TOLGA SARUHAN ELECTROCHEMICAL DETERMINATION OF SOME WATER SOLUBLE AND FAT SOLUBLE VITAMINS BY USING PENCIL GRAPHITE ELECTRODE NEU 2021

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A THESIS SUBMITTED TO INSTITUE OF GRADUATE STUDIES

OF

NEAR EAST UNIVERSITY

By

TOLGA SARUHAN

In Partial Fulfillment of the Requirements for

the Degree of Master of Science

In

Food Engineering

NICOSIA, 2021

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Approval of the Institute of Graduate Studies

Prof. Dr. K. Hüsnü Can Başer

We certify that this thesis is satisfactory for the award of the degree of a Master of Science in Food Engineering

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct. I have fully cited and referenced all material and result that are not orginal to this work.

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To my family…

ABSTRACT

Vitamins are a group of organic compounds needed in small quantities that by the body to perform all of its essential functions like growing tissues, regulating metabolism and maintain a healty immune system. In recent years, substantial efforts have been focused on development of simplified, fast and cost-effective approaches for vitamin analysis. In this aspect, electrochemical techniques are very promising with their high sensitivity, simplicity, miniaturization and low cost. Aim of this study was to analyze some water-soluble and fat-soluble vitamins either as single vitamin or in a mixture by using electrochemical methods with pencil graphite electrode. For this purpose, we have selected vitamin C, B1, B6, B12, A, D, and E. These vitamins were electrochemically analyzed in pharmaceutical preparates and in some representative foods such as apple juice, spinach and salmon. In simultaneous analysis of water-soluble vitamins by using differential pulse voltammetry with PGE can be promising technique for the determination of vitamin C and vitamin B6 or vitamin B1 + B12 and B6. In the case of fat-soluble vitamins, resolution between vitamin D and E peaks was not satisfactory, therefore they cannot be determined quantitatively. However, vitamin A and D or vitamin A and E can be detected in a single run.

Keywords: Fat-soluble vitamins; water-soluble vitamins; vitamin analysis; electrochemical methods; differential pulse voltammetry; PGE

ÖZET

Vitaminler, vücut dokularının gelişmesi, metabolizmanın düzenlenmesi ve sağlıklı bir bağışıklık sistemini sürdürmek gibi tüm temel işlevlerin yerine getirmesi için küçük miktarlarda ihtiyaç duyulan organik bileşiklerdir. Son yıllarda, vitamin analizi için basit, hızlı ve uygun maliyetli metotların geliştirilmesinde önemli çabalar gösterilmiştir. Bu açıdan elektrokimyasal teknikler, yüksek hassasiyetleri, basitlikleri, minyatürleştirilebilmeleri ve düşük maliyetleri ile çok ümit vericidir. Bu çalışmanın amacı, bir kalem grafit elektrot ile elektrokimyasal yöntemler kullanarak, suda çözünen ve yağda çözünen bazı vitaminleri tek bir vitamin veya karışım halinde analiz olanaklarını ortaya koymaktır. Bu amaçla C, B1, B6, B12, A, D ve E vitaminleri seçilmiştir. Bu vitaminler, farmasötik preparatlarda ve elma suyu, ıspanak ve somon gibi bazı temsili gıdalarda elektrokimyasal olarak analiz edilmiştir. PGE ile diferansiyel puls voltammetri kullanılarak suda çözünen vitaminlerin eşzamanlı analizinde, C vitamini ve B6 vitamini veya B1 + B12 ve B6 vitamini tayini için umut verici bir teknik olabilir. Yağda çözünen vitaminler söz konusu olduğunda, D vitamini ve E pikleri arasındaki ayırım yeterli olmadığından kantitatif olarak belirlenemezler. Bununla birlikte, A ve D vitamini veya A ve E vitamini birlikte tespit edilebilir.

Anahtar Kelimeler: Yağda çözünen vitaminler; suda çözünen vitaminler; vitamin analizleri; elektrokimyasal metotlar; diferansiyel puls voltammetri; PGE

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABS:	Acetate Buffer Solution
AdsDPV:	Adsorptive Stripping Differantial Pulse Voltammetry
CE:	Counter Electrode
CN-:	Cyano Group
CV:	Cylic voltammetry
DC:	Direct Current
DPV:	Differantial Pulse Voltammetry
EIS:	Electrochemical Impedance Spectroscopy
ELISA:	Enzyme, Linked Immunosorbent Assay
GC:	Gas Chromatography
GCE:	Glassy Carbon Electrode
HCL:	Hydrochloric Acid
HPLC:	High Performance Liquid Chromatography
I.U.:	International Unit
ID-LC:	Isotope Dilution- Liquid Chromatography
IUD:	Introuterine Device
KCL:	Potassium Chloride
LC:	Liquid Choromatography
LOD:	Limit of Detection
MS:	Mass Spectrometry
OH:	Hydroxyl
PBS:	Phosphate Buffer Solution
PGE:	Pencil Graphite Electrode

PLF:	Pyridoxal 5 Phosphate
Py:	Pyrrole
RDA:	Recommended Dietary Allowance
RE:	Referance Electrode
RSD:	Relative Standard Deviation
SWV:	Square Wave Voltammetry
WE:	Working Electrode

CHAPTER 1
INTRODUCTION

Vitamins are a group of organic compounds needed in small quantities that body to perform all of its essential functions like growing tissues, requlating metabolism and maintain a healty immune system (Ball, 2006). There are thirteen vitamins important for human nutrition and these can be divided into two groups according to their solubility (Ball, 2006). Vitamins A, D, E, and K are called fat-soluble vitamins; It also includes up to 50 carotenoids with varying degrees of vitamin A activity. The water-soluble vitamins contain vitamin C and the members of the B complex vitamins, namely thiamin (vitamin B1), riboflavin (vitamin B2), niacin, vitamin B6, pantothenic acid, folate, and vitamin B12. In addition to that, the solubility properties are directly related to the distribution of vitamins in different food groups and the analytical methods to be used.

Most of the vitamins are absolutely essential in human nutrition, because the tissues in the human body cannot synthesize them (Combs & McClung, 2017). There are two notable exceptions that are vitamin D and niacin. The cutaneous synthesis of vitamin D depends on adequate exposure of the skin to sunlight; niacin synthesis depends on adequate intake of tryptophan, the protein-bound amino acid precursor (Cagetti et al., 2020). Plants are considered the primary source of essential nutrients for the human body due to their ability to synthesize vitamins other than vitamin B12.

Several B group vitamins act as coenzymes for enzymes involved in the catabolism of foodstuffs to produce energy for the organism. In the situation of subclinical vitamin deficiency, some people can benefit from vitamin supplements (Ball, 2006).

Various diseases can occur when vitamins are taken in small amounts. These disorders which are generally related to fat-soluble vitamins are night vision for vitamin A, dry eyes and skin, the slowdown in growth and reproduction. Vitamin D is essential for bone health. In cases of insufficient intake of vitamin D, rickets, osteoporosis, and osteomalacia can occur. Vitamin E and K deficiencies have been associated with bleeding, anemia and neurological changes. When water-soluble vitamin intake is inadequate, symptoms of high fever, malaise, anorexia, instability, headache, and muscle weakness are observed. Taking high or

inadequate in sufficient amounts of vitamins is not healthy. For this reason, human beings should pay attention to the amount of vitamins they should take daily.

Table 1.1: The amounts of vitamins to be taken daily by various age groups.

Vitamins	Age Groups	Recommended Daily Intake
A	0-12 months old's babies	0.375 mg
	1-3 years olds child's	0.4 mg
	7-10 years olds child's	0.7 mg
	11-14 years olds child's	0.8 mg
	15-18 years olds child's	1.0 mg
	for adult male	1.0 mg
	for adult female	0.8 mg
D	0-12 months olds babies	10 µg
	1-13 years olds child's	15 µg
	14-18 years olds child's	15 µg
	19-50 years olds adults	15 µg
	51-70 years olds	15 µg
	>70 years olds seniors	20 µ
E	for child's	3-10 mg
	for adult male	10 mg
	for adult female	8 mg
K	for child's	15-20 µg
	for adult male	80 µg
	for adult female	65 µg
B1	for all ages	0.4 mg
B6	for all ages	1.5-2 mg
B12	for all ages	2 µg
C	for all ages	75-80 mg

Individuals' metabolic processes must respond to the urgent needs of their bodies. Therefore, the vitamin requirements of humans are subject to constant change between certain limits as can be seen in Table 1.1, the recommended dietary allowance (RDA) of a vitamin is the average daily dietary intake level sufficient to meet the needs of nearly all healthy individuals (97-98%) in a given life stage and gender group (Flecther et al., 2020).

Vitamins may have a toxic effect when taken more than enough. Toxic effects of fat-soluble vitamins are mainly; anorexia, painful swelling in long bones, hair loss and itchy rashes, headache, abnormal development in bones. Water-soluble vitamins have generally no toxicity since they do not get stored within the body. However, vitamin C may have toxic effect. As a result of its toxicity in the human body, it can be experienced severe headache, vomiting, diarrhea, and developing calcium oxalate in the kidney. Vitamins are physiologically active substances and a true assessment of the nutritional value of a food commodity or diet with respect to a particular vitamin can only be obtained by determining the vitamin’s biological activity. The only direct means of determining biological activity is a bioassay based on a biological function. Vitamin determinations in foods are routinely performed using microbiological assays or physicochemical methods of analysis.

Best approach to ensure we get a variety of vitamins and minerals in the proper amount is to adopt a balanced diet. This involves an emphasis on fruits and vegetables, whole grains, beans and legumes, low-fat protein and dairy products. Most of the foods contain multiple vitamin and mineral sources (Table 1.2 and Table 1.3).

Table 1.2: Dietary source of fat-soluble vitamins.

Vitamin Name	Function	Dietary Sources
A	Helps with (1) healthy mucous membranes; (2) skin, vision, tooth and bone growth; (3) health of the immune system.	Food of animal origin (retinol): liver, eggs, fortified margarine, butter, cream, cheese, fortified milk. Food of plant origin (beta-carotene): dark orange vegetables (pumpkin, sweet potatoes, winter squash, carrots), fruits (cantaloupe, apricots), dark green leafy vegetables.
D	Required to properly absorb calcium.	Fortified margarine, fortified milk, fatty fish, liver, egg yolks; the skin can also produce vitamin D when it is exposed to sunlight.
E	Helps to protect the cell walls.	Nuts and seeds, egg yolks, liver, wholegrain products, wheat germ, leafy green vegetables and polyunsaturated plant oils.
K	Required for correct blood clotting.	Vegetables from the cabbage family, leafy green vegetables, milk; it is also produced in the intestinal tract by the bacteria.

Table 1.3: Dietary source of water-soluble vitamins.

Vitamin Name	Benefits	Dietary Sources
Ascorbic Acid (Vitamin C)	Ascorbic acid is an antioxidant, and it is a portion of an enzyme that is required for protein metabolism. It also helps with iron absorption and is important for the health of the immune system.	Vegetables and fruits, especially: kiwifruit, mangoes, papayas, lettuce, potatoes, tomatoes, peppers, strawberries, cantaloupe and so on.
Thiamine (Vitamin B1)	Thiamine is a portion of an enzyme that is required for energy metabolism, and it is important for nerve function.	In all nutritious foods: nuts and seeds, legumes, wholegrain/ enriched cereals.
Riboflavin (Vitamin B2)	Riboflavin is a portion of an enzyme that is required for energy metabolism. It is also important for skin health and normal vision.	Enriched, wholegrain cereals and breads, leafy green vegetables, milk products.
Niacin (Vitamin B3)	Niacin is a portion of an enzyme that is required for energy metabolism. It is also important for skin health as well as the digestive and nervous systems.	Peanut butter, vegetables (particularly leafy green vegetables, asparagus and mushrooms), enriched or wholegrain cereals and breads, fish, poultry and meat.
Pantothenic Acid (Vitamin B5)	Pantothenic acid is a portion of an enzyme that is required for energy metabolism.	It is widespread in foods.

Pyridoxine (Vitamin B6)	Pyridoxine is a portion of an enzyme that is required for protein metabolism. It also helps with the production of red blood cells.	Fruits, vegetables, poultry, fish, meat.
Folic Acid (Vitamin B9)	Folic acid is a portion of an enzyme that is required for creating new cells and DNA.	Liver, orange juice, seeds, legumes, leafy green vegetables.
Cobalamin (Vitamin B12)	Cobalamin is a portion of an enzyme required for the production of new cells, and it is important to the function of nerves.	Milk, milk products, eggs, seafood, fish, poultry, meat. It is not present in plant foods.
Biotin (Vitamin H)	Biotin is a portion of any enzyme that is required for energy metabolism.	It is widespread in foods and can be produced by bacteria in the intestinal tract.

Unfortunately, this is far from being achievable everywhere since it requires universal access to adequate food and appropriate dietary habits (Coffin et al., 2006), and some losses of certain vitamins during food processing are inevitable. Another point to consider is that natural variations in the vitamin content of a raw food material may effect the content of vitamins in the final product more than the processing itself (Ball, 2006). From these standpoints, food fortification can have advantage to deliver nutrients to large segments of the population without requiring radical changes in food consumption patterns. In fact, fortification has been used for more than 80 years in industrialized countries as a means of restoring micronutrients lost with food processing, in particular, some of the B vitamins (Coffin et al., 2006). Because of increased awareness of the widespread prevalence and harmful effects of micronutrient malnutrition and changes in food systems increasing numbers of developing countries are also committed to fortification programmes.

Consequently;

- To determine raw food product-nutritional quality
- To asses the effect of food processing on the nutritive quality of final product
- To use vitamins reasonably in our diets
- To combat micronutrient deficiencies and to ensure that the target population will benefit food fortification programme,

it is essential to have or depelope quick, easy efficient and safe analytical methods. Great progress in analytical chemistry has been achieved and some new analytical instruments have been developed since 2010 (Zhang et al., 2018). Considering few comprehensive reviews of pretreatment and determination of vitamins has been published systematically. Ultrasonic assisted extraction (UAE), supercritical fluid extraction (SFE), SPE, LLE, dispersive liquid-liquid microextraction (DLLME) and various analysis methods, including chromatographic methods, electrophoretic methods, microbiological assays, immunoassays, biosensors and others have been reported and used to analyze vitamins since 2010 (Zhang et al., 2018).

Among many techniques, electrochemical methods have drawn attention due to its easy operation, sensitivity and low cost (Parvin et al., 2018).

Aim of this study is to investigate possibilities of analyzing some water-soluble and fat-soluble vitamins either as single vitamin or in a mixture by using electrochemical methods with pencil graphite electrode. For this purpose, we have selected vitamin C, B1, B6, B12, A, D, and E. These vitamins were electrochemically analyzed in pharmaceutical preparates and in some representative foods such as apple juice, spinach and salmon, to investigate whether simultaneous analysis of water-soluble or fat-soluble vitamins can be performed by using differential pulse voltammetry with PGE.

CHAPTER 2
THEORETICAL FRAMEWORK

Vitamins are essential substances that must be taken from outside, as the body cannot synthesize. They are regulatory substances and form the coenzyme part of compound enzymes. Vitamins do not provide energy and are not used as building units in cells. Since they are large enough to pass through the cell membrane, they enter the bloodstream without being digested. Vitamin determinations in foods can be performed using spectrophotometric, chromatographic, electrochemical methods, microbiological or immuno assays. The selection of proper analytical method depends on objective of the measurement. Physicochemical properties of vitamin and food matrix play an important role in selection of methods.

2.1 General Properties of Vitamins

2.1.1 Vitamin A

Is illustrative for compounds with the biological efficiency of retinol, formally having to contain five C-C double carbon bonds. It is regenerated from the non-cyclic parent compound and a functional group at the end of the acyclic part (Imad et al., 2017). These are called retinoid because their vitamin A structure is similar to retinol. There are three structures that naturally have vitamin A activity; alcohol retinol, aldehyde retinal (retinaldehyde) and acid retinoic acid. Beta-carotene type vitamin A is an antioxidant. Antioxidants protect the publication from the services it provides, called free radicals (Combs & McClung, 2017).

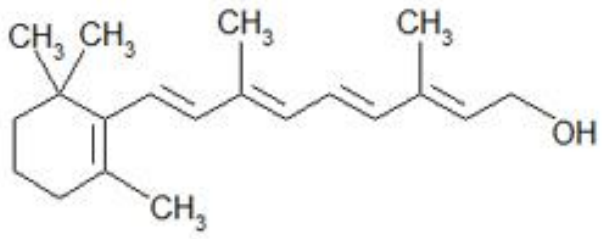

Figure 2.1: Chemical structure of retinol.

2.1.2 Vitamin D

Is it descriptive in order to all steroids showing the biological efficiency of cholecalciferol. These compounds derive 7-dehydrocholesterol according to photolysis of the B link in vivo. It has a nine-carbon lateral-chain having a single double bond. There are two primary forms of vitamin D as Ergocalciferol (D2) and Cholecalciferol (D3) (Price & Preedy, 2020). People can create enough vitamin D endogenously from the sterols in the body in order to the effect of ultraviolet light on the skin by stocking sunlight as necssary. For this reason, vitamin D is named "sunlight vitamin" (Combs & McClung, 2017).

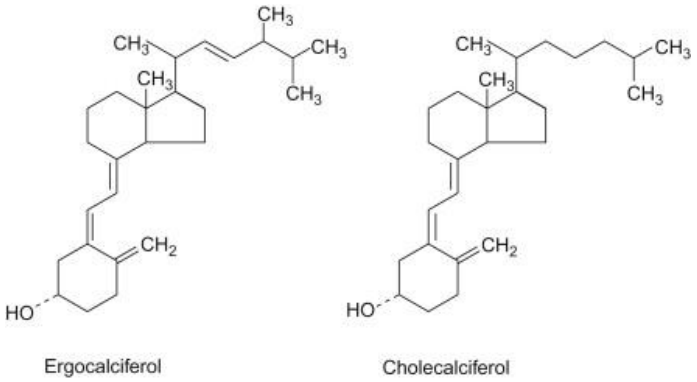

Figure 2.2: Chemical structure of vitamins D2 and D3.

2.1.3 Vitamin E

Is the common assign for all tocol and tocotrienol derivatives that qualitatively show the biological activity of α-tocopherol "(Combs & McClung, 2017). These compounds are isoprenoid lateral-chain differentiation of 6-chromanol, tocols with lateral-chains be forming of three fully satiated isopentyl units, tocopherols comprising the mono-, di-, and trimethyl tocols; and tocotrienols being 6-chromanol derivatives with like lateral-chain containing three double bonds.

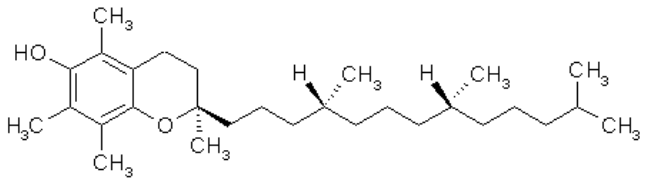

Figure 2.3: Chemical structure of tocopherol.

2.1.4 Vitamin C

Is revealing for compounds that show qualitatively the biological activity of ascorbic acid, i.e., 2,3-dihydro-l-three-hexane-1,4-lactone.

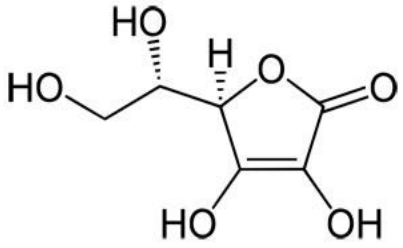

Figure 2.4: Chemical structure of ascorbic acid.

2.1.5 Vitamin B1 (Thiamine)

Vitamin B1 is more commonly known as thiamine. It was the first vitamin to be discovered and was first separated from rice husks in 1926. And "And 3- [(4-amino-2-methyl-5-pyrimidinyl) methyl] -5- (2-hydroxyethyl)- trivial description of 4-methylthiazolium (Price & Preedy, 2020).

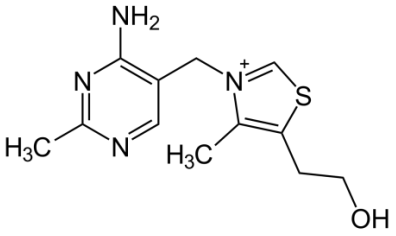

Figure 2.5: Chemical structure of thiamine.

2.1.6 Vitamin B6 (Pyridoxine)

Is also defined as pyridoxine. It is not stored in the body and is a water-soluble vitamin. Usable forums are pyridoxine hydrochloride and pyridoxal 5 phosphate components. Its active form is pyridoxal 5 phosphate (PLF). Pyridoxine needs cofactors such as riboflavin and magnesium to transform into PLF. The phrase of pyridoxine is an insignificant description of sole vitamin B6-active compound, 3-hydroxy-4, 5-bis (hydroxymethyl) -2-methylpyridin, previously named adermine either pyridoxal.

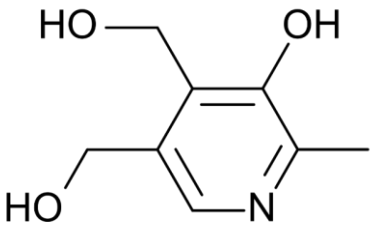

Figure 2.6: Chemical structure of pyridoxine.

2.1.7 Vitamin B12 (Cobalamin)

This vitamin a water-soluble vitamin and it's could be stocked in the body, albeit in small amounts. It is a vitamin also known as cobalamin due to its cobalt mineral content. There are two forms of the cobalamin. One of them is methylcobalamin, which has a methyl group. The other is adenosylcobalamin, which is a 5′-deoxyadenosyl grouping. Some synthetic vitamins are metabolically effective as they are converted into any of the forms; These contain cyanocobalamin with a cyano group (CN−), aqua cobalamin by a connected water molecule, hydroxocobalamin by hydroxo (OH) group and nitritocobalamin with a nitrite group.

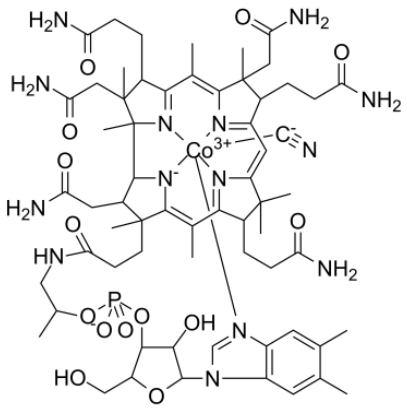

Figure 2.7: Chemical structure of vitamin B12.

2.2 Various Extraction and Clean-up Techniques in Vitamin Analysis

It is essential for a successful assay that the vitamins be quantitatively extracted from the food matrix in a form that can be accurately measured by the particular high-performance liquid chromatography (HPLC) technique to be used. Methods of extracting the fat-soluble vitamin from food matrices include alkaline hydrolysis, enzymatic hydrolysis, alcoholysis, direct solvent extraction, and supercritical fluid extraction of the total lipid component.

2.2.1 Direct Solvent Extraction

The fat-soluble vitamins can be extracted from the food matrix without chemical change using a solvent system that is capable of effectively penetrating the tissues and breaking lipoprotein bonds.

2.2.2 Open Column Chromatography

The more recent applications of open-column chromatography in fat soluble vitamin assays utilize liquid–solid (adsorption) chromatography using gravity-flow glass columns dry-packed with magnesia, alumina, or silica gel. Such columns enable separations directly comparable with those obtained by thin-layer chromatography to be carried out rapidly on a preparative scale.

2.2.3 Ultrasonic Assisted Extraction (UAE)

As a high efficiency pretreatment method, UAE can save time and increase the yield and the quality of an extract dramatically. The extraction efficiency can be enhanced by ultrasonic energy through induced cavitation. The information block diagram of UAE is shown in Figure 2.9.

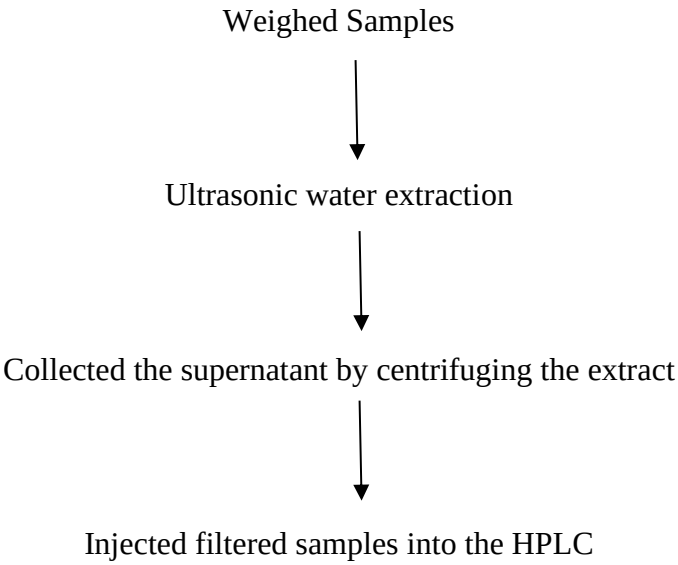

Figure 2.8: Information block diagram of UAE.

After ultrasonic, we can collect the solvents by filtration or centrifugation easily. In general, after extraction, centrifugation or filtration is inevitable (Zhang et al., 2018). Compared with the other pretreatment methods (e.g., heating reflux), the UAE methods mentioned above have shorter extraction times with good recovery. However, the methods still consume large volume of solvents

2.2.4 Supercritical Fluid Extractin (SFE*)*

Supercritical fluid carbon dioxide is an excellent extraction medium for nonpolar compounds and is beginning to replace the use of organic solvents in analytical methods for determining fat-soluble vitamins in foods (Ball, 2006). Analytical supercritical fluid extraction (SFE) using supercritical fluid carbon dioxide is a welcome technology in view of the environmental and health problems associated with the use of solvents, especially chlorinated ones.

These unique properties make supercritical fluids particularly suitable for extracting compounds from solid or semisolid food samples.

SFE can be employed either as an offline method, in which a “stand alone” extraction instrument is used to collect the sample extract for subsequent analysis, or an online method, in which the extraction instrument is coupled directly to an analytical chromatographic instrument (Zhang et al., 2018).

2.2.5 Solid-Phase Extraction

Solid-phase extraction, a refinement of open-column chromatography, uses disposable prepacked cartridges to facilitate rapid cleanup of sample extracts prior to analysis by HPLC. SPE is one of the most common methods to pretreat samples and has been applied to analyze vitamins in different matrices. For liquid samples, SPE is generally directly used to treat real samples (Zhang et al., 2018).

In the SPE process, solid phase materials, which are useful for extraction, concentration and clean-up, are available in a wide variety of chemistries, adsorbents and sizes. The sorbent selected in SPE controls analytical parameters such as selectivity, affinity and capacity. For this reason, different SPE materials have been used. Because of the different chemical properties of water-soluble and fat-soluble vitamins, SPE methods using different columns have been established.

SPE methods have shown good behavior in the process of purification producing the desired clean-up effect and achieving automation (Ball, 2006).

SPE has many advantages compared with other extraction methods, e.g., complete phase separation, high recovery and low consumption of organic solvents (Zhang et al., 2018).

2.2.6 Liquid-Liquid Extraction

Another complementary method for the extraction and purification of vitamins is LLE. LC method for fat-soluble vitamins using the LLE process. When do some water-soluble analysis in this method, multivitmain syrup, n-hexane–DEE solution and DMSO should be mix with vortex. This can be taken as the normal procedure of LLE (Zhang et al., 2018).

2.2.7 Dispersive Liquid-Liquid Micro-Extraciton

Different miniaturized pre-treatment techniques based on LLE were developed prior to 2010, including SDME, HF-LPME and so on. Since 2010, DLLME has become a very popular environmentally benign sample preparation technique, because it has a lot of advantages, such as low solvent cost and high enrichment factor.

In the work of (Viñas et al., 2013) DLLME with HPLC-PDA detection and a comparison with MS/MS detection for vitamins D and K in foods were combined. For the DLLME procedure, the targets were extracted with acetonitrile (3 mL) which was also used as dispersive solvent. Then, an extractant solvent (carbon tetrachloride, 150 μL) was added, the mixture was injected into water directly using a micropipette, and after being shaken and centrifuged, the demented phase was collected and evaporated to dryness. The residue was reconstituted and injected into the LC.

This method eliminates interfering compounds in the matrix, is sensitive and has an improved limit of detection (LOD) compared to other methods.

Compared with traditional LLE methods, the abundant contact surface of fine droplets and analytes speeds up the mass transferring processes of analytes from the aquatic phase to the organic phase in a DLLME process, which not only greatly enhances the extraction efficiency but also overcomes the time-consumption problem (Zhang et al., 2018). However, the recoveries obtained by the DLLME method are usually not high enough compared with those of other methods. This may be caused by the use of a dispersive solvent which usually decreases the partition coefficients of analytes into the extraction solvents.

2.3 Detection Methods

2.3.1 HPLC Method

HPLC stands for as follows; (High-Performance Liquid Chromatography. High-The meaning of high performance; It is known as high resolution (separation). It is also named High-Pressure Liquid Chromatography for it enables the mobile phase to move with loud pressure (Eser & Burcu, 2018). HPLC is situated on the result that the components reduced in a liquid enter into dissimilar interplay with the constant phase, usually on strict assistance in a column, and move at dissimilar accelerate for the column, resulting in the separation of

the ingredients leaving the column at a dissimilar while. HPLC has recently been recognized as an inevitable tool in many fields. It is utilization to separate as well as identify types of a variety of organic, inorganic, and biological examples. HPLC is mainly used in many fields. These areas are; medicine (e.g. antibiotics), biochemicals (e.g. proteins), foodstuffs (e.g. antioxidants), industrial chemicals (e.g. dyes), pollutants (e.g. pesticides), clinical medicine (e.g. drug metabolites), drugs (e.g. poisons) (Eser & Burcu, 2018). HPLC is generally classified according to the different contraptions or type of constant phase. These contain dispersion either liquid-liquid chromatography, suction either solid-liquid chromatography, ion-change chromatography, dimension separator chromatography, proximity chromatography, as well as chiral chromatography. HPLC occurs in four parts: Degasser, pump, autosampler, column, a detector. Degasser; Provides removal of reduced gases available in mobile phases. HPLC is quite expensive, but in many ways, it is the most essential tool and method for analysis. HPLC is required for comprehensive analysis.

Determination of the Fat-Soluble Vitamins by HPLC

The lipid fraction of foods containing the fat-soluble vitamins is composed mainly of triglycerides, with much smaller amounts of sterols, carotenoids, phospholipids, and minor lipoidal constituents. A proportion of the indigenous fat-soluble vitamin content of a food is bound up with a lipoprotein complex, and hence the fat-protein bonds must be broken to release the vitamin. The protective gelatine coating used in certain proprietary vitamin premixes will need to be dissolved before commencing the analysis of supplemented foods.

Determination of the Water-Soluble Vitamins by HPLC

HPLC columns used for the analysis of water-soluble vitamins are of the same type as those used in fat-soluble vitamin assays (Ball, 2006).

Application of HPLC

In this section, applications are arbitrarily divided into single vitamin analyses and multiple vitamin analyses. The requirement to determine the naturally occurring vitamin of a foodstuff allows little scope for determining more than one vitamin at a time (Ball, 2006). This is because of difficulties of quantitatively extracting the vitamins from their various

bound forms, the need to measure low indigenous concentrations in the presence of a complex matrix, and the requirement to determine several vitamers of some vitamins.

2.3.2 ELISA Method

ELISA method was found as an alternative to radioimmunoassay methods in the 1960s, and since then its use has spread all over the world. Because the reagents used in the ELISA method are long-lasting and have no radiation hazard related to waste materials, they are rapidly being preferred over the IUD (Radioimmunoassay) method (Imad et al., 2017). The term ELISA is named after the first letters of some English words. The abbreviation for this procedure is Enzyme, Linked Immunosorbent Assay. This procedure is popularly common as tests used in the identification of AIDS (HIV) contamination. In addition, these analysis procedures can diagnose many illnesses. With this ELISA method, many tests are carried out to investigate and diagnose dozens of infections, from Hepatitis, Rubella, Measles, Herpes virus infections to bacterial infections such as Brucella (Imad et al., 2017). The ELISA method is a group of tests used by doctors to diagnose not only infectious diseases but also many health problems, especially autoimmune diseases that affect the immune system. This method can also be used in the analysis of vitamin and drug amounts. The fundamental rationale of this method to reveal the effect among the antigen and antibody special to the test to be researched and detected utilization of the enzyme. All practices and assay methods utilization enzymes to indicate antigen-antibody reactions are usually called enzyme immunoassay (enzyme immunoassay, EIA, ELISA). In this method, the antibody-antigen to be investigated is evident by the enzyme. Tests that require the use of enzymes also color substrates. The coloration that occurs due to the reaction taking place in these tests is measured and interpreted by the spectrophotometer apparatus. Consequently, unmarked antigen and antibody searching owing to the tagged conjugate, as well as this method is given result by appraising the enzyme action generated by adding a substrate to the reaction. Today, ELISA tests are very important and it is necessary to be able to perform these tests macro and micro in laboratories (Hamblin et al., 1986).

What is a Macro ELISA?

In the Macro ELISA method, many more samples are studied and finalized in a much faster and faster manner by self-acting apparatus. This procedure serves a quick conclusion according to the Micro ELISA method. (Hamblin et al., 1986).

What is a Micro ELISA?

This procedure is performed by hand, with manual straws, test reactive, as well as a spectrometer where a conclusion is read. This procedure performed by hand is usually preferential while a few samples come to the laboratory. There are also automatic devices that work with Micro ELISA tests. Laboratories with intensive tests use automatic devices (Hamblin et al., 1986).

Biospesific Methods for Some of the B-Group Vitamins

Biospecific methods of analysis for selected vitamins of the B group can be broadly classified as immunoassays and protein-binding assays (Ball, 2006). Immunoassays are based on the specific interaction of an antibody with its antigen, and are represented by the enzyme-linked immunosorbent assay (ELISA). Biospecific assays can be performed on complex biological matrices, so they require minimal sample cleanup. The analytical stages can be automated using equipment that is commercially available, but the methods can only be described as semiautomated, as it is necessary to liberate the vitamins from their bound forms using manual extraction procedures.

Principle of ELISA

An ELISA is an enzyme-linked immunoassay in which one of the reactants is immobilized by physical adsorption onto the surface of a solid phase. In its simplest form, as used in food analysis applications, the solid phase is provided by the plastic surface of a 96-well microtiter plate. The ELISA can be performed manually, with the aid of push-button dispensers, or it can be totally automated, complete with computer for calculation of standard curves, statistical analysis of data, and data storage. In the direct competitive ELISA, the analyte vitamin molecules and added enzyme–vitamin conjugate compete for a limited number of binding sites on the immobilized antibody (Ball, 2006).

The amount of bound enzyme is then determined by addition of substrate and spectrophotometric measurement of the colored product. The generally preferred ELISA format for vitamin assays in food analysis is a two-site noncompetitive assay used in the indirect mode.

This format employs two antibodies: a primary antivitamin antibody raised against a hapten–protein conjugate, and an enzyme-labeled, species specific second antibody, which binds specifically to the primary antibody.

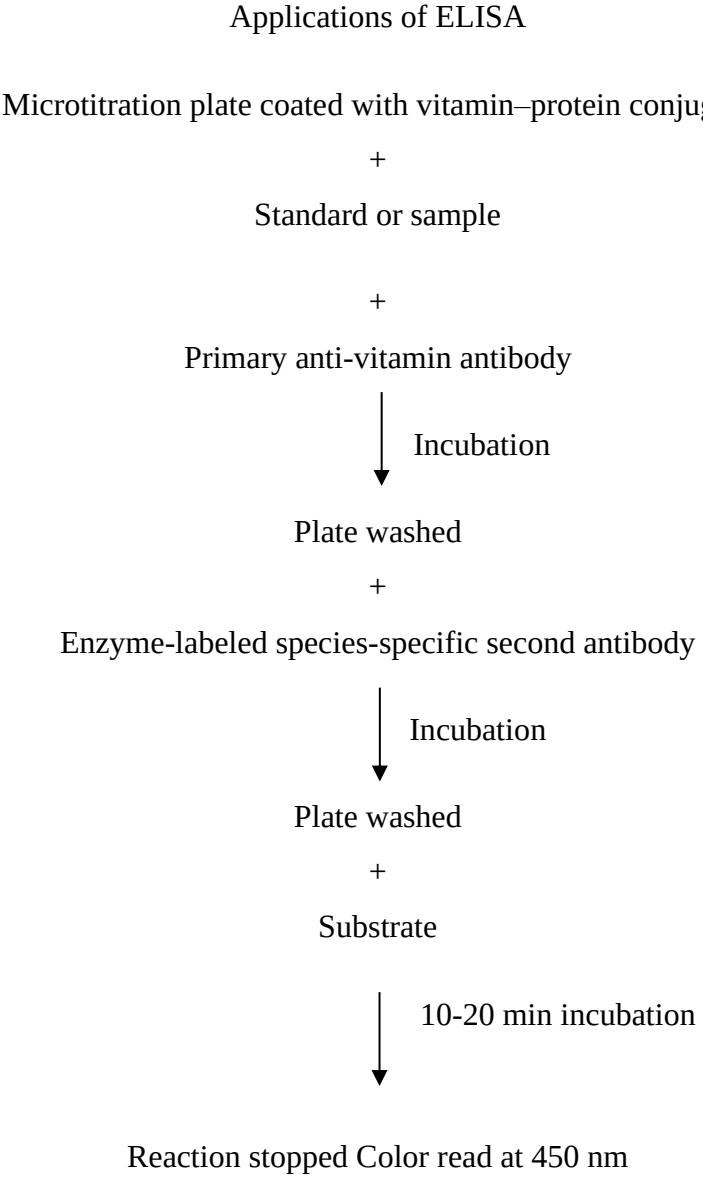

Figure 2.9: Methodology of ELISA

2.3.3 Electrochemical Methods

In recent years, substantial efforts have been focused on development of simplified, fast and cost-effective approaches for vitamin analysis. In this aspect, electrochemical techniques are very promising with their high sensitivity, simplicity, miniaturization and low cost. These techniques can provide a non-specific fingerprint of oil samples. The employment of voltammetric techniques for the detection of olive oil adulteration is very rarely described in the literature (Apetrei et al., 2014). These researches used chemically modified carbon paste electrodes using three virgin olive oils of different quality, a refined olive oil and two seed oils. Based on their previous work on chemically modified electrodes (Apetrei et al., 2005; 2006), they developed also voltammetric e-tongues for the detection of olive oil adulteration with seed oils (Apetrei et al., 2014). Oliveri and friends also achieved a discrimination of olive from maize oils as well as classification of olive oils according to their geographical origin (Oliveri et al., 2009). Tsopelas and friends were used glassy carbon electrode for voltammetric fingerprinting of extra virgin olive oil with olive pomace oil as well as the most common seed oils, such as sunflower, soybean and corn oil, by either direct analysis of diluted oils or using methanolic extracts of them (Tsopelas et al., 2018).

Theory of Electrochemistry and Some Electrochemical Techniques

Electrochemical techniques are related to the interaction between electricity and chemistry, expressly the measurements of electrical quantities, such as current, potential, or charge, and their relationship to chemical parameters. There are variable applications of using electrochemical measurements such as industrial quality control, and biomedical analysis.

Electrochemical processes take place at the electrode-solution interface. Potentiometric and potentiostatic measurements are the two principal types of electrochemistry. Both of them need at least two electrodes as conductors and a contacting sample (electrolyte) solution, which compose the electrochemical cell. One of these electrodes which called working electrode responds to the target analyte. Other one called reference electrode which is of constant potential due to independent of the properties of the solution.

Potentiometry is a static (zero current) technique in which information about the sample composition is obtained from measuring the potential through a membrane.

Potentiostatic also known as controlled potential, techniques relate to the study of charge transfer processes in the electrode-solution interface and are based on dynamic (no zero current) conditions. The electrode potential is used to derive an electron transfer reaction and the resulting current is measured while chemical species gaining or losing an electron. The resulting current demonstrates the rate at which electrons move along the electrode solution interface. Any chemical species that is electroactive (reduce or oxidize) can be measured by potentiostatic techniques.

The purpose of controlled-potential electrochemical experiments is to obtain a current response which is related to the concentration of the target analyte. This accomplished by monitoring the electron transfers during the redox process of the analyte.

$$O + ne^- \leftrightharpoons R \qquad (2.1)$$

Where O is oxidized and R is reduced forms of redox couple. Such a reaction occurs in a potential region which makes the transfer of electrons thermodynamically or kinetically suitable. The potential of the electrode can be used to determine the concentration of the electroactive species at the surface [CO (0, t) and CR (0, t)] according to the Nernst equation for systems controlled by the laws of thermodynamics.

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Nernst equation:

$$E = Eo + 2.3\frac{RT}{nF} \log \frac{CO\ (0, t)}{CR\ (0, t)} \qquad (2.2)$$

Where *Eo* is the standard potential for the redox reaction, *R* is the universal gas constant (8.314 J K-1 mol-1), *T* is the temperature (Kelvin), *n* is the number of electrons transferred in the reaction and *F* is the Faraday constant (96,487 coulombs).

The current caused by a change in the oxidation state of the electroactive species is called the faradaic current. Faradaic current is a direct measure of the rate of the redox reaction. Voltammogram is the resulting current-potential plot and it is a display of current signal on vertical axis versus the excitation potential on horizontal axis. The exact shape and magnitude of the voltammetric response is governed by the processes involved in the electrode reaction. The total current is the summation of the faradaic currents for the sample and blank solutions.

The path of the electrode reaction can be quite complex and is carried out in a row containing several steps. The rate of such reactions is determined by the slowest step in the sequence. Mass transport of the electroactive species to the electrode surface, the electron transfer across the interface and the transport of the product back to the bulk solution are the simple reactions that involve. Mass transport occurs by diffusion, convection and migration. The spontaneous movement under the influence of concentration gradient which aimed at minimizing concentration differences called diffusion. Transportation of the electrode by a gross physical movement (stirring, rotating or vibration of electrode) called convection. Movement of charged particles along an electrical field called migration.

When three modes of mass transport occur simultaneously, it becomes complicated to relate the current to the analyte concentration. This condition can be substantially simplified by suppression of electromigration or convection by the addition of excess inert salt or by the use of a quiescent solution, respectively and the movement of the electroactive species is limited by diffusion. The reaction on the surface of the electrode produces a concentration gradient adjacent to the surface which causes a diffusional flux (Wang, 2000).

Cyclic Voltammetry

Cyclic voltammetry used to acquire qualitative information on electrochemical reactions. Significant information on the thermodynamics of redox processes, on the kinetics of heterogeneous electron transfer reaction, and on coupled chemical reactions or adsorption processes can rapidly provided by cyclic voltammetry results. Cyclic voltammetry is usually the first experiment performed in an electrochemical study due to offering a rapid location of redox potentials of the electroactive species, and convenient evaluation of the effect of media upon the redox process. Cyclic voltammetry is a linear scan of the potential of a stationary working electrode (in an unstirred solution) using a triangular potential waveform as shown in the Figure 2.8. During the sweep potential, the resulting current is measured from the applied potential by the potentiostat. Cyclic voltammogram is the resulting plot of current versus potential.

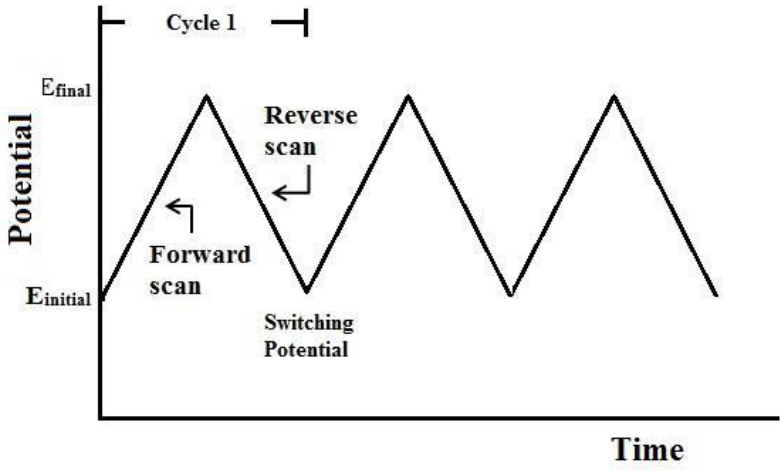

Figure 2.10: Potential-time excitation signal of cv experiments

The expected response of a reversible redox couple (O+ne^-⇋R) during a single potential cycle is shown in Figure 2.9.

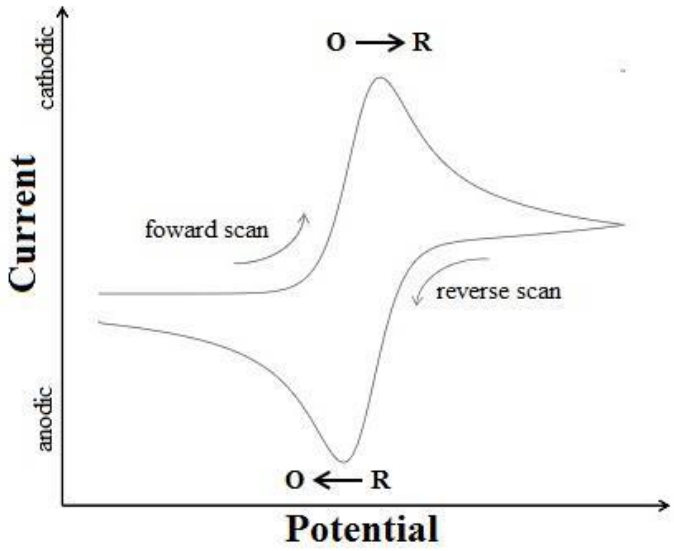

Figure 2.11: Cyclic voltammogram of reversible redox process

Initially it is assumed that only the oxidized O form is present. For the first half a negative-going potential scan is chosen, starting from a value where no reduction occurs. A cathodic current begins to increase until a peak is reached as the potential approaches the characteristic *Eo* for the redox process. The direction of the potential sweep is reversed when the potential region where the reduction process has taken place traversed. R which is generated in the forward half cycle is reoxidized back to O and an anodic peak result during the reverse scan. Formation of the diffusion layer near the electron surface causes the characteristic peaks of cyclic voltammogram (Wang, 2000).

Differential Pulse Voltammetry

The aim of pulse voltammetric techniques are lowering the detection limits of voltammetric measurements. Measuring trace levels of organic and inorganic species makes the differential pulse voltammetry very useful technique. Fixed magnitude pulses which are superimposed on a linear potential ramp, are applied to the working electrode at a time just before the end of the drop in differential pulse voltammetry as shown in Figure 2.10.

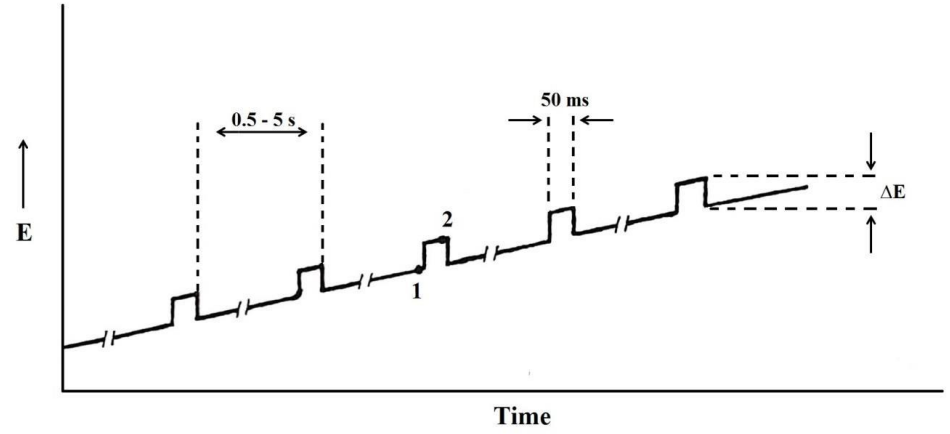

Figure 2.12: Excitation signal of differential pulse voltammetry

In differential pulse voltammetry, the current is sampled twice. One is just before the pulse application (at point 1 in Figure 2.10) and other one late in the pulse life (at point 2 in Figure 10) when the charging current has decayed. The first current is instrumentally subtracted from the second current. This current difference [$\Delta i = i(t2) - i(t1)$] is plotted versus the applied potential. The resulting differential pulse voltammogram consists of the current peaks whose height is directly proportional to the concentration of the corresponding analytes:

$$p = nFAD1/2C\sqrt{\pi tm}\ (1-\sigma 1+\sigma) \qquad (2.3)$$

Where $\sigma = \exp[(nF/RT)\ (\Delta E/2)]$ and ΔE is the pulse amplitude. Large pulse amplitudes obtained from the maximum value of the quotient $(1 - \sigma) / (1 + \sigma)$ which is unity. If species occur near the polarographic half wave potential, they can be identified by using the peak potential (Ep):

$$Ep=E1/2-\Delta E/2 \quad (2.4)$$

The differential pulse process results in a very efficient correction of the charge background current. The charging current contribution to the differential current is negligible. It can be described as:

$$\Delta ic \simeq -0.00567Ci\ \Delta Em2/3t-1/3 \quad (2.5)$$

Where *Ci* is the integral capacitance.

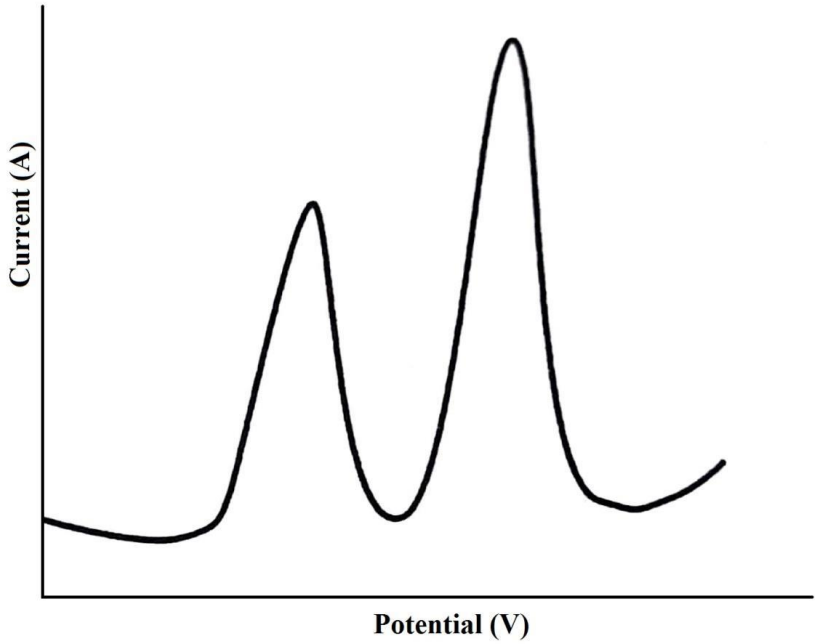

Figure 2.13: A typical differential pulse voltammogram

The selection of pulse amplitude and potential scan rate generally requires a balance between sensitivity, resolution and speed. Large pulse amplitudes cause larger and wider peaks. Pulse amplitudes of 0.025V coupled with a 0.01V/s scan rate are commonly used. Besides the improvement of sensitivity and resolution, information about the chemical form of analyte appear can be provided by differential pulse technique (Wang, 2000).

CHAPTER 3
RELATED RESEARCH

Vitamins are of great importance for human health. We can get most of the vitamins from foods or as supplements. Vitamins are studied in two groups, the first of which are fat-soluble (lipophilic vitamins) and the others are water-soluble (hydrophilic vitamins). Since vitamins have a great role in our lives, researchers are working over the analysis of fat-soluble and water-soluble vitamins by developing / using various chromatographic and electrochemical methods. Some of these studies will be summarized below.

Sys et al. (2016) studied Determination of lipophilic vitamins using adsorptive stripping voltammetry technique. The aim of the researchers in this study was to inspect whether the adsorptive stripping differential pulse voltammetry (AdsDPV) is an appropriate technique for the simultaneous & precise electrochemical determination of lipophilic vitamins. Researchers chose retinol (A_1 vitamin) as the representative fat-soluble vitamin. Due to the lipophilic character of analyte, they performed all electrochemical measurements in two stages. They took the first steps by immersing the backlog of lipophilic vitamins over the Glassy Carbon Electrode (GCE) surface, with dipping each processing vitamin (50.0 μmol. L^{-1}) in aqueous acetonitrile solutions (50%, v / v) at 400 rpm for 5 min. They performed the second steps in the differential pulse voltammetry potential step (Estep) of 5 mV, amplitude potential (Eampl) 25 mV of the accumulated vitamins, interval time (t) inside 0.01 moles of L^{-1} acetate (pH 4.5) buffer, 0.1 s and sweep speeds (ν) 50 $mV.s^{-1}$. The results obtained by the researchers inside this work indicated that the simultaneous determination of some lipophilic vitamins requires much more optimization studies. Accordingly, Sys et al. say that in this study we have to understand first peak position in the simultaneous determination of lipophilic vitamins from whichever chromatographic methods application.

Law et al. (2020) carried out a research on realization of the methods of vitamins A and E on a multiplexer liquid chromatography-tandem mass spectrometry platform to simplify the laboratory workflow. In this study, researchers included the most common approach to vitamin A analysis. These were liquid-liquid extraction and high-performance liquid chromatography (HPLC) analysis. They used liquid chromatography and mass spectrometry (LC/MS) in sample preparation. For, vitamin A analysis, they performed a comparison between LC/MS and HPLC methods by measuring patient samples in the vitamin A

concentration range of 11–81 μg / dL. According to their results, the use of LC / MS downloadable sample analysis time for vitamin A was determined. Thus, this method further optimizes the clinical workflow through the consolidation of previously stand-alone machines.

Lim et al. (2011) realized out improvement of accuracy for the determination of vitamin A in infant formula by isotope dilution-liquid chromatography/tandem mass spectrometry. In this study, the researchers determined the isotope dilution-liquid chromatography / mass spectrometric (ID-LC / MS) technique, this, the nominee reference technique in order to confirm of vitamin A in the baby formula. They discovered this vitamin A is an important fact that prevents the reliability of the measurement results of oxidative degradation in sample preparation processes. Therefore, they are attentively ruminated to minimize the oxidative breakdown of vitamin A with stopping example exposure to the oxygen. They then applied same warnings in order to arrange for calibrations solutions. By using the ID-LC / MS technique, the researchers showed repeatability experiments on the same day be smaller than %1,0 relative standard deviation, as well as with this method they estimated the measurement uncertainty to be less then %1,0. The results of repeatability/reproducibility studies and uncertainty assessment at the end of this study proved that the proposed technique was metrologically sufficient to be used as a reference technique. They used experimental design to minimize sample exposure to the atmosphere in sample preparation processes to develop the performance of other HPLC-based technique for A vitamin analysis.

David et al., (2015) studied voltammetric analysis of B1 and B2 vitamins using a pencil graphite electrode. Because of the importance of vitamins for human health, they advocated the development of new cheap and fast methods to determine. David et al. investigated the voltammetry attitude of vitamins over a lead graphite electrode using cyclic voltammetric inside dissimilar media. In the presence of each other, they made it by quantitatively determining the two vitamins directly with differential pulse voltammetry. B1 was just electroactive inside a NaOH solution that produced two irreversible oxidation peaks; the first peak at 250 mV is finely described as well as used inside quantitative determinations. Inside the status of B6, the researchers identified finely and observed an oxidation peak inside all the supportive electrolytes studied apart from HCL. The deviation limits obtained in their results were 5.34×10^{-6} M and 2.81×10^{-6} M for B1 and B6, in order of. They found how

simple and fast this method they developed and successfully implemented the definition of two vitamins in drugs.

Parvin et al. (2018) actualized to the detection of vitamin B_{12} using a sensitive and selective electrochemical sensor, Au & PPy & FMNPs and TD-modified electrode for analysis. In this study, the researchers constructed a recent electrochemical sensor to precisely and selectively to detect vitamin B_{12} by electropolymerization of pyrrole (Py) inside the entity due to a triazine dendrimer containing ferromagnetic (FM) nanoparticles on the gold electrode (Au). They investigated the efficiency and coaction of Au / PPy / FMNPs with TD with B_{12}, cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS), differential pulse voltammetry (DPV), UV-vis Spectro electrochemistry as well as density functional theory computations. This instrument they created excelled for a large linear range (2.50 nM - 0.5 M), large-scale reproducible reaction (%2.3 RSD), low noise percentage, as well as long-term steadiness due to B_{12}. The researchers applied the structured sensor to B_{12} analysis in food samples, and they found that the modified electrode excelled in detecting B_{12} by a large linear range, high reproducible response, as well as long-term steadiness. This electrochemical instrumental was also tried for other target molecules selected, as well as the conclusion showed that the proposed sensor is only appropriate for B_{12} detection. Additionally, Parvin et al. recommended this electrochemical technique for selected and conflicting species.

Khaleghi et al. (2016) studied the production of new electrochemical sensors to detect the presence of vitamin C in vitamin B9 in food and drug samples. For the voltammetric analysis of vitamin C, they planned to synthesize and apply NiO - multiwalled carbon nanotube nanocomposite and 1-butyl - 3 - methylimidazolium tetrafluoroborate (Bmim [BF4]) in a carbon sensitive matrix. In their analysis, the researchers found that the peak potential due to vitamin C oxidation at the surface due to the NiO & MWCNT carbon paste electrode (NiO & MWCNTs & (Bmim [BF4] & CPE) was at 440 mV. Under a similar condition, a simple carbon paste reduces the oxidation peak potential on the electrode surface by more than about 200 mV. When analyzing vitamin C and B_9, they observed that the oxidation peak stream rise by regarding 3.5 times at the NiO & MWCNT (Bmim [BF4] & CPE) surface class with to the carbon paste electrode. The researcher decided that at the 0.85 V point, Vitamin C was in Vitamin B9. As a result, Khaleghi et al. creatures found that this sensor is suitable for vitamin C analysis in food and drug samples.

Chauhan et al. (2019) developed an effective electrochemical biosensor using gadolinium oxide nanorods functionalized with aspartic acid for the detection of Vitamin D3. The purpose of the researchers in this study was to add gadolinium oxide nanorods (Gd2O3NRs) to the electrochemical biosensor they would use for the analysis and detection of vitamin D3. They first synthesized Gd203NRs hydrothermally and functionalized them by aspartic acid (ASP-Gd203NRs). This acid functionalization can't change the phase, figure, as well as the structure of [Gd2O3NRs]. They then performed on the surface of the ASP-Gd2O3NRs & ITO electrode according to find the blocking of the D3 vitamin monoclonal antibody [Ab-VD] and to identify the D3 vitamin. The researchers then performed the BSA & Ab-VD & Asp-Gd2O3NRs & ITO immunoelectron response study by dissimilar D3 vitamin concentrations using differential pulse voltammetry techniques. Inside the conclusion due to this study, a 0.09 ng mL^{-1} detection limit was obtained, while they obtained a sensitivity worth of 0.35 A ng - 2 mL cm - 2 by a linear range of 15–90 ng mL^{-1} for D3 vitamin.

Anusha et al. (2020) developed and manufactured electrochemical nanosensors using fullerene-C60 as well as bimetallic nanoparticles composite film to detect vitamin D3 in blood cells. They wanted to increase vitamin D to determine the concentration in blood cells, and they recommended fullerene-C60 and copper-nickel for this. Before starting the work, they manually reduced the Fullerene-C60 to GCE by drop-casting technique, as well as CuPs and NiPs left electrochemically in the fullerene-modified GCE. For the detection of vitamin D, a nanocomposite film of reduced fullerene-C60 of CuNPs-NiNPs on GCE was then set with a high dynamic concentration range of 1.25-475μM as well as a detection limit of 0.0025μM. In addition, they evaluated the effect of supporting electrolytes and the solvent composition sensor on the electrochemical performance on various kinetic parameters. According to the results of the researchers, this sensor they created for the detection of vitamin D in clinical and drug samples showed that they could achieve the results they wanted.

Carlucci et al., (2013) studied various analysis methods using surface plasmon resonance and electrochemical affinity biosensors for the detection of vitamin D. In this study, researchers discussed different approaches to D vitamin determination handling affinity-based biosensors. First, they developed an immune sensor situated on SPR transduction for through the detection of D vitamin and obtained a limit of detection of 5 mg/ml. This value obtained was away from the wants of researchers on the clinical determination. Later,

Carlucci and his friends connected 25O HD with Au NPS and determined the amplification of the SPR signal. They then reduced LOD of 2,5 mg / ml, twice the susceptibility. As a result, they resorted to the D vitamin binding protein (VDBP) pathway and developed an alternative method of SPR based on indirect vitamin D determination and delivered it to a 45ng / ml LOD that is really close to constant success. In their results, the researchers performed an electrochemically converted biosensor situated in the reaction of vitamin D with 4-ferrocenylmethyl-1, 2, 4 - triazoline - 3, 5-Dion [FMTAD].

Sys et al. (2017) studied the tocopherol amount of vitamin E in margarine and edible oils. Their system was consisting of a glassy carbon paste electrode as a processing electrode, Ag & AgCl with 1M KCL salt bridge as a reference electrode, as well as platinum wiry as a counter electrode and they, used square wave anodic stripping voltammetry (SWASV) on 0.2 M HNO3 for detection. The method used by the researchers is to use a lipophilic binder of the glassy carbon paste electrode found in silicone oil extraction and biologically active compounds. They determined the linear ranges for α-tocopherol 5×10−7 - 4×10−5 and 5×10−8 - 1×10−5 mol/L with 1.0×10^{-7} & 3.2×10^{-9} mol/L detection limits for 10 & 20 minutes respectively.

Bakre et al. (2014) studied the presence of sunflower in olive oil and used alpha-tocopherol as a discriminator to detect adulteration. They prepared blends of olive oil and sunflower oils of 5%, 10%, 15%, and 20% respectively, and analyzed them using reverse-phase and high-pressure liquid chromatography with a fluorescence detector. Chromatographic system was composed of C18 column by methanol-acetonitrile [50-50] mobile phase. They set the fluorescence detector at 290 nm the excitation wavelength and the emision wavelength at 330 μm. They observed that concentration of α-tocopherol increased linearly in olive oil complicated by sunflower oil. Their technique was basic, picky, sensitive, and exact [RSD = %2,7] for alpha-tocopherol and precisely detected %5 sunflower oil on olive oil.

Diaz et al. (2004) studied the voltammetric analysis of tocopherols found in different vegetable oil samples and vitamin E in olive oil. The researchers used platinum wire as a counter electrode, then Ag & AgCl as a reference electrode. Then they used the glassy carbon electrode as the electrode working in a hexane and ethanol environment. They applied direct current (DC), differential pulse voltammetry (DPV), as well as square wave voltammetry (SWV) methods. After applying these methods, they examined changes in sulfuric acid

concentration, instrumental parameters, and the ratios of hexane and ethanol. Then Diaz et al. obtained separate voltammetric peaks of A-tocopherol and δ-tocopherol while the peak of β-tocopherol and γ-tocopherol overlopped. Finally, they used the PLS multivariate calibration technique, then compared the conclusions of DC & DPV voltammograms. In their results, they announced that they found the best data range for DPV.

Pardakhty et al. (2016) studied the sensitivity of vitamin C in drug samples and a technique based on a nanostructured carbon paste electrode as a sensor for an efficient voltammetric determination. In this study, the researchers found a square wave voltammetric technique for trace analysis of vitamin C. They modified 1-butyl-3-methylimidazolium tetrafluoroborate followed by NiO nanoparticle and carbon paste electrode as the binder. The researchers then used the sensor they developed for quantitative analysis of vitamin C in drug and food samples. The LOD was as low as 0.04 μM in the results. The researchers then studied the electro-oxidation attitude of the vitamin C in the modified electrode. As a result, nanostructured electrode was found to effectively promote electro catalytic oxidation of ascorbic acid.

Luo et al., (2018) designed the N, S doped carbon dot-based fluorescent "on-off" sensor to analyze the presence of vitamin C in fruits. In this study, the researchers changed this sensor based in N and S doped carbon dots (N, S-CDs), N, S-CDs advanced to assign the content of vitamin C in fruits by means due to Fe3 + (closed) fluorescence quenching and recovery with vitamin C. In its results, the sensor allowed vitamin C to be detected with a limit of 4.80 μmol / l on a linear range of 15–100 μmol / l. The researchers then expressed that it is compatible by the reference technique (P> 0.07), which states that it is a practical fluorescent sensor for the detection of vitamin C in fruits detected with this sensor.

Wang et al. (2017) applied a new ratio electrochemical sensor to find the sensitivity of vitamin C. According to the researchers in this study, electron communication features are preferred for nanomaterials broadband range [3.40 eV], major stimulation binding energy [65 eV], toxicity, biocompatibility, chemical & photochemical stability as well as the production of elevated-impact sensors. If the performance of the catalyst system is severely degraded, it is due to the lack of any of these components. So, the use of ZnO / Al_2O_3 nanocomposite as catalytic materials is very important. The researchers then expressed that ZnO / Al_2O_3 / SPE showed benefits in terms of selectivity, repeatability, as well as precision.

CHAPTER 4
MATERIALS AND METHODS

4.1 Materials

4.1.1 Vitamin Standards

In this study, 7 vitamins were used namely A, D, E, B_1, B_6, B_{12}, and C. Vitamin standards as pharmaceutical pure preparats were obtained from drug stores and stored at room temperature figure (4.1).

Figure 4.1: Vitamin samples

Fat-soluble Vitamins

Vitamin A standard of 30.000 I.U. (30 mg/capsule) was supplied by KOÇAK FARMA A.Ş and used for coating pencil graphite lead.

Vitamin D standard of 300.000 I.U. (7,5 mg/ml) was supplied by DEVA HOLDING and used for coating pencil graphite lead.

Vitamin E standard of 200 I.U. (13 mg/capsule) was supplied by KOÇAK FARMA and used for coating pencil graphite lead.

Water-soluble Vitamins

B1+B6 vitamin mixed standard of 33.333 I.U. (100 mg/ampoule) B1 + 33.333 I.U. (100 mg/ml) B6 was supplied by DEVA HOLDING and working solution of B1+B6 mixture was prepared by diluting with PBS. However to figure out B6 peak position

in the mixture, vitamin B6 standard of 94.400 I.U. (50 mg/tablet) was also used supplied by HEALTHAID LTD.

Vitamin B12 standard of 4000 I.U. (1 mg/ampoule) was supplied by DEVA HOLDING and working solution of B12 was prepared by diluting with PBS.

Vitamin C standard of 99% purity was supplied by SIGMA and working solution of vitamin C was (2mg/ml) prepared by diluting with PBS.

4.1.2 Buffer Solutions

Acetate Buffer (ACB) 0.5 M – pH 4.8

a) 28.9 ml of concentrated glacial acetic acid was measured and diluted to 250 ml with distilled water.

b) 10 g of NaOH was dissolved in distelled water and volume was made to 250 ml (1N NaOH).

c) pH of the diluted acetic acid was adjusted top H 4.8 with 1N NaOH by measuring pH meter.

d) 1.68 g of NaCl was added and volüme was made to 1000 ml.

Phosphate Buffer (PBS) 0.1 M – pH 8.0

a) 1.3608 g of monobasic potassium phosphate (KH_2PO_4) was dissolved in distilled water and volume was made to 100 ml.

b) 4.3545 g dibasic potassium phosphate (K_2HPO_4) was dissolved in 250 ml distilled water. Then, 6 ml of monobasic pottasium phosphate (KH_2P0_4) was added into voltammetric flask and stored in a refigerator.

4.1.3 Reference Electrode Cleaning Solutions

Reference electrode was cleaned and refreshed during the analysis of samples. Reagents used in this cleaning process were 3M KCL and 1M HCL. The frit at the top of the reference electrode was supposed to be wet at all times, therefore this electrode was kept in 3M KCL (Figure 4.2). 1 M HCL solution is to clean the silver wire that enables the transmission of electricity and signals inside the reference electrode and also to coat this wire with silver. In addition, this reagent is the liquid inside the reference electrode.

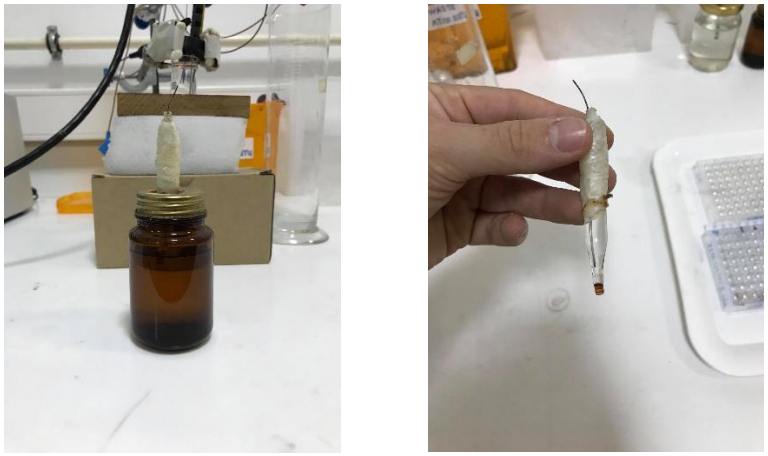

Figura 4.2: Home-made reference electrode (RE)

Preparation of 3M KCL

55.9125 g of KCL is dissolved with 200 ml of distilled water, then poured into a 250 ml volumetric flask and distilled water were added to the mark.

Preparation of 1M HCL

1 M of HCL solution was prepared from concentrated HCL (37%) by using $M_1V_1 = M_2V_2$ formula for calculating the dilution. The HCL of 37% purity corresponds to 12 M. From the formula ‘’12 M x V_1 = 1M x 250 ml’’, V_1 was calculated as 20.8 ml. 20.8ml of 37% HCL was added into 200 ml of distilled water and then brought to the mark.

4.1.4 Equipments

Autolab Potentiostat

Cyclic voltammetric and differential pulse voltammetric measurements were performed using AUTOLAB PGSTAT 204 (Utrecht, The Netherlands) potentiostat with NOVA 2.1.2 software (Figure 4.3). Reference electrode (RE) was an Ag/AgCl with 3M KCl, counter electrode (CE) was platinum wire and working electrode (WE) was pencil graphite lead. 0.5mm Pentel P205 model pencil and Tombow HB 0.5mm pencil leads obtained from bookstore and used as pencil graphite electrode (PGE). Pencil leads are composed of ~65% graphite, ~30% clay, and a binder (wax, resins, or high polymer).

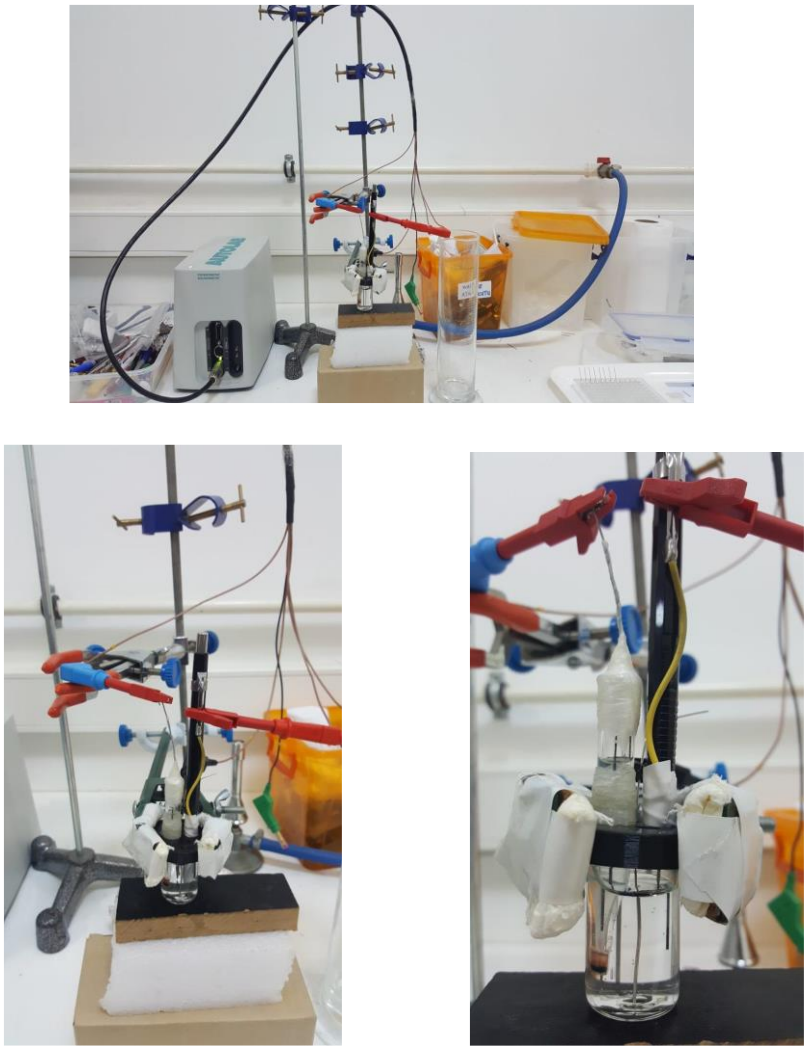

Figure 4.3: AUTOLAB PGSTAT 204 potentiostat and electrodes.

pH Meter, Balances and Glassware

353 ATC pH-meter was used to determine and adjust the pH value of the buffer solutions (Figure 4.4). LA 114 model Digital (110g/0.1mg) balance was used for weighing chemicals. Beakers were used to contain reagents and the samples. Distilled water was used to prepare solutions and clean the equipment.

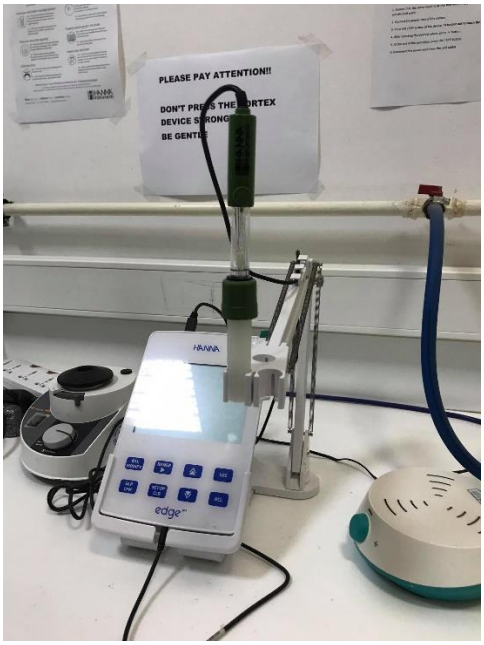

Figure 4.4: pH meter

4.2 Methods

4.2.1 Preparation of Pencil Graphite Electrode (PGE) and Chrono Amperometry

3 cm long graphite pencil tip 0.5 HB was placed in the Rotring Tikky Model pencil which is used as a holder for graphic lead (figure 4.5). One end of pencil tip was connected to a copper wire for electrical contact, and a 1.5cm pencil graphite tip was inserted into the solution to be analyzed while holding the holder upright position to create a short circuit during analysis. Chrono Amperometry was applied to bare PGE at +1.4V for 30 seconds in 0.5M acetate buffer solution (ABS) or 0.1 M phosphate buffer solution (PBS) for activation and cleaning its surface. These leads were then used for vitamin analysis.

Figure 4.5: Pencil graphite electrode (PGE)

4.2.2 Cyclic voltammetric (CV) and Differantial Pulse Voltammetric (DPV) Measurements

The electrochemical behavior of vitamins was examined by using cyclic voltammetry in negative and positive region in acetate buffer or phosphate buffer depending on vitamins types. Cyclic voltammetry (CV) measurements were performed with 1.5 V of upper vertex potential -1.5V of lover vertex potential, 0.005 V of step potential and 10mV/s scan rate.

Based on CV results, selected fat-soluble and water-soluble vitamins, and their calibration standards were analyzed using differential pulse voltammetry techniques in acetate buffer or phosphate buffer systems. Differential pulse voltammograms were performed by potential range -0.2 to + 1.2 V with 0.005 V step potential and 10 mV/s scan rate.

4.2.3 Electrochemical Analysis of Fat-Soluble Vitamins by Coating Method

200 µL of vitamin samples from the working solution into were transfered Eppendorf tubes. PG tips were dipped in the vitamin solution, and kept for 30 minutes. At the end of this period, tips were taken from solution and dried for 30 minutes. These coated and dired tips were used for differential pulse voltammetric detection of fat-soluble vitmains (Figure 4.6). 30 min was chosen as optimum immobilization time from previous studies.

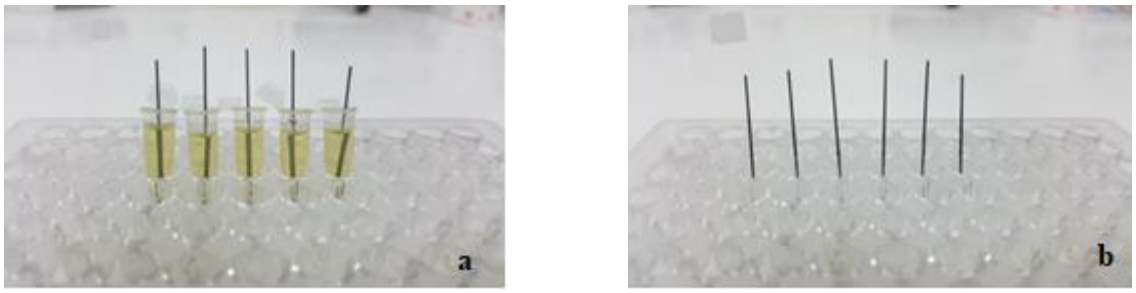

Figure 4.6: Coating (a) and drying after coating of pencil graphite leads (b) with analyte in eppendorf tubes.

4.2.4 Electrochemical Analysis of Water-Soluble Vitamins by Immersion Method

For the voltammetric detection of water-soluble vitamins, working solutions of vitamins were prepared in PBS and oxidation signals of vitamins were measured in this medium

4.2.5 Extraction of Vitamins From Representative Food Samples

We weighed the salmon that we used for the analysis of fat-soluble vitamins on a 10 g analytical scale. Then we crushed the fish and added 100 ml acetate buffer to it and put it to stand by mixing. After mixing well, we filter it with filter paper and put it in a 10 ml beaker. And then these processes were completed, we took it differantial pulse voltammetric analysis (0.1g/ml sample equivalent).

We took 1 ml of the apple juice sample used for water-soluble vitamin analysis into a 10 ml beaker and added 9 ml of phosphate buffer to it. Then we did the pulse voltammetric analysis (0.1g/ml sample equivalent).

Another example of a representative food used for water-soluble vitamin analysis is spinach. 10 grams of sample was taken from this spinach and 100 ml of phosphate buffer was added on it and it was crushed and mixed thoroughly. After mixing the spinach and buffer, we wait and filter it with filter paper and put it in a 10 ml beaker. Then we applied pulsed voltammetric analysis (0.1g/ml sample equivalent).

CHAPTER 5
RESULTS AND DISCUSSION

5.1 Cyclic Voltammetric (CV) and Differential Pulse Voltammetric (DPV) Measurements of Vitamin A, D and E

First, cyclic voltammetry was used to obtain electrochemical fingerprint of each vitamin.

Based on cyclic voltammetry results differential pulse voltammetry measurements of these fat-soluble vitamins were performed in the range of -0.2-1.2V.

5.1.1 CV and DPV of Vitamin A

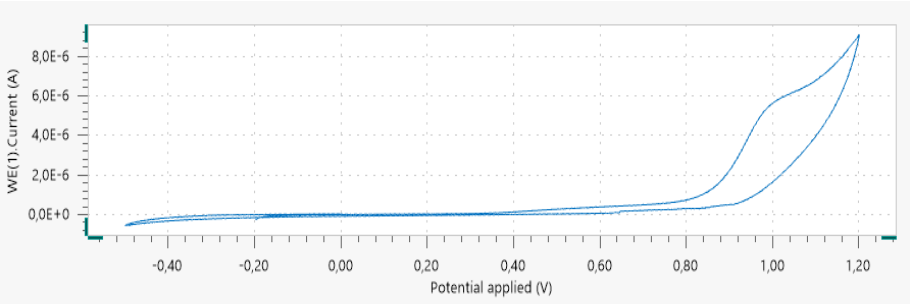

Figure 5.1: Cyclic voltammogram of vitamin A

Characteristic cyclic voltammogram of vitamin A was obtained in acetate buffer medium. As can be seen in Figure 5.1, vitamin A has an oxidation peak at 0.93 V hence, -0.2 – 1.2 V range was selected for DPV measurements of vitamin A.

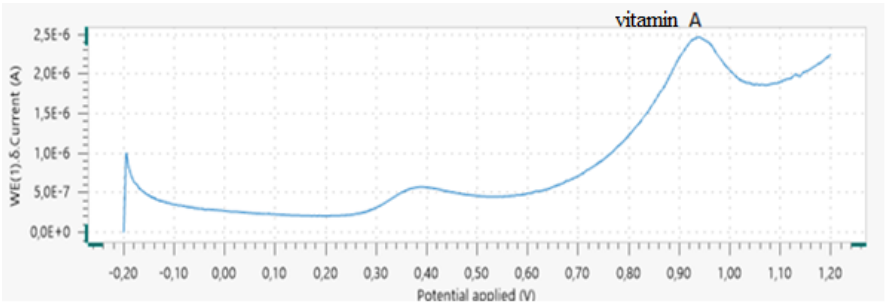

Figure 5.2: Differential pulse voltammogram of vitamin A

The oxidation peaks were measured at 0.48 and 0.93 V. Peak height was recorded as 1.074×10^{-6} A at 0.93 V for vitamin A (Figure 5.2).

5.1.2 CV and DPV of Vitamin D

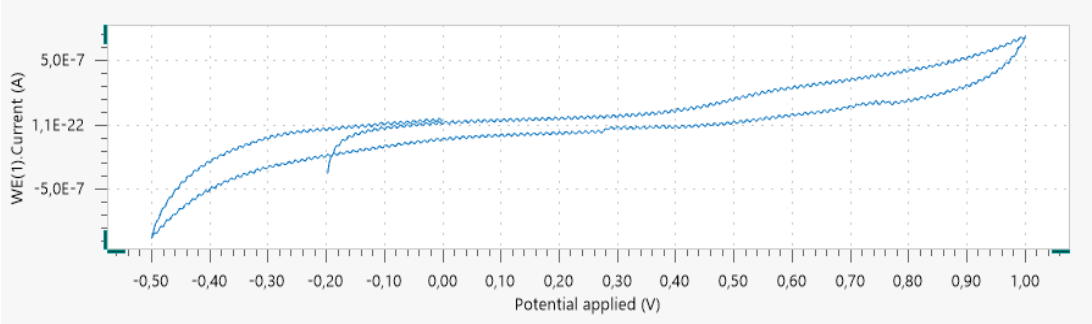

Figure 5.3: Cyclic voltammogram of vitamin D

In the presence of vitamin D in ABS medium, characteristic cyclic voltammogram was obtained. As can be seen in Figure 5.3, vitamin D has an oxidation peak at 0.48 V and -0.2 – 1.2 V range was selected for DPV measurements of vitamin D.

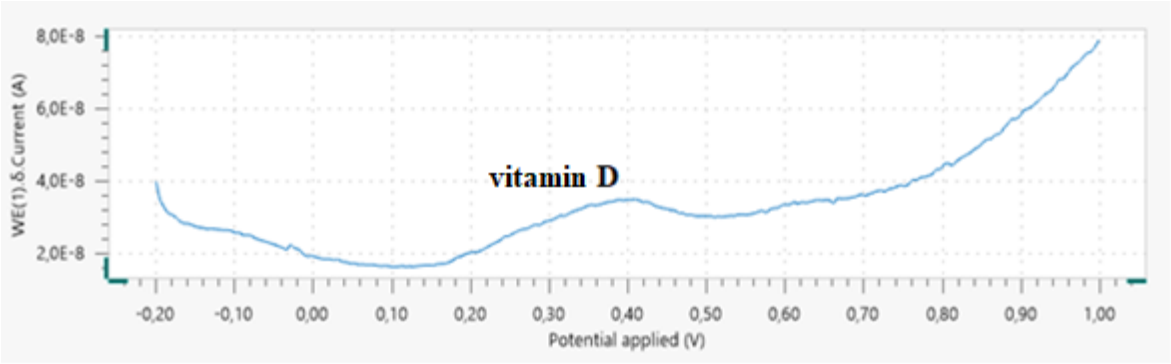

Figure 5.4: Differential pulse voltammogram of vitamin D

The oxidation peak were measured as 0.48 V and peak height $9{,}55 \times 10^{-9}$ A (Figure 5.4).

5.1.3 CV and DPV of Vitamin E

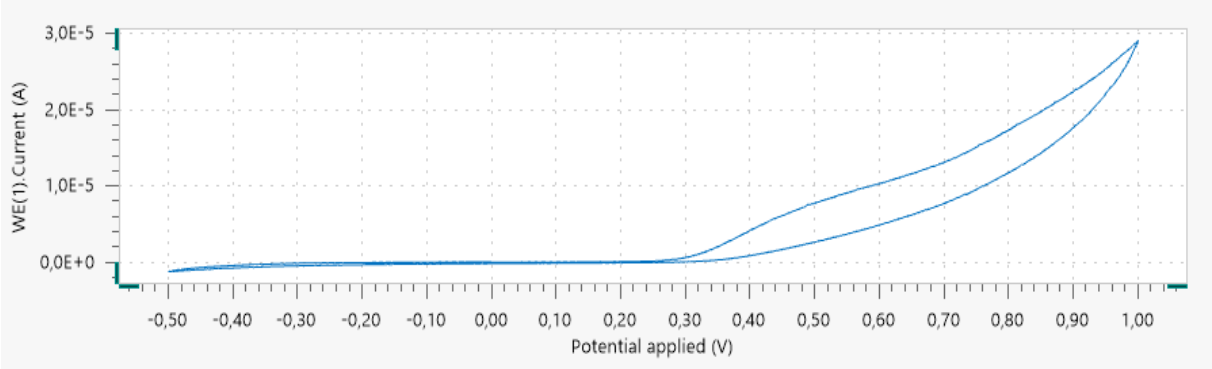

Figure 5.5: Cyclic voltammogram of vitamin E

In the presence of vitamin E in ABS medium, characteristic cyclic voltammogram was obtained. As can be seen in Figure 5.5, vitamin E has an oxidation peak at 0.40 V and -0.2 – 1.2 V range was selected for DPV measure ments of vitamin E.

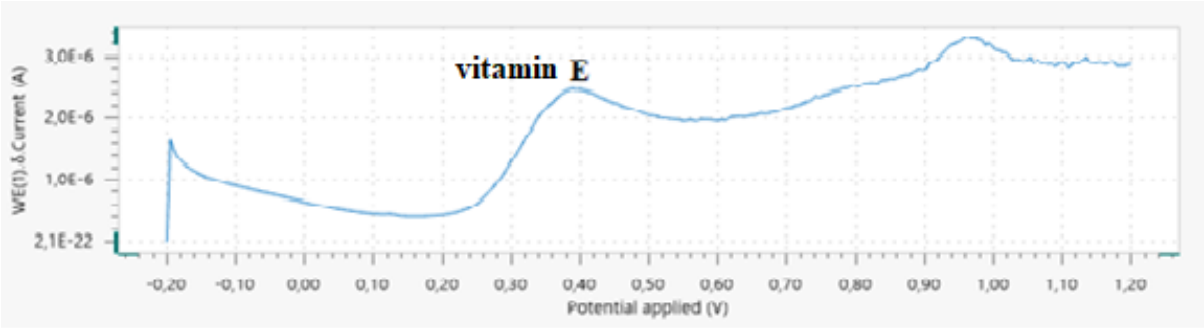

Figure 5.6: Differential pulse voltammogram of vitamin E

The oxidation peak were measured as 0.4 V and peak height 4.8098×10^{-7} A (Figure 5.6).

5.1.4 Simultaneous DPV Measurement of Vitamin A, D and E.

Since vitamin A and D have almost some peak position of 0.50 V by DPV, mixtures of vitamin A and D, mixture of vitamin A and E were analysed in different runs.

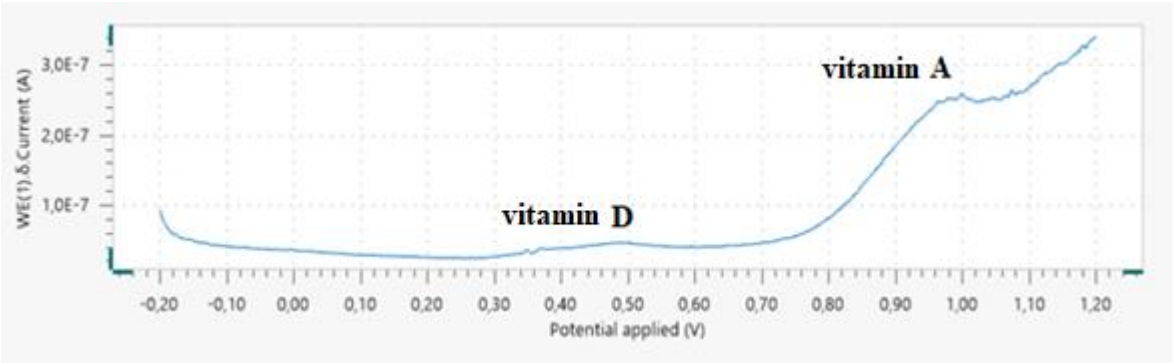

Figure 5.7: Differential pulse voltammogram of the mixture of vitamin A+D

The oxidation peaks of vitamin D and A were measured as 0.48 and 0.93 V respectively. Vitamin D peak height was 1.0279×10^{-8} A at 0.48 V; Vitamin A peak height was 6.4592×10^{-8} A at 0.93 V (Figure 5.7).

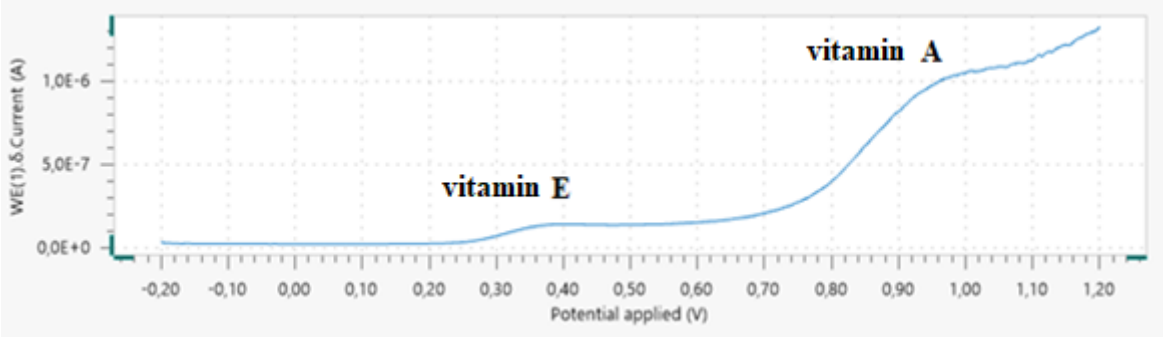

Figure 5.8: Differential pulse voltammogram of the mixture of vitamin A+E

The oxidation peaks of vitamin E and A were measured as 0.4 and 0.93 V respectively. Vitamin E peak height was 6.1819×10^{-8} A at 0.4 V; Vitamin A peak height was 6.4592×10^{-8} A at 0.93 V (Figure 5.8).

5.2 Cyclic Voltammetric and Differential Voltammetric Measurments of Vitamin B1+B6, B12 And C

First, cyclic voltammetry was used for each selected water-soluble vitamin to obtain electrochemical fingerprints.

Based on cyclic voltammetry studies of vitamins above, differential pulse voltammetry measurements were performed in the range of -0.2-1.2V.

Water-soluble vitamins was immobilized onto PGE surface during the analysis.

5.2.1 CV and DPV of Vitamin B6

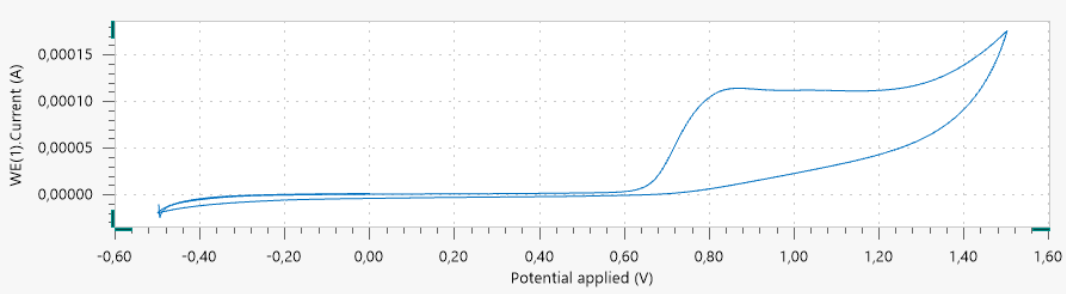

Figure 5.9: Cyclic voltammogram of vitamin B6

Characteristic cyclic voltammogram of vitamin B6 was obtained in PBS medium. As can be seen in Figure 5.9, vitamin B6 has an oxidation peak at 0.70 V therefore, -0.2 – 1.2 V range was selected for DPV measurments of vitamin B_6.

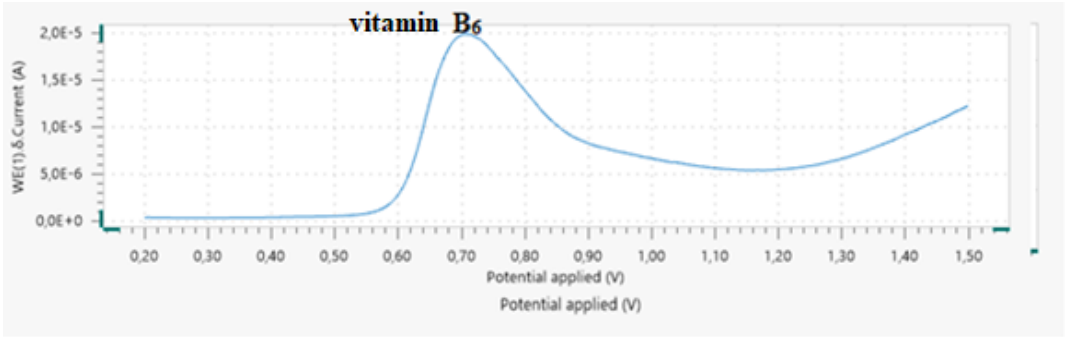

Figure 5.10: Differential pulse voltammogram of B6

The oxidation peak of vitamin B6 were measured at 0.70 V, and peak height was $1.3x10^{-5}$ A (Figure 5.10).

5.2.2 Mixture of B1+B6 Vitamins

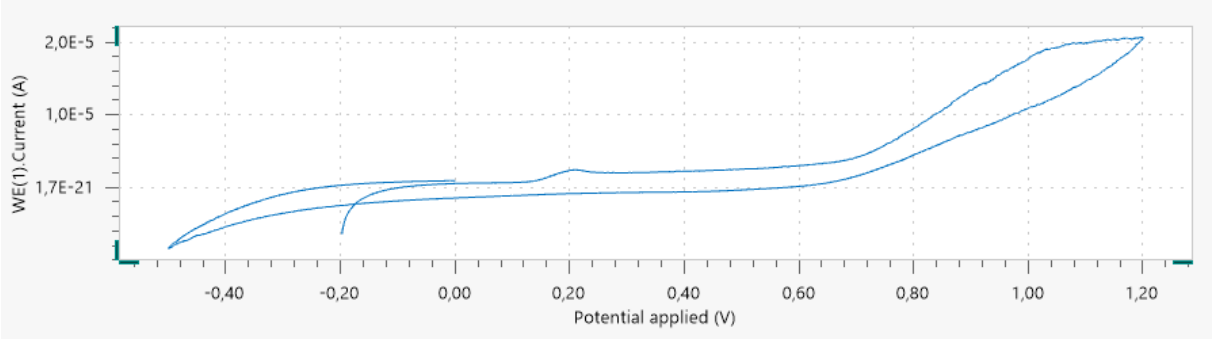

Figure 5.11: Cyclic voltammogram mixture of vitamin B1+B6

In the presence of vitamin B1+B6 in PBS medium, characteristic cyclic voltammogram was obtained. As can be seen in Figure 5.11, vitamin B1 has an oxidation peak at 0.14 V and vitamin B6 has an oxidation peak at 0.70 V based on cylic volttamogram results, -0.2 – 1.2 V range was selected for DPV measurments of vitamin B1+B6.

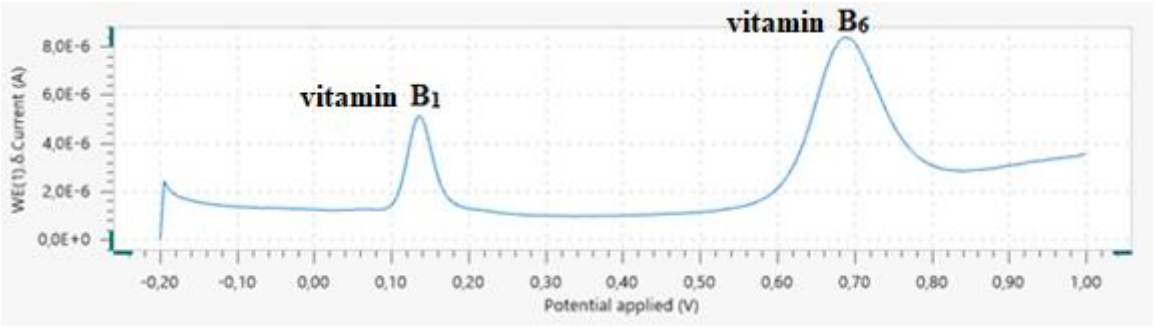

Figure 5.12: Differential pulse voltammogram mixture of vitamin B1+B6

The oxidation peaks were measured as 0.14 V and 0.70 V. Vitamin B1 peak position of 0.14 V and peak height, 8.3×10^{-6} A; Vitamin B6 peak position of 0.70 V and peak height, 1.3×10^{-5} A, respectively (Figure 5.12).

5.2.3 CV and DPV of Vitamin B12

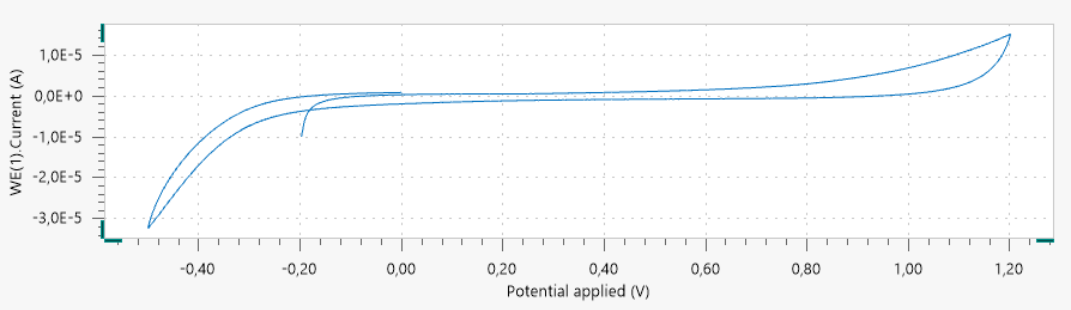

Figure 5.13: Cyclic voltammogram of vitamin B12

Characteristic cyclic voltammogram of vitamin B12 was obtained in PBS medium. As can be seen in Figure 5.13, vitamin B12 has an oxidation peak at 0.14 V therefore, -0.2 – 1.2 V range was selected for DPV measurments of vitamin B12.

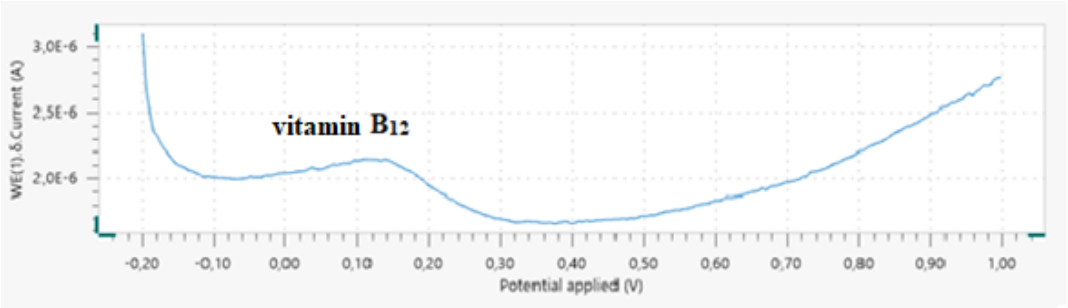

Figure 5.14: Differential pulse voltammogram vitamin B12

The oxidation peak of vitamin B12 were measured at 0.14 V and peak height $1.7x10^{-6}$ A (Figure 5.14).

5.2.4 CV and DPV of Vitamin C

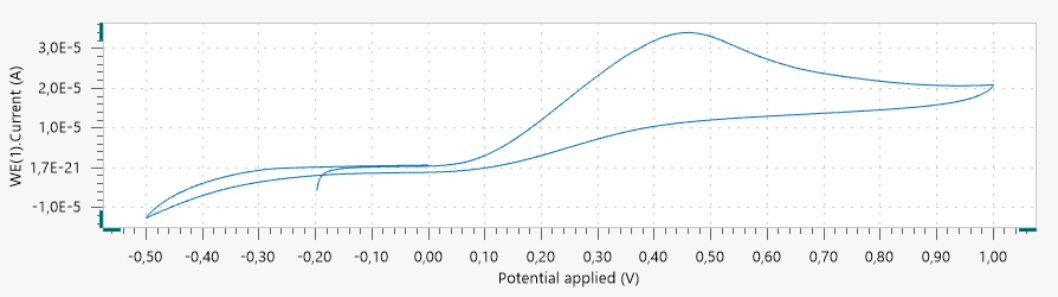

Figure 5.15: Cyclic voltammogram of vitamin C

Characteristic cyclic voltammogram of vitamin C was obtained in PBS medium. As can be seen in Figure 5.15, vitamin C has an oxidation peak at 0.17 V

therefore, -0.2 – 1.2 V range was selected for DPV measurments of vitamin C.

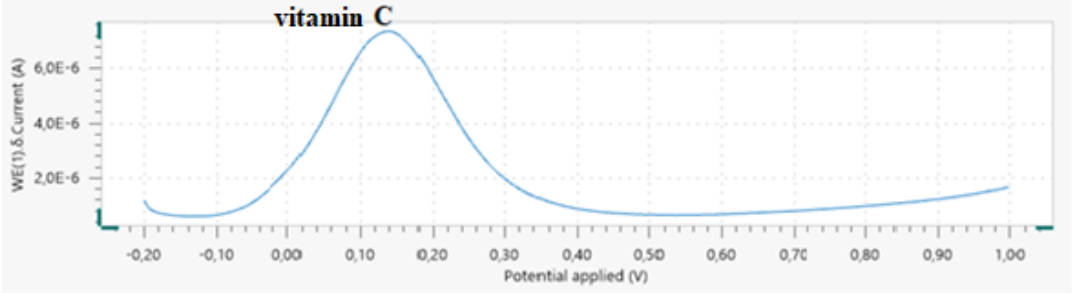

Figure 5.16: Differential pulse voltammogram vitamin C

The oxidation peak of vitamin C were measured at 0.17 V and peak height 4.2×10^{-6} A (Figure 5.16).

5.2.5 Mixture of Vitamins B1+B6+B12

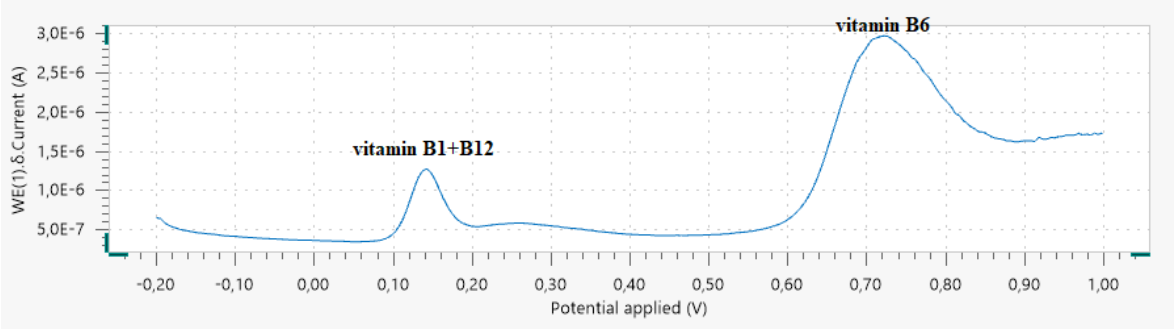

Figure 5.17: Differential pulse voltammogram of the mixture of vitamins B1+B6+B12

The oxidation peaks were measured as 0.14, 0.22, and 0.70 V. Vitamin B1 peak position of 0.14 V and peak height, $8.3x10^{-6}$ A; Vitamin B12 peak position of 0.22 V and peak height $7.4x10^{-7}$; Vitamin B6 peak position of 0.70 V and peak height, $1.3x10^{-5}$ A, respectively (Figure 5.17).

5.2.6 Mixture of Vitamins B1+B6+B12+C

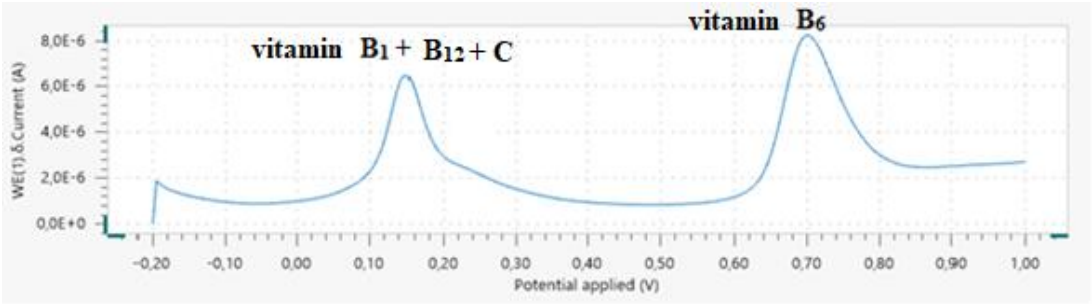

Figure 5.18: Differential pulse voltammogram of the mixture of vitamins B1+B6+B12+C

The oxidation peaks were measured as 0.14, 0.22, and 0.70 V. Vitamin B1 and C peak position of 0.14 V and vitamin B1 and C peak height, $8.3x10^{-6}$ A; Vitamin B12 peak position of 0.22 V and peak height $7.4x10^{-7}$; Vitamin B6 peak position of 0.70 V and peak height, $1.3x10^{-5}$ A, respectively (Figure 5.18).

5.3 Linearity and Limit of Detection (LOD) Values of Vitamin A, D, E, C, B1+B6 and B12

In method development studies, linearity and LOD are significant parameters to be determined. Using the calibration curves is an easy way to estimate the linearity and LOD. In the present of study, we determined linearity for vitamin A, D, E, C, B1+B6 and B12 through calibration experiments and LOD values from calibration curve by using a template. The coefficient of correlation (R^2) values of both studies were found to be 0.999. The LODs and coeffients of correlation for other vitamins studied have been summarized in Table 5.1. Calibration curves of vitamin A, D, E, C and B1, B6, and B12 can be seen in Figures 5.19, 20, 21, 22, 23, 24, 25.

Table 5.1: LOD and coefficient of correlation (R^2) values of vitamins A, D, E, C, B1, B6, B12.

Vitamin	**Peak Position (V)**	**Correlation Coefficient (R^2)**	**Limit of Detection (µg/ml)**
A	0.93	0.998	19.6
D	0.48	0.999	4.0
E	0.40	0.999	32.0
C	0.17	0.999	548.7
B1	0.14	0.999	5.7
B6	0.70	0.998	5.0
B12	0.14	0.999	4.6

LOD and coefficient of correlation (R^2) values for vitamin C had been determined in previous studies of our Department. The LOD of vitamin C in previous study (Ashel, 2019), and in our study was same as 548.7 µg/ml.

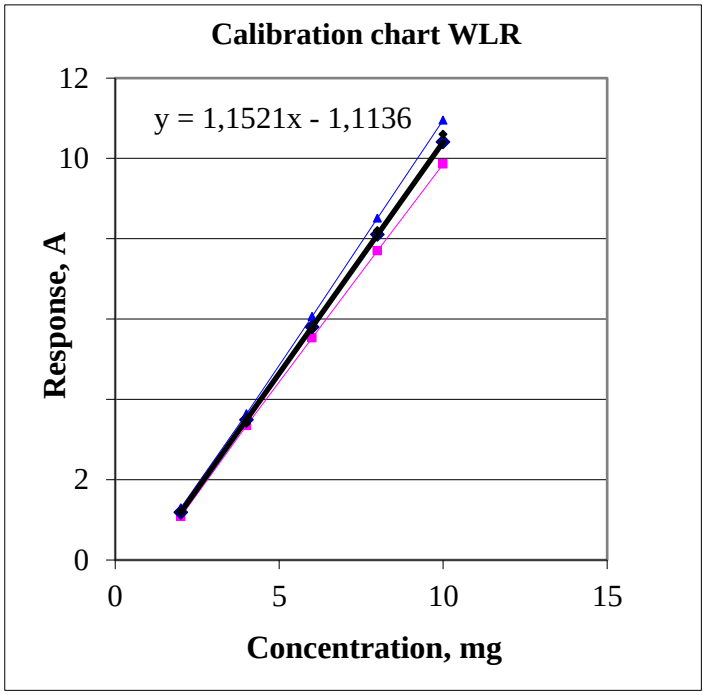

Figure 5.19: Calibration curve of vitamin A

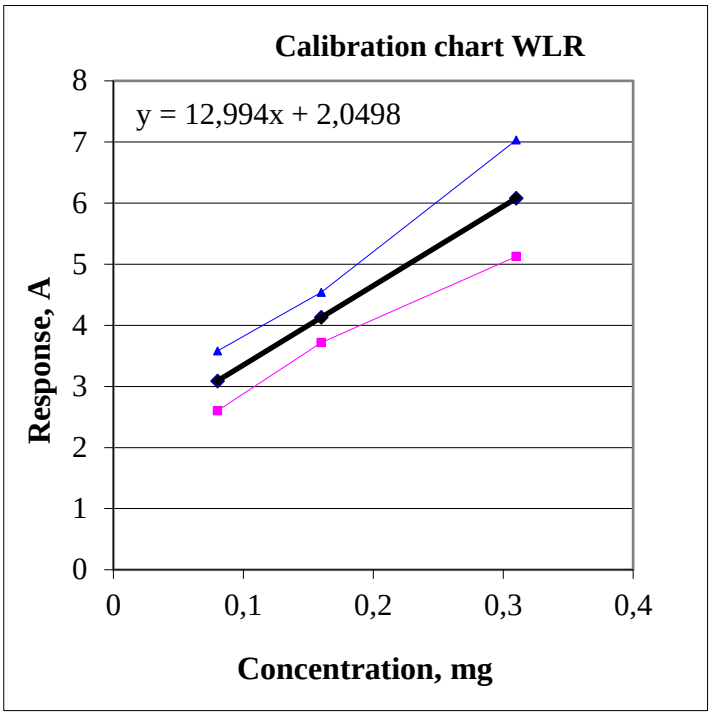

Figure 5.20: Calibration curve of vitamin D

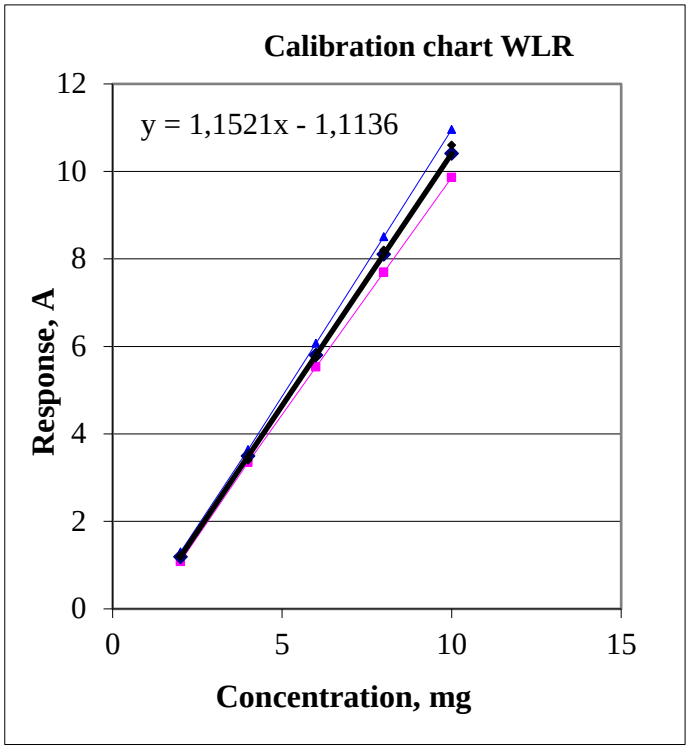

Figure 5.21: Calibration curve of vitamin E

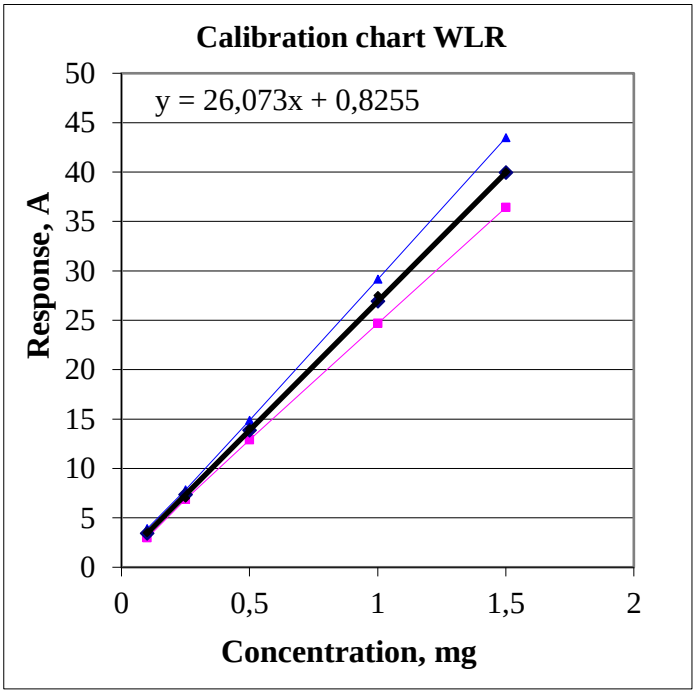

Figure 5.22: Calibration curve of vitamin C

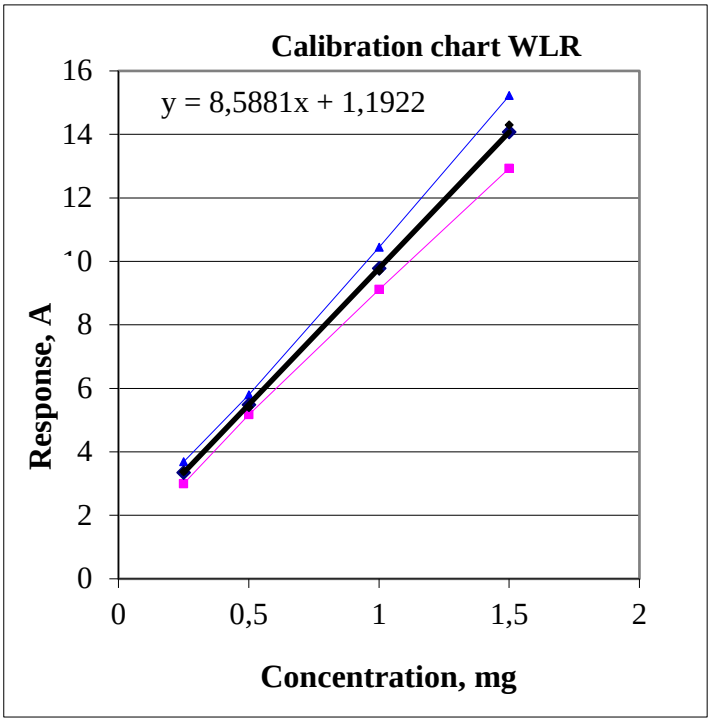

Figure 5.23: Calibration curve of vitamin B1

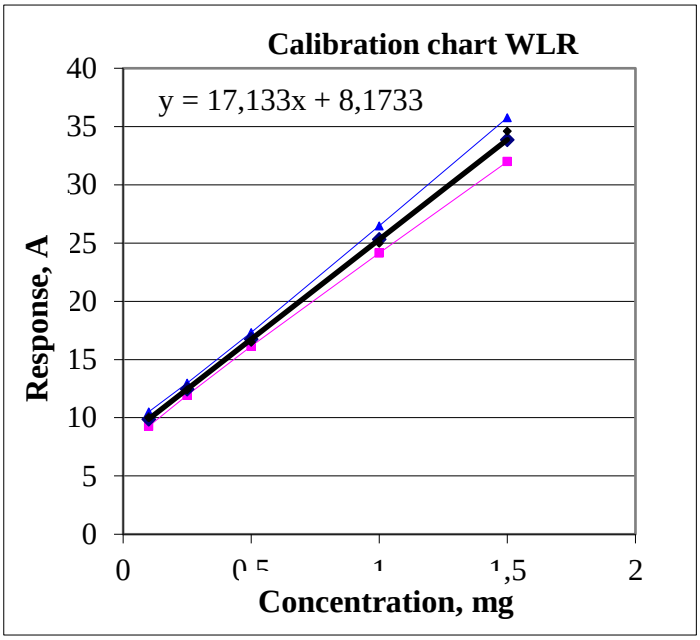

Figure 5.24: Calibration curve of vitamin B6

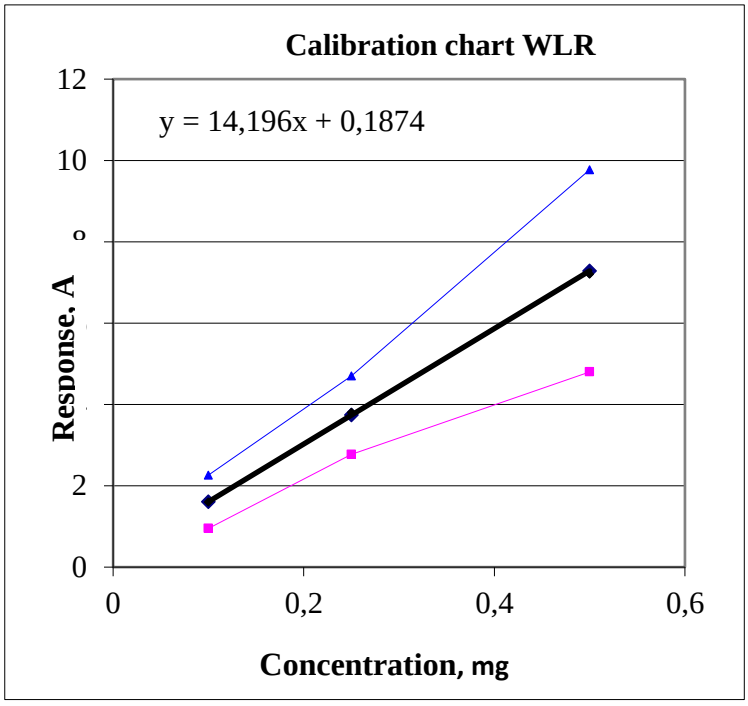

Figure 5.25: Calibration curve of vitamin B12

5.4 Electrochemical Vitamin Analysis in Selected Reprasentative Food Samples

Clear apple juice was selected as a food sample for representing low level matrix effect to determine water-soluble vitamins, and spinach for representing complex food samples.

Salmon was selected as representative food sample for fat-soluble vitamin determination.

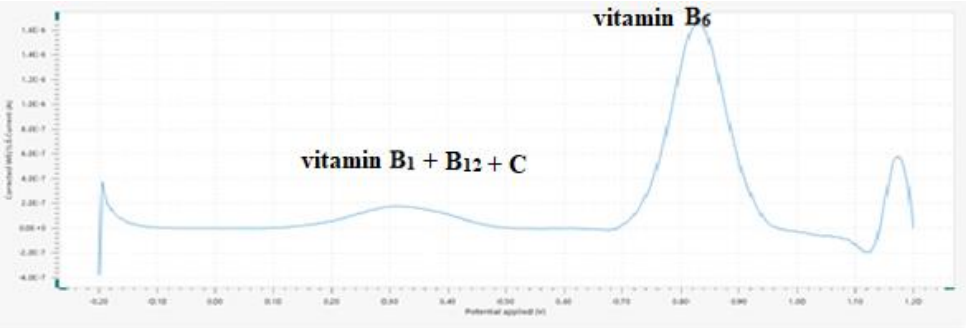

Figure 5.26: Differential pulse voltammogram of clear apple juice.

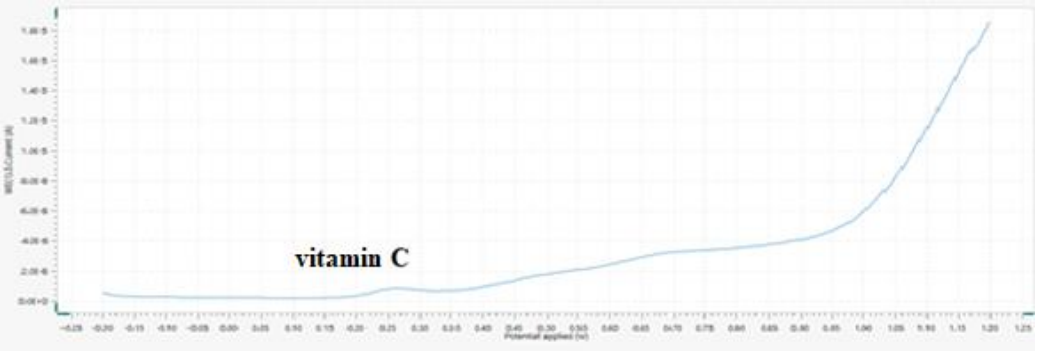

Figure 5.27: Differential pulse voltammogram of spinach.

As can be seen in Figure 5.25, two peaks were observed in apple juice sample at the peak position of vitamin B1, B12 and C and vitamin B6. However no further attempt was made for quantitation. In spinach sample, responses for expected vitamin peaks were lower than those in apple juice (Figure 5.26).

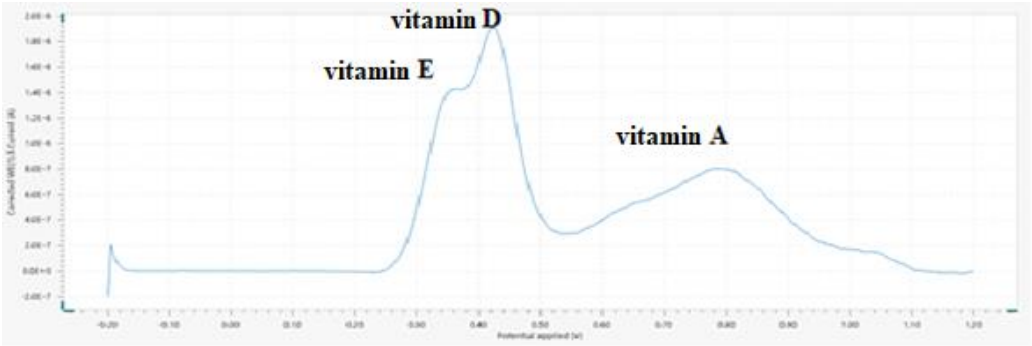

Figure 5.28: Differential pulse voltammogram of salmon fish.

As can be seen Figure 5.27, three peaks were observed in salmon fish at the peak position of vitamin A, D, and E. However no further attempt was made of quantitation.

CHAPTER 6
CONCLUSION AND RECOMMENDATIONS

A rapid, cost-effective and sensitive voltammetric determination of some fat and water soluble vitamins was performed by using pencil graphite-based ‘’single use’’ sensor. Method was also applied for vitamin detection in some representative food products such as apple juice, spinach and salmon..

As can be seen in Table 5.1, resolution between vitamin D and E peaks was not satisfactory, therefore they cannot be determined quantitatively. However vitamin A and D or vitamin A and E can be detected in a single run.

Similarly, vitamin C, B1, and B12 peaks are also overlapping. In simultaneous analysis of water-soluble vitamins by using differential pulse voltammetry with PGE can only be promising technique for the determination of vitamin C and vitamin B6 or vitamin B1 + B12 and B6. If food or drug samples are containing vitamin C, B1, B12 and B6 only B6 can be analyzed quantitatively, while others can be analyzed qualitatively.

Our recommendations based on our research results would be as following:

1. Other electrochemical techniques including various biosensors may be tried.
2. For determination of vitamins in food commodities, further method validation studies may be carried out.
3. Comparative studies by using electrochemical and other conventional techniques can be carried out to confirm results in quantitative analysis of vitamins.

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APPENDICES

APPENDIX I

APPENDIX 1: ETHICAL APPROVAL DOCUMENT

ETHICAL APPROVAL DOCUMENT

Date:14/01/2021

To the Graduate School of Applied Sciences

For the thesis 63roject entitled as "**ELECTROCHEMICAL DETERMINATION OF SOME WATER SOLUBLE AND FAT SOLUBLE VITAMINS BY USING PENCIL GRAPHITE ELECTRODE**", the researchers declare that they did not collect any data from human/animal or any other subjects. Therefore, this 63roject does not need to go through the ethics committee evaluation.

Title: Assist. Prof. Dr.

Name Surname: Perihan ADUN

Signature:

Role in the Research Project: Supervisor

APPENDIX 2

APPENDIX 2: Similarity Report