol working solution were added, respectively, to the second and third triplicates. Then the mixtures were incubated for 30 minutes at 37-40 $^{o}$C. Finally, 100 μL of imidazole solution were added to the mixtures and diluted to the mark with pH 7 phosphate buffer. The samples were then ready for CL measurement.

Another set of experiments was conducted to determine the recovery of the proposed FIA-POCL method using the butter sample (2 mg/mL). The same procedure was used to prepare the butter samples for the recovery studies except that the butter samples were spiked with 200 μL and 400 μL of standard cholesterol working solution (0.9992 mg/mL) for the second and third triplicates respectively.

Preparation of Consumer Samples for Analysis

Diverse samples were chosen to be determined for their cholesterol content using the proposed FIA-POCL method. An extraction procedure to extract the free cholesterol was tried at first. Cholesterol was extracted with 50 mL of a freshly prepared methanolic 2 M potassium hydroxide for two hours. The warmed mixtures were transferred into a 200-mL separating funnel. After the mixture had cooled down to room temperature, 100 mL of 1:1 (v/v) petroleum ether and ether were added and mixed very well. The organic phase was allowed to separate for 30 minutes. The clearly separated lowered organic phase was drained off and the extraction process was repeated. The organic phase solvent was evaporated on a rotary evaporator and the resulting solution was kept in the refrigerator. The residue obtained was redissolved by the same procedure as was used to prepare the standard cholesterol solution. The sample was made in methylene chloride. However, the extraction process did not seem to work well and so the procedure was abandoned. It was too time-consuming, contamination and loss of sample seemed to be major problems. Instead, for all subsequent determinations, the samples were prepared without the extraction procedure and better results were obtained. Samples such as beef fat, chicken fat, butter, and a number of cheeses such as Italian and sharp cheddar were used. Approximately 0.25 gram of samples were dissolved in 25 mL of 2-propanol (10 mg/mL). Further dilution had to be made because 10 mg/mL of samples caused precipitation to occur when other reagents were added. Therefore, 1-mL of each sample was diluted into 10-mL with 2-propanol to give a final working solution of 1-mg/mL concentration. Then for experiments, 500 μL and 1000 μL of the diluted food samples were pipetted into 5-mL volumetric flasks, and then 250 μL cholesterol esterase, 500 μL cholesterol oxidase, and 500 μL sodium cholate were added to them. The solutions were

incubated for 30 minutes at 37-40 $^{o}$C and 100 μL of imidazole solution added after incubation. Finally, the mixtures were diluted to the mark with the pH 7 phosphate buffer. The samples were then ready for CL measurement. The same procedure was used for all types of samples. However, for beef fat, chicken fat, and lard, 1.0 g of the stock sample was dissolved in 25 mL of 2-propanol from which the working solutions were made the same way as other samples. Theses samples had to be prepared daily and stored in the refrigerator.

# CHAPTER 5

# RESULTS AND DISCUSSION

The results of all the experiments on optimization and validation of the proposed analytical method were tabulated and discussed in this chapter. These experiments were conducted to evaluate the linear dynamic range, detection limits, precision, and accuracy of the proposed methods.

## Analytical Figures of Merit

The performance and reliability of the proposed methods were assessed by determining the important analytical figures of merit: linearity range, reproducibility, or precision (as relative standard deviation, RSD) and the recovery or accuracy of the methods. The proposed procedure was also compared with a well established method to assess its validity.

## Effect of Surfactants

Various surfactants have been used to enhance CL signal. The effect of several surfactants on CL signal was examined in this study. The results indicated that anionic surfactants such as sodium dodecylsulfate (SDS) had a slight quenching effect on the proposed procedure. The measured CL signal was very weak when SDS was added to the solution versus the non ionic surfactant Triton X-100 which improved the CL signal. Triton X-100 is often used as a lipid solubilizing agent. The amounts of the surfactants added must be adjusted carefully because the solubility of the lipid and the activity of enzyme were affected by the amount of the surfactant present. It was found that 100 μL of Triton X-100 must be added to the incubation mixtures of cholesterol oxidase and cholesterol esterase and cholesterol sample to improve the solubility of cholesterol.

Sodium cholate is also another important surfactant used in this experiment. Based on the results obtained, adding 500 μL sodium cholate (1.29 mg/mL) to the incubation mixtures improved the CL signal. Being a lipid solubilizer, the surfactants help to prevent cholesterol from precipitating out in the presence of aqueous reagents.

Optimization of Imidazole

Imidazole, a base, has been recognized as an excellent catalyst for TCPO and hydrogen peroxide reaction. For the FIA- POCL reaction the amount of imidazole used was varied to determine the optimum concentration of imidazole to obtain the highest CL signal.

Several 100-μL aliquots of standard cholesterol working solution, 250 μL cholesterol esterase, 500 μL cholesterol oxidase, and 500 μL of sodium cholate were pipetted into different 10-mL volumetric flasks. The solutions were incubated for 30 minutes in a 37 – 40 $^{0}$C water bath. After incubation 1, 5, 10, 40, 100, 250, and 500 μL of imidazole stock solution (100 mg/mL) were added into the volumetric flasks containing the cholesterol mixtures and diluted to the mark with pH 7.0 phosphate buffer. The results of the experiment are tabulated in Table 3. The CL signals were the average of three injections. These results were also plotted in Figure 10.

Table 3. Results of Optimization of Imidazole Concentration for FIA-POCL Reaction

| Volume (μL) of Stock imidazole Solution | 1.0 | 5.0 | 10.0 | 40.0 | 100.0 | 250.0 | 500.0 |
|---|---|---|---|---|---|---|---|
| CL Intensity | 0.011 | 0.028 | 0.051 | 0.094 | 0.289 | 0.420 | 0.211 |

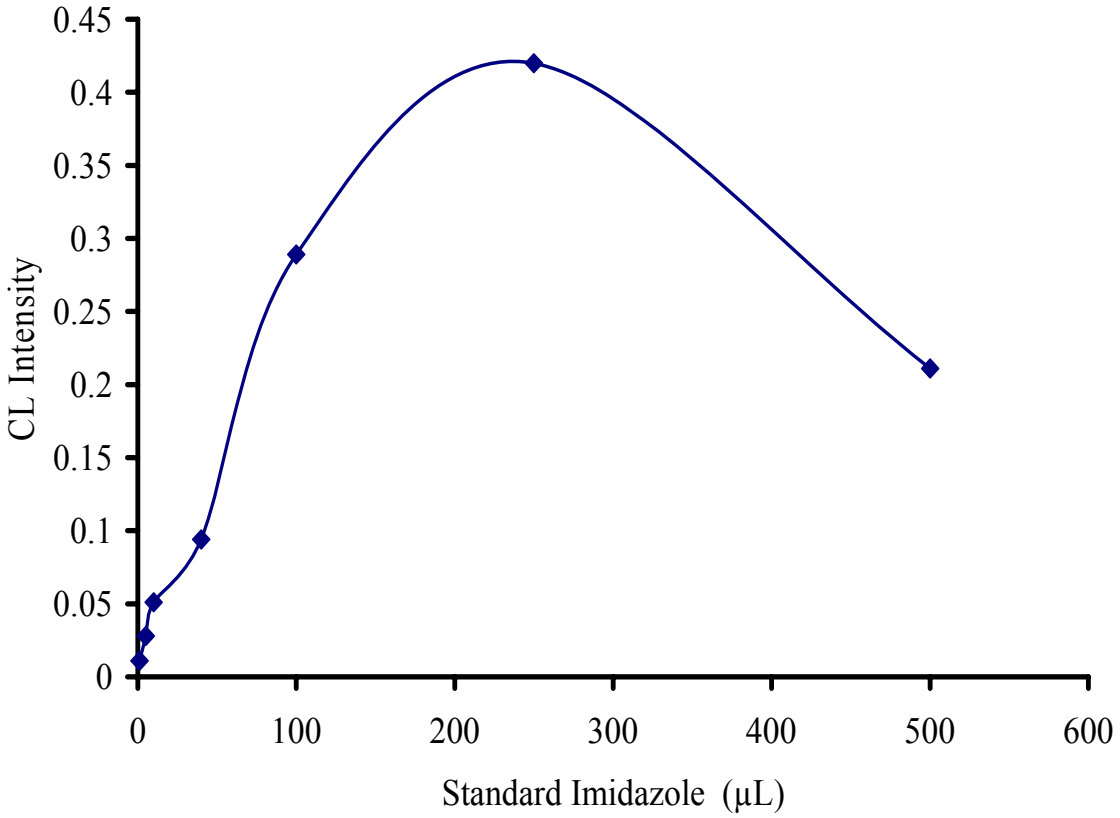



Figure 9. Plot of the results of the optimization experiment of volume of imidazole Stock solution to be used as catalyst for FIA-POCL reaction The concentration of the stock imidazole solution was 100 mg/mL.

The results showed that CL intensity increased with the amount of imidazole used up to a certain value. The maximum CL intensity occurred when 250 µL of stock imidazole solution was added. Based on the results the optimum amount of imidazole for the proposed method was chosen to be 200 µL of stock imidazole solution even though the maximum signal was at the 200-250 µL range. The 200 µL of imidazole used correspond to a concentration of 0.50 mg/mL in the mixture. As one increased the concentration of imidazole, the CL reaction may proceed at such a rate that the CL signal may have vanished by the time the reagents reach the flow cell for measurement.

Moreover, it was observed that excess amount of imidazole could lead to the precipitation of cholesterol.

Linearity Studies of CL signal with Concentration of Hydrogen Peroxide

It is well known that hydrogen peroxide is enzymatically generated by reactions of various oxidases. Sensitive and selective methods for hydrogen peroxide determination have been a requisite in biological or biomedical chemistry. A wide variety of CL reactions exists and many of these have been employed for the determination of hydrogen peroxide. Hydrogen peroxide acts as an oxidant in the reaction with TCPO and the oxidation provides the excitation energy for CL.

The CL signal was measured by varying the concentration of $H_2O_2$ reacting with fixed amounts of TCPO and imidazole at a given flow rate. The results of the linearity study of CL with hydrogen peroxide concentration is tabulated in Table 4 and also plotted in Figure 10.

Table 4. Result of Linearity Study of CL Intensity with Varying Hydrogen Peroxide Concentrations

| $H_2O_2$ (M) | 0.0009 | 0.0018 | 0.0026 | 0.0035 | 0.0044 |
|---|---|---|---|---|---|
| CL Intensity | 32.0 | 47.0 | 60.3 | 72.0 | 80.0 |

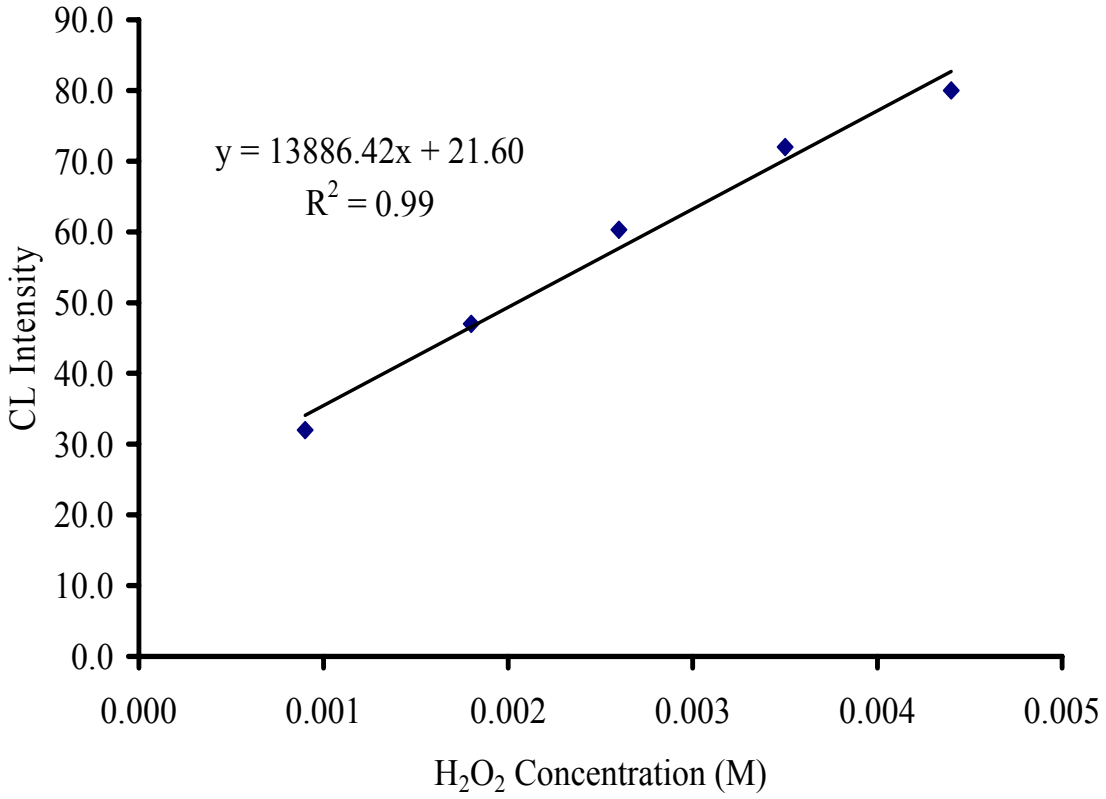



Figure 10. Plot of CL signal with varying hydrogen peroxide concentrations. The CL Intensity is given in arbitrary units

The Calibration curve was linear for hydrogen peroxide concentration from $9.0 \times 10^{-4}$ M to $4.4 \times 10^{-3}$ M. The equation of the regression line was Y = 13886.42x + 21.60 with correlation coefficient of 0.99. These result showed that CL intensity increases linearly as we increase the concentration of hydrogen peroxide. The data indicated that the linear dynamic range was wider than the concentration range of hydrogen peroxide used in the study.

Reproducibility Studies

For a proposed method to be useful and applicable for analysis, the analytical method has to be reproducible. Additionally, the proposed procedure has to have about

the same precision as the established reference method. To evaluate the reproducibility of the proposed procedure, two sets of experiments were performed. One set was conducted using the standard cholesterol solutions prepared in our laboratory. The second set was conducted on the commercial butter samples to determine the reproducibility of the measurement and the variability of the commercial item.

For the first set, eight 100-μL aliquots of standard cholesterol working solution of concentration 0.9992 mg/mL, 250 μL cholesterol esterase, 500 μL cholesterol oxidase, and 500 μL sodium cholale (129.6 mg/mL) were pipetted into 5-mL volumetric flasks. The mixtures were incubated for 30 minutes at 37-40 $^{o}$C to produce the hydrogen peroxide. Then 100 μL of imidazole were added to the mixtures and the solutions finally diluted to the mark with pH 7 phosphate buffer. The final concentration of cholesterol in all the solutions was 0.01998 mg/mL. CL measurements were then made by injecting the resulting mixture into the 20-μL loop of the FIA system containing a flowing stream of 1.20 mg/mL TCPO with 3μL/mL of perylene in acetonitrile. The results of the experiment are tabulated in Table 5.

Table 5. Results of the Reproducibility Study of the Proposed FIA-POCL Method Using Standard Cholesterol Solution. Final Concentration of 0.020 mg/mL

| Trial | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| CL Intensity | 0.330 | 0.343 | 0.336 | 0.329 | 0.329 | 0.331 | 0.313 | 0.333 |
| Average CL Intensity | | | 0.330, (0.024 mg/mL, +2.0%) | | | | | |
| Relative Standard Deviation | | | 2.57 % | | | | | |

From the data obtained the mean of eight CL measurements was 0.330, with a relative standard deviation of 2.57 %. The results were within experimental error and demonstrated that the precision of the proposed analytical procedure was satisfactory.

The second study was conducted using a commercial butter bought from a local store. The butter samples were prepared without any extraction procedure. Butter samples weighing 0.25 g each were dissolved in 25 mL of 2-propanol giving a final concentration of 10 mg butter/mL. Then 1-mL aliquots of the dissolved butter sample were pipetted into eight 5-mL volumetric flasks. Then, as described before, the same incubation procedure and FIA-POCL measurement was carried out. The final concentration of the mixtures was 2 mg butter/mL solution. The experimental results are tabulated in Table 6.

Table 6. Results of the Reproducibility Study of the Proposed FIA-POCL Method Using Butter Sample

| Trial | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| CL Intensity | 0.310 | 0.316 | 0.322 | 0.320 | 0.317 | 0.333 | 0.316 | 0.322 |
| Average CL Intensity | | | 0.320 | | | | | |
| Relative Standard Deviation | | | 2.11 % | | | | | |

For the data obtained, the mean CL intensity for the eight aliquots of the butter sample was 0.320 with a relative standard deviation of 2.11 %. These results showed that the proposed procedure was reproducible for both pure standard cholesterol solution and the cholesterol present in samples like butter.

Linearity Studies of the Proposed Method on Cholesterol Samples

To determine the linearity of our proposed procedure for cholesterol determination, two different experiments were done. The first one was described earlier indicating CL signals was linear with hydrogen peroxide concentration. The second one to be described below is to show the linearity of CL intensity with standard cholesterol concentration. For this, a series of standard cholesterol solutions were pipetted into 5-mL of volumetric flasks. As described before, the same incubation procedure and FIA-POCL measurement were carried out. The results of the study are tabulated in Table 7 and the plot shown in Figure 12. Each datum is an average of triplicate injections.

Table 7. Results of the Linearity Study of the Proposed FIA-POCL Method on CL Intensity and Cholesterol Concentrations

| Cholesterol Concentration (mg/mL) | 0.01 | 0.02 | 0.04 | 0.08 | 0.12 |
|---|---|---|---|---|---|
| CL Intensity | 0.128 | 0.281 | 0.540 | 1.20 | 1.80 |

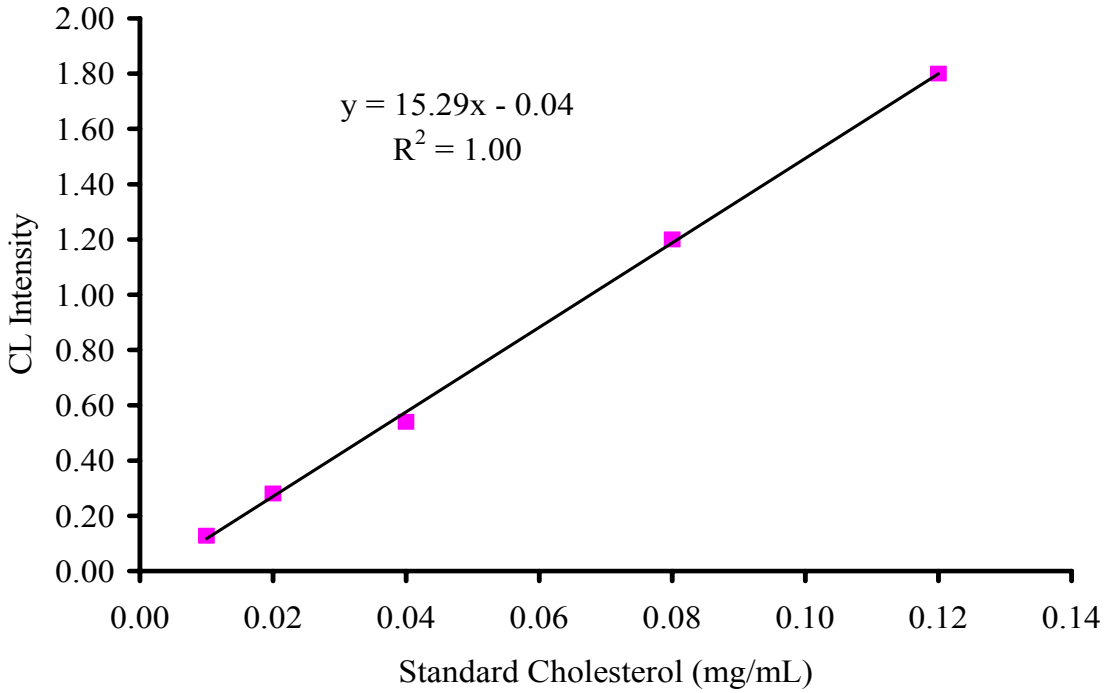



Figure 11. Plot of CL intensity versus Standard Cholesterol Concentration of 0.010 to 0.119 mg/mL The correlation coefficient for the line was 1.00

The plot shows a linear range of 10-120 µg/mL with a correlation coefficient of 1.00. The regression line is given by y = 15.29x - 0.04. These results indicate that there is a linear relationship between the CL signal and the standard cholesterol concentration for the proposed method. The upper range of the cholesterol concentration is limited by the solubility of cholesterol in the FIA system. The lower limit of quantifiable detection is about 5 µg/mL of cholesterol.

Recovery Studies

To determine the accuracy the proposed procedure, recovery studies were conducted. In these studies, two experiments were done to confirm the results. In each experiment, three sets of triplicate samples containing fixed amounts of commercial cholesterol standard samples, or purchased butter, were pipetted into each set of nine different 5-mL volumetric flasks. Then three different triplicates, but known amounts of

cholesterol working solution (0.9992 mg/mL) were added to them. To each of the spiked samples, 250 μL cholesterol esterase, 500 μL cholesterol oxidase, and 500 μL sodium cholate were added as described before, incubated, treated, and CL measurements made to determine the amount of cholesterol added to the samples. The recoveries of the spiked amount of cholesterol added to the samples were calculated based on the following equation:

$$\text{Recovery (\%) of cholesterol} = \frac{\text{Cholesterol}_{\text{commercial standard}} - \text{Cholesterol}_{\text{added standard sol.}}}{\text{Cholesterol}_{\text{added standard sol.}}} \text{ X } \mathbf{100\%}$$

The results of the recovery studies of the proposed FIA-POCL method first using the commercial standard cholesterol samples are tabulated in Table 8, and that using the purchased store butter are shown in Table 9.

Table 8. Results of the Recovery Study of the Proposed FIA-POCL Method on Commercial Standard Cholesterol Samples (0.10 mg)

| Sample | CL Signal | Cholesterol Standard added (mg ) | Total cholesterol Found (mg ) | % Recovery |
|---|---|---|---|---|
| 1 | 3.20 | 0.000 | | |
| 2 | 3.20 | 0.000 | | |
| 3 | 3.20 | 0.000 | | |
| 4 | 5.93 | 0.100 | 0.101 | 100.9 |
| 5 | 6.00 | 0.100 | 0.103 | 103.3 |
| 6 | 5.93 | 0.100 | 0.101 | 100.9 |
| 7 | 8.80 | 0.200 | 0.195 | 97.5 |
| 8 | 8.80 | 0.200 | 0.195 | 97.5 |
| 9 | 8.81 | 0.200 | 0.195 | 97.5 |
| Mean Recovery | | 99.6 % | | |
| Relative Standard Deviation | | 2.44 % | | |

The result of these studies showed that the recoveries of added pure standard cholesterol ranged from 97.5 to 103.3 % in commercial standard cholesterol samples, while that in butter were found to be in the range from 101.5 to 108.0 %. It is interesting to note that for the butter samples the recoveries were all over 100% and that the precision for butter was better (RSD of 1.5%) than using the commercial standard cholesterol samples (RSD of 2.44%). However, the results indicate that the proposed method has acceptable precision and recovery, and that the type of sample does not significantly affect the recovery.

Table 9. Results of the Recovery Study of the Proposed FIA-POCL Method on Butter Samples (0.50 mg/mL)

| Sample | CL Signal | Standard Cholesterol Added (mg ) | Total Cholesterol Found (mg ) | % Recovery |
|---|---|---|---|---|
| 1 | 1.00 | 0.000 | | |
| 2 | 1.00 | 0.000 | | |
| 3 | 1.00 | 0.000 | | |
| 4 | 6.20 | 0.200 | 0.206 | 103.0 |
| 5 | 6.13 | 0.200 | 0.203 | 101.5 |
| 6 | 6.33 | 0.200 | 0.211 | 105.5 |
| 7 | 12.00 | 0.400 | 0.431 | 108.0 |
| 8 | 11.63 | 0.400 | 0.417 | 104.3 |
| 9 | 11.67 | 0.400 | 0.418 | 104.5 |
| Mean Recovery | | 104.5% | | |
| Relative Standard Deviation | | 1.5% | | |

Analytical Applications

The proposed CL method was applied to the determination of cholesterol present in different food products with high level of cholesterol such as cheeses, butter, lard and beef and chicken fat. These samples were prepared as described in the previous chapter and no extraction was used. Also for comparison purposes, a well established enzymatic colorimetric method reported by Malike and Pundir [62] was also performed in parallel with the proposed FIA-POCL method for the determination of cholesterol in most of the same food samples. The reported method involved an enzymatic reaction step that was followed by a reaction to produce a colored product whose absorption was measured, instead of measuring the CL signal after the enzymatic reaction.

For the colorimetric procedure, the food samples and standard cholesterol solutions of various amounts were mixed with, as described before, 250 μL of cholesterol esterase, 500 μL of cholesterol oxidase, and 500 μL of sodium cholate in different volumetric flasks. However, the mixtures were incubated for only 5 minutes at 37 – 40$^{o}$C. Then 1.0 mL of color reagent was added. The color reagent was prepared by mixing 50 mg of 4-aminophenazone, 100 mg of phenol, and 1 mg of peroxidase enzyme per 100 mL of phosphate buffer. The mixtures were further incubated for 20 minutes to generate a highly colored quinoneimine dye whose absorbance was measured at 520 nm.

The sample stock solutions were the same. For the colorimetric method reaction, 500 μL and 1000 μL aliquots of the working solution of food samples were also used. Into 5-mL volumetric flasks, 500 μL cholesterol oxidase, 250 μL cholesterol esterase, and 500 μL sodium cholate salt were added to the food samples, mixed, and incubated for five minutes. Then 1.0 mL of the color reagent was added to each flask, diluted to the

mark with phosphate buffer, and further incubated for 10 minutes for color production. Finally the absorbances of the solutions were measured at 520 nm.

Table 10 shows the collected data for the construction of the calibration curve for the enzymatic colorimetric determination of cholesterol in food products. The results were obtained from the mean of triplicate injections. The regression line has the equation $y = 1.93x + 0.001$ with a correlation coefficient of 0.997 and the plot is shown in Figure 14. The regression equation is then used to determine the amounts of cholesterol in various samples as shown in Tables 11, 12, and 13.

Table 10. Absorbances of Standard Cholesterol Solutions for the Calibration Curve of Cholesterol Determination Using the Enzymatic Colorimetric Method

| Cholesterol Standard (mg) | 0.010 | 0.050 | 0.100 | 0.150 |
|---|---|---|---|---|
| Absorbance | 0.016 | 0.099 | 0.202 | 0.284 |

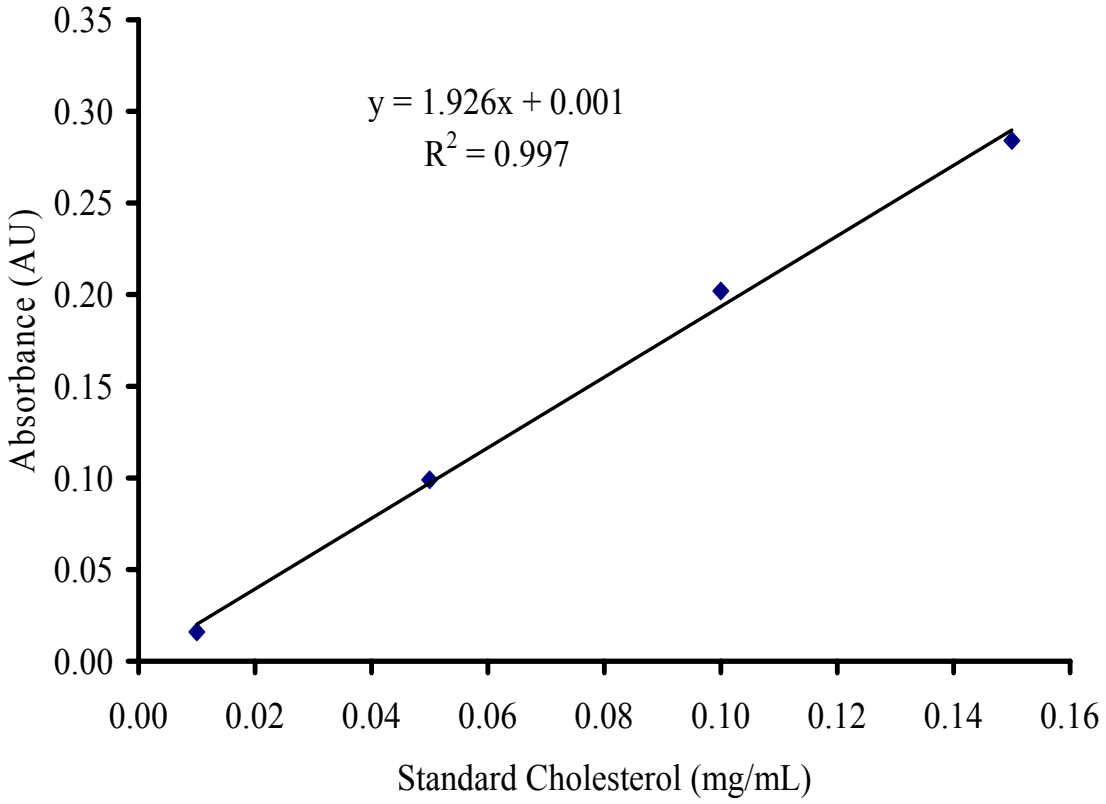



Figure 12. Plot of Absorbance Versus Standard Cholesterol Concentration (mg/mL) for the Enzymatic Colorimetric Method

Table 11. Results of the Enzymatic Colorimetric Determination of Cholesterol For Commercial Cholesterol Standard and Butter

| | Absorbance (AU) | Cholesterol Found (mg) | Cholesterol Found (mg/g) |
|---|---|---|---|
| Commercial Cholesterol Standard (2.0 mg/mL) | | | |
| 10 µL (0.02 mg) | 0.037 | 0.017 | (-15 %) |
| | 0.037 | 0.017 | (-15%) |
| | 0.039 | 0.018 | (-10%) |
| Butter (1.0 mg/mL) | | | |
| 200 µL (0.20 mg) | 0.037 | 0.017 | 8.5 |
| | 0.036 | 0.016 | 8.0 |
| | 0.039 | 0.018 | 9.0 |
| 250 µL (0.25 mg) | 0.042 | 0.019 | 7.6 |
| | 0.040 | 0.018 | 7.2 |
| | 0.041 | 0.018 | 7.2 |
| Mean (mg/g) = 8.11 Relative Standard Deviation = 8.53 % | | | |

Table 12. Results of Enzymatic Colorimetric Determination of Cholesterol in Butter

| Volume of Butter Sample (0.50 mg/mL) | Absorbance (AU) | Cholesterol Found (mg) | Cholesterol Found (mg/g) |
|---|---|---|---|
| 500 | 0.106 | 0.055 | 11.0 |
| | 0.108 | 0.056 | 11.2 |
| | 0.106 | 0.055 | 11.0 |
| 1000 | 0.21 | 0.108 | 10.8 |
| | 0.211 | 0.109 | 11.0 |
| | 0.212 | 0.109 | 10.9 |
| Mean (mg/g butter) = 11.0 Relative Standard Deviation = 0.90% | | | |

Table 13. Results of Enzymatic Colorimetric Determination of Cholesterol in Italian and Cheddar Cheese

| Cheese (µL) | Absorbance (AU) | Cholesterol Found (mg) | Cholesterol Found (mg/g) |
|---|---|---|---|
| Italian Cheese | | | |
| 500 | 0.092 | 0.047 | 9.4 |
| | 0.093 | 0.048 | 9.6 |
| | 0.095 | 0.049 | 9.8 |
| 1000 | 0.159 | 0.082 | 8.2 |
| | 0.161 | 0.083 | 8.3 |
| Mean = 9.1 mg/g cheese Relative Standard Deviation = 8.3% | | | |
| Cheddar Cheese | | | |
| 500 | 0.09 | 0.046 | 9.2 |
| | 0.086 | 0.044 | 8.8 |
| | 0.088 | 0.045 | 9.0 |
| 1000 | 0.148 | 0.076 | 7.6 |
| | 0.155 | 0.080 | 8.0 |
| Mean = 8.5 mg/g cheese Relative Standard Deviation = 8.1% | | | |

Tables 11, 12, and 13 show that the amounts of cholesterol obtained in the different types of samples using the enzymatic colorimetric methodology were quite consistent. Also, for the commercial cholesterol standard sample, the results are reported slightly differently in that the expected values were known. So the last column for it reports the % error of the value obtained relative to the expected value. Also in Table 11, the results for the same butter sample taken in different amounts for analysis gave notable different results with the larger volume samples gave lower values. Although within the same set of volumes, the results agreed well with one another. This could be due to pipeting error as the micro-pipeter had been found to suck up somewhat different

volumes unexpectedly. In Table 12, using a different butter sample, 500 μL butter sample gave a result of 11.1 mg cholesterol /g butter while 1000 μL gave about 10.9 mg/g. The results are now more consistent as can be seen with the 0.90% RSD.

The same procedure was performed for the cheeses samples whose results were tabulated in Table 13. The results of using 500 μL of the cheese sample give the mean amount of 9.6 mg cholesterol/g cheese. However the 1000 μL solutions gave only the mean of 8.3 mg/g in Italian cheese. Cheddar cheese also showed similar discrepancy. For the 500 μL the mean results were 9.0 mg cholesterol /g cheese, but for the 1000 μL solutions a lower cholesterol amount of 7.8 mg cholesterol/g cheese were obtained. No good explanation for these discrepancies exists other than to suggest that the pipets were not in calibration. In the future this needs to be carefully taken into consideration.

After the food samples cholesterol content were determined by the colorimetric method, the samples were subjected to determination by the proposed FIA-POCL method. The results obtained using the colorimetric and the proposed FIA-POCL methods have shown fairly similar results and had satisfactory agreement with each other. The results for the same butter and cheese samples using the proposed FIA-POCL method are tabulated in Tables 14 and 15. The values given are the mean of triplicate injections.

A calibration curve was first prepared using the standard cholesterol working solution with cholesterol standards of concentration of 0.02, 0.05, 0.08, and 0.120 mg/mL were obtained. The calibration curve shows a straight regression line with the equation of $y = 14.538x - 0.0855$ and correlation coefficient of 0.9929.

Table 14. Results of the Proposed FIA-POCL Method Determination of Cholesterol in Butter

| Butter (µL) | CL Signal | Cholesterol Found (mg/mL) | Cholesterol Found (mg) | Cholesterol Found (mg/g) |
|---|---|---|---|---|
| 500 | 0.10 | 0.013 | 0.0638 | 12.76 |
| 500 | 0.10 | 0.013 | 0.0638 | 12.77 |
| 1000 | 0.21 | 0.020 | 0.0999 | 9.99 |
| 1000 | 0.21 | 0.020 | 0.1006 | 10.10 |
| Mean (mg/g) = 11.4 Relative Standard Deviation = 13.9% | | | | |

Tables 14 and 15 are the results of the determination of cholesterol using the proposed FIA-POCL method. The results are quite similar to that of the colorimetric procedure. The smaller volume solutions of the samples gave higher cholesterol contents than using greater volumes of the prepared sample. This again points to the faulty pipetting volumes. However, in general it seems the proposed method gave a larger cholesterol contents in these food items than the colorimetric method. For instance the mean for the butter sample obtained by the proposed FIA-POCL method was 11.4 mg cholesterol/g sample while the colorimetric method gave 11.0 mg cholesterol/g sample. The variation for the cheeses were greater with the proposed method gave consistently higher values.

Table 15. Results of the Proposed FIA-POCL Method Determination of Cholesterol in Cheeses

| Cheese Type (μL sample) | CL Intensity | Cholesterol Found (mg/mL) | Cholesterol Found (mg) | Cholesterol Found (mg/g) |
|---|---|---|---|---|
| Italian | | | | |
| 500 | 0.120 | 0.0141 | 0.0707 | 14.14 |
| 500 | 0.139 | 0.0154 | 0.0772 | 15.44 |
| 1000 | 0.273 | 0.0247 | 0.1233 | 12.33 |
| 1000 | 0.280 | 0.0251 | 0.257 | 12.57 |
| | | Mean (RSD) | 13.62 (10.68%) | |
| Cheddar | | | | |
| 500 | 0.120 | 0.0141 | 0.0707 | 14.13 |
| 500 | 0.120 | 0.0141 | 0.0707 | 14.13 |
| 1000 | 0.263 | 0.239 | 0.1199 | 11.99 |
| 1000 | 0.280 | 0.0251 | 0.1257 | 12.57 |
| | | Mean (RSD) | 13.21 (8.32%) | |
| Harvati | | | | |
| 500 | 0.130 | 0.0148 | 0.0741 | 14.82 |
| 500 | 0.130 | 0.0148 | 0.0741 | 14.82 |
| 1000 | 0.273 | 0.0246 | 0.1233 | 12.33 |
| 1000 | 0.270 | 0.0244 | 0.1233 | 12.23 |
| | | Mean (RSD) | 13.55 (10.85%) | |

The proposed FIA-POCL method was also extended to determine the cholesterol contents in lard, beef, and chicken fat. The results of the determination are tabulated in

Table 16. The values obtained for all three kinds of sample were quite consistent in range which is quite reassuring.

Table 16. Results of the Proposed FIA-POCL Method Determination of Cholesterol in Beef and Chicken Fat and Lard

| | CL Intensity | Cholesterol Found (mg) | Cholesterol Found (mg/g fat) |
|---|---|---|---|
| Beef fat in (μL) | | | |
| 500 | 0.107 | 0.062 | 12.4 |
| | 0.105 | 0.061 | 12.2 |
| | 0.100 | 0.059 | 11.8 |
| 1000 | 0.22 | 0.112 | 11.2 |
| | 0.23 | 0.116 | 11.6 |
| | 0.23 | 0.116 | 11.6 |
| Mean = 11.8 mg/g Relative Standard Deviation = 3.8 % | | | |
| Chicken fat (μL) | | | |
| 500 | 0.11 | 0.063 | 12.7 |
| | 0.11 | 0.063 | 12.7 |
| | 0.11 | 0.063 | 12.7 |
| 1000 | 0.22 | 0.111 | 11.1 |
| | 0.23 | 0.116 | 11.6 |
| | 0.24 | 0.121 | 12.1 |
| Mean = 12.2 mg/g Relative Standard Deviation = 5.6 % | | | |
| Lard (μL) | | | |
| 500 | 0.1 | 0.059 | 11.8 |
| | 0.11 | 0.059 | 11.8 |
| | 0.11 | 0.059 | 11.8 |
| 1000 | 0.22 | 0.112 | 11.2 |
| | 0.23 | 0.116 | 11.6 |
| Mean = 11.6 mg/g Relative Standard Deviation = 2.0 % | | | |

# CHAPTER 6

## SUMMARY AND CONCLUSION

The proposed enzymatic CL procedure for the determination of free cholesterol was evaluated in terms of its precision, linearity, and accuracy. The experimental procedures once optimized, and based on the results shown in Chapter five the feasibility of the proposed FIA-POCL method for the determination total cholesterol in different food samples was feasible.

The linearity studies indicated that the proposed FIA-POCL procedure has a good dynamic range. The calibration curve for hydrogen peroxide had an equation of $y = 13886.42x + 21.60$ for hydrogen peroxide concentrations from $9.0 \times 10^{-4}$ M to $4.4 \times 10^{-3}$M. For standard cholesterol, calibration curve had a regression equation of $y = 15.29x – 0.04$ of correlation coefficient 1.00. The reproducibility of the proposed procedure was established by two different experiments. The study using standard cholesterol solution at a concentration of 0.020 mg/mL gave a relative standard deviation of 2.57% and the second set using English butter of 0.20 mg/mL gave a relative standard deviation of 2.11 %. The accuracy of the proposed study was examined using commercial standard cholesterol and the store butter sample. The recoveries found using the commercial cholesterol standard were from 97.5 to 103.3 % and that of the butter samples were from 101.5 to 108.0 %. Clearly, the proposed FIA-POCL method has figures of merits which are satisfactory.

The proposed method were applied to determined the total cholesterol in food items such as butter, cheese, lard and chicken and beef fat, which are considered to have high levels of cholesterol. For comparison, an enzymatic colorimetric method of

determination was run parallel with the proposed FIA-POCL for some of the food items. For the butter, cheese, and lard the results for the colorimetric method and the proposed procedure obtained were comparable. The FIA-POCL method found the amount of total cholesterol and the relative standard deviation are in butter, 11.4 mg/g (13.9%), beef fat, 11.8 mg/g (3.8%), chicken fat, 12.2 mg/g (5.6%), lard, 11.6 mg/g (2.0%), and in cheese, overall, 13.5% (10.0%). However, the proposed FIA-POCL method gave results which were consistently higher than the colorimetric method. The labels in all cheese and butter packages, regardless of brands and sources, all gave the same value, without stating it was free or total cholesterol. The label values are about 10 times lower than the values obtained by the enzymatic colorimetric procedure and the proposed FIA-POCL method. However, the FIA-POCL in one set of experiment on reproducibility gave us an error +2.0% from the expected value using the standard cholesterol solution. So the labeled value seemed suspect.

In conclusion, the proposed FIA-POCL method can be used for the determination of total and free cholesterol. We should conduct further study to determine if the cause of some discrepancies was due to pipeting or pipet calibration. Also, it may be better separation or extraction can be devised as comparison to the proposed method in which no extraction procedure was carried out.

BIBLIOGRAPHY


1. Christie, W. W., "*Sterols and sterol esters : Structure, Occurrence, and Biochemistry Analysis*" Lipid analysis- 3rd Edition 2003, pp. 1-7
2. Nelson, D. and Cox, M. M. "Lehninger Principle of Biochemistry" 3rd Edition 2000 pp. 799-814.
3. Campbell, Neil A. "Biology" Second Edition, The Benjamin/Cummings Publishing company, Inc. 1987, 833-834.
4. "High Blood Cholesterol and What Your Need to know" U.S. DEPARTMENT OF HEALTH AND HUMAN SERIVE, National Institutes of Health (NIH) May 2001. Publication No. 01-3290
5. Hill, W. I, and Kolb, K. D. "Chemistry for Changing Time"8th Edition, Prentice Hall, Inc. 1998, 429-430.
6. Stein EA. "*Managing dyslipidemia in the high-risk patient.*" Am. J. Cardiol 2002; 89 (5A): 50C- 57C.
7. Chamis-Pasha, H. "*Cholesterol. How low should we go*?" Saudi Med J. 2005 Vol. 26(1): 11-18
8. Levy, I. R. "*Cholesterol, Lipoprotein, Apoproteins, and Heart Disease: Present Status and Future Prospectve.*" Clin. Chem 1981, 27, 653-662.
9. Johnes, Peter H. "*Statins as the cornerstone of drug therapy for dyslipidemia: Monotherapy and combination therapy options*" Am Heart J. 2004; 148: S9-13.
10. Pearson, Thomas A. "*The Epidemiological Basis for Populations-wide Cholesterol Reduction in the Primary Prevention of Coronary Artery Disease*" The American Journal of Cardiology. 2004, Vol. 94 (9A).
11. Amundson, Drake M. nad Zhou, Mingjie, "*Fluorometric Method for the Enzyme Determination of Cholesterol*" J. Biochem. Biophys. Methods 1999, 38, 43 -52.
12. Rifai, N; et al, "*Clinical Chemistry Journal has contributed to Progress in Lipid and Lipoprotein testing for fifty years*." Clin. Chem. 2004, 50(10), 1861 – 1870.

**13.** Zak, Bennie, "*Cholesterol Methodologies: A Review"* Clin. Chem. 1977, 23, 1201 – 1212.

**14.** Zaltkis, A., Zak, B., and Boyle, A.J., "*A new Method for the Direct Determination of Serum Cholesterol*" J. Lab. Clin. Med. 1953, 41, 486-491.

**15.** Zak, B and Ressler N. "*Methodology in Determination of Cholesterol"* Am. J. Clin. Pathol. 1955, 25, 433-442

**16.** Henry, R. Cannon, D. C. and Winkelmn, J. W., "*Clinical Chemistry Principle and Technics*" Bio-Science Laboratories, 2nd edition. 1974, 1429 – 1491.

**17.** Kim, E., and Goldberg, M., "*Serum Cholesterol Assay Using a Stable Liebermann-Burchard Reagent*." Clin. Chem. 1969, 15, 1171 – 1179.

**18.** Burke, R. W. et al, "*Mechanisms of the Liebermann-Burchard and Zak Color Reaction for Cholesterol*" Clin. Chem. 1974, 20(7), 794-801.

**19.** Katan, Martijin B., Haar, Frits van der, Kromhout, Daan and Schouten, Frans J. M. "*Standardization of Serum Cholesterol Assays by Use of Serum Calibrators and Direct Addition of Liebermann-Burchard Reagent*." Clin Chem. 1982: 28/4 683 - 686.

**20.** Pearson, S; Stern, S. McGavack, T. H. "*A Rapid procedure for the determination of serum cholesterol.*" Analytical Chemistry. 1953, 25, 813-814.

**21.** Jamieson, A. "*A method for the rapid determination of serum total cholesterol using a modification of the Pearson Reaction*. "Clin, Chem Acta, 1964, 27, 530-535.

**22.** Wybenga, D. R.; Pileggi, V. J.; Dirstine, P. H.; and Giorgio, John D. *"Direct Manual Determination of Serum Total Cholesterol with a Single Stable Reagent"* Clin. Chem. 1970, 16, 980-984.

**23.** Bowman, Robert E. and Wolf, Richard C. "*Observations on the Zak Cholesterol Reaction: Glacila Acetic Acid Purity, a more Sensitive Absorbance Peak, and Bromide Enhancement*" Clin. Chem. 1962, 8(3) 296- 301.

**24.** Hau-shan Hung, Jui-chang W. Kuan, and George G. Guillbault. "*Fluorometric*

*Enzymatic Determination of Total Cholesterol in Serum*" Clin. Chem 1975, 21, 1605-608.

**25.** Amundson, Drake M. and Zhou, Mingjie, "*Fluorometric method for the enzymatic determination of Cholesterol.*" J. Biochem and Biophys. Methods 1999, 38: 43-52.

**26.** Allain, Charles C.; Poon, Lucy S.; Chan, Cicely S. G.; Richmond, W. and Fu, Paul C.; "*Enzymatic Determination of Total Serum Cholesterol.*" Clin. Chem. 1974, 20/4, 470-475.

**27.** Juan M. Fernandez-Romero. M. D. Luque De Castro and Miguel Valcarcel. *"Enzymatic determination of total cholesterol in Serum by Flow Injection Analysis*" J. Pharm. Biomed. Anal., 1987, 5 pp. 333-339.

**28.** Richman, W. "*Use of Cholesterol Oxidase for Assay using Total and Free Cholesterol in Serum by continuous-Flow Analysis*" Clin. Chem. 1979, 10, 1579 – 1588.

**29.** M. Masoon and Alan Townshend, "*Determination of Cholesterol by Flow Injection Analysis with Immobilized Cholesterol Oxidase*" Analytical Chimica Acta, 174(1985), pp. 293-297.

**30.** Sharul Bariyah, Shahul Hamid and Ibrahim Che Omar, "*Determination of Total Cholesterol in Serum by Cholesterol Oxidase and Cholesterol Esteras Co-Immobilized on to Cellulose Acetate Membrane.*" Pakistan Journal of biological Sciences 6 (2003): 1340-1344.

**31.** G. J. Moody, G. S. Sanghera and J. D. R. Thomas " *Factors Concerning the Design and Calibration of an Amperometric Enzyme Electrode System for the Flow Injection Analysis of Cholesterol*" Analyst, September 1988, vol 113, pp. 1419-1422.

**32.** Manihar Situmorang, Peter W. Alexander, D. Brynn Hibert, *"Flow Injection Potentiometry for Enzymatic Assay of Cholesterol with a Tungsten Electrode Sensor*." Talanta 49 (1999) 639 – 649.

**33.** O' Haver, T. C *"The Development of Luminescence Spectrometry as an*

*analytical Tool*." Journal of Chemical Education, Volume 55, Number 7, July 1978. pp 423-427.

**34.** Garcia-Campana, A. M. and Baeyens, W. R. G. "*Principles and recent analytical applications of chemiluminescenc"* ANALUSIS, 2000, 28 No # 8.

**35.** Garcia-Campana, A. M. and Baeyens, W. R. G. "*Principles and Recent Analytical Applications of Chemiluminescenc*" ANALUSIS, 2000, 28 No # 8.

**36.** Whitehead, Thomas P., Kricka, Larry J., Carter, Timothy J. N and Thorpe, Gary H. G., "*Analytical Luminescence: It's Potential in the Clinical Laboratory*" CLIN. CHEM. 1979, 25/9, 1531 – 1546

**37.** Guilbault, George G. "Fluorescence, Theory, Instrumentation, and Practice" Marcel Dekker, Inc., New York 1967.

**38.** G. G. Gilbault, "Practical Fluorescence, Theory, Methods and Techniques" Marcell Dekker, New York, 1973.

**39.** Douglas A. Skoog, F. James Holler and Timothy A. Nieman, "Principle of Instrumental Analysis" 5th edition, Saunders College Publishing, 1998. pp. 355 – 376.

**40.** Guilbault, George G. "Practical Fluorescence" 2nd ed., rev. and expanded, New York : M. Dekker, 1990

**41.** Cormier, M. J., Hercules, D. M. and Lee, J., "Chemiluminescence and Bioluminescence" Plenum Press, New York-London. 1973.

**42.** Radziszewski, B. Chem. Ber. 10, 1877, pp 70-75.

**43.** Albrecht, H. O.; Z. Phys. Chem. 1928, 10, 70.

**44.** Kricka, Larry K. and Thorpe "*Chemiluminescent and Bioluminescent Methods in Analytical Chemistry*. " Analyst, 1983, 108, 1274 – 1293.

**45.** Lary J. Kricka and Gary H. G. Thorpe, "*Chemiluminescent and Bioluminescent Methods in Analytical Chemistry*" Analyst, November, 1983, vol. 108, pp. 1274 – 1293.

**46.** Sasamoto, H., Maeda, M. and Tsuji, A. *"Chemiluminescent Enzymatic Assay for*

*Cholesterol in Serum using Luigenin*." Analytica Chimica Acta 310, 1995 pp 347-353.

**47.** Rost, M., Karge, E. and Klinger, W. "*What do we measure with Luminol-, Lucigenin- and Penicillin-amplified Chemiluminescence? 1. Investigations with hydrogen peroxide and Sodium Hypochlorite*" J. BIOLUMIN CHEMILUMIN 1998; 13: 355-363.

**48.** Olsson, B., "*Determination of Hydrogen Peroxide in Flow System with Microperoxidase as catalyst for the Luminol chemiluminescence Reaction*."Anal Chem. Acta, 1982, 136, 113-119.

**49.** Nussbaum, M. A; Nekimken and H. L.; Nieman, T. A. *"Luminol Chemiluminescence for Determination of Iron (II) in Ferrioxalate Chemical Actinometry"* Anal. Chem., 1987, 59, 211-213.

**50.** Tsunoda, Makot and Imai, Kazuhro, "*Analytical applications of peroxyoxalate chemiluminescenc*" Analytic chemical Acta 541 (2005) 13-23.

**51.** Lee, J. H, Rock, J. C, Park, S. B, Schlautman, M. A and Carraway, E. R "*Study of the Characteristics of Three High-energy Intermediates Generated in Peroxyoxalate Chemiluminescence (PO-CL) reactions*." J. Chem. Soc., Perkin Trans. 2, 2002. pp 802-809.

**52.** Mojtaba Shamsipur, Mohammad Javad Chaichi and Ali Reza Karami "*A Study of Peroxyoxalate-Chemiluminescence of Acriflavine*" Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, Volume 59, Issue 3, February 2003, Pages 511-517

**53.** Malin, E., Jonsson, T., and Irgum, K., *"Heterocyclic compound as catalyst in the Peroxyoxalate Chemiluminescence reaction of bis(2, 4, 6-trichlorophenly)oxalate*" Anal. Chimica Acta 1998, 361, 205-215.

**54.** Josson, T and Irgum, K. "*Very Fast Peroxyoxalate Chemiluminescence"* Acta 400 (1999) 257 – 264.

**55.** Sigvardson, K. W., Kennsih J. M. and John, B. W., *"Peroxyoxalate Chemiluminescence Detection of Polycyclic Aromatic Amine in Liquid*

*Chromatography*." Anal. Chem 1984, 56, 109-1102.

**56.** Arnous, A., Petrakis, C., Makris, D. P., and Kefalas, P., *"A Peroxyoxalate Chemiluminescence-based assay for the evaluation of Hydrogen Peroxide Scavenging activity employing 9, 10-diphenylanthracene as the Fluorophore.*" J. of Pharmacology and Toxicological Method. 2002, 48, 171 – 177.

**57.** Neuvonen, Helmi. "*A two-intermediate Model for Imidazole-promoted Peroxyoxalate Chemiluminescence.*" J BIOLUMIN CHEMILUMIN 1997: 12: 241-248.

**58.** Nozaki, O, Iwaeda, T. Moriyama, H. and Kato, Y. *"Chemiluminescent Detection of Catecholamines by Generation of Hydrogen Peroxide with Imidazole.*" Luminescence 1999: 14: 123-127.

**59.** Nazaki, O. and Kawamoto, H. "*Reaction of horseradish Peroxidase with Imidaxole for Continuous Determination of Hydrogen Peroxide using a microflow Injection-Chemiluminescence Detection System.*" Luminescence 2003; 18: 203-206.

**60.** Jaromir Ruzicka, "Flow Injection Analysis" 2nd edition, A Wiley-Interscience Publication, 1988.

**61.** Fletcher, Philip, Andrew, Kevin N., Calokerinos, Antoony C., Forbes, Stuart and Worsfold, Paul J. "*Analytical Application of Flow Injection with Chemiluminescence Detection-a Review*" Luminescence 2001; 16: 1-23.

**62.** Mohan, A. G., Turro, N. J., J. Chem Ed. 1794, 51, 528.

**63.** Kevin N. Andrew, Matthew G. Sanders, Stuart Forbes and Paul J. Worsofold, *"Flow Methods for the Determination of Polycyclic Aromatic Hydrocarbons using Low Power Photomultiplier Tube and Charge Coupled Device chemiluminescence detection.*" Acta 346 (1997) 113 – 120.

**64.** Malik, Vinita and Pundir, Chandra S. "*Determination of Total Cholesterol in Serum by Cholesterol Esterase and Eholesterol Oxidase Immobilized and Co-Immoblized on to Arylamine Glass.*" Biotechnol. Appl. Biochem. (2002) 35, 191–197.

**65.** Costello, Richard L.; Curran, George L.; "*Modified Turbidimetric Procedure for Determination of Cholesterol"* Clin, Chem Acta, 1957, 27, 108-115.

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