UNIVERSITI SAINS MALAYSIA

UNIVERSITI SAINS MALAYSIA

POLAROGRAPHIC DETERMINATION OF ASCORBIC ACID IN ROSELLE JUICE SAMPLES

DISSERTATION SUNMITTED IN PARTIAL FULFILLMENT FOR THE DEGREE OF BACHELOR OF SCIENCE (HONS) IN FORENSIC SCEINCE

ROSILA BINTI OMAR

SCHOOL OF HEALTH SCIENCES
UNIVERSITI SAINS MALAYSIA
HEALTH CAMPUS
16150, KUBANG KERIAN, KELANTAN, MALAYSIA

2015

UNIVERSITI SAINS MALAYSIA

Polarographic determination of ascorbic acid in roselle juice samples

Dissertation submitted in partial fulfillment for the Degree of Bachelor of Science (Health) in Forensic Science

Rosila binti Omar

School of Health Science

Universiti Sains Malaysia

Health Campus

16150 Kubang Kerian

Kelantan

Malaysia

2015

ACKNOWLEDGEMENTS

In the name of Allah the most Gracious and Merciful, I would like to express my gratitude to Allah for giving me strength and guidance to complete this research. A special thank you goes to my supervisor, Prof Madya Mohamad Hadzri bin Yaacob for his patience and the supervision given throughout the research.

I would like to express my sincere thanks and appreciation to Encik Zulkhairi bin Othman for his invaluable guidance and knowledge in order to use the voltammetry machine for polarography technique throughout the researach.

I would like to show my deepest appreciation to my collegues especially Nor Fatin Ayuni binti Mohamad Nor, Nurzahirah binti Mohd Rashid, Oo Kee Lian and Siti Zaharah binti Md Isa for their support and help.

Last but not least, I would like to thank my family members for helping me to find the roselle plant. Thanks also for their encouragement, support, patience and understanding

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

Ag/AgCl	Silver-silver chloride
CL	Chemiluminescence
DCIP	Dichlorophenoliodophenol
DHA	Dehydroascorbic acid
DME	Dropping mercury electrode
DOA	Department of Agricultural
EDTA	Ethylenediaminetetraacetic acid
FAMA	Federal Agricultural and Marketing Authority
Fe	Ferum
FIA	Flow injection analysis
FID	Flame ionization detector
FI-CL	Flow injection chemiluminescence
g	grams
GC	Gas chromatography
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
IA	Adequate intake
IOM	Institute of Medicine
IUPAC	International Union of Pure and Applied Chemistry
KCl	Potassium chloride
LC	Liquid chromatography
M	Mol (molarity)
mg	Miligrams
ml	Mililitres
NaOH	Sodium hydroxide

ND	Not detected
OPD	ortho-phenylenediamine
RDA	Recommended dietary allowance
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
TLC	Thin layer chromatography
UL	Tolerable upper intake level
UV	Ultraviolet
UVD	Ultraviolet detector

ABSTRAK

Asid askorbik yang juga dikenali sebagai vitamin C tidak boleh disintesis oleh badan manusia. Oleh itu, adalah penting untuk memperoleh vitamin C daripada sumber-sumber lain seperti jus rosel. Pengambilan harian yang disarankan bagi vitamin C ialah 60 mg/ hari. Teknik polarografi telah digunakan untuk menentukan kuantiti vitamin C dalam sampel jus rosel. Parameter yang digunakan dalam teknik ini adalah seperti berikut: keupayaan awal: -0.0499 V, keupayaan akhir: 0.1299 V, keupayaan pengumpulan: 0.55 V, masa pengumpulan: 15 s, masa keseimbangan: 10 s, langkah voltan: 0.005951 V, masa langkah voltan: 0.6 s, kadar imbasan: 0.0099 V/s, voltan denyut: 0.05 V, and masa denyut: 0.04 s. Electrolit yang digunakan ialah 10 ml air nyahion dan 1 ml buffer asetat pH 4.64. Puncak keupayaan bagi vitamin C ialah pada 0.063 V. Tiga daripada lima jus rosel komersil memberikan keputusan yang positif untuk kandungan vitamin C. Bagi analisa untuk sampel mentah seperti kelopak rosel dan daun, didapati daun mengandungi kandungan vitamin C lebih tinggi berbanding kelopak. Sebagai kesimpulan, teknik polarografi telah berjaya digunakan bagi menentukan kandungan vitamin C dalam sampel jus rosel.

ABSTRACT

Ascorbic acid which is also known as vitamin C cannot be synthesised in human body. Hence, it is important to get the vitamin C from other source such as roselle juice. The recommended daily intake of vitamin C is 60 mg/day. Polarography technique has been used to determine the concentration of vitamin C in the roselle juice samples. The parameters for polarography technique for ascorbic acid determination are as follows: start potential: -0.0499 V, end potential: 0.1299 V, deposition potential: 0.55 V, deposition time: 15 s, equilibrium time: 10 s, voltage step: 0.005951 V, voltage step time: 0.6 s, sweep rate: 0.0099 V/s, pulse amplitude: 0.05 V, and pulse time: 0.04 s. The electrolytes used were 10 ml deionised water and 1 ml acetate buffer with pH of 4.64. The peak potential of ascorbic acid was found at 0.063 V. From five commercial roselle juice samples, three of them give a positive result on the ascorbic acid content. In the analysis of raw samples, it was found that the leaves have higher levels of vitamin C compared to calyces. As the conclusion, polarography technique was a successful applied for the determination of ascorbic acid in commercial roselle juice samples and raw samples such as roselle calyces and leaves.

CHAPTER 1

INTRODUCTION

1.1 Roselle

1.1.1 Roselle as general

The scientific name of roselle is *Hibiscus sabdariffa* and it belongs to the family of Malvaceae (Halimatul *et al.*, 2007). The name of roselle depends on the place such as in English-speaking regions, it is known as rozelle, sorrel, sour-sour, Jamaica sorrel, Guinea sorrel, Lemon bush, Jelly okra and Florida cranberry. In the Near East, it is called *karkade* and in India it is called as *Gongura, Patwa, Pundi, Lal-ambadi, Polechi* and *Yerra gogu* (Mahadevan *et al.*, 2008).

In Malaysia, commercial planting of roselle started in 1993 in Terengganu following the promotion by the Department of Agricultural (DOA) (Mohamad *et al.*, 2009). The roselle plant is believed to be brought in from India to our country (Halimatul *et al.*, 2007). The plant consists of stems, leaves, calyces and seeds. Seeds are suitable to be planted at the beginning of the monsoon season since during the first three to four months of growth, the plant requires average rainfall of 130 to 250 mm (Mohamed *et al.*, 2012).

To strengthen the roselle industry in Malaysia, DOA has launched a special programme to promote collaboration with the Federal Agricultural and Marketing Authority (FAMA). As a result, processing, expanding and marketing of roselle products for local market mainly dominated by small companies. However, the export market has not been fully explored.

The roselle plant is not so tall. The average height of the plant is about 3.5 m. The stems of the plant are smooth, cylindrical and dark green to dark red in colour. The colour of the stem depends on the seed source (Mohamed *et al.*, 2012). Roselle plant has alternate green with reddish veins and long or short petioles leaves with 7.5-12.5 cm long. Each calyx of roselle consists of five large sepals and the calyx is red in colour (Mahadevan *et al.*, 2008). Figure 1.1 shows the roselle plant.

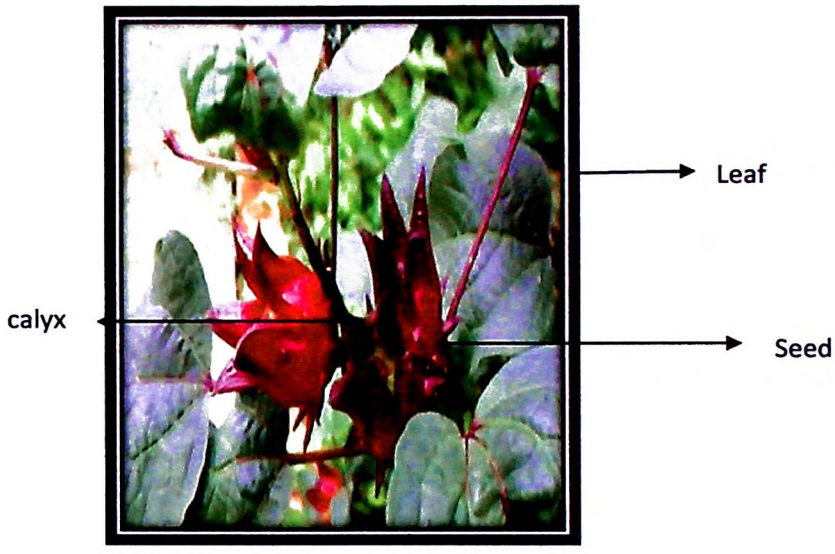

Figure 1.1 : Roselle plant

Roselle plant is suitable to be planted in the soil that is well drained. It can be harvested after the seeds have been ripen that is about five months after planting (Mohamed *et al.*, 2012). Roselle juice or concentrate is produced from fresh-harvested calyces although in many other countries produce roselle tea from dried calyces. Pure roselle juice has bitter taste therefore, many people preferred to drink roselle-fruits juice (Mgaya-Kilima *et al.*, 2014).

Roselle is suitable to be prepared as juice because it is rich in vitamin C and anthocyanin that are good for health. Roselle plants can aid in many medical applications. It helps to treat hypertension, pyrexia and liver damage (Mohamed *et al.*, 2012).

Futhermore it can help to reduce weight. The sepals are rich in protocatechuic acid and hence effective in the treatment against leukemia. Roselle juice can also be used as a remedy for cancer. Others medical applications of roselle are such as help in reducing fever, promoting kidney function, and clearing blocked nose (Mohamed *et al.*, 2012). Traditionally, the roselle plants are consumed as antiseptic, astringent, sedative and refrigerant (Mahadevan *et al.*, 2008). Table 1.1 shows the components of roselle plant and its content.

Table 1.1 : Components of roselle plant and its content (Mahadevan *et al.*, 2008)

COMPONENT	CONTENT
Leaves	Protein, fat, carbohydrate,fibre, iron, thiamine, ribiflavin, niacin, ascorbic acid
Calyces	Ascorbic acid, proteins, minerals
Seeds	Protein, unsaturated fatty acid, dietary fibre, minerals, steroids, tocopherols

1.2 Ascorbic Acid

1.2.1 Ascorbic acid in general

Ascorbic acid generally known as vitamin C has a chemical formula of $C_6H_8O_6$. It was first isolated by Albert Szent-Gyorgyi in 1928 from the adrenals and citrus fruits. He called it as hexuronic acid because it has six carbon atoms and acidic. In nature vitamin C is a strong reducing agent and is easily oxidises to dehydroscorbic acid (Hacisevki, 2009). Most mammalian species can produce their own vitamin C because they have an enzyme called gulanolactone oxidase that is essential for the synthesis of ascorbic acid immediate precursor which is 2-keto-1-gulanolactone. However, human and some primate, and guinea pigs cannot synthesize ascorbic acid due to the absence this enzyme (Kumar and Rizvi, 2012).

Since vitamin C is an essential vitamin, hence it is need to be part of our diet. It is important to determine the concentration of ascorbic acid in order to know whether it consumption will fulfill the recommended daily intake of 60 mg/day (IOM, 2000) and also important to our health (Sarkiyaki and Ikoida, 2010).

1.2.2 Chemistry of ascorbic acid

The IUPAC name for ascorbic acid is 2,3-didehydro-L-theo-hexano-1,4-lactone (Hingdon and Frei, 2002). Figure 1.2 shows the chemical structure of ascorbic acid.

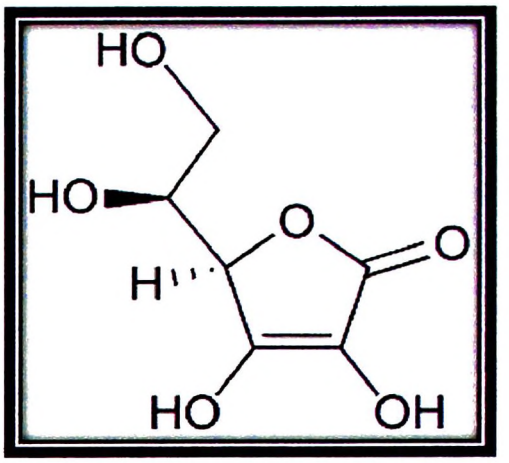

Figure 1.2 : Chemical structure of ascorbic acid

Ascorbic acid is an electron donor. Most physiological and biochemical actions of ascorbic acid are due to its role as electron donor (Padayatty *et al.*, 2003). Other than that, ascorbic acid is also an antioxidant because it prevents other compounds from being oxidise by donating its electrons. However, ascorbic acid itself is oxidised during the process of electron donation (Padayatty *et al.*, 2003). Figure 1.3 shows the oxidation process of ascorbic acid.

In human, all the ascorbic acid that is oxidised does not recover because there is only partial reduction back to ascorbic acid. Some of the dehydroascorbic acid (DHA) is metabolised by hydrolysis and is lost.

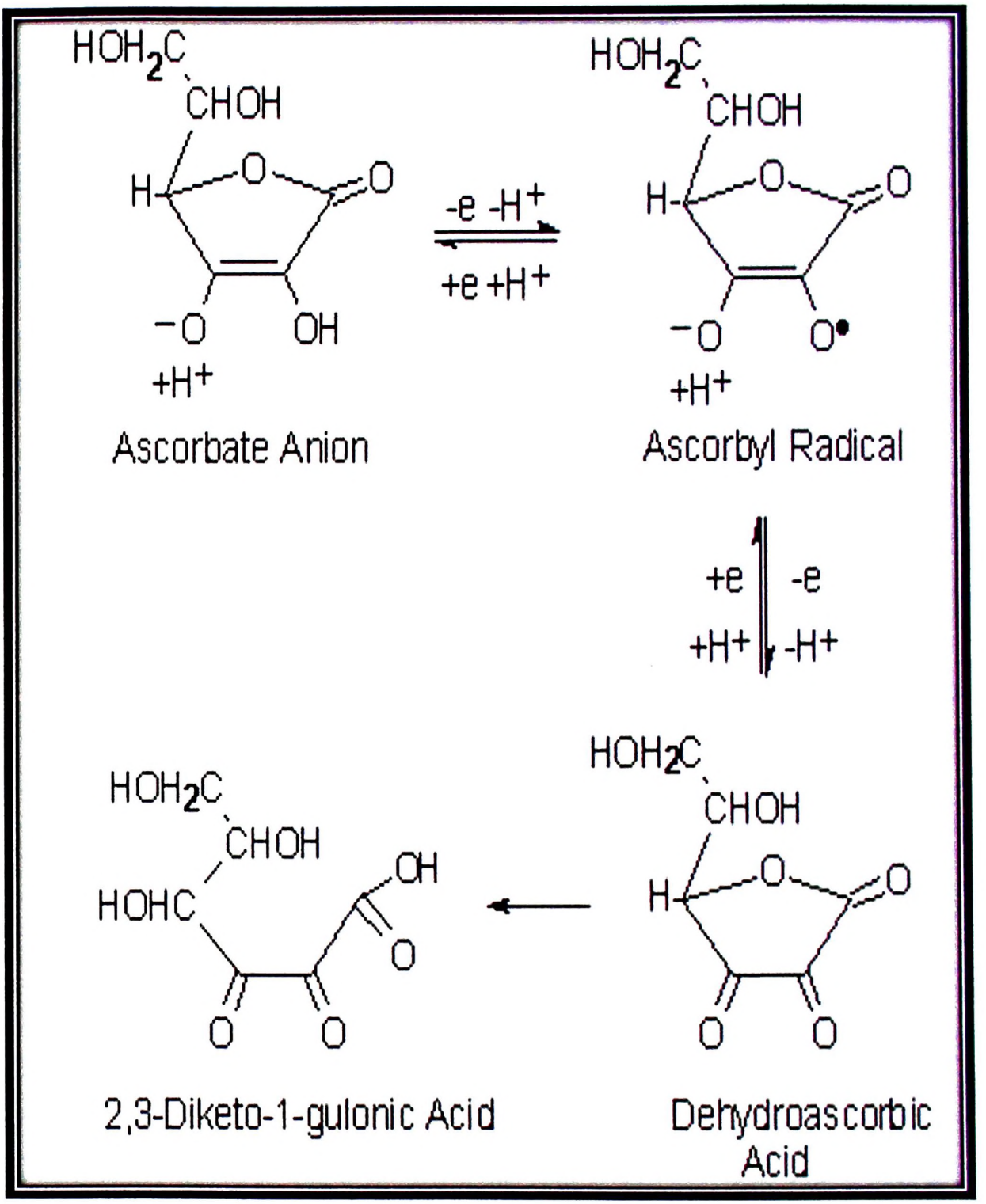

Figure 1.3 : Oxidation process of ascorbic acid.

1.2.3 Physical and chemical properties of ascorbic acid

The physical and chemical properties of ascorbic acid are as shown in Table 1.2

Table 1.2 : Physical and chemical properties of ascorbic acid

Chemical names	L-ascorbic acid, ascorbic acid, 2,3-didehydro-L-theo-hexono-1,4-lactone,3-ketp-L-gulofuranolactone.
Synonyms	Vitamin C
Chemical formula	$C_6H_8O_6$
Formula weight	176.13 g/mol
Color	White to slightly yellow
Odor	Odorless
Taste	Acid, sharp, pleasant
Physical state	Crystalline powder
Melting point	190°C with decomposition
Solubility	Freely soluble in water Sparingly soluble in ethanol Insoluble in ether
Density	1.65 g/cm^3
Condition of instability	Heat, light, air, action of oxidising agent and metal ions Resistance to freezing
Corrosivity	Non-corrosive in the presence of glass
Oxidation	Oxidised well by air oxygen

1.2.4 Biological function of ascorbic acid

1.2.4.1 Vitamin C as an antioxidant

An antioxidant is a substance which helps in neutralising free radicals by preventing the oxidation of substrates (Halliwell and Gutteridge, 1999). Vitamin C interacts enzymatically and non-enzymatically with damaging oxygen radicals and their derivatives. Vitamin C is a good antioxidant because the ascorbyl radical enables it to react with and reduces virtually all physiologically reactive oxygen species (ROS) such as superoxide, singlet oxygen and hydrogen peroxide and reactive nitrogen species (RNS) (Buettner, 1993). ROS are able to initiate cascade reactions that can lead to the production of hydroxyl radicals. The hydroxyl radical can cause protein damage, lipid peroxidation, DNA damage and cell death (Davey *et al.*, 2000).

Vitamin C plays a role in neutralizing free radicals by working in both inside and outside the cells to prevent free radical damages. Free radicals need electron pair in order to regain their stability (Kumar and Rizvi, 2012). Other than that, vitamin C also protects the DNA of the cells from the damage done by free radicals and mutagens (Du *et al.*, 2012).

Vitamin C also helps to prevent free radical damage in the lungs and central nervous system (Eerhard *et al.*, 1989). Vitamin C also plays a role as co-antioxidant in the regeneration of α-tocopherol from α-tocopheroxyl radical. The function of α-tocopherol is as prooxidant in the absence of co-antioxidant (Upston *et al.*, 1999).

1.2.4.2 Vitamin C in dietary iron absorption

Vitamin C is able to maintain metal ions in its reduced state. This is due to the function of monooxygenase and dioxygenase (Burri and Jacob, 1997). The reduction of iron by vitamin C prevent the formation of insoluble complexes with phytate and other ligands (Levine *et al.*, 1999).

Other than that, vitamin C reduces Fe^{3+} to Fe^{2+} for nonheme iron sources. This may increase the iron absorption (Goswami *et al.*, 2002). Vitamin C also acts as prooxidant *in vitro* in the presence of redox-active iron. This will contribute to the formation of hydroxyl radical and eventually lead to lipid, DNA or protein oxidation (Samuni *et al.*, 1983).

1.2.5 Excretion of ascorbic acid

Since vitamin C is a water soluble vitamin, it primarily excreted through urine (Hingdon and Frei, 2002). Ascorbate is filtered by the glomerulus in the kidney and reabsorbed by sodium dependent ascorbate transporters in the proximal tube (Rumsey and Levine, 1998). Ascorbate from intakes $\geq$ 500 mg/day will be excreted through the urine within 24 hours (Levine *et al.*, 2001). Renal reabsorption of ascorbate is saturable and human plasma ascorbate concentrations appear limited by the capacity (Hingdon and Frei, 2002). However, no information is available about the renal excretion and reabsorption of dehydroascorbic acid (DHA) because DHA is undetected in the plasma (Rumsey and Levine, 1998).

1.2.6 Health aspects of ascorbic acid

1.2.6.1 Vitamin C and cancer

Vitamin C plays a role in preventing oxidative damage in tissues and stopping the formation of carcinogens (Gorton and Javis, 1999). It intakes show protective effect for several cancers such as lung, breast, stomach and cervical (Simon et al., 2001). Simon and

colleague (2001) found that vitamin C can reduce the oxidation of guanine and increase the oxidation of adenine.

Intake of a combination of vitamin C and vitamin K prior to chemotherapy had increased survival and the effects of several chemotherapeutic agents in a murine ascetic living tumor model (Block, 1999). Other than that, vitamin C is also safe to be consumed in conjunction with radiation (Shimpo *et al.,* 1991). Various studies have shown that intake of Vitamin A, β-carotene, E and C can reduce the incidence, and delays the progression of several cancers such as skin, colon, stomach, oesophagus, mammary glands and bladder (Gorton and Javis, 1999; *Taper et al.*, 1987; Taper *et al.,* 1996). The high content of those vitamins in the plasma can decrease the incidence and risk of cancer (Walingo, 2005).

Cancer develops when there is loss of cell differentiation. However, vitamins A, β-carotene, E and C have profound influence on cell growth and differentiation (Walingo, 2005). Hence, it can help in the prevention of cancer. Moreover, vitamin C combines with vitamin E can act as scavengers for free radical in the body system.

Vitamin C affects the intracellular organelle distribution and plays vital role in cyto-differentiation of cancer cell (Walingo, 2005). It helps in cell differentiation by exerting direct cytotoxic effects, modifying membrane biogenesis, light and gap junction formation, Golgi's complex, autophagic and apoptotic activity, cell surface changes and sometimes reversing transformed cells to normal cells. The reversion of transforming cells to normal cells is important to reduce the possibility of cancer incidence (Walingo, 2005).

1.2.7 Recommended intakes of ascorbic acid

The recommended dietary allowances (RDAs), adequate intakes (IAs), and tolerable upper intake level (ULs) for ascorbic acid had been established by the Institute of Medicine (IOM) in 2000 as shown in Table 1.3. For infants, it is based on average volume of milk intake that is 780 ml. The estimated RDAs are based on relative body weight. Women require lower ascorbic acid than male because they have lower body mass. Lactating mother requires high intake of ascorbic acid to fulfill the need for both mother and infant.

Table 1.3 : Recommended dietary intakes of ascorbic acid (IOM, 2000).

LIFE STAGE GROUP	RDA/ AI (mg/day)	UL (mg/day)
INFANT		
0-6 months	40	ND
7-12 month	50	ND
CHILDREN		
1-3 years	15	400
4-8 years	25	650
MALES		
9-13 years	45	1200
14-18 years	75	1800
19-30 years	90	2000
31-50 years	90	2000
50-70 years	90	2000
>70 years	90	2000
FEMALES		
9-13 years	45	1200
14-18 years	65	1800
19-30 years	75	2000
31-50 years	75	2000
50-70 years	75	2000
>70 years	75	2000

Table 1.3 : (continued)

LIFE STAGE GROUP	RDA/ AI (mg/day)	UL (mg/day)
PREGNANCY		
≤ 18 years	80	180
19-30 years	85	2000
31-50 years	85	2000
LACTATION		
≤ 18 years	115	180
19-30 years	120	2000
31-50 years	120	2000

1.3 Polarography as general

Polarograpic technique is used to determine the concentration of ascorbic acid in samples. Polarography is a branch of voltammetry. It was invented by Jaroslave Heyrovsky in 1922. He was awarded the Nobel Prize in Chemistry in 1959 (Lawal and Etim, 2013). Polarography consists of three electrodes which are working electrode, reference electrode and auxilliary electrode. Working electrode is the electrode at which the electrochemical reaction takes place. The working electrode in polarography is a dropping mercury electrode (DME). Usually, the reference electrode is Ag/AgCl and the auxilliary electrode is platinum. Reference electrode measures current that passes through the working and auxilliary electrodes. The function of auxilliary electrode is to allow the current to flow. In polarography, current is measure when various potentials is applied. In this technique, the mercury is dropps from small capillary tube. The drop grows rapidly and finally falls from the capillary tip and immediately replace by another drop. By using DME, its accuracy is higher since dropping constantly renewed clean spherical surface of the solution. As a result, current is reproducible and independent of the previous course of analysis (Riches, 1948).

1.4 Problem statement

The benefits of roselle juice was not really well known. Roselle plant is caimed to contain high amount of ascorbic acid. This study was done in order to clarify the claimed. Deficiency in vitamin C could lead to scurvy. Hence, the amount of vitamin C need to be clarified to fullfill the requirement of dietary intake of vitamin C.

1.5 Significance of the study

There are several advantages of using polarography over other technique. The advantages are rapid, simple and straightforward analysis. The determination of ascorbic acid in roselle juice samples will help consumers to know the benefit of roselle juice. Besides, this study will help manufacturer to add precaution step during the preparation of roselle juice to maximise the amount of ascorbic acid in roselle juice. This study will also aid future researches to do analysis on roselle.

CHAPTER 2

LITERATURE REVIEW

A study done by Luvonga and colleague in 2010 had shown that ascorbic acid content in fresh calyces was 6.701 mg/100g whereas in dried calyces was 4.690 mg/100g. The determination of ascorbic acid in this study used high performance liquid chromatography (HPLC) carried out at wavelength of 265 nm. Although there were other water soluble vitamin such as niacin, thiamin, riboflavin and folic acid in roselle, it was found that ascorbic acid content was higher compared to others (Luvonga *et al.*, 2010).

Analysis of ascorbic acid in roselle leaves done by Sarkiyaki and Ikioda (2010) showed that fresh leaves content 0.1802 mg/g ascorbic acid while dried leaves content 0.00736 mg/g ascorbic acid. This result reveals that drying process has affected the concentration of ascorbic acid in roselle leaves.

Gupta (2015), studied ascorbic acid in pharmaceutical samples by polarographic method analysis. It was done by measuring the anodic wave that correspond to the oxidation of enediol system. In the absence of atmospheric oxygen, it gives good anodic wave whereby the presence of oxygen does not effect the wave.

Other than that, Masram and Jugade (2013) had used polarographic technique to estimate ascorbic acid in various marketed formulations and fruit juices. In their study, they used saturated calomel electrode as the reference electrode. The flow rate of mercury was 20 drops per minute. Based on their study, it had shown that bipthalate buffer gives a well-defined polarographic wave with the half potential of 0.150 V.

In the polarographic method, oxalic acid or EDTA is required. Generally, oxalic acid is preferred compared to EDTA, which protected the ascorbic acid during the sample preparation procedure (Gupta, 2015).

There are several advantages of polarographic method compared to other analytical methods. The principle advantages of polarographic method are that it is simple, rapid, accurate and free for any changes such as changing of colour which is difficult to be analysed by using another technique such as titrimetric or calorimetric procedure (Lento *et al.*, 1963).

The other advantages of polarographic technique is it can be carried out to detect ascorbic acid in the present of other vitamins (Gupta, 2015). When compared to 2,6-DCIP titration method, polarographic is more specific because through the diffusion current and half-wave potential, it provides both qualitative and quantitative analyses for ascorbic acid (Lento *et al.*, 1963).

There are various methods for the determination of ascorbic acid such as high performance liquid chromatography (HLPC) (Shafqat ulah *et al.*, 2012), gas chromatography (GC) (Silva, 2003), liquid chromatography (LC) (Ke *et al.*, 1994), titrimetry (AOAC, 2005), chemiluminescence (CL) (Arya *et al.*, 2000), flourometry (Arya *et al.*, 2000), and spectrophotometry (Araya *et al.*, 1998). These methods are generally applied to determine the ascorbic acid content in pharmaceutical, natural food and beverages.

In HPLC, it used gradient pump system, separation using C-18 column and commonly ultraviolet (UV) detector (Shafqat ulah *et al.*, 2012). However, dehydroascorbic acid cannot be detected using UV detector and hence need other detector (Arayne *et al.*, 2008). Other than UV detector, HPLC also equipped with other electrochemical detector

such as mass spectroscopic and flourescence detectors. Although HPLC is a selective and sensitive technique for the determination of ascorbic acid in foodstuffs and biological fluids (Iwase, 2000) , it requires complex gradient mobile phase, time consuming and poor recoveries and reproducibility (Kall and Andersen, 1999). Other than that, the stability of the technique is also affected by light, temperature, pH and the presence of oxygen or metal ions (Novakova et al., 2008).

Liquid chromatography (LC) is also commonly used in the determination of ascorbic acid. It is a succesful method when selectivity an specificity are of concerned (Oliveira and Watson, 2001). LC is good in avoiding non specific interference and ion pair (Ke *et al.*, 1994). However, the mobile phase is often very complex as it needs more than two components containing various modifiers or reagents (Novakova *et al.*, 2008). The other disadvantage of LC is that the chromatographic resolution is incomplete (Oliveira and Watson, 2001).

Other chomatographic method that can be used for determination of ascorbic acid is gas chromatography (GC) with flame ionization detector (FID) (Silva, 2003). This method is laborious and ascorbic acid tent to lose during drying step. Moreover, ascorbic acid also may lose due to oxidation in air. However, the technique is very accurate if the precaution against loss of ascorbic acid is carefully taken (Silva, 2003).

Titrimetric technique can also be used in order to quantify ascorbic acid content. The technique is usually prefer when high concentrations of ascorbic acid is considered (Tahirovic *et al.*, 2012). There are several reagents used in the titrimetric technique such as iodine solution, 2,6-dichlorophenoliodophenol (2,6-DCIP), standard cupric salt solution and *N*-bromosuccinimide. The popular reagent for direct titration of ascorbic acid is 2,6-DCIP. When using the reagent, the end point is indicate by pink colour. However, the

reagent does not specifically reduce DHA (Arya *et al.*, 2000). Hence, this technique is only applicable when the concentration of DHA is ignore. The technique is restricted for sample of citrus fruits and multivitamin tablets which do not contain minerals. The reagent is flammable, and toxic. Although titrimetric technique is a simple and rapid method, but the end point of titration is difficult to be determined if the sample is in coloured (Okiei *et al.*, 2009). This technique is not valid for packed beveraged since most of them are coloured.

Other technique that can be used to detect ascorbic acid content is fluorometric. Fluorometric technique has been used in various samples such as fruits, vegetables and human serum (Arya *et al.*, 2000). Fluorescent product is formed by the reaction of dehydroascorbic acid (DHA) with *o*-phenylenediamine (OPD) however, the technique tends to overestimate the level of ascorbic acid when there is present of other oxidizable species other than DHA such as flavin adenine dinucleotide (Okiei *et al.*, 2009). Other than that, the technique required a rigid control of pH because the fluorescent intensity is affected by the pH of the solution (Arya *et al.*, 2000).

Chemiluminescent (CL) method can also be used to analyse ascorbic acid content in samples. There are different systems that form the basis of many chemiluminecsent methods for ascorbic acid determination, examples are Cu(II)–luminol, Ce(IV)–rhodamine 6G, Fe(II)–luminol–O_2, $KMnO_4$– luminol, H_2O_2–hemin–luminol and H_2O_2–luminol–peroxidase CL (Arya *et al.*, 2000). The advantage of CL is, it requires simple instrumentation therefore appropriate for rapid and on-line analysis when combined with a flow injection system (Agater and Jewsbury, 1997). It is also tolerant to the present of excess sugar concentration and work well with real sample (Agater and Jewsbury, 1997). The other advantages of CL when compared to other methods are that it has greater sensitivity over a wide linear dynamic range and has low detection limit because no external excitation source is required hence making the background signal low (Ma *et al.*,

2002). Other than that, it is also involved in the oxidation of ascorbic acid (Agater and Jewsbury, 1997) however, the selectivity is poor and make it not suitable to analyse real samples (Arya *et al.*, 2000).

Flow injection analysis (FIA) is also useful for determination of ascorbic acid. A study done by Yebra-Biurrun in 2000 had used a flow injection technique to determine ascorbic acid (Yebra-Biurrun, 2000). The technique was based on the oxidation of 1,2-enediol group. There are several advantages of FIA, such as high sample throughput, low sample and reagent consumption, high reproducibility, simple automated operation, low contamination risks, possible enhancement in selectivity by applying kinetic discrimination and very limited laboratory bench space and apparatus required (Ruzicka and Hansen, 1988). Other than that, by using FIA, air oxidation could be minimised. Ascorbic acid content had been determined by flow injection chemiluminescence (FI-CL) coupled with on-line removal interference (Cai and Xu, 2011). The method is based on the inhibitory effect of ascorbic acid of luminol proceeded by hydrogen peroxide in Na_2CO_3-$NaHCO_3$ medium (Cai and Xu, 2011). There are several advantages of FI-CL such as simplicity of apparatus, high sensitivity, high analysis speed and good reproducibility for determination of ascorbic acid in simple samples including vitamin C injections, vitamin C tablets and foods however, this method is rarely used in determination of ascorbic acid in complex samples because of poor selectivity (Cai and XU, 2011).

Enzymatic technique can also be used to analyse the ascorbic acid. The method was simple, rapid and highly specific for ascorbic acid however, the method was costly since the purified enzyme was expensive (Salkic *et al.*, 2009). This technique has been used to analyse ascorbic acid in serum and plasma (Salkic *et al.*, 2009).

Coulometric method had also been used in ascorbic acid determination (Yebra-Biurrun, 2000). This method is very limited in its application. Coulometric is based on the quantitative oxidation of ascorbic acid at a platinum anode (Arya *et al.*, 2000). The advantages of coulometric technique are that analyte is completely electrolysed and can be determined without calibration graph (Yebra-Biurrun, 2000).

A study done by Kumar and colleagues in 2013 had used the volumetric method in order to determine the ascorbic acid content in fresh fruit juices and vegetables (Kumar *et al.*, 2013). They used the amount of dye that are needed in titration in order to make the the solution to be colored pink then, the amount of ascorbic acid content is calculated. However, the specificity of volumetric technique is lacking (O'Connell *et al.*, 2011). The technique is only limited to samples that do not contain other reducing agent.

Spectrophotometric methods are generally used for the analysis of ascorbic acid in aqueous solution. There are two types of spectrophotometric method. The first method is based on measuring the instrinsic absorption of ascorbic acid and the second method is based on measuring light absorption of products that result from reduction of various reagents by ascorbic acid (Zaporozhets and Krushinskaya, 2001). UV detector is generally used since ascorbic acid exhibits strong absorption in the UV region. UV spectrophotometric analysis has broad availability and sustainability (Mohamed Hussein, 2013). Moreover, spectrophotometric technique is a fast and simple method. Direct spectrophotometric determination of ascorbic acid in the UV region prone to matrix effect since many organic compounds in complex samples may also exhibit UV absorbances (Okiei *at al.*, 2009). Hence, it is not a suitable tecnique for the determination of ascorbic acid in vegetables and fruits (Aydogmus and Certin, 2011). Moreover, when using indirect spectrophotometric, it requires vast pre-treatment method, time consuming and high cost (Chavhan *et al.*, 2012). When compared to titrimetric technique, spectrophotometric

technique generally gives lower result, suggesting that there is a minor matrix effect (Tahirovic *et al.*, 2012). The summary of the methods used for the determination of ascorbic acid in various samples are listed in Table 2.1.

Table 2.1 : Summary of analysis method for the determination of ascorbic acid in various samples

No.	Methods	Samples	Reference
1	Bivariate	Pharmaceutical	Younes *et al.*, 2014
2	Capillary electrophoresis	Beverages	Law *et al.*, 2005
3	Capillar zone electrophoresis	Spinach, turnip, parsley	Fukushi *et al.*, 1997
4	Chemiluminescence	Fruits, juice, and vegetables	Feng *et al*, 1995
5	Cyclic voltammetry	Commercial fruit juice	Pisoschi *et al.*, 2008
6	Derivative spectrophotometry	Vegetables	Aydogmus and Cetin, 2001
7	Derivative spectrophotometry	Cauliflower	Ozgur and Sungur, 1994
8	Derivative spectrophotometry	Parsley, kiwi, grapefruit	Ozgur and Sungur, 1995
9	Direct UV spectrophotometry	Pharmaceuticals	Salkic *et el.*, 2009
10	FI-CL	Urine	Cai and Xu, 2011
11	First-derivative of ratio spectra	Pharmaceutical	Younes *et al.*, 2014