CHARACTERIZATION OF BIOACTIVE COMPOUNDS AND ANTIBACTERIAL STUDY OF PITAYA PEEL EXTRACT EXTRACTED THROUGH MICROWAVE ASSISTED EXTRACTION METHOD

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Dedicated to my parents

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ABSTRAK

Kulit pitaya dilihat sebagai sejenis sampah yang berpotensi, terutamanya di dalam industri makanan kerana jus ekstraknya boleh digunapakai sebagai pewarna semulajadi. Selain itu, ia juga mengandungi sebatian bioaktif yang bermanfaat dan mempunyai nilai komersil kerana sebatian bioaktifnya dapat menyumbang kepada sifat antioksidan dan antibakteria. Secara tidak langsung, sebatian bioaktif ini juga boleh memberi kesan yang baik kepada kesihatan manusia. Walaupun kulit pitaya mempunyai potensi, masalah dalam industri adalah kulit pitaya dibuang sebelum rawatan terutamanya dalam industri pemprosesan makanan tanpa memberi pertimbangan terhadap kesan sampingan ke atas alam sekitar. Walau bagaimanapun, kajian adalah terhad dalam menganalisa sebatian bioaktif kulit pitaya dengan menggunakan kaedah hijau, iaitu menggunakan air dan pengekstrakan berketuhar (MAE) sebagai pelarut dan alat untuk proses pengekstrakan masing-masing. Oleh sebab itu, objektif utama kajian ini adalah untuk mengkaji keadaan terbaik MAE untuk memperolehi nilai tertinggi kandungan fenolik (TPC). Seterusnya keadan terbaik MAE di gunakan untuk pengekstrakan sebatian bioaktif daripada kulit pitaya iaitu sebatian mineral dan phenolic. Kemudian, mencirikan kandungan kimia dan analisis aktiviti antibakteria daripada ekstrak kulit pitaya boleh dikaji. Teknik pengering beku telah digunakan untuk menghilang semua kandungan air yang ada di dalam kulit pitaya sebelum meneruskan pengekstrakan bagi memastikan kulit pitaya dapat disimpan untuk masa yang lama sebelum dianalisa. Kesan parameter MAE seperti kuasa, suhu, masa, dan berat sampel telah ditentukan. Induktif bergandingan spectrometer pancaran plasma-optik (ICP-OES) dan kromatografi cecair berprestasi ultra tinggi bergandingan spectrometer jisim (UHPLC-ESI-QTRAP-MSMS) telah digunapakai untuk mencirikan kandungan mineral dan jenis sebatian fenolik daripada ekstrak kulit pitaya. Analisis antibakteria ekstrak kulit pitaya dilakukan terhadap Gram-positif, *Staphylococcus aureus (S. aureus)* ATCC 6538 dan Gram-negatif, *Escherichia coli (E. coli)* ATCC 8739 untuk mengukur perubahan zon inhibitasi. Selain itu, asai masa perencatan kinetik telah digunakan untuk memerhati lengkung pertumbuhan bakteria. Dua jenis perisian digunakan iaitu SPSS dan CCLASS untuk menentukan keadaan MAE yang terbaik berdasarkan signifikasi nilai TPC dan untuk mengesahkan data kandungan mineral dari ekstrak kulit pitaya masing-masing. Di samping itu, perubahan struktur mikroskopik kulit pitaya untuk sebelum dan selepas pengekstrakan melalui MAE telah diperhatikan. Hasilnya mendedahkan bahawa nilai TPC maksimum diperhatikan pada kuasa 400 W, suhu 45 °C, dan masa hubungan 20 minit untuk ekstrak 1.2 g kulit pitaya dalam 50 mL air dengan nilai TPC maksimum yang diperoleh adalah masing-masing 5.808, 5.800, 5.723, dan 5.708 mg GAE/g kulit kering. Parameter keadaan terbaik ini telah disahkan dengan menggunakan analisis statistik, SPSS dengan Bonferroni post hoc. TPC yang ditunjukkan dalam ekstrak cecair diukur dalam mg GAE/g. Selain itu, unit IC_{50} (Perencatan Penghalang) ditentukan daripada larutan ekstrak dengan menggunakan parameter keadaan terbaik MAE dan 2,2, diphenyl-1-picrylhydrazil (DPPH) sebagai radikal bebas sintetik. Nilai IC_{50} yang didapati daripada kajian ini ialah 0.52 mL/mL. Tambahan pula, 12 daripada 24 unsur telah dikenalpasti, termasuk Ba, Ca, Cu, Cd, Fe, K, Mg, Mn, Na, Ni, Sr dan Zn. Selain itu, 13 sebatian fenolik adalah padanan yang bagus dengan pangkalan pada spectrometer jisim. Walau bagaimanapun, ekstrak kulit pitaya tidak memberi sebarang kawasan zon inhibinasi, tetapi mempunyai kesan kecil terhadap kajian asai masa perencatan kinetik. Ringkasnya, ekstrak kulit pitaya diperkaya dengan kandungan mineral berharga dan sebatian fenolik, bersama-sama dengan sifat antibakteria yang rendah. Selain itu, mikroskop pengimbasan elektron (SEM) menunjukkan bahawa kemusnahan dinding sel kulit pitaya yang disebabkan oleh radiasi gelombang mikro dari MAE adalah punca utama untuk pengekstrakan yang lebih cepat bagi sebatian bioaktif. Kesimpulannya, pengambilan sebatian bioaktif dari kulit pitaya mempunyai aplikasi yang berpotensi, di mana pada masa yang sama dapat mengurangkan sisa yang dihasilkan oleh industri pemprosesan makanan.

ABSTRACT

Pitaya peel is a potential form of fruit waste, especially within the food industry, mainly because its juice extract can be applied as natural coloring. It also contains beneficial bioactive compounds with commercial value, along with antioxidant and antibacterial properties that have a good impact upon the human health. With such potentials of the pitaya peel, unfortunately, it is discarded without treatment in food processing industries by ignoring the side effects it has towards the environment. Only a handful of studies have analyzed the bioactive compounds of pitaya peel extract via green method, which applies water and microwave assisted extraction (MAE) as solvent and tool for the respective extraction processes. Thus, the key objective of the current study is determining the best conditions of MAE in attaining the maximum total phenolic content (TPC) value. These conditions have been applied in extracting bioactive compounds from the pitaya peel, specifically mineral and phenolic compounds. Then, the chemical contents are characterized before the antibacterial activity of the extracts can be analysed and studied. Freeze dryer technique was used to remove the water content in the pitaya peel before extraction so that the pitaya peel can be stored for a longer period prior to analysis. The effects of MAE parameters, such as power, temperature, sample weight, and time, were determined. Inductively coupled plasma-optical emission spectrometry (ICP-OES) and ultra-high performance liquid chromatography coupled mass spectrometer (UHPLC-ESI-QTRAP-MSMS) had been utilized to analyze the mineral content and the type of phenolic compounds found in pitaya peel extract. The antibacterial analysis of pitaya peel extract was performed against Gram-positive, *Staphylococcus aureus (S. aureus)* ATCC 6538, and Gram-negative, *Escherichia coli (E. coli)* ATCC 8739, to determine the modification that took place at the inhibition zone. Time-kill kinetics assay was applied to monitor the bacterial growth curve. Additionally, two software programs were employed; SPSS and CCLASS, in order to determine the best condition of MAE based on significant variance between TPC mean value and validated data of mineral content from pitaya peel extract, respectively. The microscopic structural changes of pitaya peel before and after extraction on MAE had been observed as well. The outcomes revealed that the maximum TPC values were retrievable at 400 W power, 45 °C temperature, and 20 min contact time to extract 1.2 g of pitaya peel in 50 mL of water at 5.808, 5.800, 5.723, and 5.708 mg GAE/g dried peel, respectively. These best condition parameters were verified via SPSS with Bonferroni post hoc. The TPC values recorded from the liquid extract was measured in mg GAE/g, while Inhibitory Concentration unit (IC_{50}) was determined from the extract by applying the best condition parameters of MAE and 2,2, diphenyl-1-picrylhydrazil (DPPH) reagents as synthetic free radicals. The IC_{50} value recorded in this study was 0.52 mL/mL. Furthermore, 12 out of 24 elements were identified, including Ba, Ca, Cu, Cd, Fe, K, Mg, Mn, Na, Ni, Sr, and Zn, whereas 13 phenolic compounds significantly matched the mass spectral database. Nevertheless, the pitaya peel extract had no inhibition zone area, but displayed a small effect on the time-kill kinetics analysis. In short, pitaya peel extract seems to be enriched with valuable mineral contents and phenolic compounds, along with low antibacterial properties. The scanning electron microscopy (SEM) demonstrated that cell wall disruption of pitaya peel caused by microwave radiation from MAE appeared to be the main reason for rapid extraction of bioactive compounds. As a conclusion, the extraction bioactive compounds from pitaya peel exhibited potential application that could substantially reduce wastes produced by the food processing industry.

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%	Percentage
°C	Degree Celcius
mm	Millimetre
mL	Millilitre
rpm	Rotation per Minute
°C	Degree Celcius
min	Minute
g	Gram
W	Watt
hr	Hour
w/v	Weight per Volume
nm	Nanometre
Mg/l	Milligram per Litre
s	Second
mM	Milimole
µL	Microlitre
m/z	Mass To The Charge Ratio
µm	Micrometre
R_T	Retention Time
cm	Centimetre
mg/ml	Milligram per Millilitre
ug/ml	Microgram per Millilitre
CFU/mL	Coliform Unit per Millilitre
hr	Hour

LIST OF ABBREVIATIONS

OFAT	One Factor At The Time
IC_{50}	Inhibitory Concentration
DPPH	2,2, diphenyl-1-picrylhydrazyl
QC	Quality Control
ICP-OES	Inductively Coupled Plasma-Optical Emission Spectrometry
UHPLC-ESI-QTRAP-MSMS	Ultra High Performance Liquid Chromatography Coupled Mass Spectrometer
HPLC	High Performance Liquid Chromatography
SEM	Scanning Electron Microscopy
Al	Aluminium
As	Arsenic
Ba	Barium
Be	Berylilium
Bi	Bismuth
Cd	Cadmium
Ca	Calcium
Cr	Chromium
Co	Cobalt
Cu	Copper
Fe	Iron
Pb	Lead
Li	Lithium
Mg	Magnesium
Mn	Manganese
Mo	Molybdenum
Ni	Nickel
K	Potassium
Na	Sodium
Sr	Strontium
Ti	Titanium

V	Vanadium
Y	Yittrium
Zn	Zinc
SPSS	Statistic Package for Social Science
ANOVA	One-Way Analysis of Variance
SD	Standard Deviation
SE	Standard Error
RSM	Response Surface Methodology
RDA	Recommended Daily Amount
AAS	Atomic Absorption Spectroscopy
GFAAS	Graphite Furnace Atomic Absorption Spectroscopy
MAE	Microwave Assisted Extraction
UAE	Ultrasound Assisted Extraction
CSE	Conventional Solvent Extraction
ASE	Accelerated Solvent Extraction
GRAS	Generally Recognized As Safe Solvent
TPC	Total Phenolic Content
MAD	Microwave Assisted Distillation
MIS	Microwave Integrated Soxhlet Extraction
SFME	Solvent Free Microwave Extraction
Uv-Vis	Ultra Violet Visible Spectrometer
GAE	Gallic Acid Equivalent
HCL	Hydrochloric Acid
DV	Dual View
RF	Radio Frequency
TSA	Tryptone Soya Agar
TSB	Tryptone Soya Broth
OD	Optical Density
N.D	Not Detected
LOD	Limit Of Detection
IEC	Inter Element Correction
MSF	Multi Spectral Fitting

CLSI	Clinical and Laboratory Standard Institute
LQSI	Laboratory Quality Services International
MS	Mass Spectrometer
S.aureus	*Staphylococcus aureus*
E.coli	*Escherichia coli*
H. undatus	*Hylocereus undatus*
H. polyrhizus	*Hylocereus polyrhizus*
S. megalanthus	*Selinecereus megalanthus*

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Dragon fruit, which is also called 'pitaya', is an emerging fruit commonly linked to food products. In the context of Malaysian market, the fruit can be classified into three species, namely: *Hylocereus undatus (H. undatus)*, *Hylocereus polyrhizus (H. polyrhizus)*, and *Selinecereus megalanthus (S. megalanthus)*. They may be differentiated according to their respective red skin with white flesh, red skin with red flesh, or yellow skin with white flesh (Grimaldo *et al.*, 2007; Lebellec & Vaillant, 2011). *H. polyrhizus*, in particular, is selected and highlighted in this current work due to its higher phenolic compound and antioxidant activity compared to the other species (Ruzlan *et al.*, 2010). Typically consumed directly as is or prepared as a juice, pitaya peels is rendered as waste products in the juice processing industry as discarded peels cause various environmental issues, especially water pollution (Ismail *et al.*, 2012). It also facilitates the breeding ground for bacteria due to the extra nutrient stress coupled with water body acidity that triggers bacterial growth, causing water pollution and spread of plague (Dhillon *et al.*, 2013). Nevertheless, it may also be utilised as animal feedstock especially as poultry feed, due to it containing the necessary nutrients for healthy poultry growth like anthocyanin and beta-carotene. These elements have been reported to display antioxidant functions and lower the cholesterol level in blood serum (Mahlil *et al.*, 2018). Moreover, the peels may also be extracted to retrieve numerous bioactive compounds, including mineral contents and phenolic compounds.

General interest in fruit peels has escalated since the past few years due to their industrial applications and potential in the food and cosmetic industries. Phenolic compounds are commonly and frequently found in both edible and inedible plants alike (Wojdyło *et al.*, 2007). However, evidence has shown that the peels contain a higher phenolic concentration in comparison to the flesh, as the compounds are preferentially located in the peel rather than the flesh (Ayala *et al.*, 2011; Fattouch *et al.*, 2008; Kalt, 2005). Furthermore, TPC and radical scavenging activity of *H. polyrhizus* and *H. undatus* peels have been revealed and proven to be significantly higher than in the flesh (Cheok *et al.*, 2018; Ruzlan *et al.*, 2010). As a rule, phenolic compounds boast of various valuable properties, including antioxidant (Ali *et al.*, 2015), antibacterial (Mocan *et al.*, 2014), anti-carcinogenic (Zhang *et al.*, 2015), and anti-inflammatory (Correa *et al.*, 2016; Zhang *et al.*, 2016) characteristics. The peels, in particular, are also composed of essential minerals vital for human health (Marqués *et al.*, 2015). Therefore, obtaining the highest extraction and retaining the integrity of the properties of the bioactive compound have called for the use of microwave assisted extraction (MAE) technique plus water as the extraction solvent in extracting the pitaya peel (Desai *et al.*, 2010).

Technique-wise, MAE plus water, Chaiwut *et al.* (2012) has shown to be utilised in experimental extraction of TPC from pitaya peel. However, no information is available regarding the best condition for MAE parameters that generates the highest TPC value from the pitaya peel extract. Thus, the impacts of four parameters related to MAE (i.e. power, temperature, time, and sample weight) have been investigated and determined in this study. An SPSS analysis has been subsequently carried out to identify the significant variance between the TPC mean values in establishing the best condition values of the MAE parameters. Moreover, the best condition MAE may also be used to extract other bioactive compounds, including mineral contents and phenolic compounds that are previously unreported. As of currently, there is no validated and published data on mineral content extracted from pitaya peel using the CCLASS software as reported in this research that the author is aware of.

Hence, it can be concluded that MAE and water are the most preferable combined tools in the extraction of bioactive compounds from pitaya peel, which includes pectin, betalain (Thirugnanasambandham & Sivakumar, 2017) and phenolic

compounds (Ferreres *et al.*, 2017) from pitaya peel. The presence of potential in improving the method is beneficial, as the technique is environmental-friendly and cost-effective. This is especially true in investigating bioactive compounds, antioxidant activity, and antibacterial properties of pitaya peel extract.

1.2 Problem Statement

By-products that turn into wastes are inevitable in the food processing line, thus disposal of these wastes has emerged to be an issue due to their negative impact upon the environment and the human health (Ferreres *et al.*, 2017). Hitherto, fruit wastes may frequently encompass numerous useful elements with essential health importance, which could be applied in food and cosmetic industries (Gudiña *et al.*, 2016; Kabir *et al.*, 2015; Matharu *et al.*, 2016). For instance, around 150 tons of pitaya fruits are harvested annually, where massive solid wastes in the form of peels are generated by industries (Tongkham *et al.*, 2017). Consequently, this leads to global environmental issues due to dumping of wastes (Thirugnanasambandham & Sivakumar, 2017). According to Prakash *et al.* (2013), these peels contain high contents of natural antioxidants that possess positive health benefits. Hence, extraction of bioactive compounds from pitaya peels may be a promising solution to minimize waste and to gain useful compounds.

Pitaya peel had been selected in this study mainly because its color (red) that can be easily disseminated with water as extracting solvent, in comparison to other fruits (Lourith & Kanlayavattanakul, 2013). Many researchers have focused on pitaya for color extraction and revealed that pitaya contained higher TPC value and antioxidant activity. The 33% of pitaya weight that derives from the peel indicates the abundance of bioactive compounds present within the peel (Ferreres *et al.*, 2017). Besides, fruit peel has several advantages, such as inexpensive, in abundance, and a sustainable resource (Rodrigues *et al.*, 2013; Vinardell *et al.*, 2008). In this light, it is crucial to investigate the pitaya peel discarded by the food industry so as to bring awareness regarding its vast benefits to human health.

Prior studies showed that the extraction method exhibited several shortcomings, such as safety hazards, high energy input, low product quality, low extraction yields, long extraction duration, and environmental risk. In addition, the final product of the extraction technique always encompasses remnants of organic solvent that jeopardize the product quality (Yang *et al.*, 2001a). In this research, MAE was employed to prepare the samples due to its ability in extracting the bioactive compounds in a rapid manner (Azmir *et al.*, 2013), aside from limiting the degradation of bioactive componds (Flórez *et al.*, 2015). It is also feasible in extracting phenolic compounds with higher yield, when compared to other conventional methods, such as soxhlet (Karabegović *et al.*, 2013; Lou *et al.*, 2012), as well as other advance extraction method, such as ultrasonic-assisted extraction (UAE) (Fang *et al.*, 2015). The optimum conditions of MAE were determined to obtain pectin and pigment. However, no study has analyzed the effect of MAE on TPC value and antibacterial properties for pitaya peel extract. Hence, this study identified the best conditions of MAE and the maximum TPC value. This study also investigated the impacts of MAE parameters, including power, temperature, time, and sample weight.

The selection of solvent extraction for MAE is vital to ascertain higher yield of bioactive compounds. Several researchers employed a variety of organic solvents mixed with water for extraction that endanger the environment, such as emitting odor, hazardous, flammable, and toxic effects. Hence, this analysis ensures that the bioactive compounds are free from deleterious effects to promote the use of natural colorant in food and cosmetic products. As such, only water was used in this study as the extracting solvent. Michel *et al.* (2011) asserted that non-toxic solvents, such as water and ethanol, are increasing in usage for extraction process due to higher extraction yield. Water enhances swelling of cell material and extends the contact surface area in plant matrix and solvent (Hayat *et al.*, 2009). Notably, due to the consistency of water as solvent in MAE, the usage of organic solvents can be excluded (Li *et al.*, 2017). MAE with water as solvent is a superior tool in extracting bioactive compounds from pitaya peel.

Additionally, information pertaining to mineral contents and types of phenolic compounds in pitaya peel extract is in scarcity. Both elements have vital functions for the human health. Besides, there is no available data concerning determination of mineral contents and phenolic compounds of pitaya peel of *H. polyrhizus* via MAE

extraction method. With that, this study identified the mineral contents and phenolic compounds via ICP-OES and UHPLC-ESI-QTRAP-MSMS, respectively. Based on the researcher's knowledge, no study has analyzed pitaya peel extract from MAE against bacterial activity. Thus, both inhibition zone and time-kill kinetic tests were performed to investigate activities of pitaya peel extract against Gram-positive bacterium, *Staphylococcus aureus*, and Gram-negative bacterium, *Escherichia coli*.

1.3 Objectives

The current study is aimed to extract bioactive compounds of pitaya peel using water as well as determine its antioxidant content and antibacterial activity. In light of this, the study is comprised of the following objectives:

(i) To determine the best condition for MAE with highest TPC value.

(ii) To characterize the chemical contents of pitaya peel extract

(iii) To analyze the antibacterial activity of the pitaya peel extract against Gram-positive and Gram-negative bacteria.

1.4 Scope

In pursuance of achieving the study objectives, the scope of the study was divided into three sections as below:

(i) To investigate the effects of power (200-800 W), temperature (35-50 °C), time (10-30 min), and sample weight (1.2-3.2 g) to obtain the highest TPC value in laboratory scale.

(ii) To determine antioxidant activity, mineral contents, and type of phenolic compounds of pitaya peel extract.

(iii) To analyze the antibacterial activity of pitaya peel extract against Gram-positive bacterium, *S. aureus* and Gram-negative bacterium, *E. coli* through zone of inhibition and time-kill kinetic analysis.

1.5 Significant of study

The outcome of this research has significantly contributed towards better knowledge in the relevant area. The contributions are as follows:

(i) The available conventional techniques for fruit peel extraction incorporate toxic solvents, such as methanol. The utilization of water as natural solvent for extraction of pitaya peel extract implies its potential application in food as a natural colorant. This is because; water is non-toxic and environmental-friendly.

(ii) This is the first study that has detailed the best MAE conditions with water as the solvent for bioactive compound extraction using pitaya peel. The TPC value was determined based on the significant variance between the TPC mean values. MAE appears to be appropriate in this regard as it is cost-effective and environmental-friendly.

(iii) This is the first study that validated mineral contents by using the CCLASS software program. The analysis of mineral contents using this program seems to be at satisfactory level. Most industries have utilized this software program to analyze their laboratory data. This program allows the calculation of z-score, which refers to a simple method that measures mineral data content.

(iv) This is the first study that demonstrated solution extraction via MAE against antibacterial activities, particularly against *S. aureus* and *E. coli*. The pitaya peel extract exhibited modest antibacterial activity, as determined by the time-kill kinetics test. Hence, pitaya peel extract could be used as a natural colorant in food.

CHAPTER 2

LITERATURE REVIEW

2.1 History of Pitaya

Pitaya is a tropical fruit classified under the family of cactus, *Cactaceae.* The cactus fruit is also identified as pitahaya, pitajaya, pitaya roja, and pitahaya de Cardón in Mexico and northern South America. In Vietnam, the fruit is called Pitaya or Thanh Long. Originally, the French introduced pitaya to Vietnam about a century. To date, Vietnam has emerged as the main producer of pitaya within the South East Asia region (Wichienchot *et al.*, 2010). At the same time, pitaya has gain popularity globally due to its alluring nature such as tempting color, sweet and luscious. Presently, pitaya is exported over the local Asian markets such as Singapore, Hong Kong, Taiwan, Philippines, Malaysia and Thailand (Hoa *et al.*, 2006).

Now pitaya is harvested in relatively 22 countries inclusive of Australia, Cambodia, China, Malaysia, Thailand, Srilanka and Bangladesh (Inglese *et al.*, 2002; Mizrahi & Nerd, 1999). Previous studies have indicated that a mature pitaya is rich in total soluble solids, organic acid (Stintzing *et al.*, 2003), protein (Bellec *et al.*, 2006) and other minerals such as potassium, magnesium, calcium and vitamin C. According to Taiwan Food industry Development and Research Authorities, 2005, the pitaya pulp is composed of 82.5-83% moisture, 0.16-0.23% protein, 0.21-0.61% fat, and 0.7-0.9% fiber. Moreover, pitaya also rich in nutritious values of 8-9mg of vitamin C, 6.3-8.8 mg of Calcium, 30.2-36.1 mg of phosphorus and 0.55-0.65 of mg iron.

The tropical region is conducive for harvesting pitaya plants. Nonetheless, the plants should be protected from intense solar radiation and subfreezing temperatures for cultivation in subtropical environments (Nerd & Mizrahi, 1999). The appropriate environment for harvesting pitaya fruit is a dry tropical climate with an average temperature of 21 to 29 °C. Meanwhile, the fruit can resist temperature of 38 to 40 °C, and up to 0 °C for short period. Furthermore, rainfall value of 600 to 1300 mm with changing wet and dry season is favored for pitaya cultivation. Moreover, daylight is preferred by Pitaya plant, while it can be damaged by high level of light intensity. As such, certain amount of shading is necessary for its cultivation. After harvesting, the pitaya must be kept at 7 to 10 °C and 90 to 98 percent relative humidity for up to two to three months. Normally, the fruit is consumed fresh and chilled. It is sliced into two parts to display its alluring flesh. Moreover, it can also cut or scooped out using a spoon. The flesh is soft, sweet, crunchy and refreshing. The fruit can be used in fruit salads, marmalades, jellies, ices and soft drinks (Luders & Mcmahon, 2004).

2.1.1 Types of Pitaya

2.1.1.1 Yellow Pitaya

Yellow Pitaya (*S. megalanthus*) is a fresh farm grown harvest emerged from the northern part of South America. Yellow pitaya is cultivated in Colombia and Israel (Mizrahi *et al.*, 1997). The main producer of yellow pitaya is Colombia, a country in South America. Majority of the farms in Colombia have been relocated due to the massive infestation of fungi (Ruzlan *et al*., 2010). It is a medium-sized fruit with yellow peel bearing tubercles and thorns that are shed during ripening. The fruits are smaller than the other vine cacti fruits. The pulp is white and soft, where it comprises many small digestible black seeds (Nerd & Mizrahi, 1999). Nevertheless, the taste is superior. Hence, it has the higher demand and expensive compared to the others. The yellow pitaya can be kept up to four weeks at 10 °C and a week or more at 20 °C.

Figure 2.1 Yellow Pitaya

Sources: Mizrahi *et al.* (2004)

2.1.1.2 The Red Pitaya

There are two species of pitaya frequently found in the markets, which are *H. undatus* (red peel with white flesh) and *H. polyrhizus* (red peel with red flesh). Images of these species are shown in Figure 2.2 (a) and (b). They are member of the *Cactaceae family* under the *subfamily Cactoidea* (Esquivel *et al.*, 2007b). The specifications of the two types of pitaya fruit are: bulky; oblong with a red peel; big green scales. It should be noted that the fruit size is determined by the seed number (Weiss *et al.*, 1994). Moreover, the scales become yellow during ripening. The color of the skin starts to transform within 25 to 30 days after flowering in the two fruits, *H. undatus* and *H. polyhizus*. Simultaneously, the firmness of the flesh reaches the lowest. As such, eating quality is achieved within is 33 to 37 days after flowering. In general, the fruit can be harvested from 25 to 45 days after flowering. Nonetheless, 32 to 35 days was recommended by Nerd and Mizrahi (1999).

(a)

(b)

Figure 2.2 Red Pitaya with (a) White (b) Red Flesh

Sources: Moshfeghi *et al.* (2013)

2.1.2 Benefits of Pitaya

The red flesh of *H. polyrhizus* has higher antioxidant properties. Moreover, it is expensive than *H. undatus* in Malaysia due to higher costumers' demand. Consequently, the fruits are harvested on a large and commercial scale (Khalil *et al.*, 2012). Usually, consumers produce juice from the fruit or consume it directly as a fruit by peeling the skin. Evidence has shown that consumption of red pitaya may improves overall digestive system, lowers cholesterol levels and prevents constipation (Ramli *et al.*, 2014).

The red layer of pitaya contains rich sources of valuable components: vitamins including B1, B2, B3, and C; minerals such as potassium, sodium, calcium, iron and phosphorus; nutrients such as fat, protein, carbohydrate, flavonoid, crude, fiber, thiamine, phytoalbumin, niacin, pyridoxine, kobalamine, glucose, betacyanin, phenolic,

carotene and polyphenol (Bellec *et al.*, 2006). Furthermore, it is also rich in phytoalbumins that are valuable source of antioxidant compared to other subtropical fruits (Moshfeghi *et al.*, 2013). Hence, pitaya juice is manufactured as a functional drink to fulfill the consumers' demand. Importantly, the drink is recognized as a natural supplement in promoting good health condition (Dartsch *et al.*, 2009). Therefore, pitaya is recognized as a functional food with high commercial value (Kim *et al.*, 2011a).

2.1.3 Application of Pitaya Peel as Natural Food Colorant

The usage of colorant as additive agent is popular in the food processing industry. Nonetheless, the price of food increases with addition of coloring. It more readily available in the market, where there is no alternate substitute in the market for the consumers. The issue now is the production of synthetic colorants has affected the human immune system and health. Recently, there are several food colorants that have been banned in United States. According to the report from Centre for Science in the Public Interest, the food colorants contain potential cancer-causing chemicals (Moshfeghi *et al.*, 2013). The millennial consumers are well-informed and attentive to the source of the healthy food in the market (Cai *et al.*, 2005). Resultantly, the natural food coloring has received increased attention from the consumers, where production of natural food additives may rise compared to synthetic food additives. Essentially, pitaya is introduced as natural food additives due to its attractive characteristics and nutritional benefits (Ding *et al.*, 2009). In light of this, the production cost of natural food additives could be lowered with the use of disposable part (peel) rather than edible portion of the fruit. Apart from its health benefits, the usage of peel is environment-friendly as it reduces the amount of waste generated from industry.

Pitaya peel consists of various minerals and antioxidant compounds compared to other subtropical fruits (Yien *et al.*, 2012). The pitaya peel of *H. polyrhizus* comprised of outer layer and inner layer that are referred as albedo. The two layers have red and yellow pigments known as betacyanin and betaxanthins. Moreover, *H. polyrhizus* extracted using 70% ethanol via Soxhlet extraction has high phenolic content compared to other species of pitaya fruit as shown in Table 2.0 (Ruzlan *et al.*, 2010). Moreover, the red colour of *H. polyrhizus* is appealing to the consumers (Moshfeghi *et al.*, 2013), where it receives high demand in the market as alternative to synthetic dye (Esquivel *et*

al., 2007a). In addition, pitaya fruit possess anti-inflammatory and antidiabetic properties along with protective effect on cardiovascular disease such as cancer prevention (Stintzing & Carle, 2004).

Table 2.0 Summary of Antioxidant Properties of Pulps and Peel of Pitaya Fruit

Sample	**Phenolic content**	**DPPH scavenging activity**
Peel of *H. polyrhizus*	Higher than pulps	High
Peel of *H. undatus*	Higher than pulps	High
Pulps of *H. polyrhizus*	Moderate/lower	Low
Pulps of *H. undatus*	Lower than peels	Low

Sources: Ruzlan *et al.* (2010)

In addition to its function as the food colorant, previous studies have investigated other benefits of the pitaya extract (Table 2.1). These studies demonstrated the details of valuable bioactive compounds related to current study.

Table 2.1 Summary of the Previous Research on Pitaya Peel Extraction

Raw material	**Finding of the Study**	**Authors**
H. polyrhizus	Betacyanin (150 g/mL)	Ferreres *et al.* (2017)
H. undatus	TPC (4.21 mg GAE/g sample)	Phongtongpasuk *et al.* (2016)
H. polyrhizus	Betalain (9 mg/L)	Thirugnanasambandham and Sivakumar (2017)
H. polyrhizus	Pectin (7.5%)	Thirugnanasambandham *et al.* (2014)
H. polyrhizus	Betalain	Moshfeghi *et al.* (2013)
H. polyrhizus	TPC (1.351 mg GAE/g extract)	Lourith and Kanlayavattanakul (2013)
H. undatus	TPC (0.29 mg GAE/g sample)	Chaiwut *et al.* (2012)
H. polyrhizus & *H.undatus*	TPC of H. undatus lower than TPC of H. polyrhizus	Ruzlan *et al.* (2010)
H. polyrhizus	Betacyanin (25.74 mg/L)	Harivaindaran *et al.* (2008)

2.2 Phenolic Compounds

Phenolic compounds are secondary metabolites produced by plants. Commonly, phenolic compounds are found in fruits, vegetables, herbs, roots, leaves, and seeds. They are synthesized in plants as results of response towards ecological and physiological pressures including pathogen and insect attack, UV radiation and wounding (Kennedy & Wightman, 2011; Napal *et al.*, 2010). These compounds are identified as natural defense elements with reproduction or sensorial properties (Balasundram *et al.*, 2006). Many factors including physiological disparities, environmental settings, geographic differences, genetic factors, and evolution affect their concentration in each plants (Figueiredo *et al.*, 2008). Furthermore, phenolic compound contains an aromatic ring with one or more hydroxyl substituents. Phenolic compounds is categorized into various classes, where phenolic acids and flavonoid are the main groups (Aludatt *et al.*, 2017).

2.2.1 Phenolic Acid

Phenolic acids belong to a main class of phenolic compounds in plant. They present in free and bounded form. The two subgroups of phenolic acid are hydroxybenzoic acids and hydroxycinnamic acids. The hydroxybenzoic acid and hydroxycinnamic acid are composed of seven carbon atoms structure (C6-C1) and aromatic compounds with three-carbon side chain (C6-C3), respectively (Araújo *et al.*, 2014). The number and position of the hydroxyl groups attached to the aromatic ring determine the variations in the structure of phenolic acids (Yang *et al.*, 2001b). Phenolic acids are sensitive to their surroundings, where exposure to high temperature and pressure can cause degradation or modification of their biological activities (Mhiri *et al.*, 2015). Furthermore, optimization of the parameters of MAE could prevent the degradation of the phenolic compounds. Moreover, MAE has demonstrated highest yield of all components with lower usage of solvent and energy compared to conventional extraction techniques (Fang *et al.*, 2015). Therefore, MAE is chosen to extract phenolic acid from the plant. The classification and several examples of the phenolic acids are illustrated in Figure 2.3

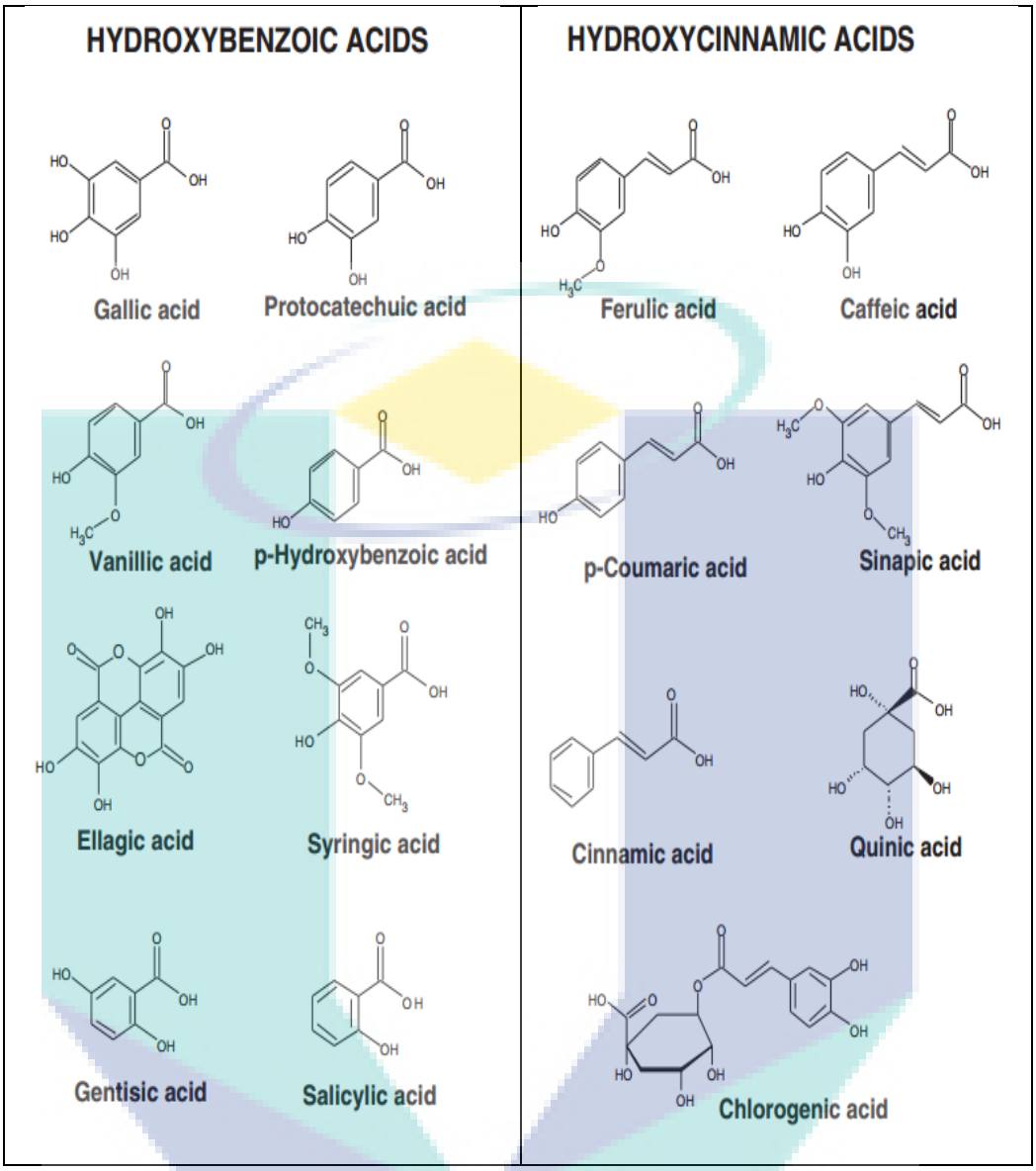


Figure 2.3 The General Formula and Names of the Subgroups of Phenolic Acid

Sources: Martins *et al.* (2011)

2.2.2 Flavonoids

Flavonoids are the major group of phenolic compounds found in plants. Flavonoid has 15 carbon atoms formed in three rings (C6-C3-C6) in which two aromatic rings are attached with three-carbon Bridge. It commonly found in the form of a heterocyclic ring. The flavonoids are categorized into six subgroups such as flavonols, flavones, flavanones, isoflavones, flavanonols, and anthocyanins. For example, rutin, quercetin, and epicatechin exist in herbs (Wu *et al.*, 2012). To date, over 8000 phenolic compounds are found, where more than 4000 compounds are categorized as flavonoid (Cong *et al.*, 2017).

2.3 Water as a Solvent

Water is plentiful, cheap, environment-friendly and an ideal solvent for industrial application (Kiamahalleh *et al.*, 2016). Moreover, water is a substitute organic solvent in the extraction process. This is because organic solvent might cause negative effects on the environment and food constituent. Water is a highly polar solvent with high dielectric constant (ε) because it contains many hydrogen bonds at ambient temperature and atmospheric pressure. In general, polar solvent has the ability to dissolve polar compounds (Chew *et al.*, 2011). For instance, phenolic acid is frequently polar in fruit and easily soluble in the water. On the other hand, water is improper for extracting non-polar compounds (Duba *et al.*, 2015).

The selection of extraction solvent for MAE is based on its ability to absorb the microwave energy and convert the energy into heat. This ability is determined by the dielectric properties of the solvent (Zhang *et al.*, 2011). In this case, water possess high dielectric constant, where it is able to absorb high microwave energy (Fang *et al.*, 2015). Furthermore, type and polarity of extraction solvent influence the extraction yields, phenolic content and antioxidant activities (Simić *et al.*, 2016). For instance, the walnut green husk had highest extraction yield using water as the solvent through maceration process compared to methanol and ethanol as shown in Table 2.2 (Fernández *et al.*, 2013).

Table 2.2 Extraction Yield of Walnut Green Husk Using Different Solvents

Solvent	Extraction Yield (%)
Methanol	11.26
Ethanol	3.90
Methanol 50%	17.66
Ethanol 50%	20.21
Water	44.11

Sources: Fernández *et al.* (2013)

Therefore, water was selected to extract bioactive compounds from pitaya peel due to its higher polarity compared to methanol and ethanol (Fernández *et al.*, 2013). As water is non-toxic, it could be applied directly as extraction solvent in the food and cosmetic industry. Furthermore, water is recommended as substitute for organic solvents due to safety and abundance. Similarly, ethanol can be utilized as a extraction solvent in contrast to methanol. Ethanol has been categorized as a generally recognized as safe solvent (GRAS) and it is safe for utilization in the food industry (Kiamahalleh *et al.*, 2016). Nonetheless, it is crucial to remove ethanol from solution as it exhibits inhibition effect on the samples during the downstream applications for ICP-OES and UHPLC-ESI-QTRAP-MSMS. Consequently, removal process of ethanol from pitaya peel extract causes degradation of several bioactive compounds under high temperature and longer evaporation time (Ruzlan *et al.*, 2010). Hence, ethanol is inappropriate for this study despite its low toxicity effect in relation to human health and the environment (Cacace & Mazza, 2003; Denev *et al.*, 2010; Karacabey & Mazza, 2010; Li *et al.*, 2012; Pérez & Castro, 2011). On the other hand, water was reported as the best solvent together with ethanol for pitaya peel extraction via maceration process to obtain TPC and 2,2, diphenyl-1-picrylhydrazyl (DPPH) (Lourith & Kanlayavattanakul, 2013). The relevant data are presented in Table 2.3.

Table 2.3 Antioxidants Activities Using DPPH and TPC of the Pitaya Peel Extract.

No	Solvent	TPC (mg GAE/g extract)	DPPH (IC_{50}, ug/ml)
1	Ethanol	1.193+/-0.011	823.580+/-13.250
2	Water	1.351+/-0.021	261.520+/-0.980

Sources: Lourith and Kanlayavattanakul (2013)

2.4 Identification of Method

2.4.1 Method Determination of Phenolic Content

Folin-Ciocalteu method is commonly used to measure the phenolic content (John *et al.*, 2014), where gallic acid is applied as the standard reference. The Folin-Ciocalteu method involves calibration with a pure phenolic compound, absorbance analysis followed by the colour reaction using spectrophotometer at 760 nm of wavelength (Hafsa *et al.*, 2016). Moreover, many analytical techniques can be applied to determine the phenolic content including gas chromatography (Anli *et al.*, 2008), thin layer chromatography (Hawrył *et al.*, 2002) and capillary electrophoresis (Kvasnička *et al.*, 2008).

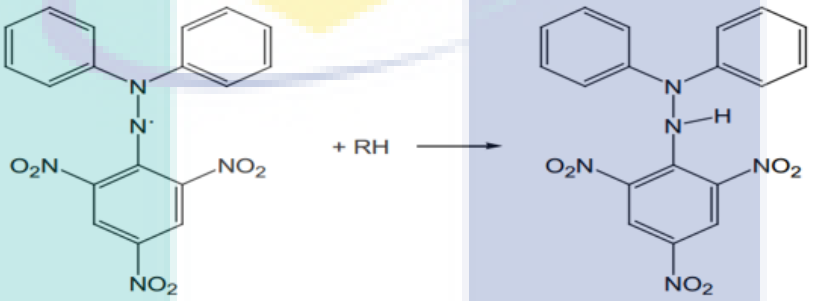
2.4.2 Method for Determination of Antioxidant Activity

Antioxidants are well-known as elements that can decelerate or inhibit the oxidation by preventing the initiation or propagation of oxidation chain reaction. Antioxidants are categorized according to their protective properties at various stages of the oxidation process. In addition, antioxidants are classified into two main categories namely primary and secondary antioxidants based on diverse mechanisms of action (Lim *et al.*, 2007). Primary antioxidant involves in scavenging of free radical, where it suppress chain initiation and break chain propagation by donating hydrogen atoms or electrons. Consequently, the radical form of antioxidant is converted into a stable form. On the other hand, secondary antioxidants inhibit the formation of radicals and protect the oxidative damages. Moreover, secondary antioxidant is also active in binding with metal ions and scavenging oxygen (Ruzlan *et al.*, 2010).

2.4.2.1 Radical Scavenging Activity

The capacity to eliminate or scavenge free radicals is the key function of primary antioxidant (Wang *et al.*, 2008). DPPH is reduced as it reacts with an antioxidant compound that donates hydrogen. This process is illustrated in Figure 2.4. The color of the mixture containing antioxidant is switched from purple to yellow. This antioxidant activity can be determined at 517 nm by using UV-vis spectrophotometer

(Ajila *et al.*, 2007). Ethanol is used as blank by excluding the test sample under the similar condition. In this study, the antioxidant activity of *H. polyrhizus* extracts over the stable DPPH is measured using the same method. It should be noted that DPPH can only be dissolved in organic media, particularly in alcoholic media and not in the aqueous media. The primary antioxidant exhibits higher potential with the rapid decline in the absorbance value (Siddhuraju *et al.*, 2002). Moreover, lowest value of IC_{50} implies the strongest DPPH scavenging capacity (Ajila *et al.*, 2007; La *et al.*, 2013).



2, 2-diphenyl-1-picrylhidrazil (Free radical form)

2, 2-diphenyl-1-picrylhidrazil (nonradical form)

Figure 2.4 Structure of DPPH and Its Reduction Form by the Antioxidant

Sources: Rohman *et al.* (2010)

DPPH scavenging activity can be quantified using the equation (2.1) (Rohman *et al.*, 2010) as expressed below:

$$\text{DPPH Scavenging activity (\%)} = [1 - (\frac{Atest}{Acontrol})]\ X\ 100\% \qquad 2.1$$

where,

$A_{control}$= absorbance of the control (2mL DPPH solution +1mL of 70% ethanol solution)

A_{test}= absorbance of the DPPH solution in the presence of extract (2ml DPPH+ 1mL of test compound)

2.4.3 Determination of Mineral Content

The mineral constituents of food play an essential role in human health (Marqués *et al.*, 2015). Sufficient amount of many essential elements forms a healthy diet to sustain normal physiological functions. Thus, it is crucial to measure mineral contents inclusive of essential and toxic elements in food. As such, pitaya peel extract is a promising natural food colorant and cosmetic ingredient for industrial applications as it contains many essential elements (Soomro *et al.*, 2016).

Analysis of mineral contents in fruit can be performed using many methods including flame atomic absorption spectroscopy (FAAS) or graphite furnace atomic absorption spectroscopy (GFAAS) (Gorinstein *et al.*, 2001; Radwan & Salama, 2006). Nonetheless, ICP-OES was utilized in this study to measure total mineral contents as observed in previous studies (Rojas *et al.*, 2010; Terrab *et al.*, 2004). It should be noted that ICP-OES has broad linear dynamic range and it enables simultaneous analysis of numerous elements in a single sample.

2.4.4 UHPLC-ESI-QTRAP-MSMS for Identification of Phenolic Compounds

The UHPLC refers to a cutting-edge method that is categorized based on the stationary phase packing material, which can be grouped into two types, namely reverse and normal phases. Commonly, the reverse and normal phases in UHPLC incorporate the separation of polar and non-polar molecules, respectively. Frequently, in the reverse phase of UHPLC, the polar solvents are applied as mobile phase, while non-polar for stationary phase. On the contrary, in the normal phase of UHPLC, the stationary phase is comparatively polar in nature (silica), whereas the mobile phase is non-polar, including hexane. Numerous composites/constituents of a sample are segregated by passing a mobile phase through a column, which is also called the stationary phase. The separation of compounds can be determined by looking into the varied affinities of stationary phase packing material against several constituents in the sample. As for this study, the phenomenex synergy fusion column (stationary phase) was utilized as it can be produced by using any non-polar material. Besides, the separation is based on the hydrophobic interaction between the compounds and the stationary phase (Moldoveanu & David, 2013).

The main antioxidant compounds found in plants are flavonoids and phenols, which are polar and readily soluble in water. UHPLC, which is coupled with a mass spectrometer (MS), has been widely used to identify phenolic compounds (López *et al.*, 2016). Meanwhile, the reverse phase of UHPLC with a phenomenex synergy fusion column has been commonly employed to segregate the antioxidant compounds. The acidic aqueous formic mixture and the acetonitrile are some of the common mobile phases employed in this separation. Moreover, the mobile phase of gradient elution (binary Gradient) has been proposed as an alternative for isocratic due to the benefits it have to offer. First, it enhances the peak shape for tailed peaks, aside from generating narrower and taller peaks for most of the constituents. Second, it lowers the retention of the later-eluting components, thus enabling rapid elution.

A number of researchers have applied the HPLC or UHPLC technique, along with MS in order to determine phenolic compounds, either by scanning MS in full or by targeting compounds in the sample, as presented in Table 2.4. It also shows that MS is a widely-used and advance new technology that determines phenolic compounds in sample extraction rapidly.

Table 2.4 Summary of Past Studies On The Application of UHPLC-MSMS To Determine Phenolic Compounds

Instrument Name	**Type of column**	**Mobile Phase**	**Raw Material**	**References**
HPLC-DAD-MSMS	Phenomenex RP- 18 (2 mm x 4.6 mm, 2 µm)	A:Water-formic acid (1%) B: Acetonitrile	Pitaya peel (*H. undatus*)	Ferreres *et al.* (2017)
UHPLC-ESI-QTOF-MSMS	Poroshell 120 EC-C18 (50 mm x 4.6 mm, 2.7 µm)	A: Water B: Methanol	Pitomba Fruit (*Talisia esculenta Radlk*)	Souza *et al.* (2016)
UHPLC-ESI-QTRAP-MSMS	Venusil C18 (100 mm x 2.1 mm, 3 µm)	A: Water-formic acid (0.1%) B: Acetonitrile-formic acid (0.1%)	Honeydew honey	Seraglio *et al.* (2016)

Table 2.4. Continued

Instrument Name	Type of column	Mobile Phase	Raw Material	References
UHPLC-ESI-MSMS	ODS-H80 (150 mm x 2 mm, 4 µm)	A: Acetonitrile-water (2:7, v/v) containing 0.1%(v/v) formic acid B: Acetonitrile-water (9:1, v/v) containing 0.1%(v/v) formic acid	Brown rice	Ogawa *et al.* (2017)
UHPLC-ESI-QTOF-MSMS	C18 (100 mm x 2.1 mm, 1.8 µm)	A: Water-formic acid (0.1%) B: Acetonitrile	Crude Drug (*Sheng-ma*)	Fan *et al.* (2016)
UHPLC-ESI-QTOF-MS	Knauer BlueOrchid C18 (100 mm x 2 mm, 1.8 µm)	A: Water B: Methanol	Dry seed (Sunflower)	Ghisoni *et al.* (2017)

2.4.5 Antibacterial Activity

Natural products like plant and fruit are major source of antibacterial compounds discovered till date (Berdy, 2005). Moreover, there has been increased attention on the plant and fruit extracts as promising antibacterial agents (Mabona *et al.*, 2013; Nazzaro *et al.*, 2013). In addition, several studies have indicated antibacterial activity from by-product of plant. These studies suggest the potential of by-product of plants as antibacterial agents (Pereira *et al.*, 2008; Rauha *et al.*, 2000).

Phenolic compounds are synthesized by plants for defense mechanism. These compounds act by interacting with the microorganism's cell membrane or cell wall and causes in alterations in membrane permeability. Consequently, this leads to cell destruction (Taguri *et al.*, 2006; Tian *et al.*, 2009). Moreover, phenolic compound can infiltrate into the bacterial cells and stimulates the coagulation of their constituents. Alternatively, phenolic compounds can react as natural antibacterial compounds that could increase the shelf life of diverse products and prevent the growth of pathogenic microorganism (Rains & Jain, 2011).

A substantial number of laboratory techniques have been made available to evaluate the in vitro antibacterial activity of an extract or a pure compound. The zone of inhibition and agar dilution are some of the well-established methods in this regard. Initially, the zone of inhibition technique was initially introduced by Smith, Laudicina and Rufo (1985) to assess the antibiotic sensitivity of some clinical specimens (Fathilah, 2011). In the zone of inhibition method, the growth media, the temperature, the incubation period, and the inoculum size should meet the specifications set by the Clinical and Laboratory Standard Institute (CLSI) for bacteria testing (Espinel *et al.*, 2011). Furthermore, the zone of inhibition technique is frequently used for the antibacterial screening of plant and fruit extract (Das *et al.*, 2010; Fguira *et al.*, 2005; Konaté *et al.*, 2012). In addition, time-kill kinetics technique is applied to determine the antibacterial activity. The time-kill kinetics test has time-dependent or a concentration-dependent antibacterial effect (Pfaller *et al.* (2004). Therefore, zone of inhibition and time-kill kinetics test are selected to analyse the antibacterial properties of pitaya peel extract.

2.5 Microwave Assisted Extraction (MAE)

In recent years, there has been increased interest in discovering effective and ground-breaking extraction methods to acquire natural bioactive compounds. These innovation are aimed towards achieving higher yield, lower extraction time and minimal solvent consumption (Simić *et al.*, 2016). As conventional methods exhibit several limitation drawbacks such as extensive extraction period, contamination of the product by solvent and relative low yield (Wang & Weller, 2006). In light of this, numerous studies have demonstrated advantages of MAE against the conventional extraction method: lower processing period, reduced solvent and energy demand; increased yield of TPC and antioxidant activity (Chen *et al.*, 2008; Hemwimon *et al.*, 2007). Moreover, MAE is cost-effective compared to other advanced extraction techniques as it needs lower extraction time (Li *et al.*, 2011). Higher TPC contents was extracted using MAE compared to conventional solvent extraction (CSE) and other advanced extraction techniques like ultrasound-assisted extraction (UAE) and accelerated solvent extraction (ASE) (Nayak *et al.*, 2015) (Refer Table 2.5).

Table 2.5 Comparison of the TPC Values From Citrus Peels Using Various Methods with Extraction Solvent of 51% Acetone

Extraction Method	TPC (mg GAE/g extract)
MAE	12.09+/-0.06
UAE	10.35+/-0.04
ASE	6.26+/-0.23
CSE	10.21+/-0.01

Source: Nayak *et al*. (2015)

Evidence has shown that samples extracted using MAE method displayed higher antioxidant activities compared to other advanced extraction techniques. Moreover, extract obtained via MAE using 50% ethanol as solvent had the highest radical scavenging activity with IC_{50} (Table 2.6). In short, MAE is appropriate to extract the bioactive compounds from plants and fruits as it results in higher TPC and antioxidant activity.

Table 2.6 The Antioxidant Activity of Different Extracts of *Eclipta Prostrate*

Extraction method	Solvent	IC_{50} (ug/mL)
MAE	50% ethanol	7.15
UAE	60% methanol	8.03
UAE	70% ethanol	8.69

Souce: Fang *et al.* (2015)

MAE allows rapid extraction of different phenolic compounds and other components with highest yields compared to traditional extraction techniques (Karabegović *et al.*, 2013). Nevertheless, there is no data on usage of MAE for extraction of mineral contents from plants and fruits. Various methods that are suitable to extract phenolic compounds are summarized in Table 2.7.

Table 2.7 Comparison of Different Extraction Methods of Phenolic Compounds

Extraction method	**Raw Material**	**Type of compound**	**References**
MAE	*Eclipta prostrata*	Caffeic acid, 5-*O*-caffeoylquinic acid, quercetin-7-*O*-glucoside, 4,5-Dicaffeoylquinic acid and 3,5-Dicaffeoylquinic acid	Fang *et al.* (2015)
UAE	*Cimicifugae rhizoma*	Caffeic, Isoferulic, Ferulic acids	Liu *et al.* (2015)
ASE	*Epilobium angustifolium*	Quercetin-3-*O*-glucuronide, Flavonol-3-*O*-glycosides	Monschein *et al.* (2015)
CSE	*Garden tea leaves (Camellia sinensis L.)*	Catechins	Gadkari *et al.* (2014)

The utilization of microwave can be merged with other extraction methods to improve its performance. For instance: microwave assisted distillation (MAD) used for the isolation of essential oils from herbs and spices (Chemat *et al.*, 2004); microwave-integrated soxhlet extraction (MIS), which is a merging of microwave heating and soxhlet (Virot *et al.*, 2007); solvent-free microwave extraction (SFME), which is the merging of microwave heating and distillation that is conducted at atmospheric pressure (Chemat *et al.*, 2009). There should be further investigation on these techniques to ensure their effectiveness in terms of guaranteeing the quality of herbal medicines (Mandal *et al.*, 2007).

On the other hand, MAE has several shortcomings. First, it requires further filtration or centrifugation to discard solid residue generated from the extraction process. Second, the efficacy of microwaves can be decreased if the target compounds or solvents are non-polar. In addition, exposure to excessive temperature and power in MAE degrades the heat sensitivity of bioactive compounds (Veggi *et al.*, 2012).

2.5.1 Operation of MAE

MAE in this particular study had been equipped with a power sensor (power range 0-800 W), an infrared temperature sensor (0-140 °C), and a time controller (0-100 min). The microwave radiation from the MAE was generated by electric and magnetic fields (Rodríguez *et al.*, 2017). The microwave is a non-ionizing radiation with no effect on the molecular structure of bioactive compounds (Farhat *et al.*, 2011; Shazman *et al.*, 2007), while the effect on extraction process is strictly associated to the conversion of electromagnetic energy to heat. Microwave heating can act directly on the sample via two mechanisms: ionic conduction and dipole rotation, in which both may occur simultaneously, hence increasing extraction yield (Lidström *et al.*, 2001; Thirugnanasambandham *et al.*, 2014).

Based on the environmental standard, MAE reflects the green technology that possesses the ability to extract a variety of phenolic compounds, such as carotenoid, flavonoids, and phenolic acids (Li *et al.*, 2013). Phenolic has a polar structure formed on natural sources, such as fruits and plants that are soluble in water and alcohol (Benedek & Kopp, 2007). Thus, numerous solvents, such as ethanol and methanol with distinct percentages of water, can be applied to extract bioactive compounds from diverse fruits and plants (Dai & Mumper, 2010).

Some of the crucial physical parameters for MAE are dielectric constant, extraction time, microwave power, solubility, and solvent property. The MAE is comprised of an infrared temperature sensor, power sensor, and a temperature controller, as illustrated in Figure 2.5. The microwave energy and the polar solvent elevate both the temperature and the pressure of sample during the MAE operation due to the penetration of microwave energy into cell matrix. As a result, the tissues of plants and fruits are ruptured and the target compounds are freed into the solvent (Cong *et al.*, 2017; Pap *et al.*, 2013). Based on a report regarding MAE, the increase in extraction yield could be due to a synergistic combination of two transfer phenomena, which are mass and heat, which function in an identical path from inside to the outside of the sample. This seems to contradict the conventional extraction, such as soxhlet extraction, which refers to mass transfer that is operated from inside to the outside. On the other

hand, transfer of heat is operated from outside to the inside sample, thus decreasing extraction yield (Farhat *et al.*, 2011; Flórez *et al.*, 2015).

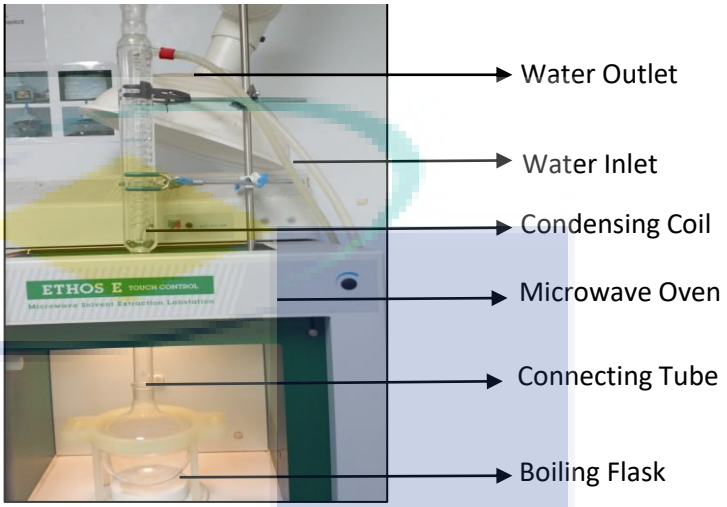


Figure 2.5 Microwave Assisted Extraction

Sources: Analytical Lab, FKKSA

The performance and the efficacy of MAE are influenced by a wide range of parameters, such as extraction period, sample particle size, power, temperature, and solid-liquid ratio (Pinela *et al.*, 2016). The optimization of these parameters is crucial to assure higher yield of bioactive compounds via extraction process. Both power and extraction period had been proven to directly correlate with yield of bioactive compounds. However, degradation can take place at high microwave power levels and longer extraction period (Dahmoune *et al.*, 2015; Vuong *et al.*, 2013). Table 2.8 depicts the use of MAE for extraction of a variety of bioactive compounds.

Table 2.8 Comparison Between Different Parameters of MAE In Terms of The Extraction Yields

Sample weight (solid/ liquid ratio)	**Volume of solvent (mL)**	**Optimal condition (P=power, t=time, T=temperature)**	**Solvent**	**Raw material**	**Bioactive compound**	**References**
24 g/L	50	P=400 W,T=45 °C, t=20 min	95% ethanol	Pitaya peel (*H. polyrhizus*)	Pectin (7.5%)	Thirugnanasambandham *et al.* (2014)
2.5 g	100	P=450 W, T=70 °C, t= 5 min	Water	Pitaya peel (*H. polyrhizus*)	Pectin (21.68%)	Tongkham *et al.* (2017)
1g	20	P=477 W, T=50 °C, t=2.13 min	Water	Watermelon (*Citrullus lanatus*)	Pectin (25.79%)	Maran *et al.* (2014)
1:10 (w/v)	100	P=810 W, T= 40 °C, t=30 min	Water	Pitaya peel (*H. undatus*)	Total Phenolic acid (0.29 mg GAE/g sample)	Chaiwut *et al.* (2012)
40 g/L	50	P=100 W,T=35 °C, t= 8 min	Water	Pitaya peel (*H. polyrhizus*)	Betalain (9mg/L)	Thirugnanasambandham and Sivakumar (2017)
45 g/L	25	P=200 W,T=146.69 °C, t= 5.51 min	Water	Tomato (*Lycopersicon esculentum)*	Phenolic acid (8.99mg GAE/g extract)	Pinela *et al.* (2016)
45 g/L	25	P=200 W, T=144.64 °C, t=3.15 min,	water	Tomato (*Lycopersicon esculentum)*	Phenolic acid (24.8mg GAE/g extract)	Pinela *et al.* (2016)

2.5.1.1 Parameters Studied

Bioactive compound extraction from the pitaya peels has been undertaken using the MAE technique in this study, specifically by applying the best condition of MAE parameters. The best condition has been distinguished by determining the highest TPC value from the pitaya peel extract. In addition, one-factor-at-a-time (OFAT) and SPSS analyses have displayed functional significance in determining the variance between the TPC mean values towards identifying the best condition values of MAE parameters. The OFAT method in particular looks into the behaviour of the model outputs and model input changes, whereby each time a design variable is changed over its entire range. Meanwhile, other parameters are held fixed at their initial mode concomitantly (Delgarm *et al.*, 2018; Giap & Kosuke, 2014). In this study, the effects of the some MAE parameters have also been studied, encompassing power, temperature, time and sample weight.

From Table 2.8, the preliminary range for MAE parameters determined in this study is based on previous research on MAE in extracting other types of bioactive compounds from pitaya peel, namely pectin and betalain from pitaya peel. The experiment has commenced with the selected power of MAE, which includes a lower and higher power of 100 W and 800 W respectively, based on literature by Thirugnanasambandham and Sivakumar (2017) and Chaiwut *et al.* (2012). The works have revealed that the bioactive compound yield has started to increase at the power of 100 W, before gradually decreasing when the power reaches 800 W. Meanwhile, this study has evaluated the power MAE range at the levels of 200, 400, 600, and 800 W. These values are linked with the direct effect of microwave radiation, which loosens the cell wall matrix and skin tissues. Then, it accelerates the extraction of bioactive compounds, whereby excessive microwave power will degrade the elements (Gfrerer & Lankmayr, 2005). Then, the minimum and maximum temperature value in MAE are set at 35°C (Thirugnanasambandham & Sivakumar, 2017) and 45 °C (Thirugnanasambandham *et al.*, 2014) respectively so as to elicit bioactive compounds yield. Further temperature increments exceeding 45 °C has revealed negligible effect on the extraction processes using the MAE technique (Maran *et al.*, 2014). Thus, this study has opted for temperature ranges for MAE to be evaluated at 35, 40, and 45 °C respectively. Moreover, extraction time in MAE has been explained previously,

whereby the initial stages of microwave radiation have promoted thermal accumulation of the reaction mixture. This has led towards effective dissolution of bioactive compound, but excessive time exposure in the microwave field may them to degrade (Pinela *et al.*, 2016). Besides, the lower and upper limits for time MAE is 8 min and 30 min respectively as per Thirugnanasambandham and Sivakumar (2017) and Chaiwut *et al.* (2012) accordingly. This has called for this study to opt for a range of time in MAE set at 10 min, 15 min, 20 min, 25 min, and 30 min respectively. Additionally, the sample weight range has been evaluated at the levels of 1.2, 2.2, and 3.2 g in this study, as per the lower and higher weight of 1.2 g and 2.5 g advocated by Thirugnanasambandham *et al.* (2014) and Tongkham *et al.* (2017) respectively. The solvent is then saturated with the large sample, negatively affecting the mass transfer rate and barricaded bioactive compound penetration into the solution and decreasing the extraction yield (Pinela *et al.*, 2016).

For MAE technique, freeze dried samples (pitaya peel) has been mixed with 50 mL of water as a solvent extraction and placed in the extraction vessel. The volume of solvent extraction has been maintained constant for all four parameters studied. The initial conditions are as follows: power 200 W, temperature 45 °C, sample weight 1.2 g and 20 min contact time for pitaya peel extraction. Meanwhile, the subsequent condition for MAE has called for the same parameters to be selected differently according to the range set in this experiment. They are microwave power (200-800 W), temperature (35-45 °C), time (10-30 min) and sample weight (1.2-3.2 g), whereby the influence of each parameter has been investigated in OFAT. Each trial has been carried out in triplicate, with each factor influence upon TPC yield has been assessed statistically using SPSS with Bonferroni's post-hoc test.

2.6 Scanning Electron Microscopy (SEM)

Cell wall disruption effect occurred in the sample due to microwave radiation from MAE is observable scanning electron microscopy (SEM) (Hu *et al.*, 2018). Over the past few years, SEM usage in the fields of life science, chemistry, food and fruit analysis have increased in a stronghold (Dudkiewicz *et al.*, 2011). It also allows the sample tilting process, allowing observation from different angles (Pouchou *et al.*, 2002), whereas optical imaging using the technique is cheap, repeatable, enables very simple sample preparation, and offers the advantages of fast scanning images (Lyman *et al.*, 2012). Furthermore, microscopic structural changes of a sample before and after extraction can be observed to understand the characteristics of different extraction methods. The effectiveness of SEM in observing the fruit peel structure after extraction has been demonstrated in various studies. Xu *et al.* (2014) have extracted pectin from grapefruit peel using ultrasound heating method, while Hu *et al.* (2018) have extracted oil from tiger nut (*Cyperus esculentus L.*) using MAE and Soxhlet extraction. In this research, SEM has been utilised to elucidate morphological changes of pitaya peel extracted via MAE method.

The SEM is comprised of two major parts, namely the column and the cabinet. The column refers to the extension that the electrons traverse from emission until reaching the sample, where the installed detectors capture the signals scattered due to the electron-sample interaction. Meanwhile, the detectors are energy transducers that transform any type of signal into an electrical signal, which is then sent to the control cabinet. The control cabinet is equipped with an electronic system that enables quantification of electrical signals sent by the detector, which converts them into analysable information like images and graphs (Assumpção & Ferri, 2017). Figure 2.6 illustrates the compartments of SEM, consisting of vacuum, electron gun, electron column, and sample chamber.

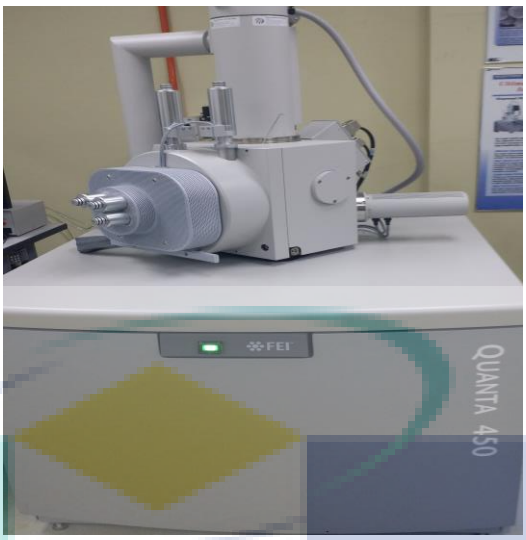


Figure 2.6 Scanning Electron Microscopy (SEM)

Sources: Central Lab, UMP

Generally, samples examined via SEM must be electrically conductive to minimize charge build-up on them due to the electron beam. Any charge build-up may potentially degrade the sample, subsequently distorting the image data (Sawyer *et al.*, 2008). During imaging, the electrons continuously bombard the sample, allowing negative charges to be generated under the beam. An adequately large negative charge buildup may deflect the incident and emit electrons to ruin the image. Hence, this may be prevented by ensuring the sample is electrically conductive so that the current deposited by the electron beam can pass through it and to the electrical ground (Lyman *et al.*, 2012). In fact, some samples, such as metals, are natural conductors, whereas ceramics, polymers, and biological materials are not conductive. Hence, the sample surface is coated with a thin layer of inert conductive substance, such as platinum, as undertaken by the pitaya peel samples in this analysis (Hu *et al.*, 2018).

The development in SEM has brought upon significant improvisation for surface morphology observation, which includes the use of low-voltage scanning electron microscope (LVSEMs), environmental scanning electron microscope (ESEMs), sources capable of providing greater brightness than field emission scanning electron microscope (FESEM), and electron detectors within the lenses (Danilatos, 2013). Prior studies have also used SEM to observe the external structure of samples after extraction

by utilising varied extraction methods, as portrayed in Table 2.9. The table thereby substantiated SEM as a great tool in comprehending the characteristics of various extraction techniques in observing the external structure of various samples.

Table 2.9 Analysis of Microscopic Changes On SEM Test

Extraction method	**Raw material**	**References**
Microwave assisted extraction (MAE)	Tiger Nut (*Cyperus esculentus L.*)	Hu *et al.* (2018)
Hydrodistillation	Herb (*Dodartia orientalis L.*)	Wang *et al.* (2017)
Microwave assisted extraction (MAE)	Brown seweed tallus	Flórez *et al.* (2015)
Microwave assisted hydrodistillation	Thyme leaves	Golmakani and Rezaei (2008)
Steam distillation	Lavender	Sahraoui *et al.* (2008)
Microwave hydrodiffusion and gravity	Rosemary leaves	Bousbia *et al.* (2009)
Microwave dry-diffusion and gravity	Caraway seed	Farhat *et al.* (2010)

2.7 Summary of the Literature Study

This chapter describes the pitaya peel extract as a potential natural agent for application in the food and cosmetic industries. The advantages of pitaya peel are elaborated in detail. The benefits concerning the usage of non-toxic solvent, such as water, are discussed in-depth. Water had been selected as the extraction solvent in this study mainly due to its non-toxic effect and high polarity attributes. The use of polar solvent is suitable for MAE as it is equipped with a sensor. Moreover, this chapter depicts the selection of research methodology, which is MAE, along with the vast advantages it has to offer. In this light, several parameters, such as lower processing time, smaller solvent volume, reduced energy demand, elevated yield of TPC, and increased antioxidant activity linked to MAE, are described in this chapter.

In short, this study determined the best conditions for extraction of pitaya peel extract of *H. polyrhizus* using the MAE technique. Two types of bioactive compounds, which are mineral contents and phenolic compounds, had been measured for the first time in this study. The antibacterial activities against pitaya peel extract from MAE were evaluated in this research for the first time as well. Finally, in order to comprehend

the characteristics of extraction sample after MAE, SEM was employed for it is an exceptional instrument used to observe external structure of samples, as explained in great length in this chapter.

CHAPTER 3

METHODOLOGY

3.0 Overview of Research Methodology

The methodology of this study is divided into five stages, as illustrated in Figure 3.1 that summarizes the flow chart of research design of this study in the attempt to achieve the three objectives outlined in this study. The five stages incorporated in the research design are as depicted in the following: Stage One, preparation of the pitaya peel in powder form via freeze-drying method; Stage Two, determination of the best conditions for four MAE parameters, namely, power, temperature, time, and sample weight of sample, based on TPC values; Stage Three, extraction of the bioactive compounds of pitaya peel based on the best conditions of MAE parameters determined in Stage Two; Stage Four, evaluation of pitaya peel extract via ultraviolet-visible (UV-Vis), inductively coupled plasma (ICP-OES), and ultra-high performance liquid chromatography coupled mass spectrometer (UHPLC-ESI-QTOF-MSMS); and lastly, Stage Five, evaluation of antibacterial activities displayed by pitaya peel extract against Gram-positive and Gram-negative bacteria.

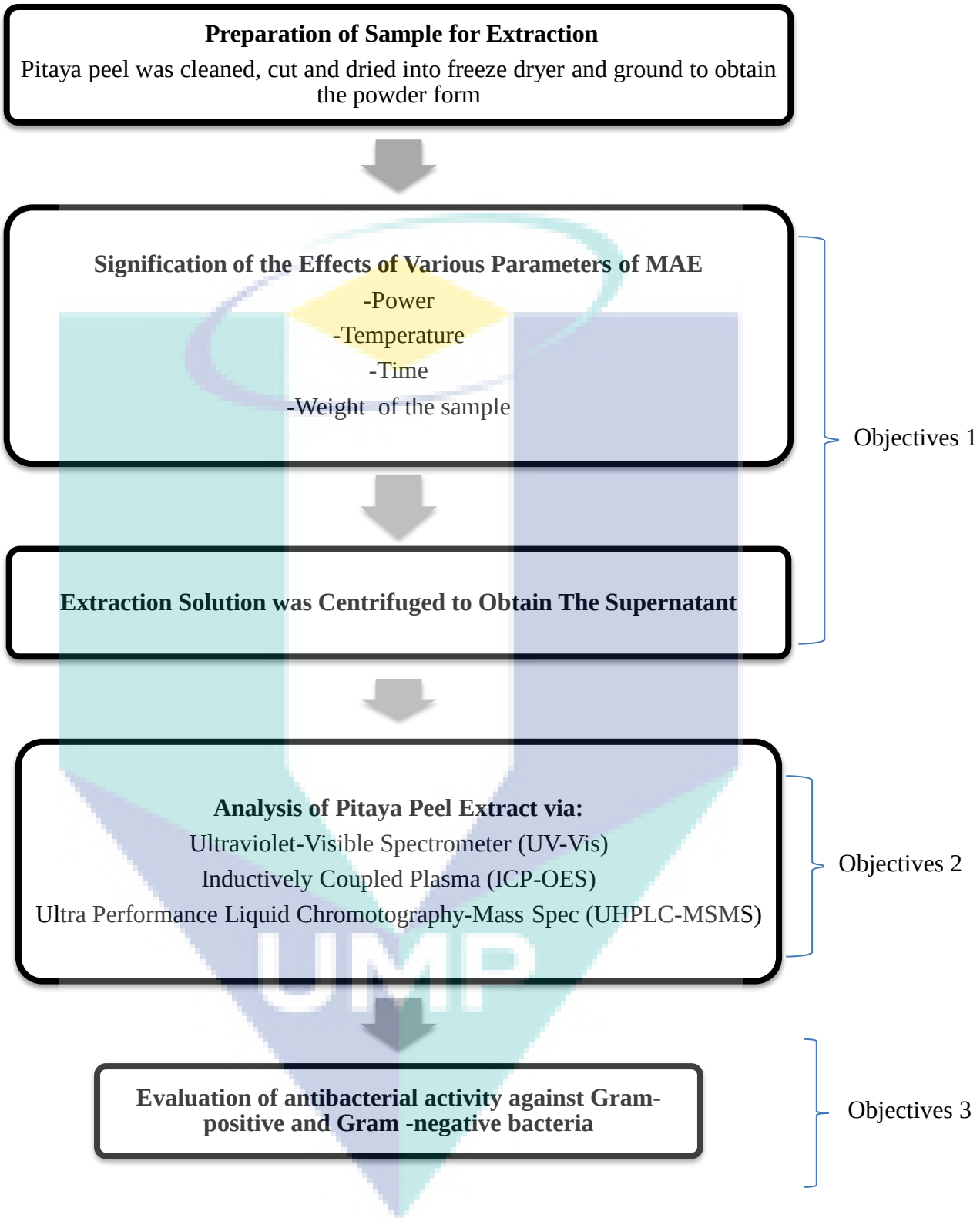


Figure 3.1 Flow Chart of Research Design of the Study

3.1 Sample Preparation and Chemicals

The pitaya fruits of *H. polyrhizus* species have been purchased during a dry season from a store in Temerloh, Pahang. The peels were separated, weighed and washed with distilled water. Subsequently, the peels were sliced into 2 cm pieces. The samples then subjected to the freeze-drying process, where the samples were frozen overnight at -80 °C and then kept in the freeze dryer (Alpha 2-4LDplus, German) for 96 h. Later, the freeze-dried samples were grounded and sifted through a 40 mesh (0.42 mm) sieve to form the powdered samples. These samples were kept in sealed bags and stored in a dry place for subsequent experiments (Chaiwut *et al.*, 2012).

Pure standard of Folin-Ciocalteu's reagent, Gallic acid, and DPPH were purchased from Sigma-Aldrich, while the standard components for mineral analysis were purchased from Perkin Elmer. Other reagents applied from analytical to chromatographic grades were obtained from Merck.

3.2 Extraction Procedure

3.2.1 Microwave Assisted Extraction (MAE)

The extraction of pitaya peels was performed by employing the MAE (ETHOS 1, Milestone SRL, Sorisole, Italy) technique, along with the OFAT method. The MAE was equipped with a time controller, a temperature sensor, a power regulator, and a circulating water-cooling system. At the time of the experiment, power, temperature, and time parameters were controlled by using an electronic control panel. Liquid-solid extraction was performed by adding the freeze-dried sample with 50 mL of distilled water into a 1000 mL extraction flask. Next, the extraction flask was placed in the MAE and was linked to the cooling system through a hole found at the top of the microwave extraction apparatus. After that, the MAE was turned on and the experimental condition, including microwave power, as well as extraction temperature and time, had been set by the digital panel. The conditions of MAE, specifically power, temperature, time, and sample weight, were assessed throughout the process.

The homogenate was centrifuged at 9000 rpm for 40 min at 25 °C shortly after the extraction process. The supernatant was collected after the centrifugation. The similar process was repeated twice so as to allow maximum extraction of bioactive compounds. All experiments were performed in triplicates (Thirugnanasambandham & Sivakumar, 2015).

3.2.2 Extraction of Bioactive Compound

Pitaya peel in the powder form was obtained through freeze-drying method prior to the extraction process via MAE. A total volume of 50 mL distilled water was used as a solvent for the extraction process (Cardoso *et al.*, 2014a).

3.2.3 Effect of Parameters

The experimental parameters determine the yield of bioactive compounds from the extraction of pitaya peel. As such, the effects of power, temperature, time of MAE, and weight of the pitaya peel were investigated in this study. It should be noted that these four parameters have been shown to significantly affect the yield of bioactive compounds from the extraction process (Prakash *et al.*, 2013).

3.2.3.1 Effect of Power in MAE

The effect of extraction power was determined through MAE analysis of four values of power (200, 400 , 600 , and 800 W). Meanwhile, other parameters were fixed as constant (Chaiwut *et al.*, 2012; Thirugnanasambandham & Sivakumar, 2015; Thirugnanasambandham *et al.*, 2014). Concurrently, the temperature, extraction period, and weight of the pitaya peel were fixed at constant values of 45 °C, 20 min, and 1.2 g respectively. Subsequently, remaining residues in the liquid extract were removed through centrifugation for 40 min. Finally, the TPC yield was measured using UV-VIS.

3.2.3.2 Effect of Temperature in MAE

The effect of temperature was determined through MAE analysis of three extraction temperature points (35 , 40 , and 45 °C). Meanwhile, other parameters were fixed as constant with minor variation (Chaiwut *et al.*, 2012; Thirugnanasambandham & Sivakumar, 2015; Thirugnanasambandham *et al.*, 2014). Simultaneously, the power, extraction period and weight of the pitaya peel was maintained at constant values of 400 W, 20 min, and 1.2 g, respectively. Subsequently, the remaining residues in the liquid extract were removed through centrifugation for 40 min. Finally, the TPC yield was measured using UV-VIS.

3.2.3.3 Effect of Time in MAE

The effect of extraction period was determined through MAE analysis using five extraction periods (10, 15, 20, 25, and 30 min). Meanwhile, other parameters were fixed as constant with small adjustment (Chaiwut *et al.*, 2012; Thirugnanasambandham & Sivakumar, 2015; Thirugnanasambandham *et al.*, 2014). Other parameters were at the same time, power, temperature, and weight of the pitaya peel were maintained at constant values of 400 W, 45 °C, and 1.2 g respectively. Subsequently, remaining residues in the liquid extract were removed through centrifugation for 40 min. Finally, the TPC yield was measured using UV-VIS.

3.2.3.4 Effect of Weight of Sample in MAE

The effect of weight of sample was determined through MAE analysis of three weights pitaya peel extracts (1.2, 2.2, and 3.2 g). Meanwhile, other parameters were maintained constant with slight alteration (Chaiwut *et al.*, 2012; Thirugnanasambandham & Sivakumar, 2015; Thirugnanasambandham *et al.*, 2014). Subsequently, remaining residues in the liquid extract were removed through centrifugation for 40 min. Finally, the TPC yield was measured using UV-VIS.

3.3 Analysis of Solution Extraction

3.3.1 Determination of TPC

The antioxidant content of each sample was quantified using the TPC assay with the Folin-Ciocalteu method, which was adapted by Lim *et al.* (2007). A total volume of 0.3 mL of each extract was added in each test tube. Subsequently, 1.5 mL of the Folin-Ciocalteu reagent plus 1.2 mL of 7.5 % w/v of sodium carbonate solution were added to the test tubes. The test tubes were then shaken and incubated at room temperature for 30 min in the dark. Consequently, the absorbance was measured at 765 nm via Shimadzu Lambda. All samples and readings were performed in triplicates. A calibration curve was depicted using the regression equation of the calibration curve for gallic acid as Equation 3.1. Finally, the antioxidant contents were expressed as mg gallic acid equivalent (GAE)/g of the dried peel.

$$(y = 0.094x, r2 = 0.9985) \quad 3.1$$

3.3.2 Free Radical Scavenging Activity Assay

The DPPH assay was utilized to measure the free radical scavenging activity of the antioxidants (Khamsah *et al.*, 2006) with minor adjustments. In this study, DPPH is a synthetic free radical that reacts with the antioxidant content of the pitaya peel to form the DPPH complex. The reaction was visible, where the colour transformed from purple to yellow due to the hydrogen-donating ability of the antioxidant (Ajila *et al.*, 2007).

The reagent and solution for this experiment were prepared using 70 % ethanol. Each sample was prepared in serial dilution of 0.2, 0.4 , 0.6 , 0.8, and 1.0 mL/mL with a final volume of 10 mL.

In a test tube, 1 mL pitaya peel extract was added to 2 mL DPPH reagent (0.1 mM). Subsequently, the solution was mixed using a vortex mixer for 20 seconds and incubated at room temperature for 30 min in the dark. The reduction of DPPH was quantified at 517 nm against 70 % ethanol as a blank. Importantly, all tests were conducted in triplicates. The percentage of the scavenging activity was quantified

through the absorbance rates. The antioxidant activity of the samples was measured using the Equation 3.2 as follows:

$$\text{DPPH Scavenging activity (\%)} = [1 - (\frac{Atest}{Acontrol})]\ X\ 100\% \qquad 3.2$$

Where, $A_{control}$ was the absorbance of the DPPH solution excluding the extract. Contrarily, A_{test} was the absorbance of the DPPH solution in the presence of the extract encompassing 2 mL DPPH and 1 mL sample compound.

3.4 Determination of Mineral Contents

3.4.1. Sample Preparation

The best conditions of MAE obtained from TPC study was applied to extract mineral from pitaya peel. A mass of 1.2 g freeze-dried sample was weighed precisely in a extraction vessel and 50 mL of water added to the vessel. The extraction of the mineral using MAE was performed for 20 min at 45 °C, and 400 W. Subsequently, the pitaya peel extract was centrifuged to get the supernatant. The supernatant were then filtered using Whatman filter paper with size of 5C. Later, 5ml of filtered solution was measured using a measuring cylinder and was poured into 10 mL volumetric flask. Then, 10 % hydrochloric acid (HCL) was mixed into the volumetric flask up to 10 mL. Lastly, the mixed solution was transferred from volumetric flask into the vial tube. The mixture was immediately subjected to ICP-OES (Marqués *et al.*, 2015).

3.4.2 Operating Condition on ICP-OES

The mineral content analysis was performed via SGS (Malaysia) method in agreement with the SGS MINE-MG SOP 010 (2017). This technique is applied for elemental analysis of ICP-OES in the food sample. The Perkin Elmer Optima 8300 DV Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) system (German) furnished with an autosampler plus Mira mist nebulizer was utilized for mineral content analysis. The calibration standard for the analysis contains the stock solutions of a multi-element standard solution (1000 ppm) with 24 elements

(Aluminium (Al), Arsenic (As), Barium (Ba), Berylilium (Be), Bismuth (Bi), Cadmium (Cd), Calcium (Ca), Chromium (Cr), Cobalt (Co), Copper (Cu), Iron (Fe), Lead (Pb), Lithium (Li), Magnesium (Mg), Manganese (Mn), Molybdenum (Mo), Nickel (Ni), Potassium (K), Sodium (Na), Strontium (Sr), Titanium (Ti), Vanadium (V), Yittrium (Y) and Zinc (Zn)) dissolved in 5% nitric acid .

All elements were measured in the radial mode of ICP-OES, excluding As and Pb, which were measured in the axial mode. All experiments were performed in triplicates. The quality control (QC) standard in solution with a concentration of 10 ppm that comprises 24 elements was applied for data verification. The QC of the pitaya peel extract was also performed. Table 3.1 demonstrates the operating conditions of the ICP-OES.

Table 3.1 The Operating Conditions of The ICP-OES Instrument

Parameter	Flow Rate
Argon plasma	12 L/min
Auxiliary gas	0.4 L/min
Nebuliser gas	0.5 L/min
Radio Frequency (RF)	1500 Watts
Pump	1.5 mL/min

Source: SGS MINE-MG SOP 010 (2017)

3.5 Determination of phenolic compounds by UHPLC-ESI-QTRAP-MSMS

3.5.1 Sample Preparation

The best conditions for MAE from TPC study was utilized for extraction of phenolic compounds from pitaya peel. A mass of 1.2 g freeze-dried sample was weighed precisely in extraction vessel, where 50 mL of water was added. MAE was used for extraction of phenolic compounds from the samples under the following conditions: 20 min at 45 °C; 400 W. Subsequently, the supernatant was obtained after centrifugation of pitaya peel extract from MAE. The supernatant was then filtered using Whatman filter paper with size of 5C. Later, the filtered solution were diluted with 5 mL methanol (50%) UHPLC grade and filtered with 0.45 µm nylon syringe filter to inject with volume 20 µL into LCMSMS (Souza *et al.*, 2016).

3.5.2 Operating Condition on UHPLC

The analysis was performed using a Flexar FX 15 ultra-high-performance liquid chromatography (UHPLC, PerkinElmer, Inc, Massachusetts, USA) coupled with an AB SCIEX 3200 QTrap hybrid linear ion trap triple quadruple mass spectrometer furnished with a turbo ion spray source. Chromatographic separation was performed using a Phenomenex Synergi Fusion (100 mm x 2.1 mm x 3 µm) column (polar). The detector applied is photomultiplier to determine phenolic compounds. Mobile phase A consisted of water with 0.1 % (v/v) formic acid plus 5 mM ammonium format, while the mobile phase B composed of acetonitrile containing 5 mM ammonium formate. Elution was conducted through a linear gradient from 5-95 % B (0.01-10 min) for 2 min, returned to 10 % B in 0.1 min, and then re-equilibrated for 3 min prior to the next injection. Ionisation was performed through electrospray ionization on the AB Sciex Turbo V source with an ionization temperature of 500 °C and purified nitrogen gas (99 %) as the collision gas via nebulization. Collision energy was fixed at 35eV aimed at mass fragmentation. Complete scan with MS/MS data collection evaluation was carried out in negative mode. Moreover, data analysis, processing, and interpretation were conducted via AB SCIEX Analyst 1.5 and Advance Chemistry Development, Inc (ACD/Labs, Ontario, Canada) MS Processor software. Principal component analysis (PCA) was performed using Marker View Software (AB SCIEX, Massachusetts, and USA). The following parameters were applied for PCA: retention time (R_T) range: 0-15 min; tolerance : 0.5 min; mass range: m/z 100-1000; mass tolerance: 0.01 Da; noise threshold: 5 (Lau *et al.*, 2014).

3.6 Antibacterial Testing

3.6.1 Preparation of Sample

The best conditions for MAE from TPC study was utilized for extraction of antibacterial compounds from pitaya peel. A mass of 1.2 g freeze-dried sample was weighed precisely in extraction vessel, where 50 mL of water was added. MAE was used for extraction of antibacterial compounds from the samples under the following conditions: 20 min at 45 °C, and 400 W. Subsequently, the supernatant was obtained

after centrifugation of pitaya peel extract from MAE. The supernatant was then filtered using Whatman filter paper with size of 5C. Subsequently, the filtered solution was utilized for the antibacterial testing (Chaiwut *et al.*, 2012).

3.6.2 Preparation of Medium

3.6.2.1 Preparation of Broth

A total of 500 mL of broth in two conical flasks, each containing 250 mL of broth was prepared. Thus, 15 g of Tryptone Soya Broth (TSB) was added to 500 mL of distilled water and transferred to a Schott bottle. The solution was then stirred using magnetic stirrer and well dissolved through boiling on the hotplate. Each flask contained 100 mL of broth, which were sterilized in the autoclave at temperature of 121 °C for 20 minutes (Park *et al.*, 2016).

3.6.2.2 Preparation of Agar

A total of 20 g Tryptone Soya Agar (TSA) was added to 500 mL of distilled water and transferred into a Schott bottle. The mix solution was well dissolved through boiling on hotplate. Petri dishes, TSB and TSA were autoclaved at 121 °C for 15 min. The cap of the Schott bottles was left unfastened to endure high pressure. At the same time, the laminar flow cabinet was sprayed and wiped with methanol. Subsequently, UV rays were turned on for 15 min to sterilize the workspace. After the temperature of the autoclave is reduced, the agar medium was taken out and poured on Petri dishes and allowed to harden. Upon hardening, the Petri dishes containing agar medium were sealed with parafilm to avoid any contamination. Finally, the Petri dishes were stored in the freezer upside down (Jayaprakash *et al.*, 2017).

3.6.3 Preparation of Bacterial Culture

3.6.3.1 Inoculum

In this study, *S. aureus* and *E. coli* were used as test organisms, which have been purchased from Team Medical & Scientific Sdn Bhd (TMS), Selangor, Malaysia. The samples were prepared from fresh colonies on TSA. One loopful of the each bacteria was inoculated in various conical flasks comprising 100 mL of TSB. The flasks were then incubated in an incubator shaker at 37 °C and 120 rpm for 18 h till the exponential phase. A total of 100 µL of each bacteria culture were reinoculated into new sterilized conical flasks comprising 100 mL of TSB for additional 22 h to get 10^8 CFU/mL (Hafsa *et al.*, 2016).

3.7 Analysis of Antibacterial

3.7.1 Zone of Inhibition

Agar plate TSA was inoculated with standardized inoculum of the test bacteria. The indicator cultures were utilized in this study, *S. aureus* and *E. coli*. A total of 100 µL inoculum with bacterium was transferred to the Petri dish comprising TSA. A T-spreader was utilized to spread the bacteria suspension uniformly on the agar. Subsequently, a total of 20 µL of pitaya peel extract were released on the centre of filter paper disc (around 7 mm in diameter). Later, all discs were placed on the nutrient agar plate. Moreover, positive control was prepared through addition of 20 µL Dettol as a substitute for pitaya peel extract and negative control was used by excluding material on the centre of filter paper disc to smear on the nutrient agar plate. Subsequently, plates were incubated at 33 °C for 24 h. Later, the diameter of inhibition zone was quantified in millimeter. The antibacterial activity was expressed as the diameter of inhibition zones produced by the extracts against the test bacteria. It should be noted that three independent experiments were carried out with each strain (Balouiri *et al.*, 2016).

3.7.2 Time-kill Kinetics

The time-kill kinetics test is a suitable method to determine a time-dependent or a concentration-dependent antibacterial effect (Pfaller *et al.*, 2004). The test is conducted in broth culture medium (TSB) using three conical flask comprising a bacterial suspension of *S. aureus* and *E. coli*, respectively. A total of 50 mL of sterilized TSB were poured into a conical flask, where three flask were prepared for each bacterial suspension against the extracted sample. A total of 100 µL of bacterial suspension were added into each flask. The first and second conical flask of TSB encompassing a bacterial suspension, which were prepared by adding pitaya peel extract of two volume levels of 5 mL and 10 mL, respectively. The third conical flask was considered as the growth control, prepared by excluding pitaya peel extract. To obtain the initial optical density (OD), the sample were taken immediately after addition of pitaya peel extract. Afterwards, the samples were taken every two-hourly up to a total of 14 hours (2, 4, 6, 8, 10, 12 and 14 hours) with the incubation being done under a suitable condition (30 °C and 150 rpm) (Konaté *et al.*, 2012; Pfaller *et al.*, 2004). The OD reading was measured using the samples from each conical flask with time intervals of 2 h. The samples were diluted to suitable concentrations for the OD measurement. Subsequently, the absorbance of each sample was measured using a spectrophotometer at 600 nm (Bernardez & Andrade, 2015). Finally, a calibration curve is depicted by plotting the absorbance and incubation time was depicted.

3.8 Scanning Electron Microscopy (SEM) Observation

The SEM (FET QUANTA-450, Netherlands) was employed to observe the morphological changes of pitaya peel samples before and after MAE extraction. The samples were oven-dried at 60 °C for 15 minutes and sputtered with a thin layer of platinum. Next, the samples were observed under vacuum at an accelerating voltage of 8.0 KV and 500 magnification (Hu *et al.*, 2018).

3.9 Statistical Analysis

3.9.1 SPSS

All experiments were conducted in triplicates, where the results were expressed as mean ± standard deviation (SD) or standard error (SE). The data were analysed using the IBM SPSS Statistic 21.0 (Zain *et al.*, 2014). One-way analysis of variance (ANOVA) was used measure the mean values between the groups. The multiple correction using Bonferroni test was applied. The significant level of ($p \leq 0.05$) was used in this study.

3.9.1.1 One-way Anova

The One-way Anova approach had been applied in this study to compare the TPC mean values. The significant variance was determined by comparing the *p*-values with 5 % error fixed in SPSS. In fact, two conditions can be derived from *p* (practicalities) values, which could be either small or large. The test of TPC mean values is significant if H_0 (null hypothesis) is rejected as the *p*-value would be smaller than 5 % error, but insignificant if H_0 (null hypothesis) is accepted as the *p*-value exceeds the 5 % error (Hox *et al.*, 2017).

3.9.2 CCLASS

Data interpretation of mineral content was performed using CCLASS in agreement with the SGS (Malaysia) manual record (SGS KLG WI 013, 2017). This software was applied to validate the mineral contents of pitaya peel extract that was acquired from the ICP-OES instrument. The data were analyzed based on quality control (QC) standard as the reference material. In this study, QC standard with concentration 10 ppm was chosen to recover all elements in the mineral contents. Tolerance for each element at QC standard was fixed into the software. This allows the users to check the accuracy of data through the use of the value of z-score. According to the laboratory quality standard international (LQSI), the QC data is acceptable range if the value is within the range of the three sigma limit, which is $-3 \leq z \leq 3$. The z-score value was calculated using the Equation (3.2)

$$\text{Z-score} = \frac{Experiment\ Value - Expected\ Vale}{Standard\ Deviation\ (SD)} \qquad 3.2$$

In Cclass, tolerance value is available for each element and the user has to provide the expected value in the software. The standard deviation for each element is calculated using the tolerance information, in which the z-score will be calculated based on the tolerance and standard deviation. As such, LQSI specified that a process is outside the control or unacceptable if a single point falls outside the three-sigma control limit, which is a z-score more than +3 or -3.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Effect of Various Parameters on MAE

The MAE had been selected in this study as a novel and green extraction technique. This particular selection had been made on the basis of the benefits that MAE has to offer, where it enables good yield of compounds within a shorter time, lesser solvent consumption, and minimum energy input (Naczk & Shahidi, 2004). Hence, the MAE appears to be a promising method to release bioactive compounds from waste food resources (Thirugnanasambandham & Sivakumar, 2017). In this research, the effects of several MAE parameters, namely, power, temperature, time, and sample weight, had been investigated to determine the best conditions. The findings of this study may lead to a significant solution to address several rising environmental issues associated to waste disposal and extraction of beneficial by-products from fruit wastes.

4.2 The Effect of Power in MAE

The best condition of power had been determined by generating the concentration of TPC value, where the MAE power contacted had been varied within the range of 200 until 800 W. The effects of power on TPC values are displayed in Figure 4.1.

Figure 4.1 shows that the TPC value increased at the initial stage with the power between 200 and 400 W. Prakash *et al.* (2013) asserted that increment of microwave power is linked to the direct effects of microwave energy on the sample, which could accelerate the extraction of phenolic compounds. As such, the cell wall matrix and the skin tissues of the sample would rapidly loosen due to microwave radiation and extensively tear (Kratchanova *et al.*, 2004). Therefore, the interaction between solvent extraction and sample is increased during the extraction process so as to enhance solvent penetration into the sample (Maran & Prakash, 2015). More electromagnetic energy is transferred on the bioactive compounds via ionic conduction and dipole rotations, thus resulting in power dissipation in the solvent and sample that further generates molecular movement and heating on the extraction system rapidly, apart from improving the efficiency of the extraction (Gfrerer & Lankmayr, 2005).

The TPC values also began to decrease when the power was at 600 to 800 W, perhaps due to the degradation of compounds with higher microwave power during the extraction (Proestos & Komaitis, 2008). Based on the statistical analysis, 200 and 800 W indicated insignificant difference with TPC values at 3.411 and 3.503 mg GAE/g dried peel, respectively. At 400 and 600 W power, significant variances had been noted with TPC values at 5.808 and 4.784 mg GAE/g dried peel, respectively. As a result, the optimum power to obtain maximum yield of TPC value was 400 W. This condition was further used to study the effects of time, weight, and temperature.

The outcomes of this study is in agreement with that reported by other researchers who investigated the extracts of pitaya (Thirugnanasambandham *et al.*, 2014) and orange peels (Prakash *et al.*, 2013), which exemplified increment in pectin, a bioactive compound, after the power was increased, but the yield seemed to decrease

when the power exceeded 400 W in MAE. Thus, in order to save energy and to avoid degradation, 400 W was utilized for further analysis of extraction.

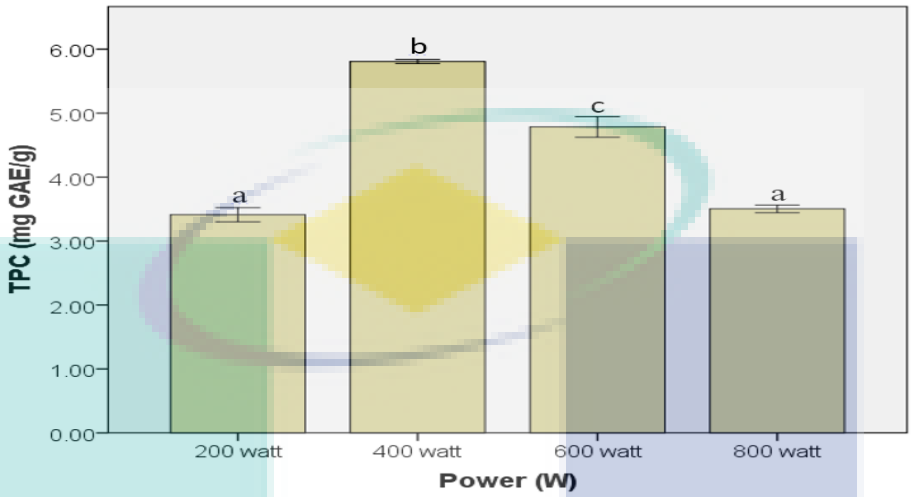


Figure 4.1 The Effects of Various MAE Power Values Against TPC Yields. Error Bars Represent the Standard Deviation from the Mean Values of Triplicate Readings. Different Letters Indicate Significant Differences at $p \leq 0.05$.

4.3 The Effect of Temperature in MAE

The best condition of temperature was determined based on the concentration of TPC value, where the MAE temperature had been varied in the range of 35 until 50 °C. Figure 4.2 illustrates the impact of temperature on TPC values.

Figure 4.2 shows that the TPC value increased at the initial stage as the temperature was hiked from 35 to 45 °C, mainly due to the formation of large amounts of dipole rotation (Thirugnanasambandham & Sivakumar, 2015). In fact, more compounds are distributed to the solvent if the temperature is high, as it facilitates the disruption of cell wall and skin tissues (Farsi & Lee, 2008). Elevated temperature values seemed to enhance extraction yield as a result of increased diffusivity of the solvent into the matrix of sample, as well as partitioned bioactive compounds into the solvent extraction (Tan *et al.*, 2011). Nevertheless, the TPC value in this experiment appeared to decrease at 50 °C and this is in line with that reported by Lee *et al.* (2007), who mentioned that increment in temperature reflects a negligible effect on the extraction of

phenolic compound content from pitaya peel via MAE technique. The reason is that the temperature of 45 °C is adequate to extract the bioactive compounds in the samples.

Meanwhile, the statistical analysis portrays insignificant difference for temperatures of 35 and 40 °C with TPC values at 5.126 and 5.273 mg GAE/g dried peel, respectively. However, temperatures 45 and 50 °C displayed significance variance with TPC values at 5.8 and 4.769 mg GAE/g dried peel, respectively. As a result, the best temperature to retrieve maximum yield of TPC value was 45 °C. As such, this outcomes was further used to analyze the impacts of time and weight.

The result obtained in this study is in agreement with the work conducted by Thirugnanasambandham and Sivakumar (2017), who investigated pitaya peel extract using MAE. This happens to prove that bioactive compound, which is betalain, increased as the temperature was hikes, but exhibited a negligible effect with excessive temperatures. In fact, similar finding was reported by Pinela *et al.* (2016), who showed increment in extraction yield of phenolic acid from tomato using MAE, along with elevated temperature and followed by a gradual decrease at exceeding temperature of extraction.

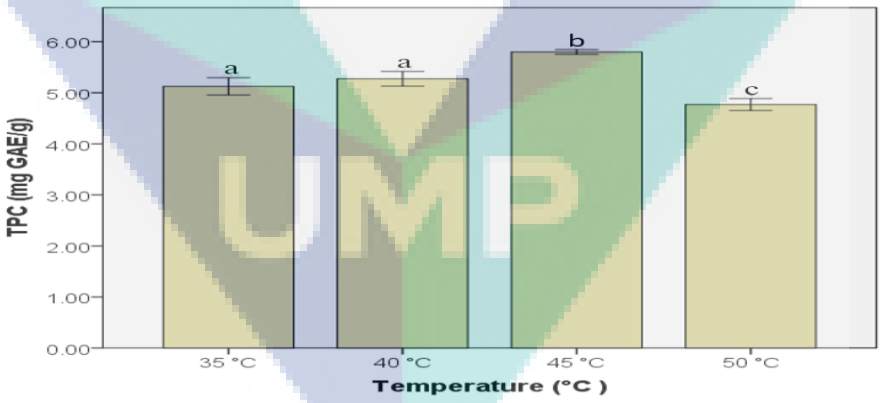


Figure 4.2 The Effects of Various Temperatures Against TPC Yields. Error Bars Represent Standard Deviation from the mean Values of Triplicate Readings. Different Letters Indicate Significant Differences at $p \leq 0.05$.

4.4 The Effect of Extraction Time in MAE

The best condition of time had been identified by the concentration of TPC value, in which the MAE time had been varied in the range of 10 until 30 mins. The effect of time on the TPC values is portrayed in Figure 4.3.

Figure 4.3 shows that the TPC value increased at the initial stage when the time was increased from 10 to 20 mins. This is attributable to the enhancement of cell wall rupture when more microwave energy was employed during the extraction process. In addition, the penetration of solvent extraction into the sample was also enhanced due to this heating phenomenon (Maran & Prakash, 2015). Therefore, the phenolic compounds that were released from the sample into the solvent increased with longer time of microwave radiation, primarily due to accumulation of heat within the solvent extraction that enhances the dissolution of phenolic compounds (Tongkham *et al.*, 2017).

Another reason to weigh in is that the MAE is composed of electric and magnetic fields, which can vibrate the polar molecule in the sample and conduct ionic molecules, which can result in rapid heat generation (Chan *et al.*, 2011) to allow the achievement of high temperature within a few minutes. This process of quickly heating material reinforces the loosening of sample tissue by the vapor within the capillary porous structure of the sample material, thus leading to effective extraction of phenolic compounds into the solvent (Maran *et al.*, 2014; Prakash *et al.*, 2013; Seixas *et al.*, 2014). Nevertheless, degradation of phenolic compound can occur when time exposure for microwave is in excess (Yang *et al.*, 2009; Zheng *et al.*, 2011). As such, the TPC value decreased from 25 to 30 min.

The statistical analysis projected that 10 and 30 mins displayed insignificant difference with TPC values at 4.806 and 4.892 mg GAE/g dried peel, respectively. In fact, 15 and 25 mins also indicated insignificant variance with TPC values at 5.136 and 5.267 mg GAE/g dried peel, respectively. The significant difference was noted at 20 mins with TPC value at 5.723 mg GAE/g dried peel. As a result, the best time to obtain maximum yield of TPC value had been 20 mins, which was employed for further analysis that embedded sample weight.

A similar trend was also reported by Cardoso *et al.* (2014b) for the time effect of MAE, which showed that betalain, a bioactive compound from red beet extract, increased due to increment in extraction time. However, a decrease in the betalain content was recorded with longer extraction time. This is also supported by Hayat *et al.* (2009), who revealed that the phenolic acid content from citrus mandarin peel extract increased rapidly with increment in extraction time, but a gradual decrease was noted in extraction yield as the extraction time was prolonged. Besides, Maran *et al.* (2014) also discovered an identical pattern with extraction yield of pectin from watermelon rind (*Citrullus lanatus)* using MAE, whereby pectin yield increased rapidly with time, but decreased gradually upon excessive extraction time.

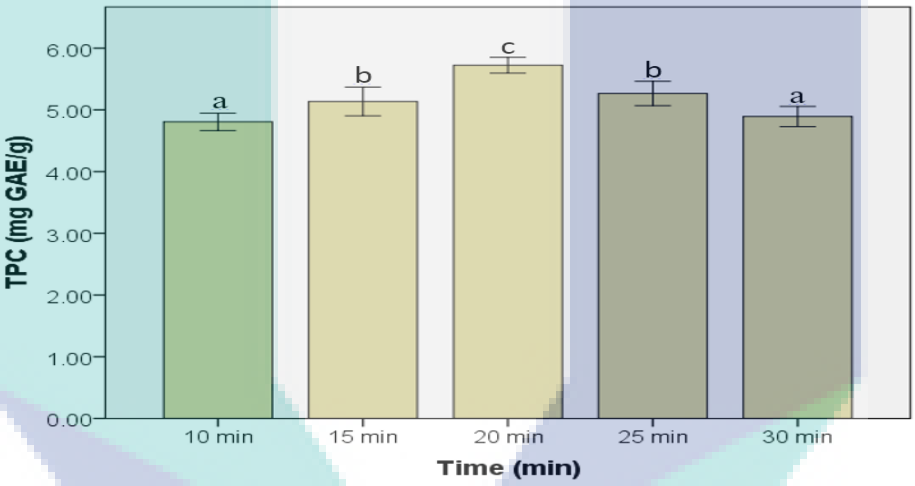


Figure 4.3 The Effects of Various Time Against TPC Yields. Error Bars Represent Standard Deviation from the Mean of Triplicate Readings. Different Letters Indicate Significant Differences at $p \leq 0.05$.

4.5 The Effect of Weight of Sample in MAE

The best condition of sample weight had been determined by the concentration of TPC value, where the sample weight values had been varied in the range of 1.2 until 3.2 g. The effects of sample weight on TPC values are displayed in Figure 4.4.

The outcomes illustrated in Figure 4.4 shows that 1.2 g of sample weight gave higher TPC value. Theoretically, increment in sample mass increases the interaction

between surface area of the sample and the solvent extraction in MAE process. Next, the volume of solvent extraction may cause excessive swelling of the sample and hence, the ability to absorb the microwave directly. This causes the cell walls to rupture, thus resulting in easy release of phenolic compounds into the solvent extraction (Guo *et al.*, 2001). In this case, the solvent volume with 50 mL of water seemed adequate to ascertain that the entire sample was immersed, especially for swelling sample during the extraction process (Dahmoune *et al.*, 2013; Eskilsson & Björklund, 2000). In this experiment, the volume of solvent extraction was constant at 50 mL.

Pinelo *et al.* (2004) mentioned that diffusivity increases as the solid-liquid ratio is increased, thus escalating the variances in phenol concentration in the solvent based on the mass transfer principle. However, in this experiment, the TPC values decreased sharply and hit a minimum of 2.2 until 3.2 g. This phenomenon could be due to the solvent extraction that had begun to saturate with the sample leading to inefficient extraction parameters. This negatively affected the mass transfer rate and could serve as a barrier to the penetration of phenolic compound into the solvent because of non-uniform distribution and exposure to microwave heating, which decreases the extraction yield (Eskilsson & Björklund, 2000; Prakash *et al.*, 2013).

The statistical analysis revealed a significant difference for sample weights of 1.2, 2.2, and 3.2 g with TPC values at 5.708, 3.779, and 3.459 mg GAE/g dried peel, respectively. As a result, the best sample weight to retrieve maximum yield of TPC value was 1.2 g.

Other researches have also reported that the solution began to saturate with the solute as the solid-liquid ratio was increased, which led to the decrease in extraction yield (Pinela *et al.*, 2016; Prakash *et al.*, 2013; Thirugnanasambandham *et al.*, 2014).

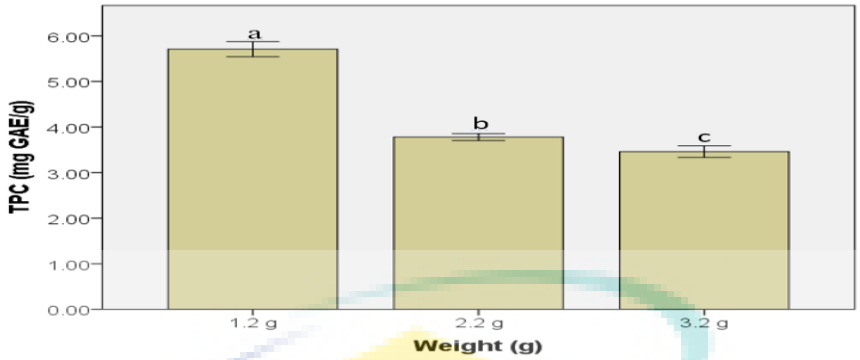


Figure 4.4 The Effects of Various Weight Values of Sample Towards TPC Yields. Error Bars Represent Standard Deviation from Mean of Triplicate Readings. Different Letters Indicate Significant Differences at $p \leq 0.05$.

4.6 Concluding Remarks

This study had successfully determined the optimum values of various parameters, namely, power, temperature, time, and sample weight at 400 W, 45 °C, 20 min, and 1.2 g, respectively. All the retrieved maximum yields of TPC values are presented in Table 4.1

Table 4.1 Maximum Values of TPC Yields For Pitaya Peel Extract

Parameter	**Result of TPC (mg GAE/g dried peel**
Power (400 W)	5.808
Temperature (45 °C)	5.800
Time (20 min)	5.723
Weight (1.2 g)	5.708

Based on Table 4.1, the best condition for power was 400 W so as to achieve the optimum yield for TPC value at 5.808 mg GAE/g dried peel. Next, the best condition for temperature was 45 °C to attain the optimum yield for TPC value at 5.800 mg GAE/g dried peel. The best condition for time was 20 min to gain optimum yield for TPC value at 5.723 mg GAE/g dried peel. Lastly, the best condition for sample weight had been 1.2 g in order to attain the optimum yield for TPC value at 5.708 mg GAE/g dried peel.

Fundamentally, power, temperature, time, and sample weight are indeed crucial for the extraction process of bioactive compounds from pitaya peel. As such, these parameters had been employed to identify antioxidant activities, mineral contents, and phenolic compounds. The next section describes the outcomes obtained from antibacterial activities upon investigation of pitaya peel extract.

4.7 Analysis of Pitaya Peel Extract and Antibacterial Study

4.7.1 Antioxidant Activity

This study had looked into the primary antioxidant activity of each sample via DPPH assay. The pitaya peel extract has been proven to be rich in phenolic content with antioxidant activity (Kosem *et al.*, 2007), where it reacts with DPPH by shifting its shade of purple to yellow (Magalhães *et al.*, 2008), as illustrated in Figure 4.5.

Figure 4.5 Antixoidant Activity of Pitaya Peel Extract

The concentration of phenolic content in the pitaya peel extract dictated the speed of color shift. The absorbance seemed to decline with the elevation in the sample concentration. As such, rapid decrease in the absorbance implies higher potential of the primary antioxidant activity (Siddhuraju *et al.*, 2002). The concentration of pitaya peel extract and the corresponding DPPH activity are portrayed in Table 4.2. The DPPH scavenging activity appeared to have elevated due to increment in phenolic content of pitaya peel extract. In fact, similar findings had been reported in a prior study, which signified that high DPPH scavenging activity is attributable to high polyphenol content (Céspedes *et al.*, 2008).

Table 4.2 DPPH Activity and Concentration of Pitaya Peel Extract

Concentration (mL/mL)	DPPH Activity (%)
0	0
0.2	29.06
0.4	39.74
0.6	57.66
0.8	79.20
1	88.21

The IC_{50} refers to the inhibitory concentration at 50 % activity of DPPH, thus demonstrating the potential capacity of the pitaya peel to reduce DPPH in an intricate manner. The lower value of IC_{50} signifies that the sample has high potential to be an antioxidant (Siddhuraju *et al.*, 2002). Figure 4.6 illustrates the IC_{50} value (mL/mL) of pitaya peel extract. As for this study, the IC_{50} of pitaya peel extract was 0.52 mL/mL, which had been derived by using the following equation: y=89.609x+3.5776. The highest DPPH scavenging activity for peels and pulps of *H. polyrhizus* and *H. undatus* had been exhibited by the IC_{50} values at 0.30 mg/mL, 0.40 mg/mL, and higher than 1.0 mg/mL for the two pulps, respectively (Ruzlan *et al.*, 2010). The IC_{50} value of pitaya peel extract used differed moderately from that reported in prior report. This could be contributed by the high antioxidant activity of pitaya peel extract, as well as the utilization of other extraction technique(s).

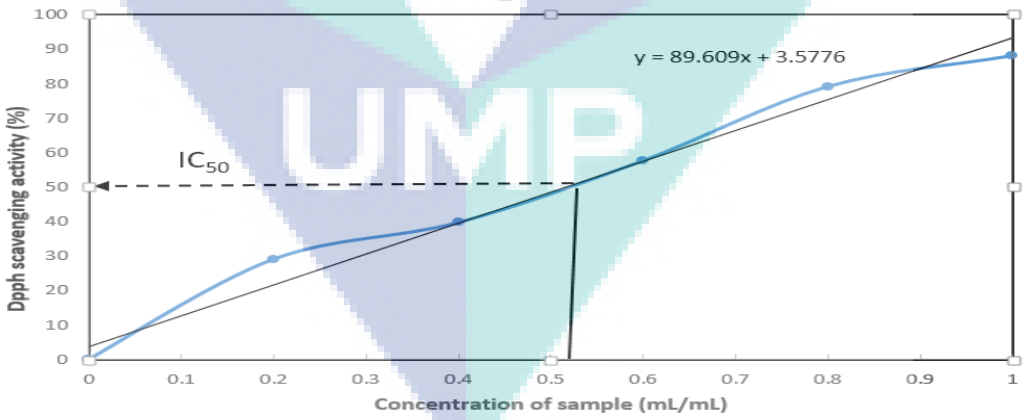


Figure 4.6 DPPH Scavenging Activity of *H. polyrhizus*.

4.7.2 Mineral Contents of Pitaya Peel

Table 4.3 Mineral Contents of Pitaya Peel Extract

Element	Wavelength (nm)	Unit	Expected Value of QC (ppm)	Tolerance (%)	Standard Deviation	Experimental Value of QC (ppm)	Pitaya peel extract concentration (ppm)	Z-Score
Al	308.215	PPM	10	35	3.50	10.50	**N.D**	0.14
As	188.979	PPM	10	12.5	1.25	10.14	**N.D**	0.11
Ba	233.527	PPM	10	11.25	1.13	10.71	**0.14**	0.63
Be	313.042	PPM	10	10.5	1.05	9.06	**N.D**	-0.90
Ca	317.933	PPM	10	22.5	2.25	10.16	**9.75**	0.07
Cd	214.440	PPM	10	10.2	1.02	9.85	**0.03**	-0.15
Co	230.786	PPM	10	11.25	1.13	8.66	**N.D**	-1.19
Cr	267.716	PPM	10	12.5	1.25	9.23	**N.D**	-0.62
Cu	327.393	PPM	10	11.25	1.13	10.12	**0.22**	0.11
Fe	273.955	PPM	10	35	3.50	10.14	**0.31**	0.04
K	766.490	PPM	10	135	13.50	10.05	**1081**	0.004
La	408.672	PPM	10	11.25	1.13	9.28	**N.D**	-0.64
Li	670.784	PPM	10	10.5	1.05	10.41	**N.D**	0.39
Mg	279.077	PPM	10	15	1.50	9.74	**56.81**	-0.17
Mn	403.075	PPM	10	12.5	1.25	10.36	**1.73**	0.29
Na	589.592	PPM	10	22.5	2.25	10.03	**2.69**	0.01
Ni	231.604	PPM	10	12.5	1.25	9.70	**0.16**	-0.24
Pb	220.353	PPM	10	15	1.50	10.46	**N.D**	0.31
Sc	361.383	PPM	10	10.2	1.02	9.93	**N.D**	-0.26
Sr	460.733	PPM	10	10.25	1.03	10.36	**0.38**	0.35
Ti	368.519	PPM	10	12.5	1.25	10.36	**N.D**	0.29
V	292.402	PPM	10	10.5	1.05	9.12	**N.D**	-0.84
Y	371.029	PPM	10	10.5	1.05	9.96	**N.D**	-0.04
Zn	206.200	PPM	10	11.25	1.125	9.60	**0.95**	-0.36

*****N.D=Not detected***

Table 4.3 demonstrates all elements detected in this study. The results indicate that a total of 24 elements were detected via ICP-OES in which each element possess a specific wavelength. Nevertheless, concentration of 12 elements, such as Ba, Ca, Cu, Cd, Fe, K, Mg, Mn, Na, Ni, Sr, and Zn were detected in pitaya peel extract at 0.14, 9.75, 0.22, 0.03, 0.31, 1081, 56.81, 1.73, 2.69, 0.16, 0.38 and 0.95 ppm, respectively. Moreover, the elements has been classified as not detected (N.D) if they are under than limit of detection (LOD) of ICP-OES method. As such, Al, As, Be, Co, Cr, La, Li, Pb, Ti Sc, V, and Y were not detected in this sample as their concentrations were lower than LOD. Throughout the ICP analysis, any interference between elements is removed via inter-element correction (IEC) and multispectral fitting (MSF), which are incorporated into the Winlab 32, ICP software. This implies that ICP-OES is a powerful method to identify multielements in a sample (Marqués *et al.*, 2015).

The findings demonstrated that all elements in QC values are within the z-score range of -3≤ z≤ 3. This indicates that the QC data were valid and within the acceptable limit. Moreover, reliable the z-score, which is within the range of -3≤ z≤ 3 is essential for QC data as it influences the final readings of the mineral contents. For instance, the final result of mineral content is invalid if the value of QC is beyond the range of z-score. It should be noted that the result of mineral contents detected in this study is valid.

Table 4.3 demonstrates that K was the element with the highest concentration (1081 ppm). It is well-known that K plays a vital role in maintaining body water content and acid balance (Marqués *et al.*, 2012). Hence, this implies that pitaya peels may be a valuable source of K. Moreover, the concentrations of Ca and Mg were 9.75 ppm and 56.8 ppm, respectively. These values were moderately higher than values reported previously (Chaiwut *et al.*, 2012). Essentially, Ca is an important element required for the formation of skeleton during childhood, where it prevents osteoporosis later in life. Furthermore, Mg is also crucial for human health as it involves in regulation of blood sugar level, energy metabolism and protein synthesis (Marqués *et al.*, 2015).

Quantity of Na detected in this study was low (2.69 ppm). Na is known an essential element that is needed in small quantities. High intake of Na is related to high blood pressure and increased risk for cardiovascular disease (World Health Organization, 2012). Moreover, the concentration of Mn, Sr, Fe, and Ti in pitaya peel extract were lower than 5 ppm. Mn and Fe are known as essential nutrients required for human health. Sufficient supply of iron is crucial for women between 14 and 50 years as well as newborn in the early months of their life. Contrarily, extreme quantity of Sr and Ti could be harmful. Thus, it is crucial to monitor Sr and Ti levels in food as these elements are unimportant for overall health.

In this experiment, the element of Pb had been lower than LOD of the ICP-OES, while the concentration of Cd was 0.03 ppm. The concentrations of these elements appeared to be lower than the reported maximum limit permitted into food set by the European Commission (EC) for Pb and Cd, which are 0.10 and 0.05 ppm, respectively (Europian Commision, 2006). Besides, Pb and Cd in extreme quantities could turn into

toxic for the human body, thus leading to cardiovascular, kidney, and bone infirmities (Järup, 2003).

The study outcomes revealed that pitaya peel extract can be used as potentially to incorporate with other products, such as food and cosmetic. This is supported by the fact that the pitaya peel extract consists of valuable mineral contents and free from harmful elements. The results also seemed to support the application of MAE for extraction of fruits, as it substantially reduces degradation of heat-sensitive components and elements (Şahin *et al.*, 2017; Wang *et al.*, 2016). In addition, the results appear to be in agreement with a past study that employed the method of MAE to extract Kaki fruits (*Diospyros kaki L.*) in the attempt to measure mineral contents (Marqués *et al.*, 2015). The vital elements required in the human body, such as Ca, Fe, K, and Mg, had been detectable in the pitaya peel extract. This is in line with that reported by Souza *et al.* (2018), who claimed the successful ICP-OES method in identifying essential elements required by the human body found in sugarcane juice. As for this study, the mineral contents did meet the requirements of QC as a certified reference material for ICP-OES concentrations, where Pb and Cd values were acceptable (Marqués *et al.*, 2012). Similarly, Andrade *et al.* (2018) stumbled upon the same trend when determining toxicity elements (Pb and Cd) in yogurt by showing that both these elements were lower than the permissible limit in food with rapid determination via ICP-OES.

4.7.3 Phenolic Acid and Flavonoid Content

Table 4.4 List of Compounds of the Pitaya Peel Extract In Accordance with UHPLC-ESI-QTRAP-MSMS

Retention time (min)	$[M-H]^-$	Mass fragment, MS/MS (m/z)	Compound identified	References
1.42	191.40	172.92, 110.98	Quinic acid	MassBank
1.98	147	56.90, 86.95, 102.97	Cinnamic acid	MassBank
3.86	191.20	172.99, 110.93	Quinic acid isomer	MassBank
3.97	135.19	116.88, 88.80	3,4-Dihydroxy vinylbenzene	MassBank
4.03	623.20	605.25, 579.00, 314.04, 299.04	Isorhamnetin 3-*O*-rutinoside	MassBank
5.03	625.32	315.10, 300.20	Myricetin rhamno-hexoside	MassBank
6.15	328.78	311.29, 229.21, 211.16, 171.15, 139.14	3,30-di-*O*-methyl ellagic acid	MassBank
6.48	314.80	300.08, 271.06, 243.12, 164.04	Isorhamnetin aglycone monomer	MassBank
6.60	269.13	251.10, 225.29, 197.04	Apigenin	MassBank
7.04	209.22	163.15	Jasmonic acid	MassBank
7.48	297.35	183.00	Oxooctadecanoic acid	MassBank
8.04	310.40	183.03, 96.91	2 (3,4-Dihydroxyphenyl)-7-hydroxy-5-benzene propanoic acid	MassBank
8.60	595.40	315.03, 279.28, 241.04	Protocatechuic Hexoside conjugate	MassBank

A total 13 compounds were detected in pitaya peel extract as shown in Table 4.4. The detection of compounds are performed through library database matching and their mass fragmentation pattern (Lau *et al.*, 2014). The results revealed that pitaya peel extract consists of apigenin, cinnamic acid, quinic acid, quinic acid isomer, isorhamnetin aglycone monomer, 3,4-dihydroxy vinylbenzene, isorhamnetin 3-*O*-rutinoside, jasmonic acid, myricetin rhamno-hexoside, protocatechuic hexoside conjugate, 3,30-di-*O*-methyl ellagic acid, oxooctadecanoic acid, and 2 (3,4-dihydroxyphenyl)-7-hydroxy-5-benzene propanoic acid. To the best of author's knowledge, the current study is the first report that has detected these compounds from pitaya peel extract of *H. polyrhizus*. It should be noted that a previous study has performed determination of phenolic contents from *H. polyrhizus*, excluding a complete analysis of phenolic compounds (Lourith & Kanlayavattanakul, 2013).

The findings revealed two types of phenolic acids namely, hydroxycinnamic acids and hydroxybenzoic acids from the analysis of the pitaya peel extract via UHPLC. The findings indicated that hydroxycinnamic acids denote cinnamic acid, quinic acid, quinic acid isomer, and 2 (3,4-dihydroxyphenyl)-7-hydroxy-5-benzene propanoic acid. Moreover, hydroxybenzoic acids denote 3,4-dihydroxy vinylbenzene, 3,30-di-*O*-methyl ellagic acid, oxooctadecanoic acid and protocatechuic hexoside conjugate. Hydroxybenzoic acid possess a C6-C1 structure, while hydroxycinnamic acids possess a C6-C3 structure with a double bond in the side chain that is exists in cis or trans configuration (Quifer *et al.*, 2015).

The peak eluting at 1.42 min of hydroxycinnamic acid and derivatives demonstrated the deprotonated molecule at m/z 191.40, which shows fragment ion at m/z 172.92 (-18 Da; neutral loss of water) and recognized as quinic acid. Additional product ions at m/z 110.98 reinforced this structure proposal to be approved as quinic acid (Bastos *et al.*, 2007). The eluting at 3.86 min and 1.42 min exhibited the identical value of m/z 191.20 for deprotonated molecule. The product ions detected for both precursors were similar (m/z 172.99, 110.93), signifying that the existence of isomer known as quinic acid isomer. Furthermore, the peak eluting at 1.98 min (m/z 147) was referred as cinnamic acid based on the fragment at m/z 102.97, following the carbon dioxide loss (-44 Da) (Ncube *et al.*, 2014). Moreover, the peak eluting at 8.04 min demonstrated the deprotonated molecule at m/z 310.40, which shows fragment ion at m/z 183.03 and m/z 96.91. As such, this peak was identified as 2 (3,4-Dihydroxyphenyl)-7-hydroxy-5-benzene propanoic acid (Bahrani *et al.*, 2014).

In general hydroxybenzoic acid and derivatives exhibited attribute similar to loss of carbon dioxide CO_2 $[M-H-44]^-$ in MS/MS experiments (Vallverdú *et al.*, 2013). The evaluation of the ESI-MS/MS data plus evidence from existing literature enabled the characterization of 3,4-dihydroxy vinylbenzene with deprotonated molecule at m/z 135.19 during eluting time 3.97 min. This shows fragment ion at m/z 116.88 and m/z 88.80 (Falcão *et al.*, 2010). Commonly, ellagic acid derivatives (m/z 328.78) at eluting time 6.15 min, shows fragment ion at m/z 311.29, 229.21, 211.16, 171.15, 139.14. Consequently, it is referred as 3,30-di-*O*-methyl ellagic acid (Weon *et al.*, 2016). Moreover, parent ion was identified at m/z 297.35 for oxooctadecanoic acid at eluting time 7.48 min, which fragment ion at m/z 183.0 (Dufour & Loonis, 2005). In addition,

compound that is considered as protocatechuic hexoside conjugate was observed at eluting time of 8.60 min, $[M-H]^-$ ion at m/z 595.40 and fragment ion at m/z 315 (Chen *et al.*, 2011).

Hydroxycinnamic acid is secondary metabolites formed with phenylalanine and tyrosine. It should be noted that phenylalanine and tyrosine are the precursors of the other polyphenol classes in plant biosynthetic mechanisms (Seedi *et al.*, 2012). The hydroxycinnamic acid has exhibited antibacterial activity, where it is active against *E. coli*, *Bacillus subtilis* and *Pseudomonas syringe* (Barber *et al.*, 2000; Guzman, 2014). Moreover, phenolic acid are useful in preventing human disease such as cancer (Ogawa *et al.*, 2017). Moreover, phenolic compounds from *H. polyrhizus* demonstrated antioxidant (Ramli *et al.*, 2014; Tenore *et al.*, 2012) and antibacterial activities (Tenore *et al.*, 2012).

The remaining compound of pitaya peel extract were categorized as flavonoid. Furthermore, flavonoid from *H. polyrhizus* exhibited good antioxidant (Esquivel *et al.*, 2007a; Kim *et al.*, 2011b; Ramli *et al.*, 2014), antiproliferative (Kim *et al.*, 2011a) and antibacterial activities (Tenore *et al.*, 2012). Moreover, isohamnetin-3-*O*-rutinoside was detected in *H. polyrhizus* (Tenore *et al.*, 2012). The results demonstrated that eluting at 4.03 min revealed the deprotonated molecule at m/z 623.20, which fragment ion at m/z 605.25, 579.00, 314.04, 299.04. As such, it is referred as isohamnetin-3-*O*-rutinoside. Moreover, isorhamentin aglycone monomer (m/z 314.80) eluting at 6.48 min with loss of one methyl group generated m/z 300.08. It also showed the fragment ion at m/z 271.06 (Hossain *et al.*, 2010). In addition, jasmonic acid has the product ion scan of m/z 209.22 at eluting time 7.04 min, which showed. It gave the fragment at m/z 163.15 as a result of carbon dioxide loss (-44 Da) (Segarra *et al.*, 2006). Furthermore, the apigenin at eluting time 6.60 min (m/z 269.13), showed the fragment at m/z 251.10 and m/z 225.29 caused by loss of water (-18 Da) and carbon dioxide (-44 Da), respectively (Lai *et al.*, 2007; Medana *et al.*, 2008). Moreover, the existence of myricetin in the extraction solution demonstrated the low degradation magnitude of the extraction process (Biesaga, 2011). Meanwhile, MS/MS analysis revealed the $[M-H]^-$ ion at m/z 625.32 with the fragment at m/z 315.10 and m/z 300.20, indicating the existence of Myricetin rhamno-hexoside (Tiberti *et al.*, 2007).

The presence of chlorogenic acid, gallic acid, and quercetin in pitaya peel extract by using the HPLC technique (Waters 2695, Agilent, USA), as reported in a past study, was not observed in this particular research (Lourith & Kanlayavattanakul, 2013). However, the study outcomes happened to be similar with a recent research on varied fruits and plants extracted via MAE (Cong *et al.*, 2017). In short, fruit and plant extracts are supplemented with phenolic compounds, where MAE is appropriate for extraction of numerous types of phenolic compounds. This present study is also in agreement with a prior research that indicated its highest yield of phenolic compounds attained with low consumption of solvent in MAE (Fang *et al.*, 2015). As a conclusion, MAE has been proven to be the best available method for instantaneous identification of phenolic compounds, as portrayed in Figure 4.7, based on the UHPLC-ESI-QTRAP-MSMS analysis.

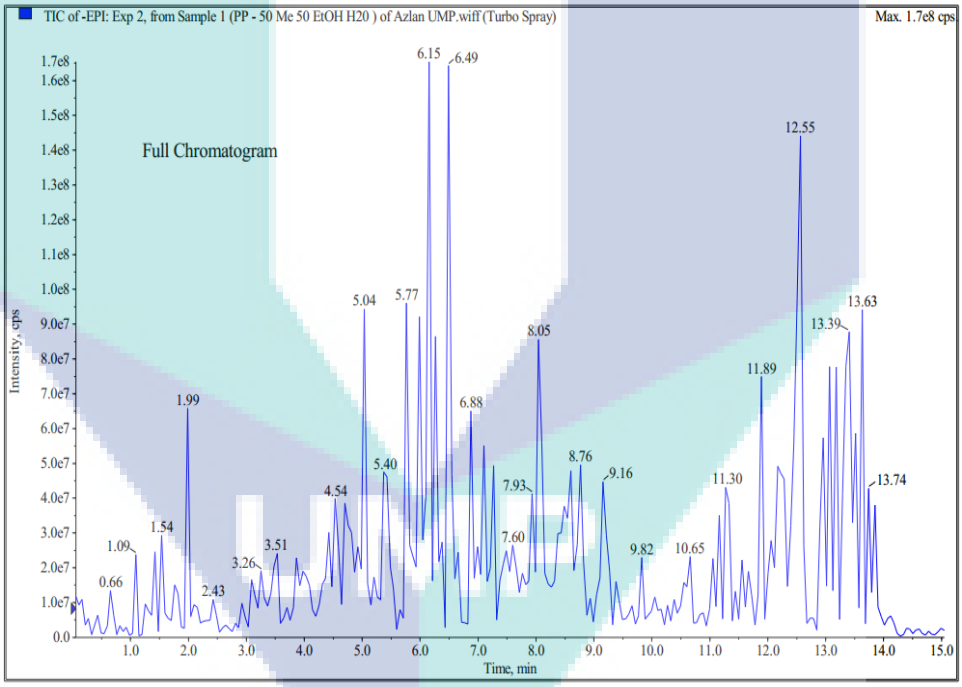


Figure 4.7 Full Chromatogram of phenolic compounds

4.7.4 Antibacterial Activity

4.7.4.1 Zone of Inhibition Test of Pitaya Peel Extract

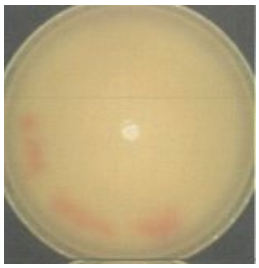
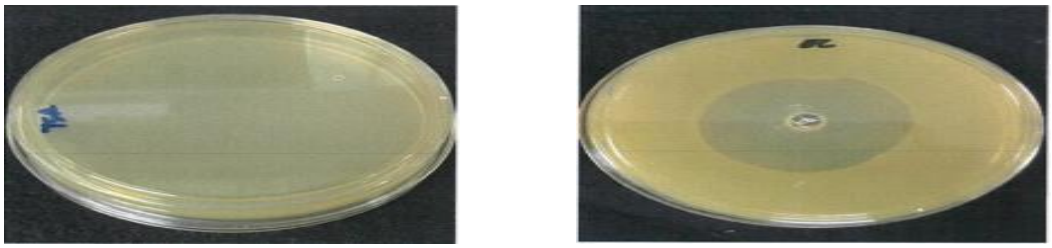
Challenge Microorganism	Diameter zone of inhibition	Results
Gram positive		
S. aureus		0 mm
Gram Negative		
E. coli		0 mm

Figure 4.8 Zone of Inhibition Test

The results obtained from the antibacterial tests are displayed in Figure 4.8. Within these recent years, more investigations have been performed upon plant extracts as potential antibacterial agents (Mabona *et al.*, 2013; Nazzaro *et al.*, 2013). From this stance, the zone of inhibition method had been employed to assess the antibacterial activities of pitaya peel extract. Generally, it is challenging to analyze the antibacterial effect against Gram-negative, mainly due to the existence of outer membrane barrier called 'lipopolysaccharide' that is lacking in Gram-positive bacteria (Fernández *et al.*, 2013). Furthermore, Gram-negative bacteria have displayed low susceptibility to phenolic compounds compared to Gram-positive bacteria due to the repulsion between phenolic compounds and lipopolysaccharide present in the surface of Gram-negative bacteria (Fattouch *et al.*, 2007; Silva *et al.*, 2018). In fact, reports oftentimes indicate that phenolic compound extracts are more efficient against Gram-positive bacteria (Klančnik *et al.*, 2009). The study outcomes demonstrated absence of inhibition zone on the discs of both *S. aureus* and *E. coli*. This implies that the pitaya peel extract could

only exhibit a small diffusivity effect that results in reduced antibacterial activity. Furthermore, the impact of antibacterial attribute can be affected by both size and shape of the bioactive compound, as these factors determine the capacity of the compounds in infiltrating the outer membrane of the bacteria (Kavak *et al.*, 2010). As such, the compounds found in the pitaya peel extract may not have completely entered the cell membrane of bacteria. Another reason could be tentatively explained by the possible insolubility of certain phenolic compounds in the solvent extraction, especially due to their polarity (Doughari, 2006). This is because; the variances in phenolic compounds of extracts, strains sensitivity of bacteria, and modification of antibacterial technique used in each test, could potentially display variation in the results of antibacterial activity in peel extracts (Mccarrell *et al.*, 2008). Figure 4.9 portrays the negative and positive controls applied in this study. The negative control was inactive against the tested bacteria, whereas the positive control, which was prepared by using Dettol, exhibited larger inhibition zone on the discs against the tested bacteria (Mccarrell *et al.*, 2008).

The experimental findings projected nil inhibitory effect on the tested bacteria. This outcome is in agreement with the report on using zone inhibition test as carried out by Phongtongpasuk *et al.* (2016), which elicited no inhibition against *S. aureus* and *E. coli* on pitaya peel *H. undatus* extract with boiling water. Table 4.4 shows the result of UHPLC-ESI-QTRAP-MSMS carried out on pitaya peel extract, whereby out of the total 13 phenolic compounds detected, only 6 have displayed antibacterial activity against *S. aureus* and *E. coli*. They include: quinic acid (Bai *et al.*, 2018), cinnamic acid (Guzman, 2014), isorhamnetin 3-*O*-rutinoside (Vagiri *et al.*, 2017), 3,30-di-*O*-methyl ellagic acid (Nono *et al.*, 2014), apigenin (Sousa *et al.*, 2006) and protocatechuic hexoside conjugate (Stojković *et al.*, 2013). These compounds may not display visible zone of inhibition on the disc despite having antibacterial activity as per Figure 4.8 due to possibilities of them being less diffusible, thus failing to enter the TSA agar. This is indicative of the consequences of high molecular weight, chemical reactivity, or the lack of solubility of these bioactive compounds (Balouiri *et al.*, 2016). However, there are other alternative and possibly better methods to measure antibacterial activity, so as to allow an observable antibacterial effect despite its small antibacterial effect as per Figure 5.0 and 5.1, via the Time-kill kinetics test.



a) Negative Control (No Growth of Bacteria)

b) Positive Control (Clearly Zone of Inhibition)

Figure 4.9 Control of Antibacterial Test

4.7.4.2 Time-kill kinetics Test of Pitaya Peel Extract

4.7.4.2.1 Time-kill kinetics against Gram-Positive Bacterium

Table 4.5 and Table 4.6 demonstrate the optical density (OD) of the samples measured at every 2 hours up to 14 hours. In this research, time-kill kinetics was selected as it is a rapid, simple and flexible technique (Mertens *et al.*, 2012).

Table 4.5 The Optical Density of *S. aureus* in the Control and Pitaya Peel Extract

Time (hr)	Optical Density		
	Control	10% pitaya peel extract	20% pitaya peel extract
0	0.03	0.02	0.03
2	0.18	0.20	0.16
4	2.45	2.42	2.18
6	4.56	4.51	4.46
8	5.26	5.20	4.88
10	5.77	5.53	5.64
12	5.93	5.86	5.73
14	5.30	5.13	4.86

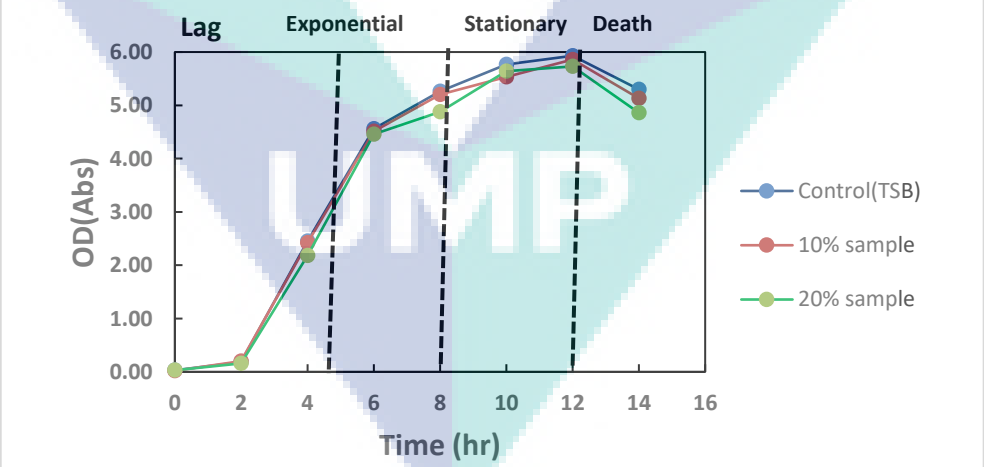


Figure 5.0 Growth Curve of the *S. aureus* at 37 °C and 120 rpm

4.7.4.2.2 Time-kill kinetics against Gram-Negative Bacterium

Table 4.6 The Optical Density of *E. coli* in Control and Pitaya Peel Extract

Time (hr)	Optical Density		
	Control	10% pitaya peel extract	20% pitaya peel extract
0	0.13	0.14	0.05
2	0.75	0.60	0.64
4	2.88	2.61	2.55
6	3.90	3.73	3.71
8	4.66	4.37	4.42
10	5.10	4.70	4.79
12	5.45	4.88	4.48
14	4.90	4.71	4.31

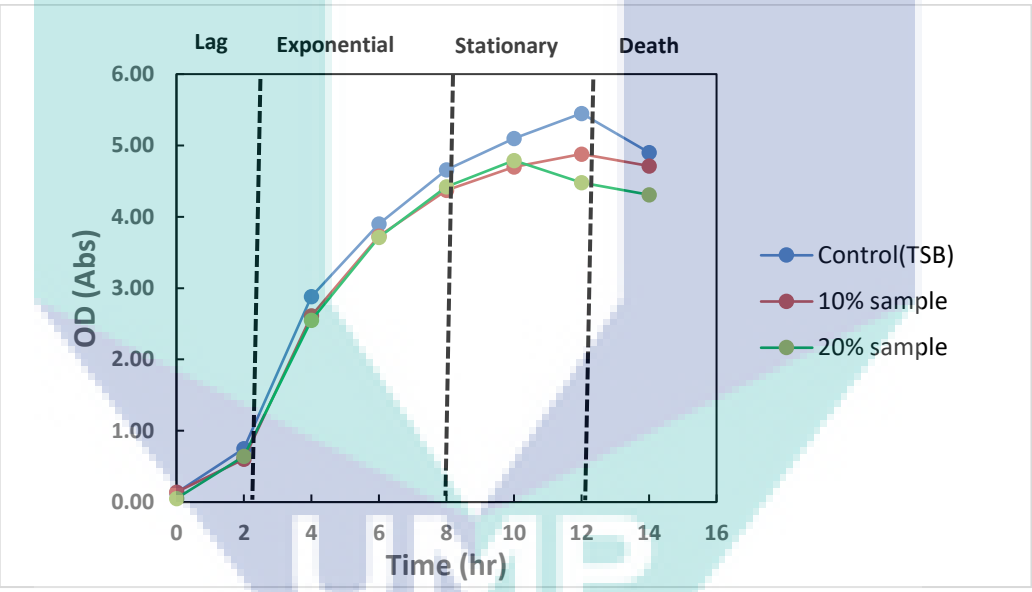


Figure 5.1 Growth Curve of the *E. coli* at 37 °C and 120 rpm

The growth curve of the *S. aureus* and *E. coli* are demonstrated in Figure 5.0 and 5.1, respectively. During the lag phase, which is between (0-2 hours), the growth of the *S. aureus* and *E. coli* would drop after inoculation. This is because the cells require sufficient period to adapt to the new environment within the conical flask. In this study, the lag phase is invisible in the graph. This could be due to the fact that the lag phase is truncated as the sample OD was measured every two-hourly interval. Furthermore, the lag phase happens instantly after inoculation, which is an adaptation period of cell to a

new environment (Métris *et al.*, 2006; Swinnen *et al.*, 2004). Furthermore, cell mass may elevate moderately during this phase without expansion of the cell number density. Generally, bacterial age and inoculum size influence the period of lag phase (Swinnen *et al.*, 2004). Biggest inoculum plus the newest inoculated parent cells could cause shortest lag phase and period to the initial division. The reduction of inoculum size could be due to longer lag phase period and the equivalent disparity (Wang & Buchanan, 2016). Therefore, the short lag phase observed in this study could be due to the age of the inoculated parent cells.

The exponential phase (log phase or growth phase) at 2 to 8 hours is demonstrated in Figure 5.0 and 5.1. The results indicated that the cells have been completely adapted to the new environment within the conical flask, where it gain capacity to grow and generate new cells (Vargas *et al.*, 2017). This study performed direct visual analysis of the various growth curve patterns. The OD value of growth curve of *S. aureus* and *E. coli* for 10% and 20% pitaya peel extract were marginally lesser than control. The bioactive compounds in pitaya peel extract demonstrated an ability to inhibit *S. aureus* and *E. coli* growth even though the effect was small. In addition, there were no inhibition zone observed during inhibition zone test adjacent to the pitaya peel extract as shown in Figure 4.8. Lastly, the growth of the *S. aureus* and *E. coli* was diminished after eight hours.

The Figure 5.0 and Figure 5.1, demonstrate that the stationary phase of the growth profile was initiated after t= 8 hours up to t= 12 hours. Meanwhile, the death phase of the growth profile started after t= 12 hours. In addition, inoculum size is an important factor that should be considered during the investigation of the transition period from exponential to stationary phase. Nonetheless, there is no existing data pertaining to inoculum size. Hence, future work should evaluate the consequence of inoculum size on late exponential and early stationary phase (Wang & Buchanan, 2016). In the course of stationary phase, the death rate is equivalent to the growth rate of the cells. The cell growth of the *S. aureus* and *E. coli* can be restricted through nutrient reduction at the stage that could not support the cell growth. During this phase, the growth curve of control should have higher OD value compared to pitaya peel extract. The results revealed that 10% pitaya peel extract resulted in higher OD value compared to 20% sample extracted. The differences observed during the eight (8^{th}) hours to (10^{th})

hours were not as expected because 20% pitaya peel extract had OD value marginally higher than 10% sample extracted. This could be due to several errors that has caused inaccurate readings of the stationary phase in growth profile graph. Moreover, it is suggested to use only two cuvettes during the OD measurement, where one is used for blank (fresh TSB) and another one is used for samples during the experiment to elude undesirable inaccuracies. This is because usage of different cuvettes might cause variation in magnitude of light intensity to pass through.

The death phase commenced after 12 hours. The OD of the cells from three growth curve decline due to reduction in the viable cell concentration. This is the results of the cell lysis, where their intracellular metabolites were released into the growth medium (Bayles, 2014; Monds & Toole, 2009). Furthermore, the cells may experience the apoptosis during the death phase. Apoptosis is also known as cell suicide, is regularly caused by the nutrient reduction and cells become smaller after the DNA cleavage (Peeters & Jonge, 2017).

For each incubation time, the OD values between examine sample and control were marginally identical. This was in agreement with a previous study (Majhenič *et al.*, 2007). In another study on time-kill kinetics test, guarana (*Paullinia cupana*) seed extract that used boiling water as the extraction solvent to obtain phenolic compounds indicated modest antibacterial activity in contrast to tested bacteria. Nevertheless, the extract is a promising antibacterial agent to be applied in food industry. Moreover, numerous studies revealed utilization of time-kill kinetics test to determine the variations in the rate and extent of antibacterial and antifungal over time (Guo *et al.*, 2011; Mback *et al.*, 2016).

4.7.5 Concluding Remark

The study demonstrated higher DPPH scavenging activity of the peel of *H. polyrhizus* compared to peel and edible portion of *H. undatus* species. The peels of hylocereus species had higher TPC compared to the pulps due to presence of various antioxidant compounds. Moreover, the IC_{50} value was 0.52 mL/mL.

The mineral content revealed that pitaya peel extract had high levels of K and Mg element, where toxic element were unfound. Hence, it is essential to determine the concentration of these toxic elements in pitaya peel extract. This would aid in obtaining information on approved maximum levels allowed in fruit as information on heavy metals for this fruit residue has been scant. Based on the study outcome, pitaya peel extract could be a good choice of fruit for the consumers. Moreover, the fruit has promising application in food, pharmaceutical, and cosmetic industries as it contains crucial mineral contents needed for human health.

The UHPLC-ESI-QTRAP-MSMS results revealed that pitaya peel extract has abundance of phenolic acid and flavonoid compounds. Meanwhile, the study did not perform the separate profiles of each phenolic acid found within the pitaya peel extract of *H. polyrhizus*.

The zone inhibition test has many advantages compared to other available antibacterial tests. Referring to this, the test is simple, economical, and able to test numerous microorganisms and antibacterial agents. Moreover, the results of zone inhibition test can be interpreted easily. Nonetheless, there was no any antibacterial activity of pitaya peel extract was observed for the zone inhibition test in this study. It should be noted that no evidence of antibacterial test from the pitaya peel extract via MAE has been demonstrated thus far.

The growth of the *S. aureus* and *E. coli* were divided into four phases namely lag phase (0-2 h), exponential phase (2-8 h), stationary phase (8-12 h), and death phase (12-14 h). Thus, the exact growth rate of cell numbers is $\mu=0$ in lag phase, where number of cells grow at constant rate, $\mu=k$ during exponential phase and the growth rate counteract

with the death rate. Consequently, the cells achieve maximum population density once the cells achieved their stationary phase. Therefore, aseptic technique is essential to prevent the contamination of pitaya peel extract.

4.8 Analysis of Microscopic Changes

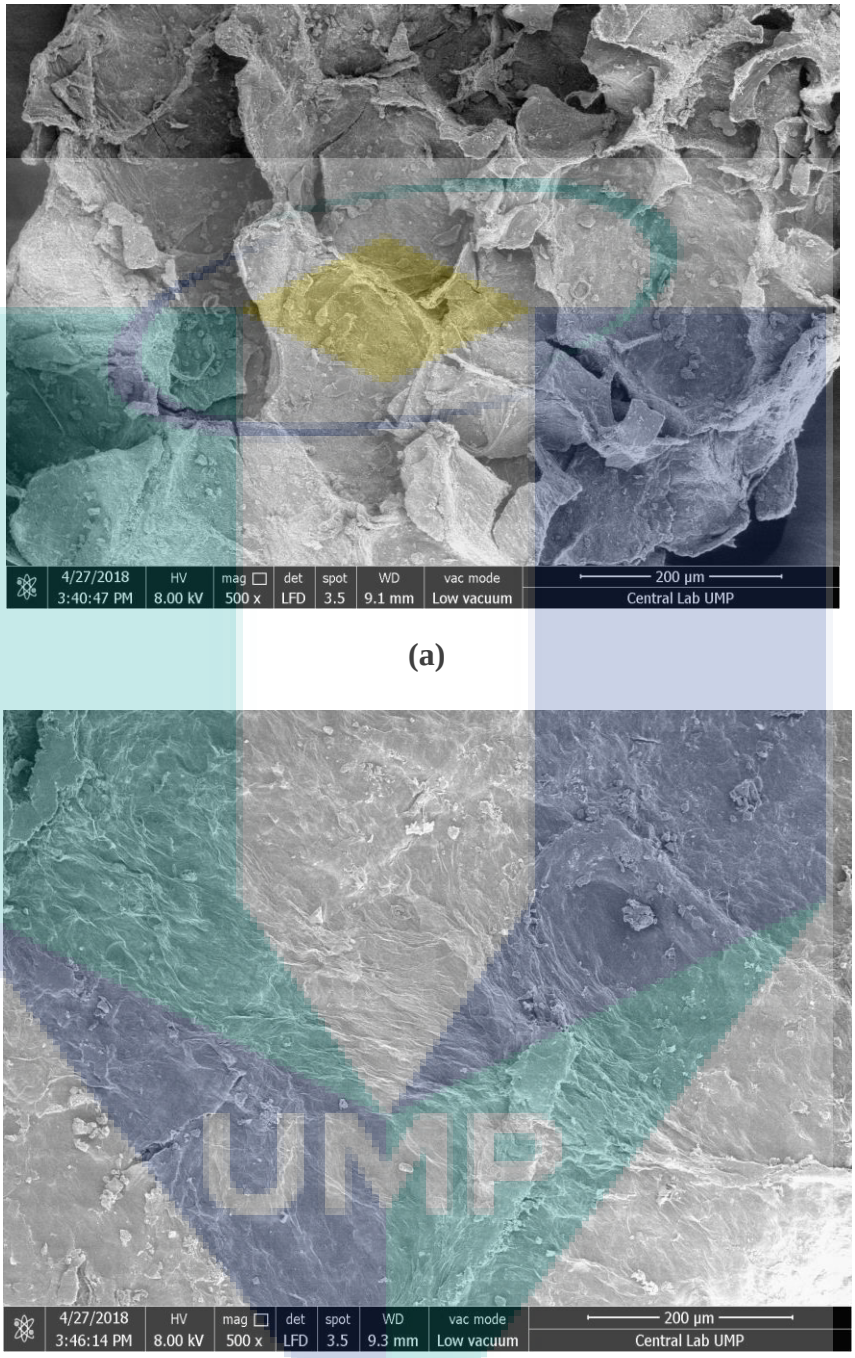


Figure 5.2 SEM of Pitaya Peel; (a) Pitaya Peel Without Treatment (500 x), and (b) Pitaya Peel By MAE (500 x).

Figure 5.2 (a) presents the untreated sample that displays an intact external structure with many folded layers. Most of the external structures in pitaya peel that were analyzed via MAE had become flat and completely ruptured, as shown in Figure 5.2 (b). This indicates that microstructural changes in the cell walls are caused by MAE *(*Flórez *et al*., 2015).

The disruption of cell wall in pitaya peel extract due to microwave radiation can promote the release of greater amounts of bioactive compounds into the solvent extraction within a short time (Bail *et al.,* 2008; Desai *et al.,* 2010). The two transport patterns pertaining to mass and heat that function in the same direction (from the inside to the outside of pitaya peel) accelerate the rates of extraction in MAE (Farhat *et al.,* 2011; Virot *et al.,* 2008). Additionally, microwave radiation increased the temperature rapidly for the sample, thus causing the cell wall to rupture and facilitate the rapid release of bioactive compounds. Hu *et al.* (2018) asserted that the effects of both cavitation and turbulence generated by microwave radiation can also destroy cell wall and promote mass transfer simultaneously, thus increasing the extraction yield.

This result is similar to that recorded by Yanık (2017), who applied MAE to extract bioactive compounds from oilive pomace. Hu *et al.* (2018) also revealed a similar pattern in extracting bioactive compounds from Tiger Nut (*Cyperus esculentus L.),* which demonstrated that cell wall disruption of the samples had been due to microwave radiation, as observed via SEM.

CHAPTER 5

CONCLUSION AND FUTURE WORK

5.1 Main Conclusion From the Study

The study outcomes revealed that MAE has emerged to be a highly appropriate instrument to extract bioactive compounds. This is because; MAE utilizes non-toxic solvent, aside from decreasing extraction period and power to attain higher TPC yields. As such, only 50 mL solvent was required to extract 1.2 g of pitaya peel powder into the MAE. Besides, MAE seems to be cost-effective in its operation, as this technique only required a low amount of solvent and sample weight. The best conditions for MAE to extract the highest amount of TPC are as follows: 400 W power, 45 °C temperature, extraction time of 20 min, and a sample weight of 1.2 g. These optimum parameters were applied in the MAE method for further analysis.

The IC_{50} value obtained via DPPH assay was 0.52 mL/mL. This value implies that pitaya peel extract does generate high antioxidant activity with small IC_{50} value.

The ICP-OES analysis had successfully detected 24 elements in the pitaya peel extract, in which a total of 12 elements had been determined in this study, namely, Ba, Ca, Cu, Cd, Fe, K, Mg, Mn, Na, Ni, Sr, and Zn. K recorded the highest concentration at 1081 ppm, followed by Mg, Ca, and Na at 56.81, 9.75, and 2.69 ppm, respectively. The toxic elements, such as Pb and Cd, were below the maximum level permissible in fruits in adherence to the European Commission. The results imply that pitaya peel extract consists of valuable mineral contents, hence projected a high potential to be utilized in the food industry.

A total of 13 phenolic compounds detected in this study displayed compatibility with the MS library. The rest of the compounds, which appeared to be incompatible with the MS library, are referred as unknown compounds, in which most of them could be flavonoids. In this experiment, the pitaya peel extract happened to comprise of apigenin, jasmonic acid, cinnamic acid, isorhamnetin 3-*O*-rutinoside, isorhamnetin aglycone monomer, quinic acid, quinic acid isomer, 3,4-dihydroxy vinylbenzene, myricetin rhamno-hexoside, oxooctadecanoic acid, protocatechuic hexoside conjugate, 2 (3,4-dihydroxyphenyl)-7-hydroxy-5-benzene propanoic acid, and 3,30-di-*O*-methyl ellagic acid. This simply implies the abundance of polyphenols and flavonoids in pitaya peel extract.

The test of inhibition zone for pitaya peel extract against Gram-positive and Gram-negative bacteria displayed negative outcome. Perhaps, this could be due to the low diffusivity of the solution extraction. Nevertheless, the pitaya peel extract exhibited modest antibacterial activities against the two types of bacteria. As such, the time-kill kinetics test is deemed appropriate to assess the antibacterial activities of pitaya peel extract.

The SEM demonstrated that cell wall disruption of pitaya peel due to microwave radiation appeared to be the main reason for rapid extraction. With that, the MAE technique can serve as an exceptional instrument for extraction of bioactive compounds in plants.

5.2 Recommendation for Future Works

The future studies should be directed towards the following recommendations:

(i) Pitaya peel extract is a promising natural food colorant and cosmetic agent as it consists of bioactive compounds and eye-catching red color. Thus, the stability of red color from water extract against pH and temperature should be elucidated to guarantee their attributes and nutritional benefits are retained when augmented to another product.

(ii) The pitaya peel extract may possibly added into a gel formulation. Subsequently, experimental analysis should be performed to determine the viscosity, odor, pH, and color. In light of this, oven stability test with different temperature points (50, 45, 40, and 25 °C) should be performed. Therefore, separation and any physical modification in textures could be detected.

(iii) Inhibitory concentration (IC_{50}) should be determined using positive control like ascorbic acid and butylated hydroxyanisole (BHA) in comparison with pitaya peel extract. A number of antioxidant activity tests like 2,2-azinobis (3-ethylbenzothaiazoline)-6-sulfonic acid (ABTS) and ferric reducing ability of plasma (FRAP) could be used for this purpose.

(iv) The optimization of TPC from pitaya peel should be performed via response surface methodology (RSM). Moreover, diverse pitaya peel species could be utilized to compare MAE conditions. Furthermore, different volumes of the solvent can be used as a optimization parameter.

(v) The concentration type of phenolic compounds in pitaya peel extract should be measured and the phenolic compounds could be verified through use of authentic standard.

(vi) The minimal inhibitory concentration (MIC) against *S. aureus* and *E. coli* should be performed on pitaya peel extract in diverse concentration values. Subsequently, log reduction should be performed to identify the total cell

number at growth curve. Moreover, environmental conditions such as available space, nutrient concentration, pH of the medium, temperature, and salt concentration should be investigated during each phase of the bacterial growth cycle (lag, exponential, stationary and death).

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APPENDIX A

Summary of the Experiment

1) Sample Preparation

(A)

(B)

(C)

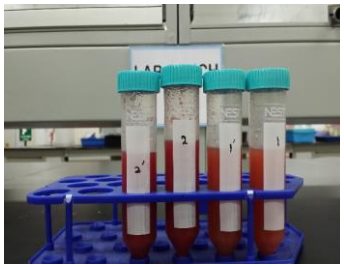
(D)

(E)

(F)

(G)

(H)



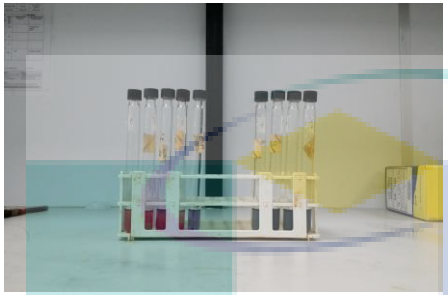
(I)

(J)

(K)

2)Analysis of Pitaya Peel Extract

(K)

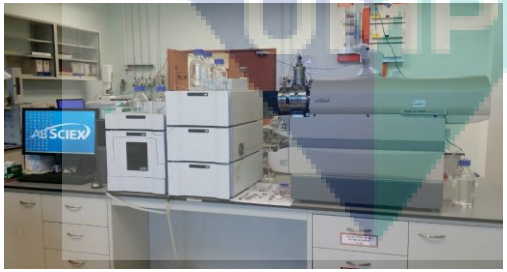


(L)

(M)

(P)

(N)



(O)

Figure 6.1 (A) Cutting (B) Loaded samples into Falcon tube (C) Freeze dried (D) Grinding (E) Sizing-40 mesh (F) Fine Powder (G) Weighing (H) MAE (I) Centrifuge (J) Filtration (K) Pitaya peel extract (L) TPC & Antioxidant Activity (M) Uv-vis (N) ICP-OES (O) UHPLC-ESI-QTRAP-MSMS (P) Inhibition zone test & Time-kill kinetic assay

APPENDIX B

TOTAL PHENOLIC CONTENT (TPC)

Gallic acid (mg/l)	Abs
0	0.005
50	0.557
100	0.995
150	1.489
200	1.896
250	2.378

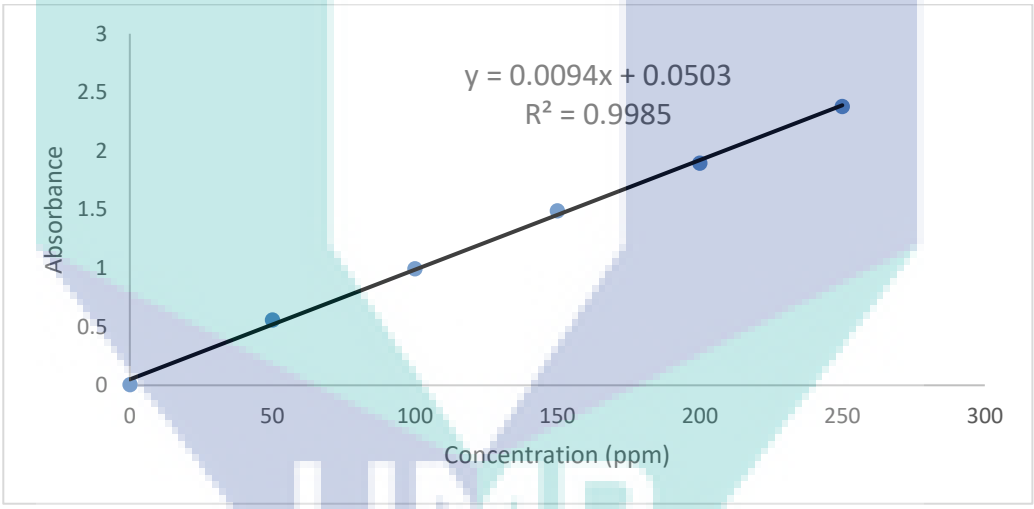


Figure 6.2 Gallic Acid Calibration with R^2=0.9985

APPENDIX C

SPSS BY STUDY EFFECT OF POWER, TEMPERATURE, TIME OF MAE AND WEIGHT OF SAMPLE

1)Effect Of Power On MAE

Multiple Comparisons

Dependent Variable: TPC
Bonferroni

(I) POWER	(J) POWER	Sig.
200 Watt	400 watt	.000
	600 watt	.000
	800 watt	.363
400 watt	200 Watt	.000
	600 watt	.000
	800 watt	.000
600 watt	200 Watt	.000
	400 watt	.000
	800 watt	.000
800 watt	200 Watt	.363
	400 watt	.000
	600 watt	.000

2)Effect Of Temperature On MAE

Multiple Comparisons

Dependent Variable: TPC
Bonferroni

(I) Temperature	(J) Temperature	Sig.
35C	40C	.136
	45C	.000
	50C	.001
40C	35C	.136
	45C	.000
	50C	.000
45C	35C	.000
	40C	.000
	50C	.000
50C	35C	.001
	40C	.000
	45C	.000

3)Effect Of Time On MAE

Multiple Comparisons

Dependent Variable: TPC
Bonferroni

(I) TIME	(J) TIME	Sig.
10 min	15 min	.010
	20 min	.000
	25 min	.001
	30 min	1.000
15 min	10 min	.010
	20 min	.000
	25 min	1.000
	30 min	.070
20 min	10 min	.000
	15 min	.000
	25 min	.001
	30 min	.000

25 min	10 min	.001
	15 min	1.000
	20 min	.001
	30 min	.004
30 min	10 min	1.000
	15 min	.070
	20 min	.000
	25 min	.004

4)Effect Of Weight On Sample

Multiple Comparisons

Dependent Variable: TPC
Bonferroni

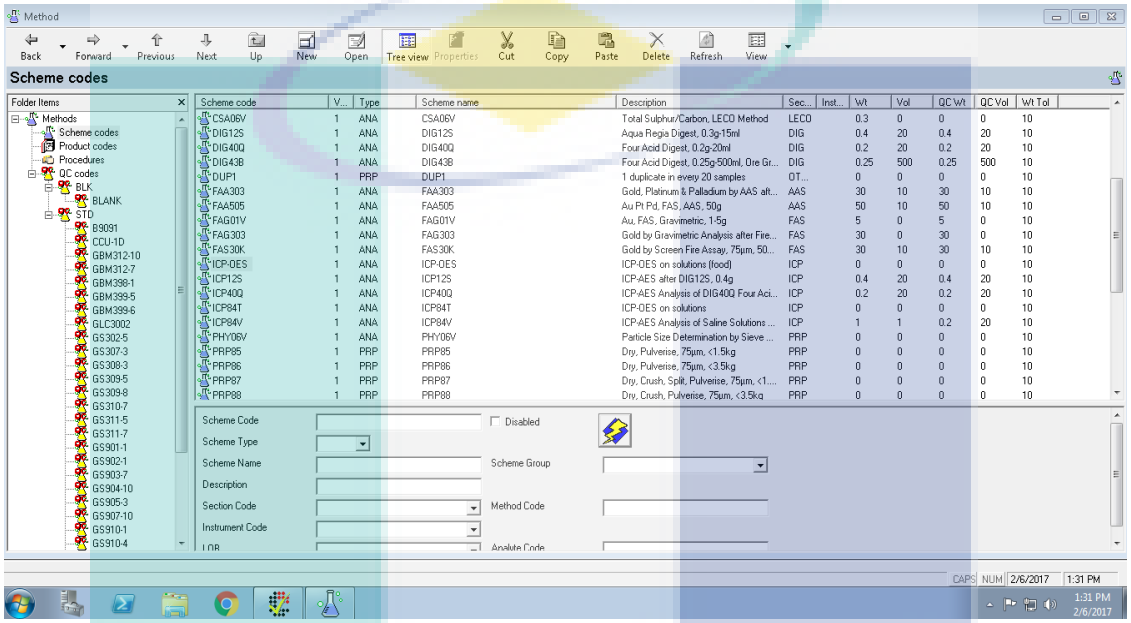
(I) Weight	(J) Weight	Sig.
1.2gram	2.2gram	.000
	3.2gram	.000
2.2gram	1.2gram	.000
	3.2gram	.003
3.2gram	1.2gram	.000
	2.2gram	.003

APPENDIX D

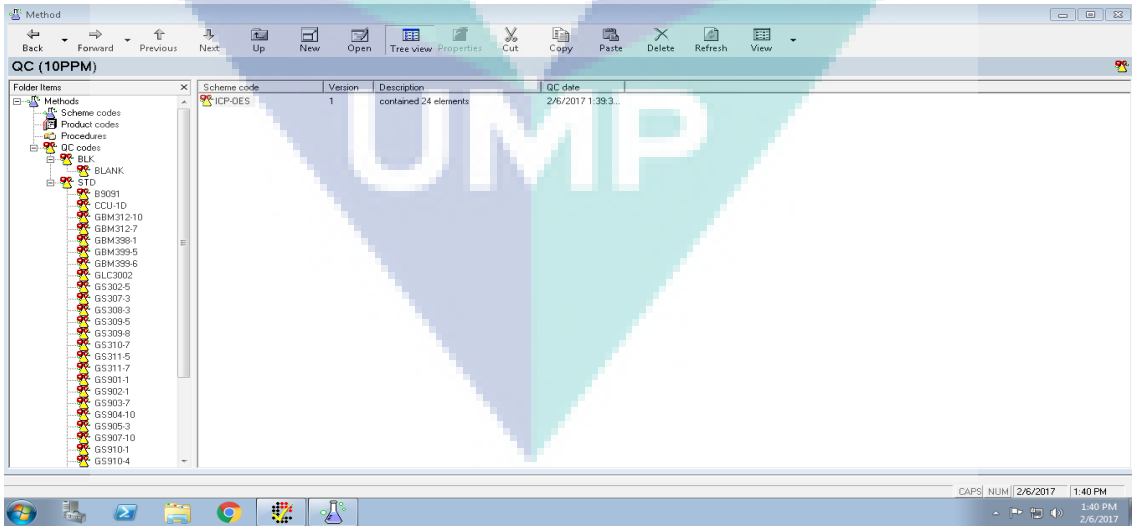
MINERAL CONTENT

USE OF CCLASS FOR QC (SGS KLG-WI 013, 2017)

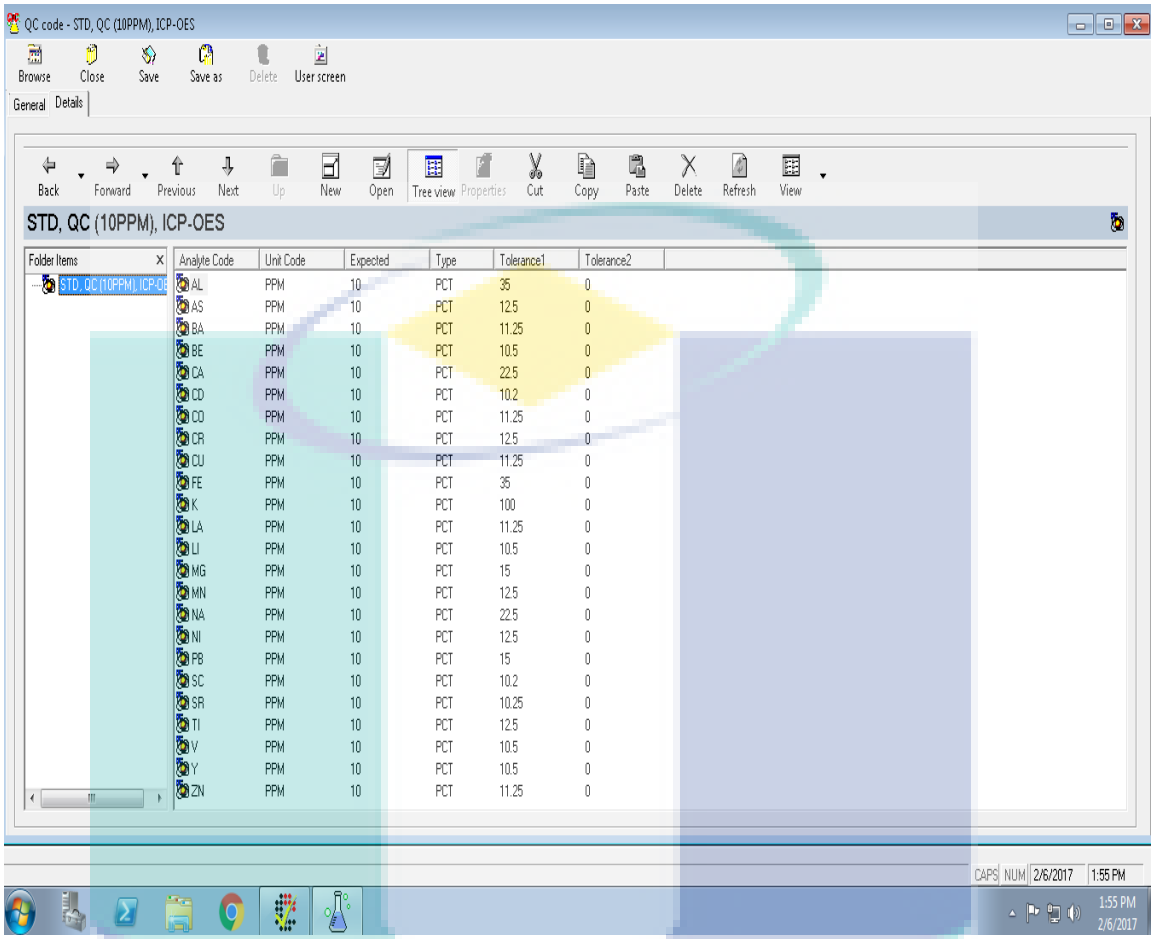
1) Method ICP-OES



2) Selection of standard/QC



3) Tolerance Value



Calculating Tolerance

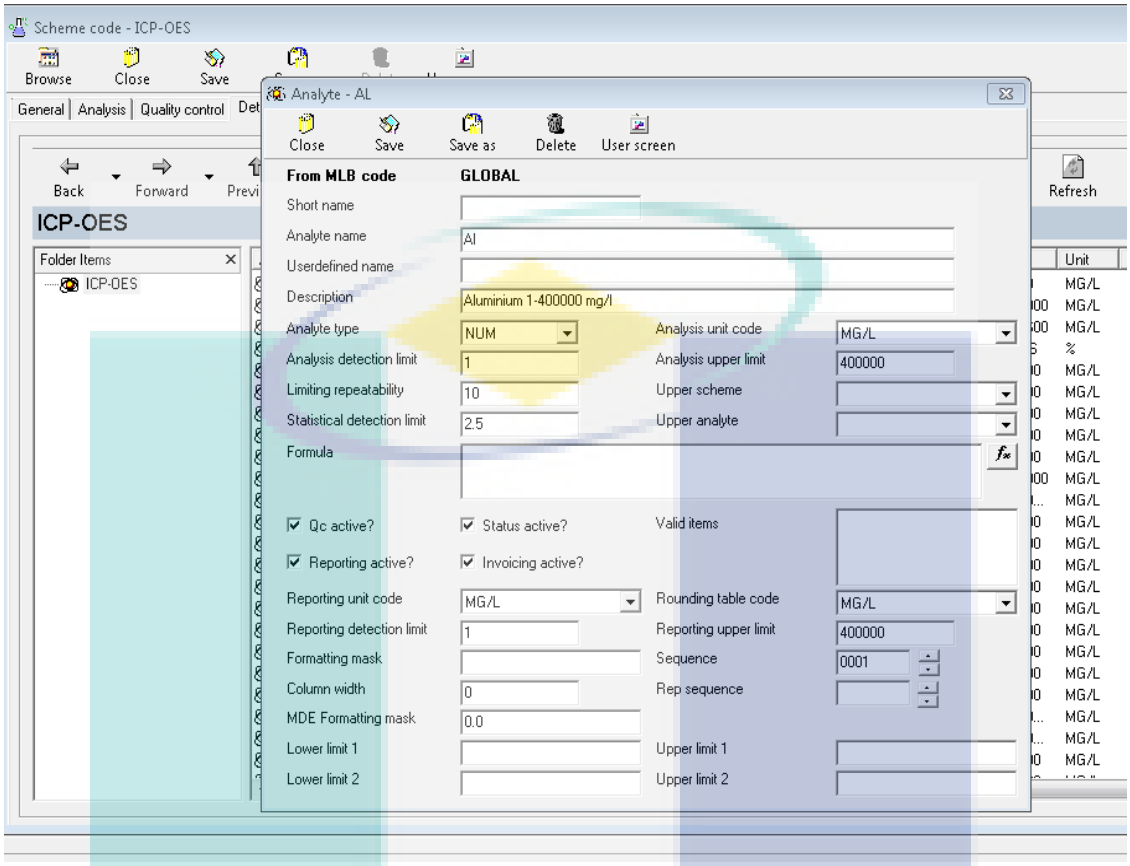
Tolerance (%)= $\frac{SDL\ X\ 100}{EV} + LR$

SDL= Statistical Detection Limit= 2.5 x Detection Limit

LR= Limit of Repeatability

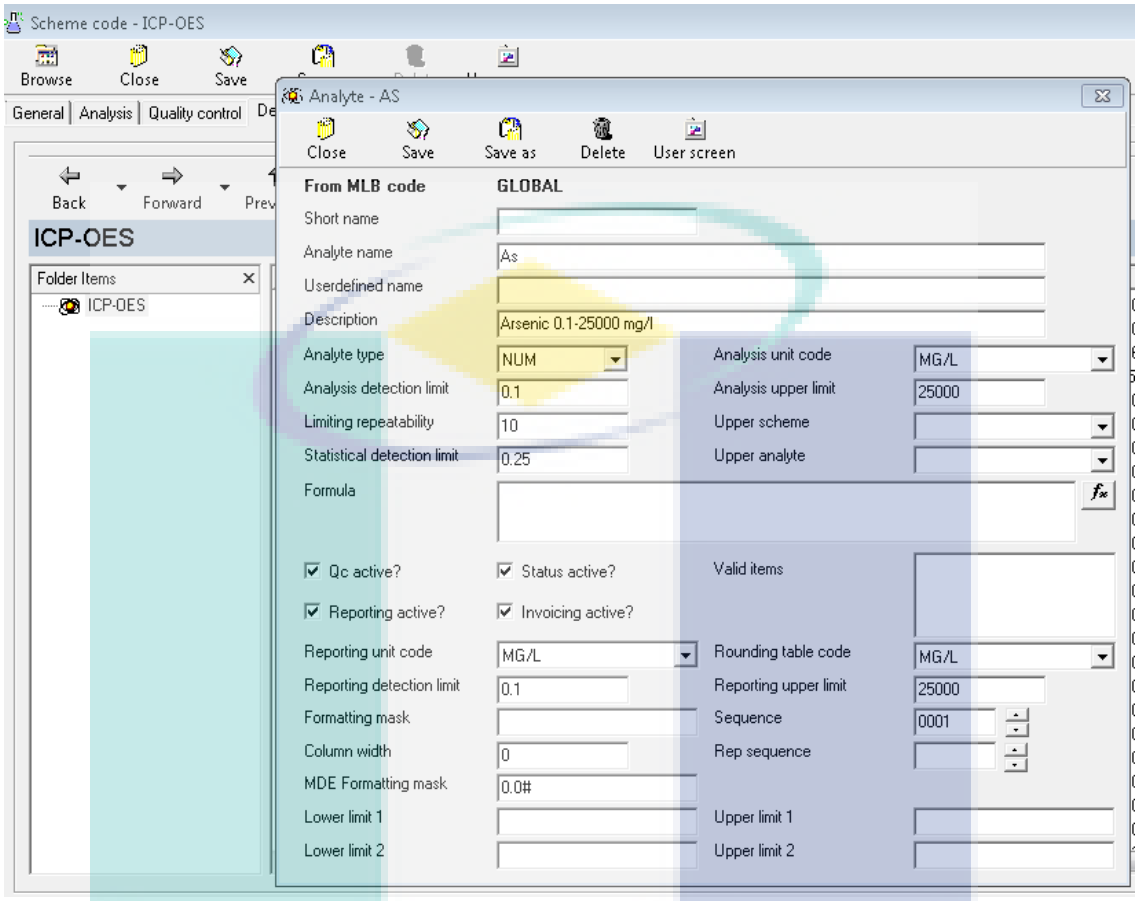
EV= Expected Value

Tolerance Value Aluminium (Al)



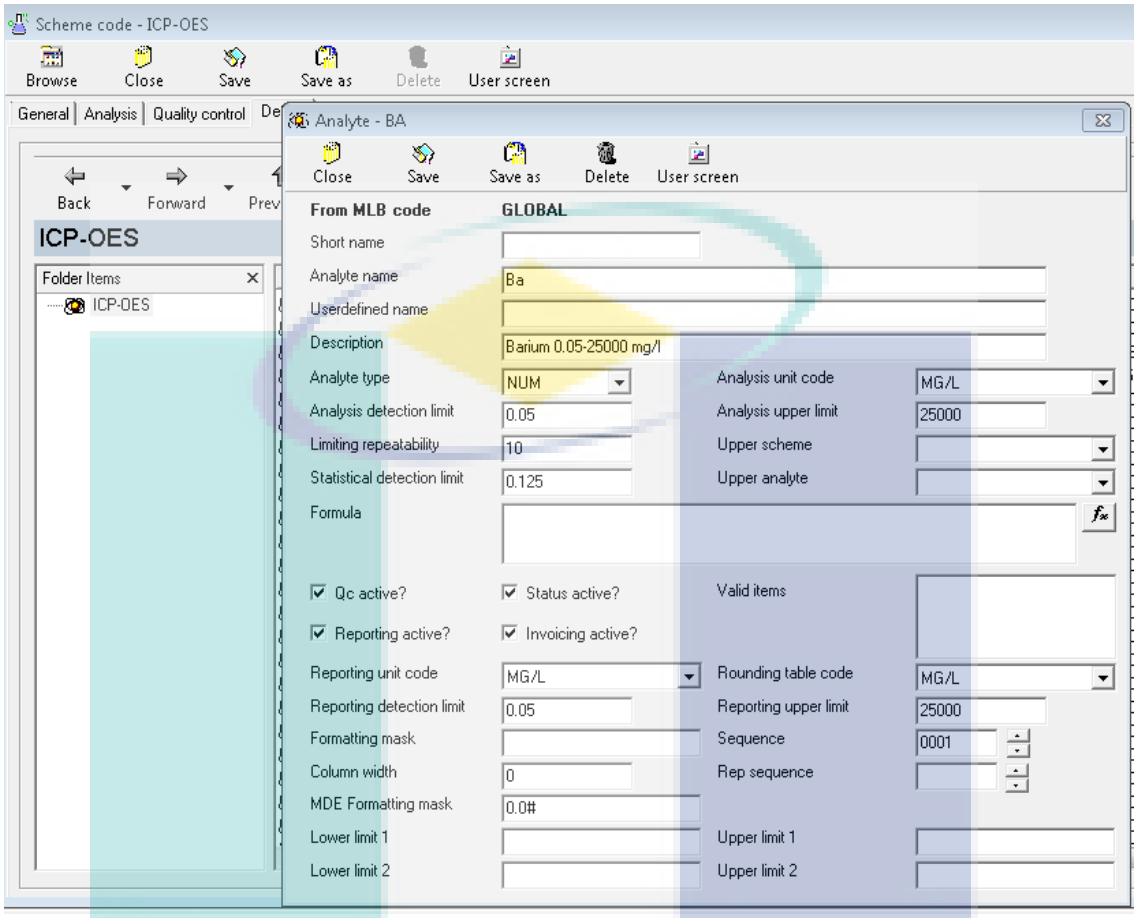
Tolerance % = $\frac{2.5}{10}$ $x100 + 10 = 35$

Tolerance Value Arsenic (As)



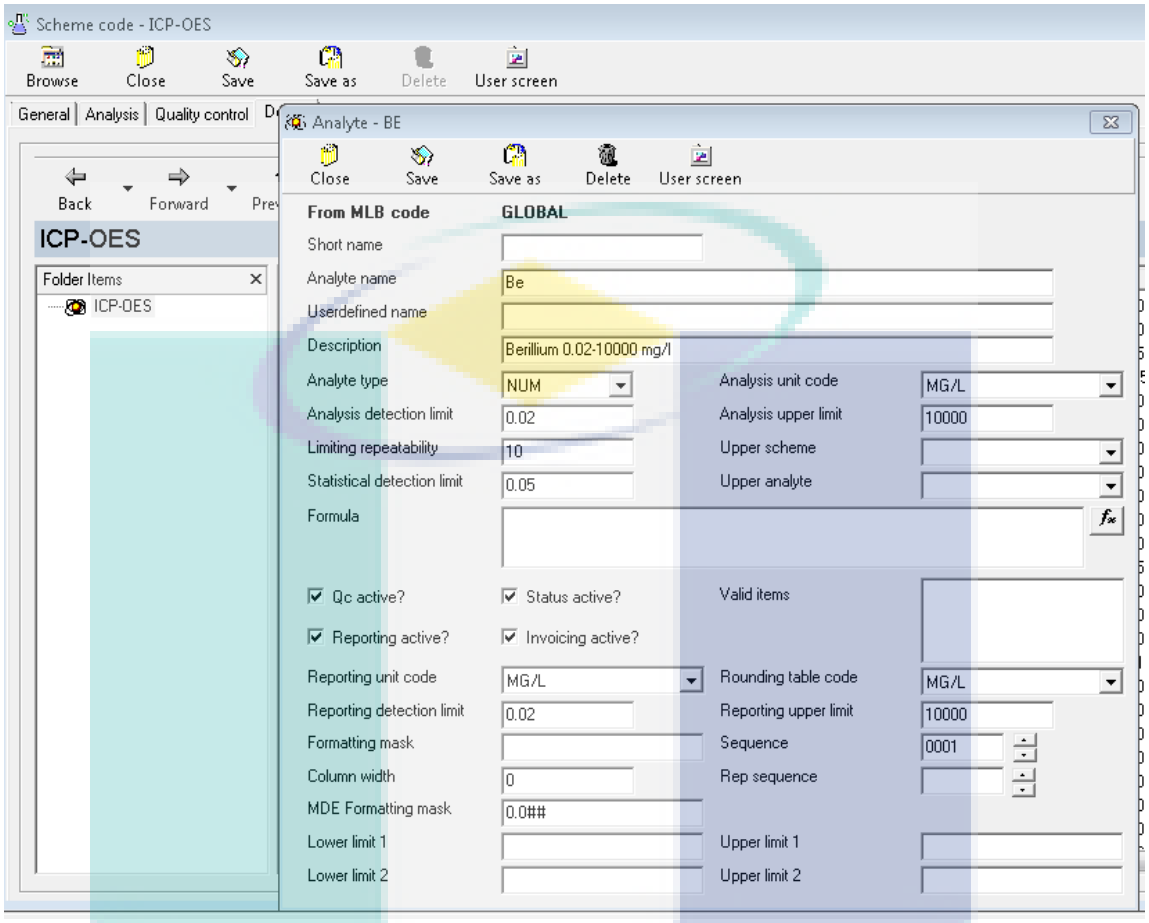
Tolerance % = $\frac{0.25}{10}\ x100 + 10 = 12.5$

Tolerance Value Barium (Ba)



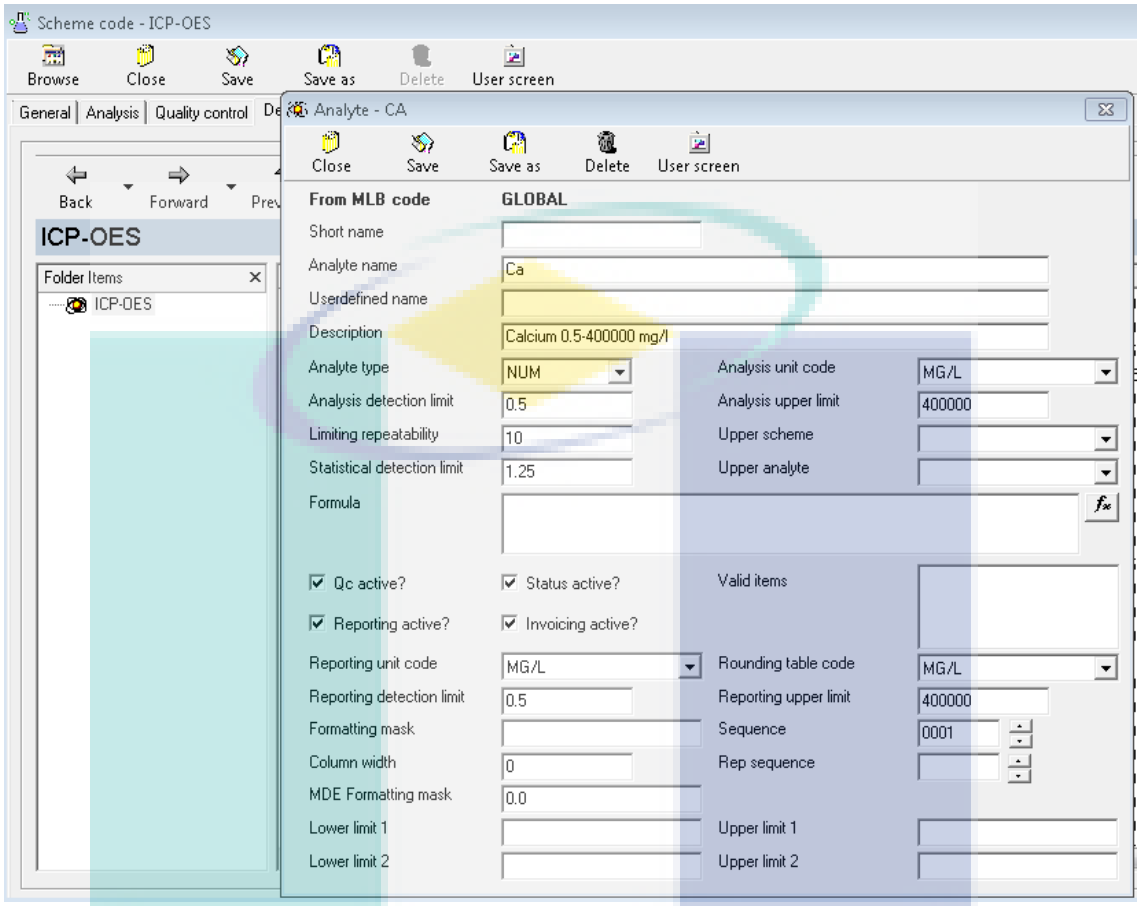
Tolerance % = $\frac{0.125}{10}\ x100 + 10 = 11.25$

Tolerance Value Berillium (Be)



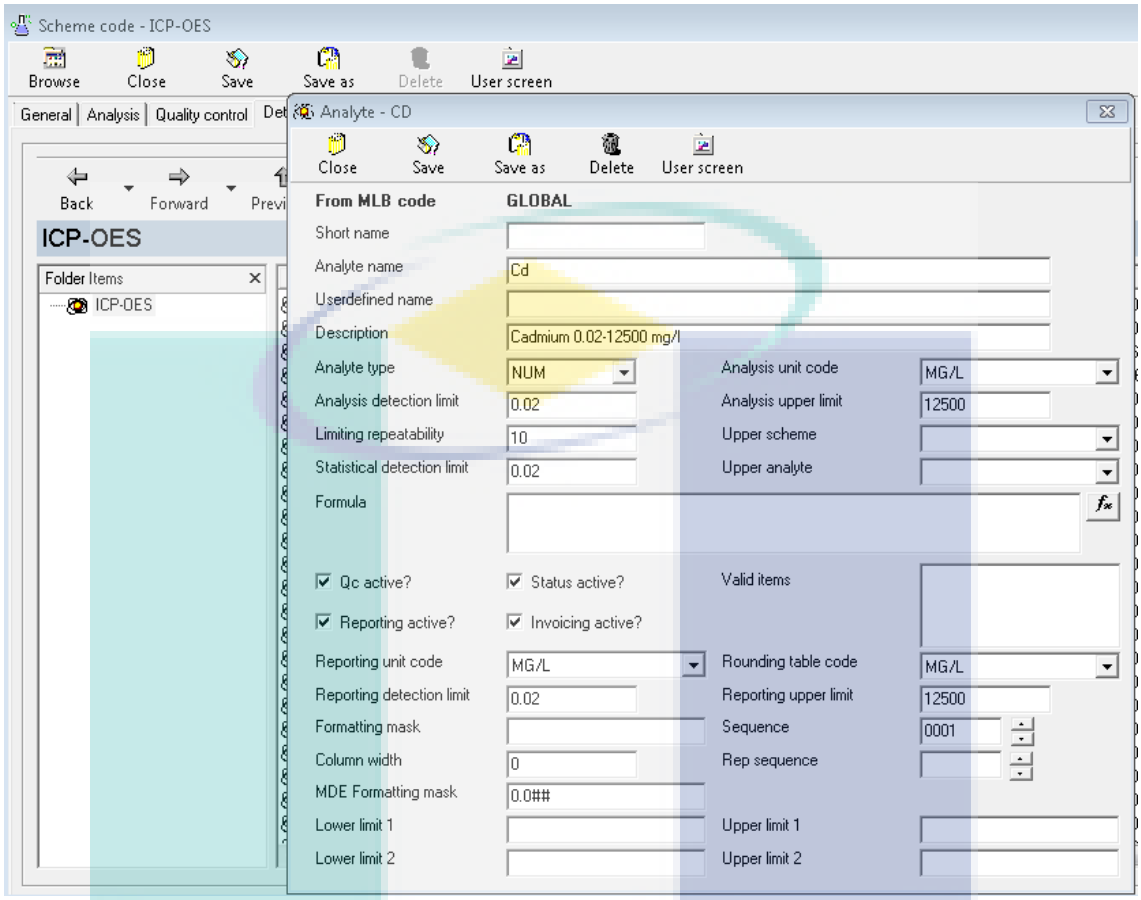
Tolerance % = $\frac{0.05}{10}\ x100 + 10 = 10.5$

Tolerance Value Calcium (Ca)



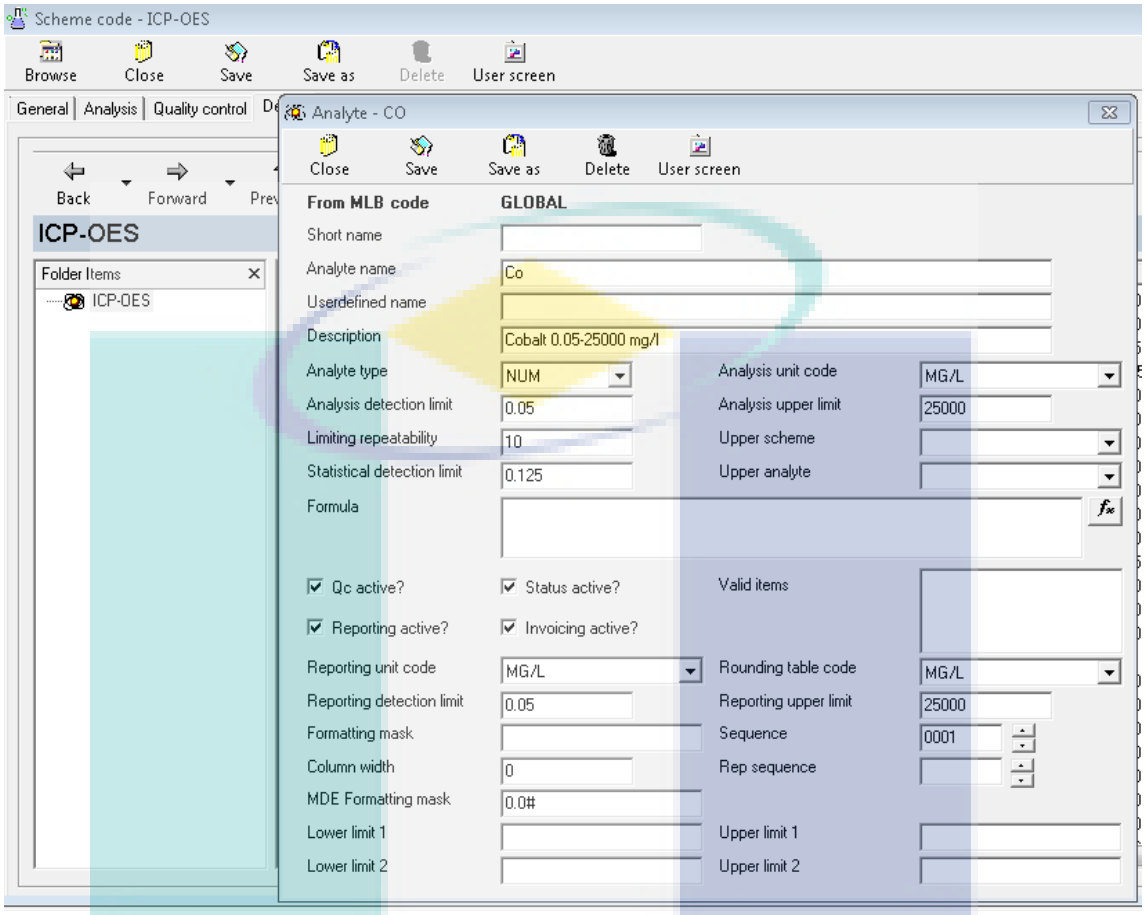
Tolerance % = $\frac{1.25}{10}\ x100 + 10 = 22.5$

Tolerance Value Cadmium (Cd)



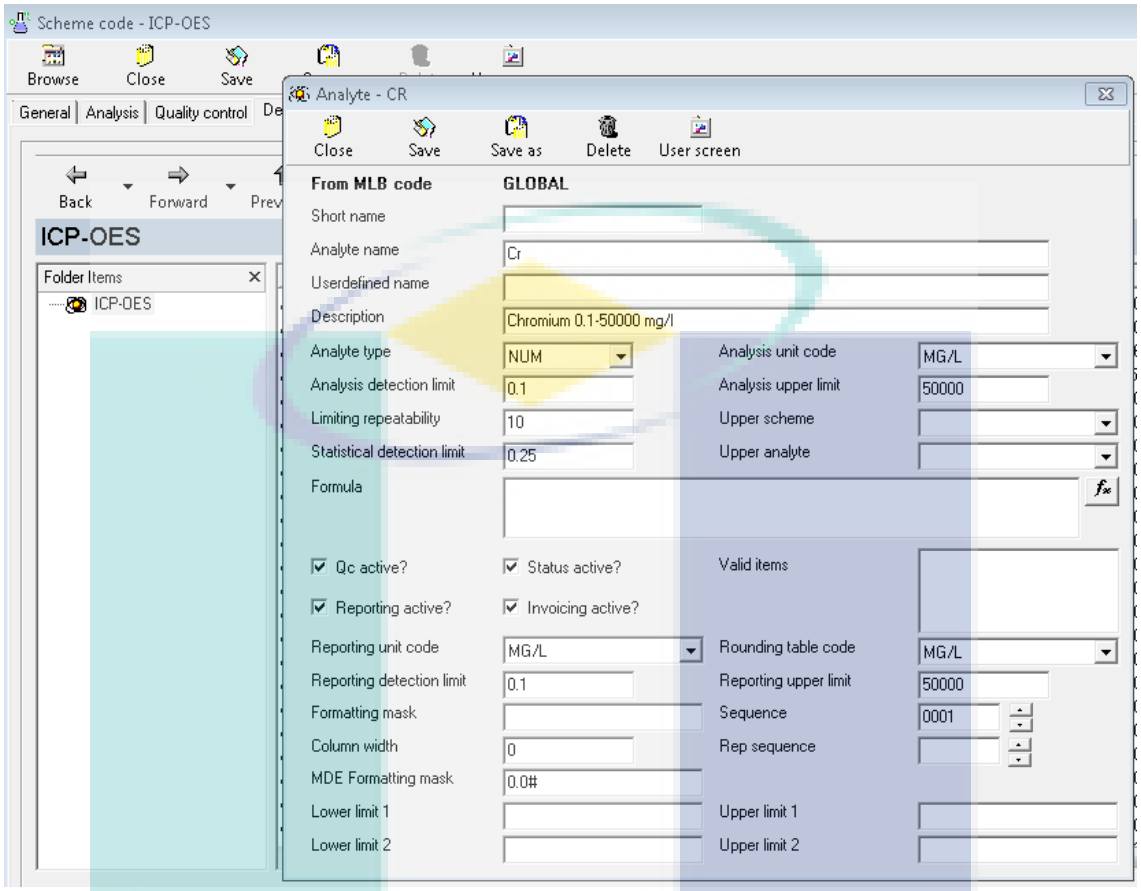
Tolerance % = $\frac{0.02}{10}\ x100 + 10 = 10.2$

Tolerance Value Cobalt (Co)



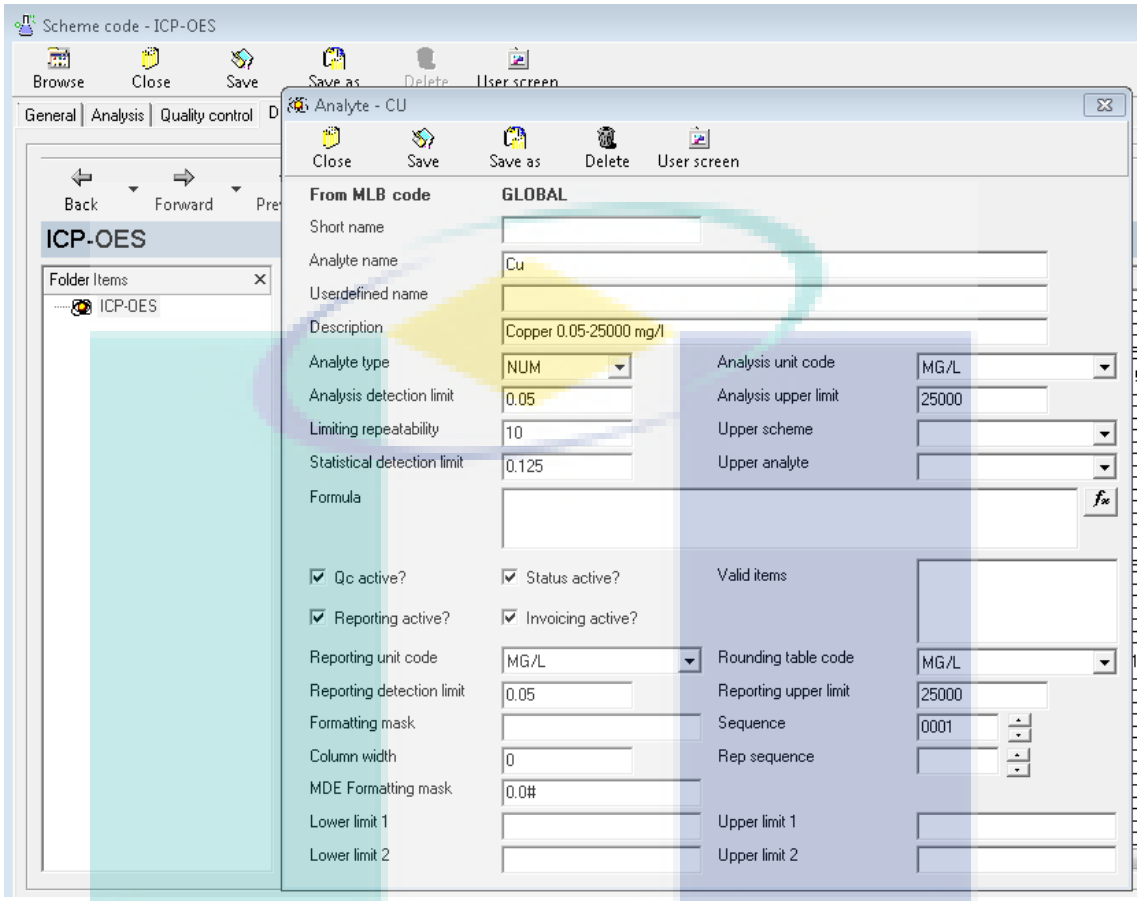
Tolerance % = $\frac{0.125}{10}\ x100 + 10 = 11.25$

Tolerance Value Chromium (Cr)



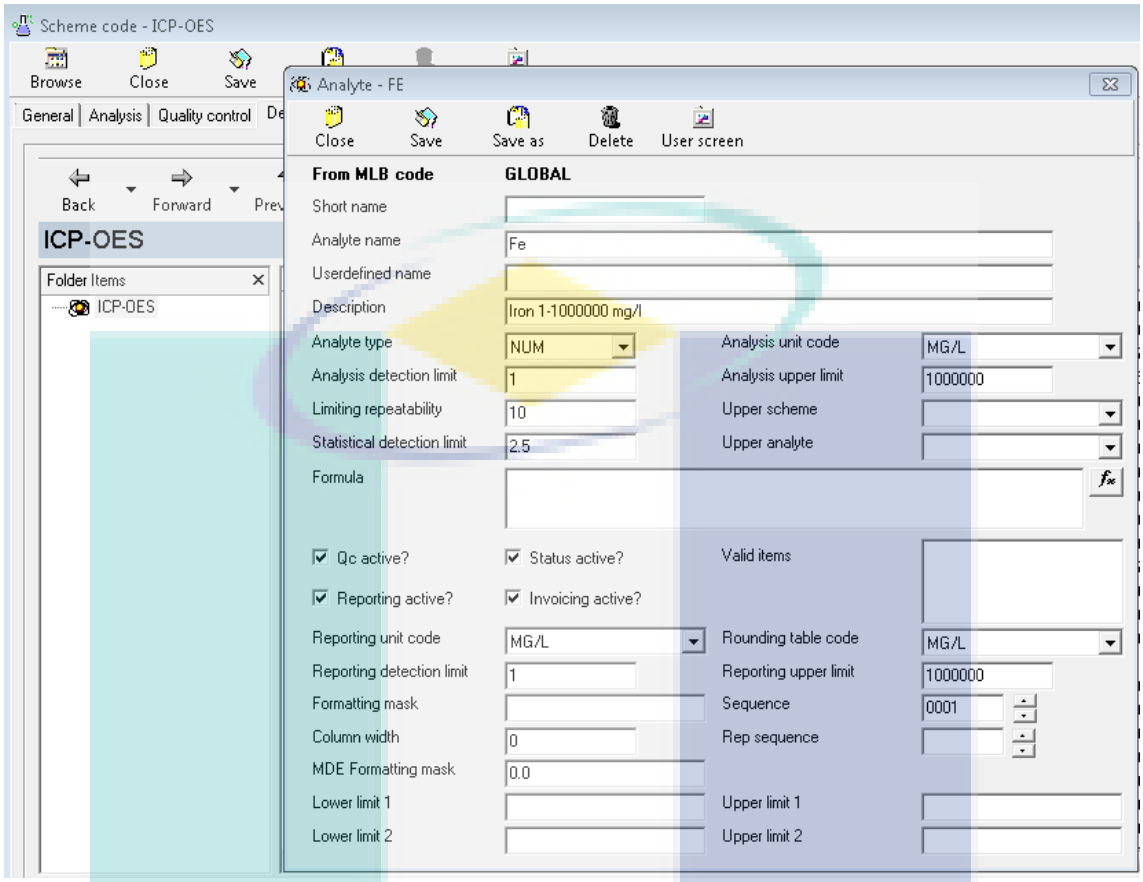
Tolerance % = $\frac{0.25}{10}\ x100 + 10 = 11.25$

Tolerance Value Copper (Cu)



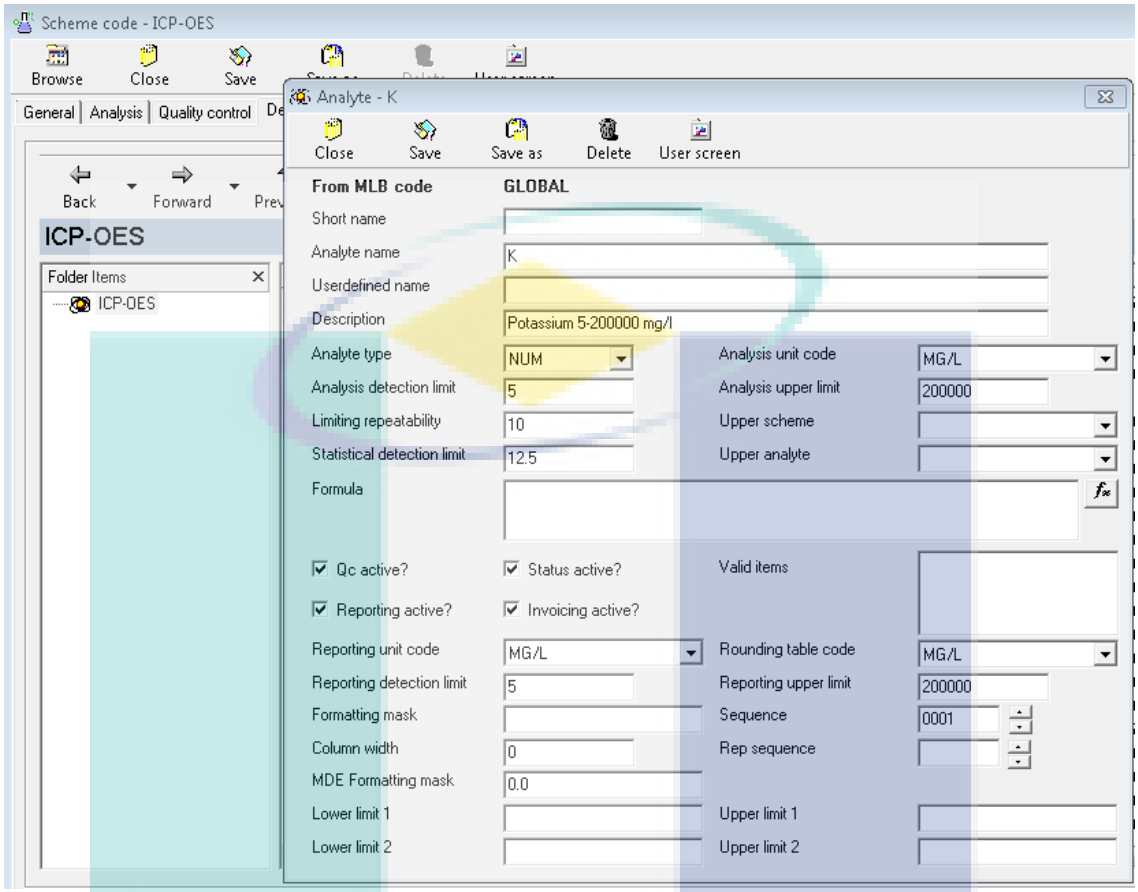
Tolerance % = $\frac{0.125}{10}\ x100 + 10 = 11.25$

Tolerance Value Iron (Fe)



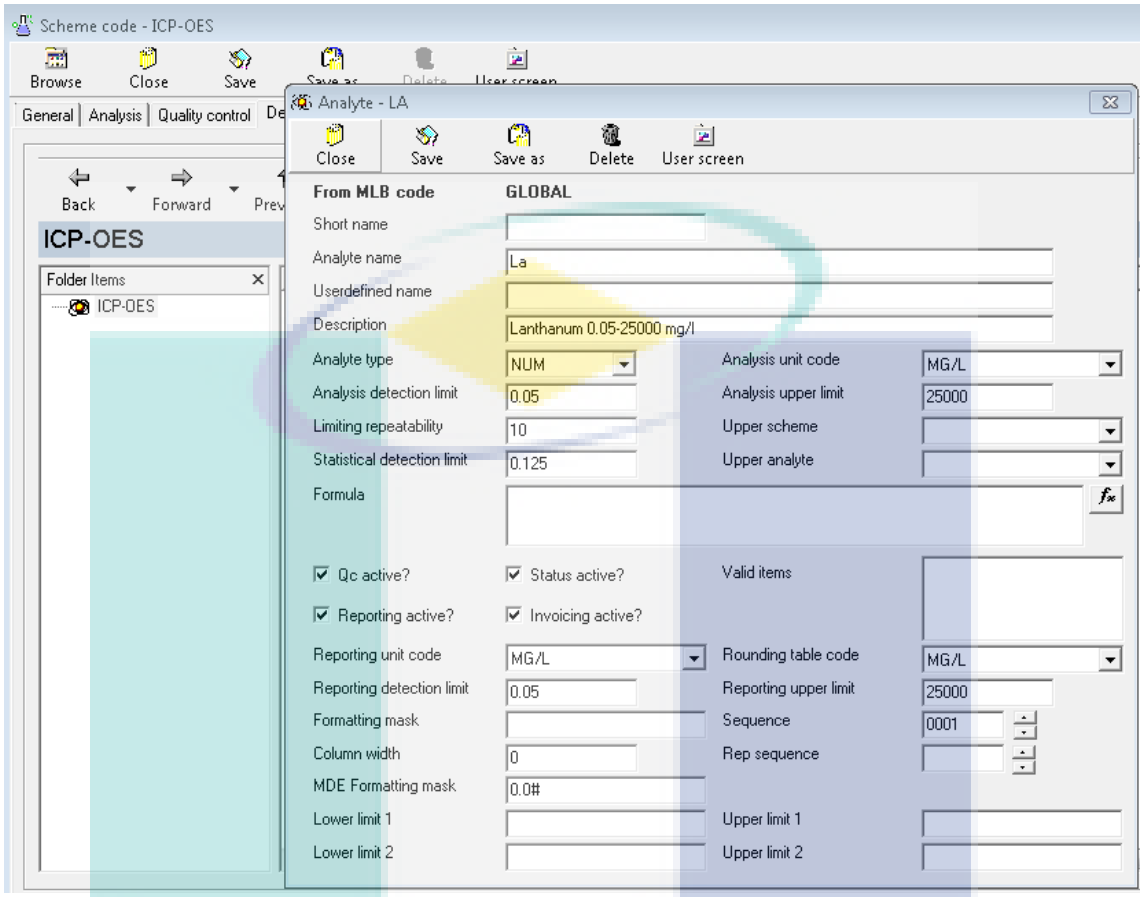
Tolerance % = $\frac{2.5}{10}\ x100 + 10 = 11.25$

Tolerance Value Potassium (K)



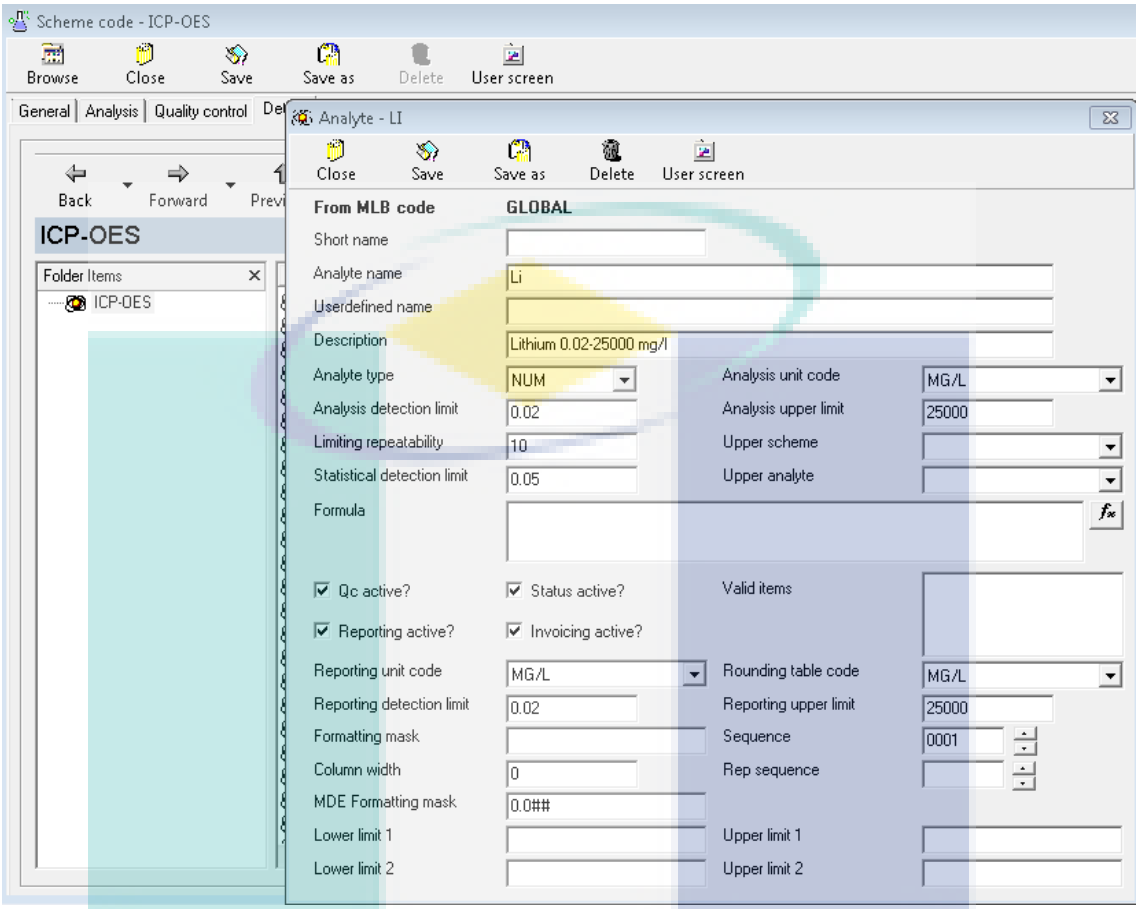
Tolerance % = $\frac{12.5}{10}\ x100 + 10 = 135$

Tolerance Value Lanthanum (La)



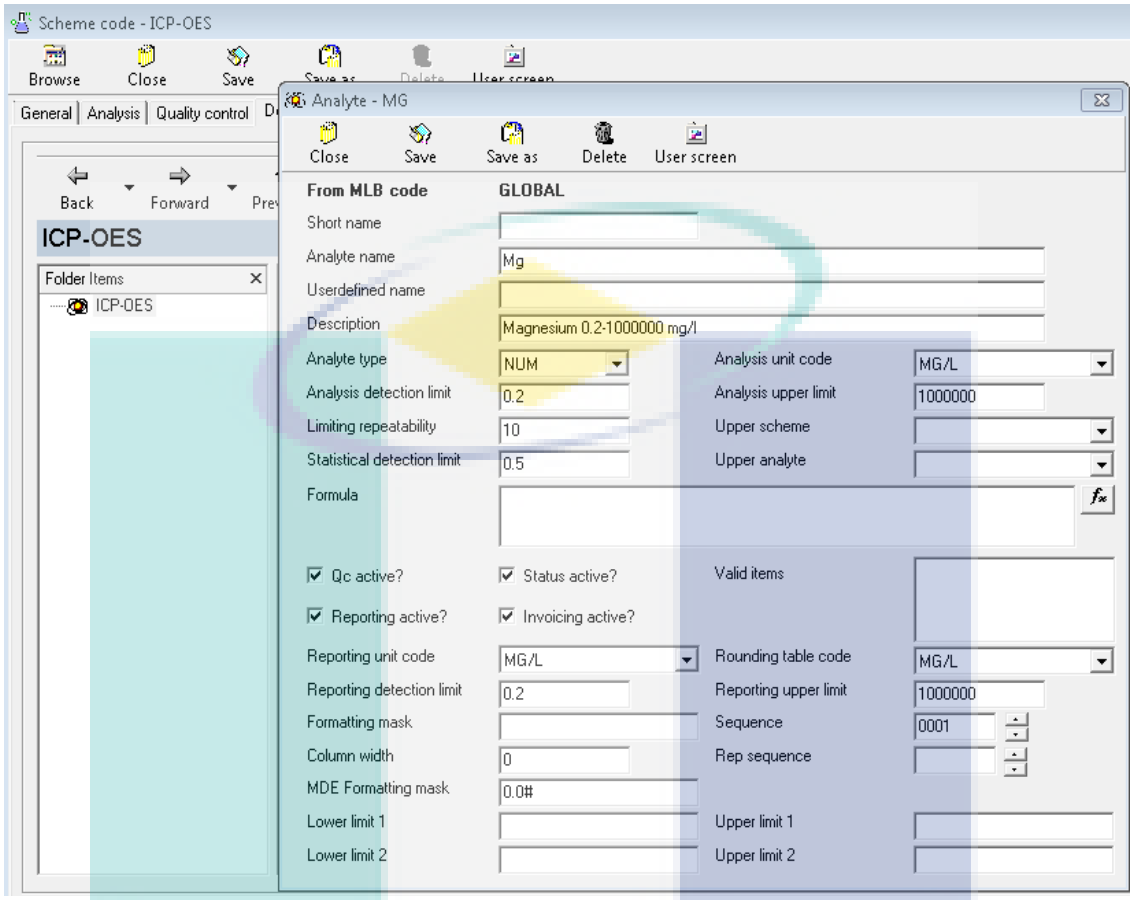
Tolerance % = $\frac{0.125}{10}\ x100 + 10 = 11.25$

Tolerance Value Lithium (Li)



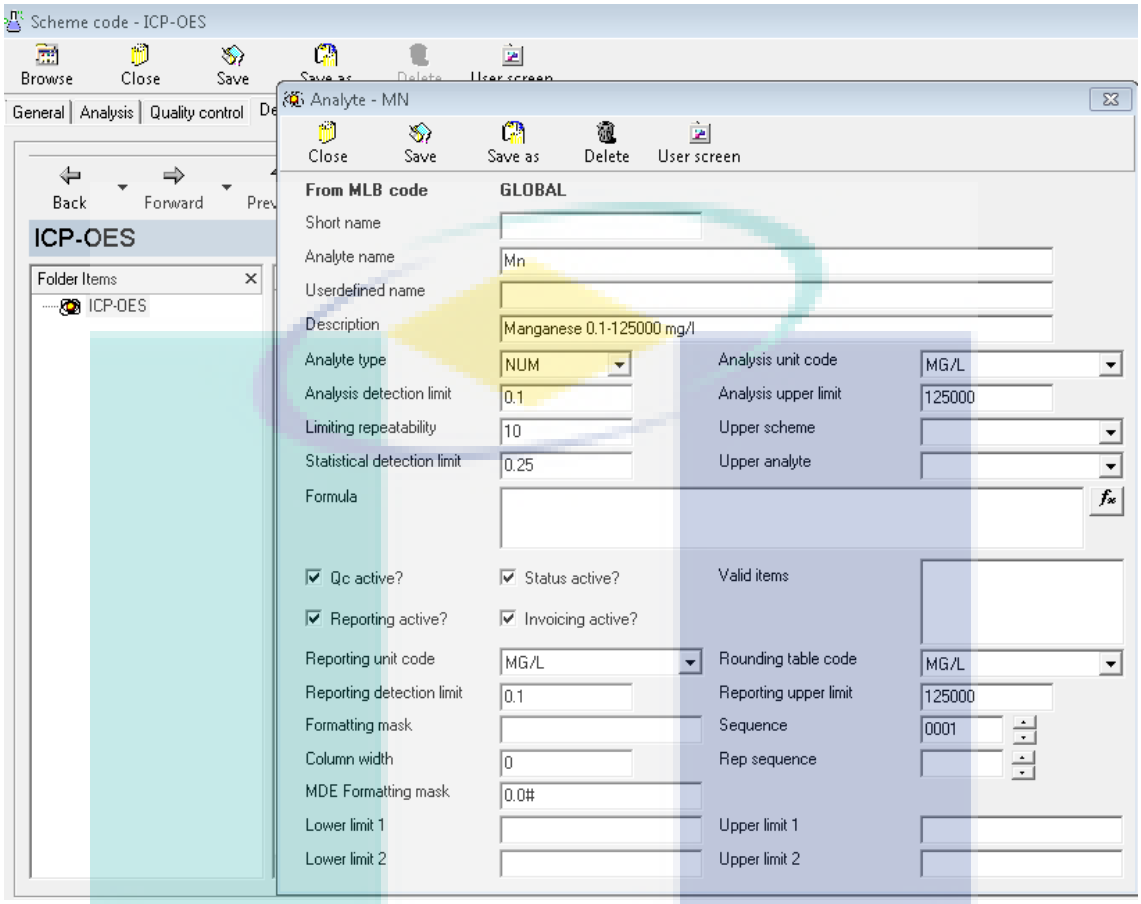
Tolerance % = $\frac{0.05}{10}\ x100 + 10 = 10.5$

Tolerance Value Magnesium (Mg)



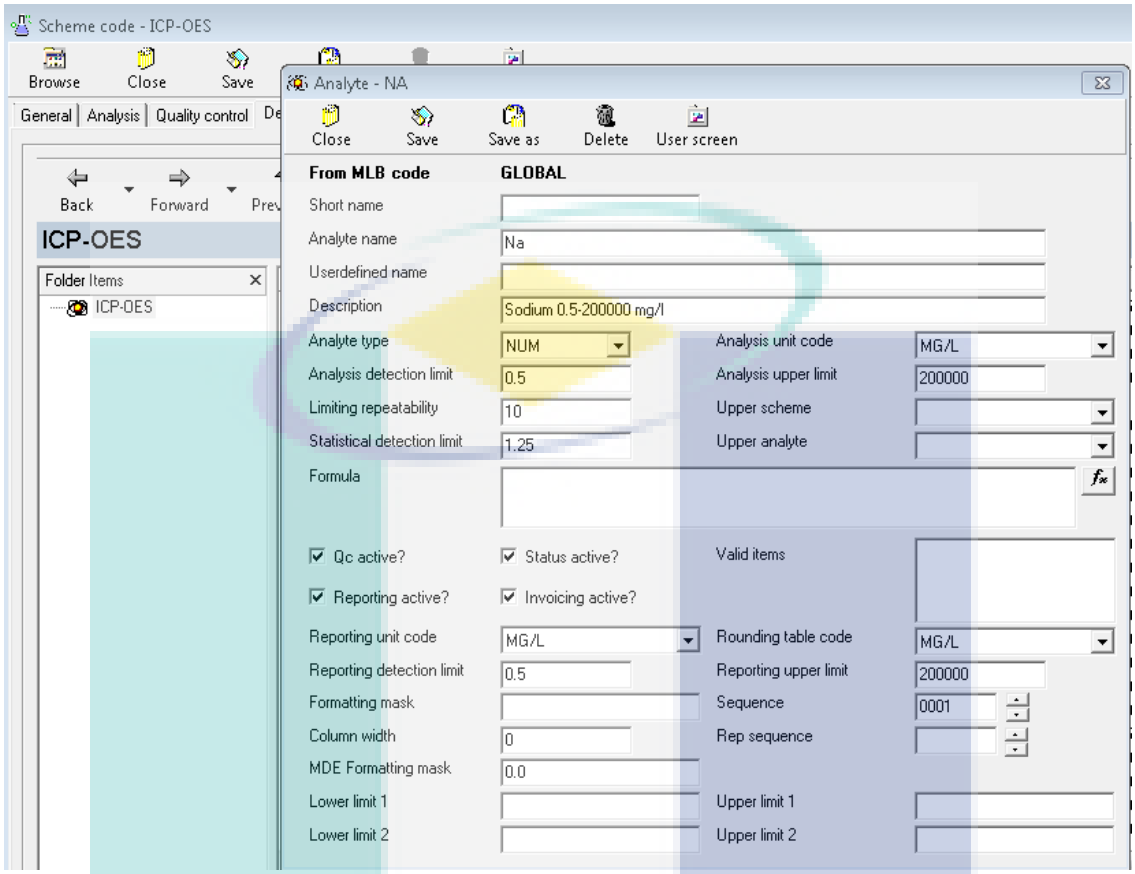
Tolerance % = $\frac{0.5}{10}\ x100 + 10 = 15$

Tolerance Value Manganese (Mn)



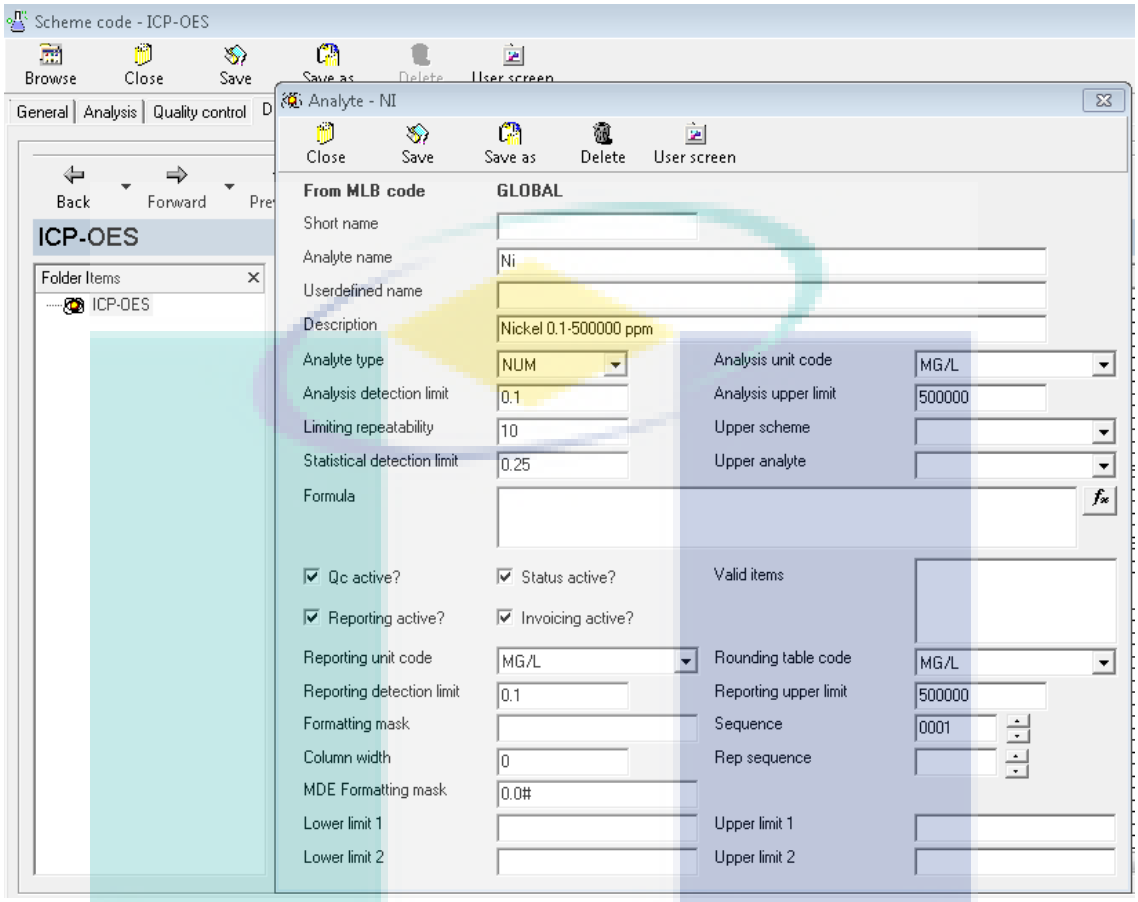
Tolerance % = $\frac{0.25}{10}\ x100 + 10 = 12.5$

Tolerance Value Natrium (Na)



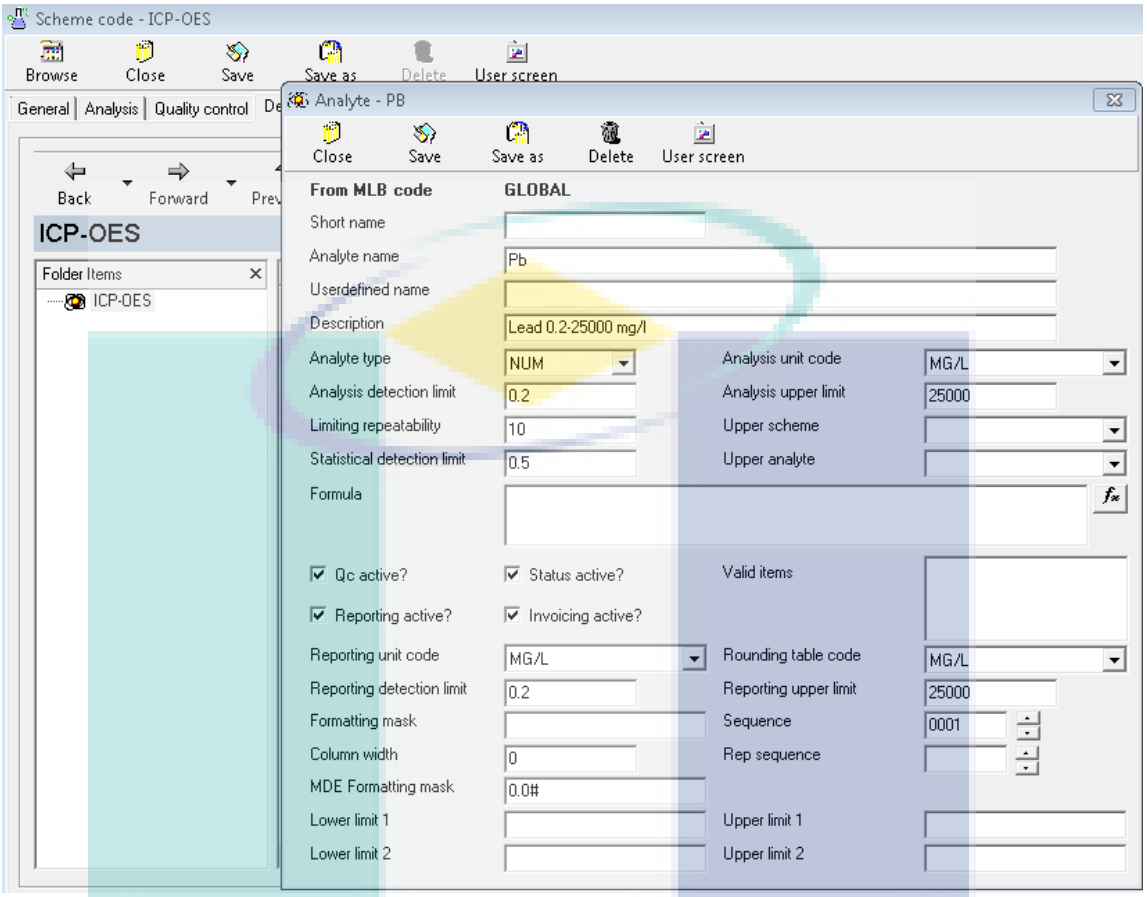
Tolerance % = $\frac{1.25}{10}\ x100 + 10 = 22.5$

Tolerance Value Nitrium (Ni)



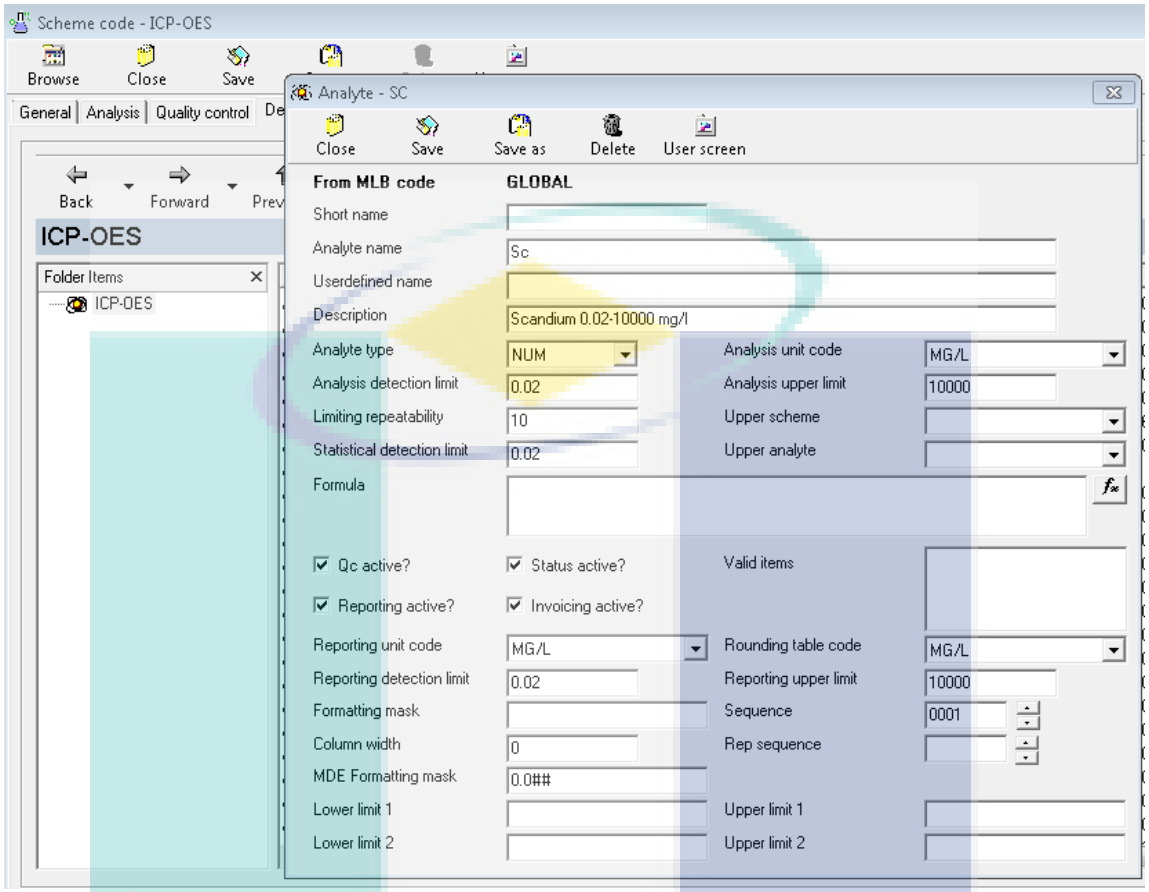
Tolerance % = $\frac{0.25}{10}\ x100 + 10 = 12.5$

Tolerance Value Lead (Pb)



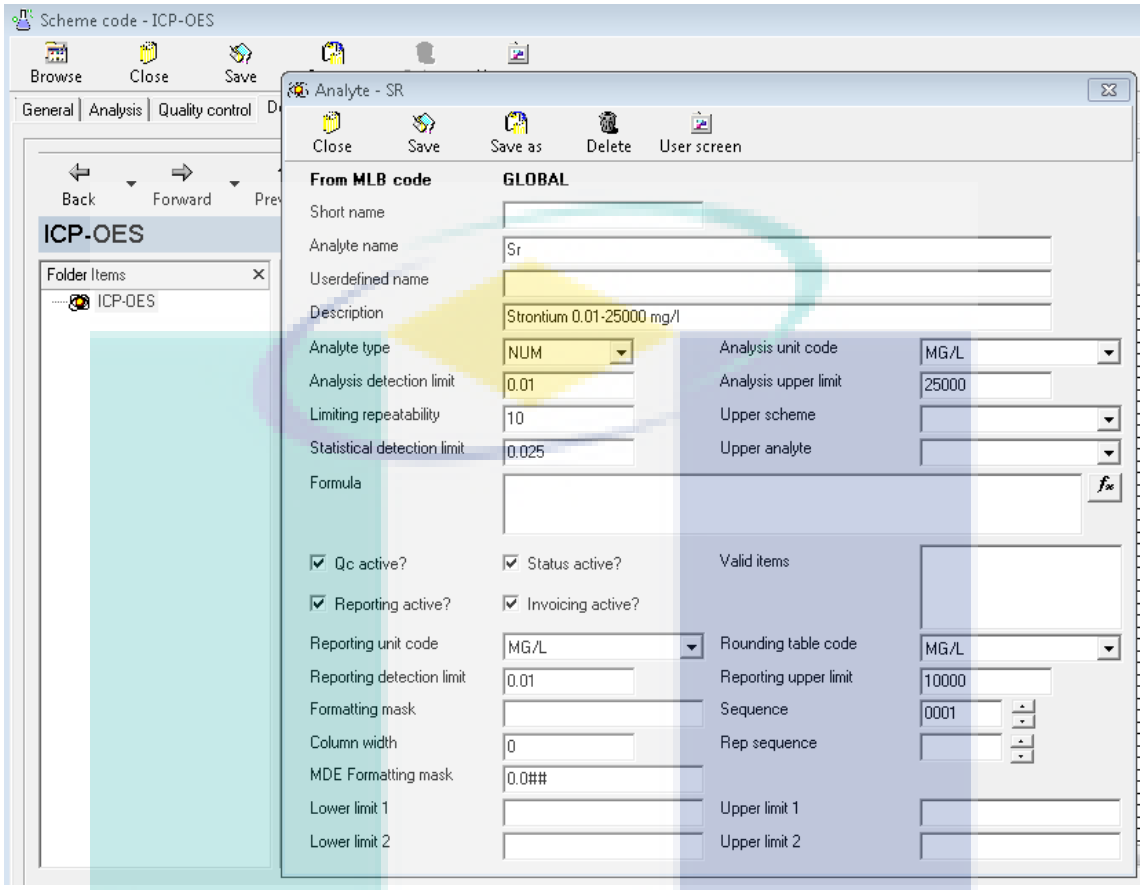
Tolerance % = $\frac{0.5}{10}\ x100 + 10 = 15$

Tolerance Value Scandium (Sc)



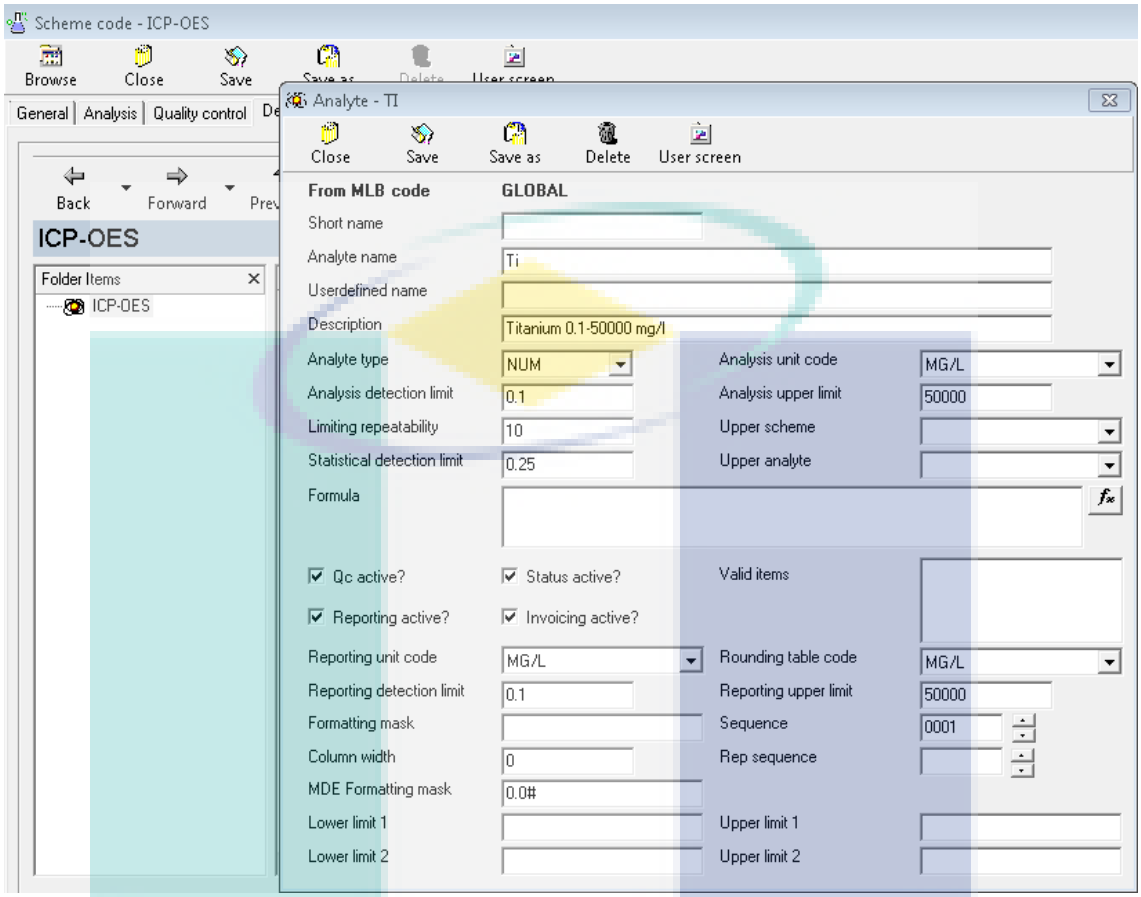
Tolerance % = $\frac{0.02}{10}\ x100 + 10 = 10.2$

Tolerance Value Strontium (Sr)



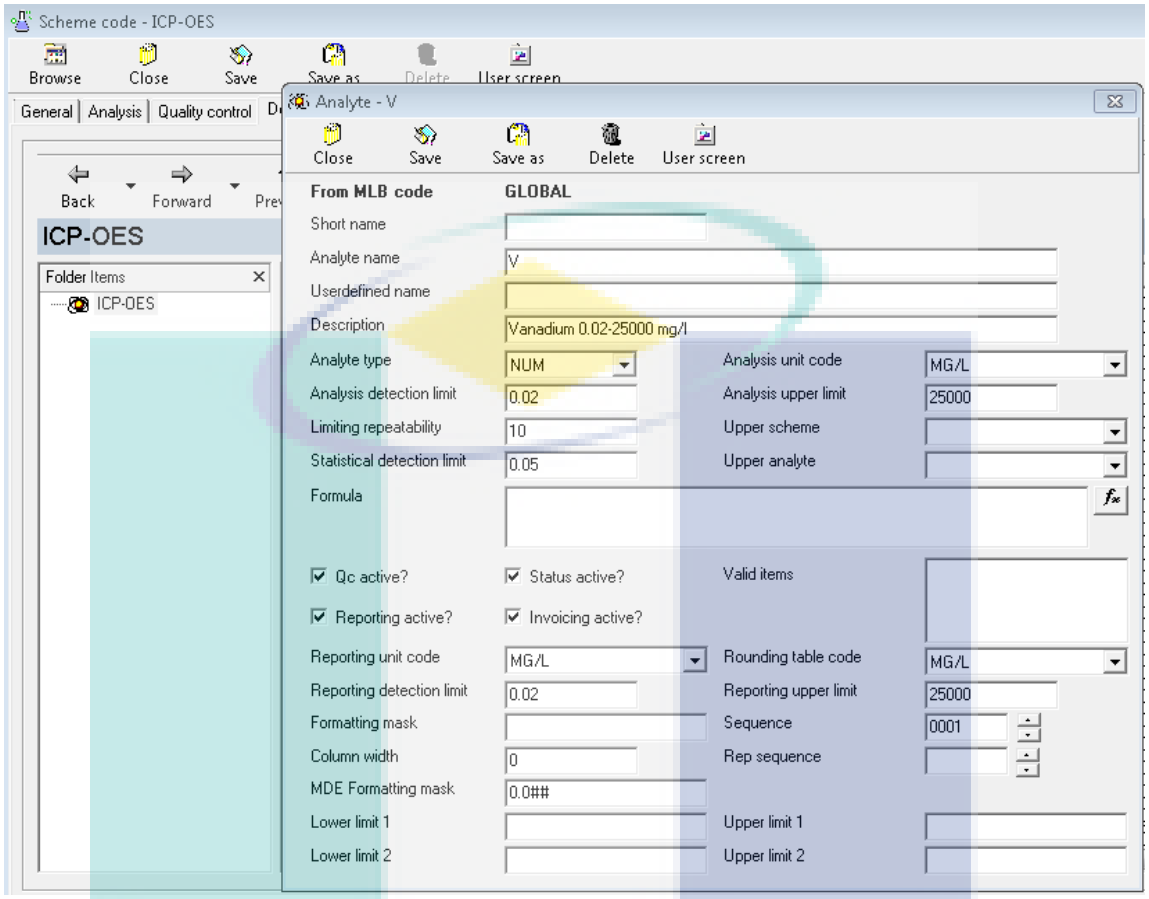
Tolerance % = $\frac{0.025}{10}\ x100 + 10 = 10.25$

Tolerance Value Titanium (Ti)



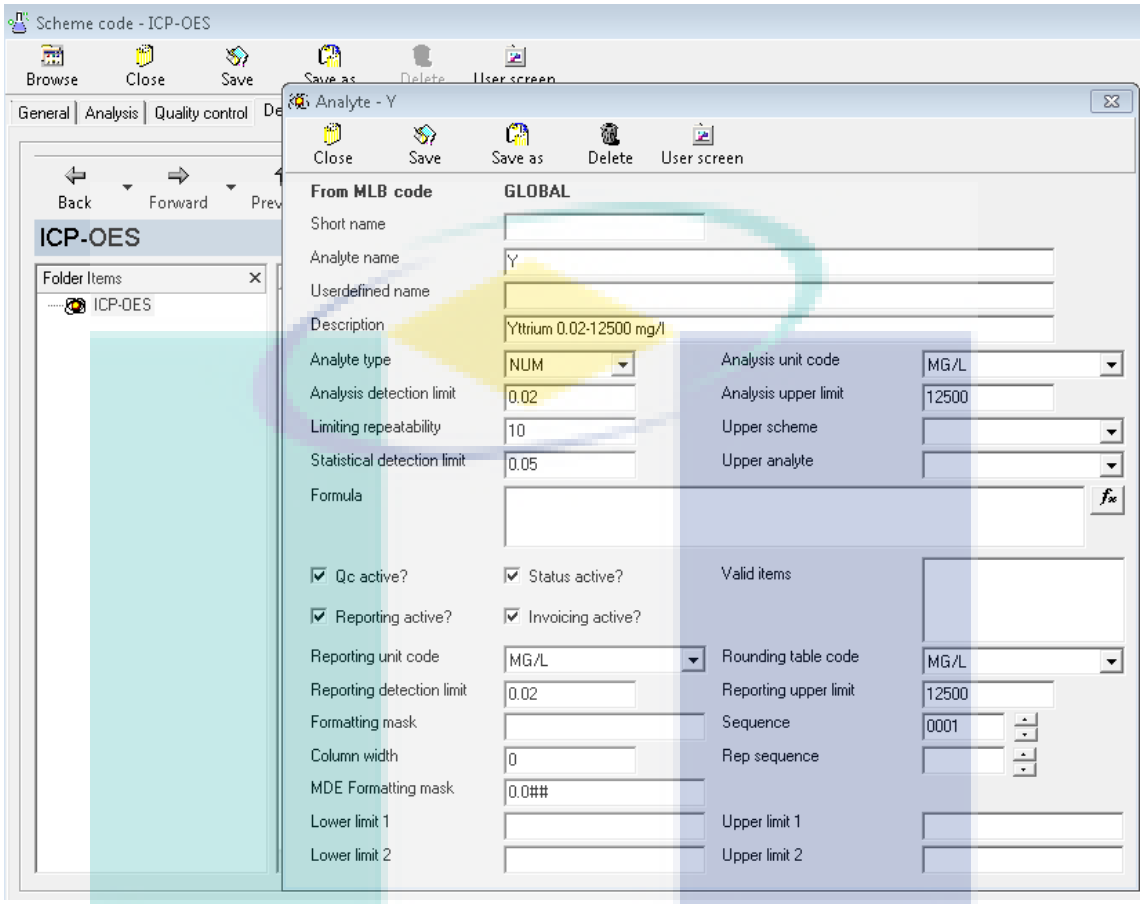
Tolerance % = $\frac{0.25}{10}\ x100 + 10 = 12.5$

Tolerance Value Vanadium (V)



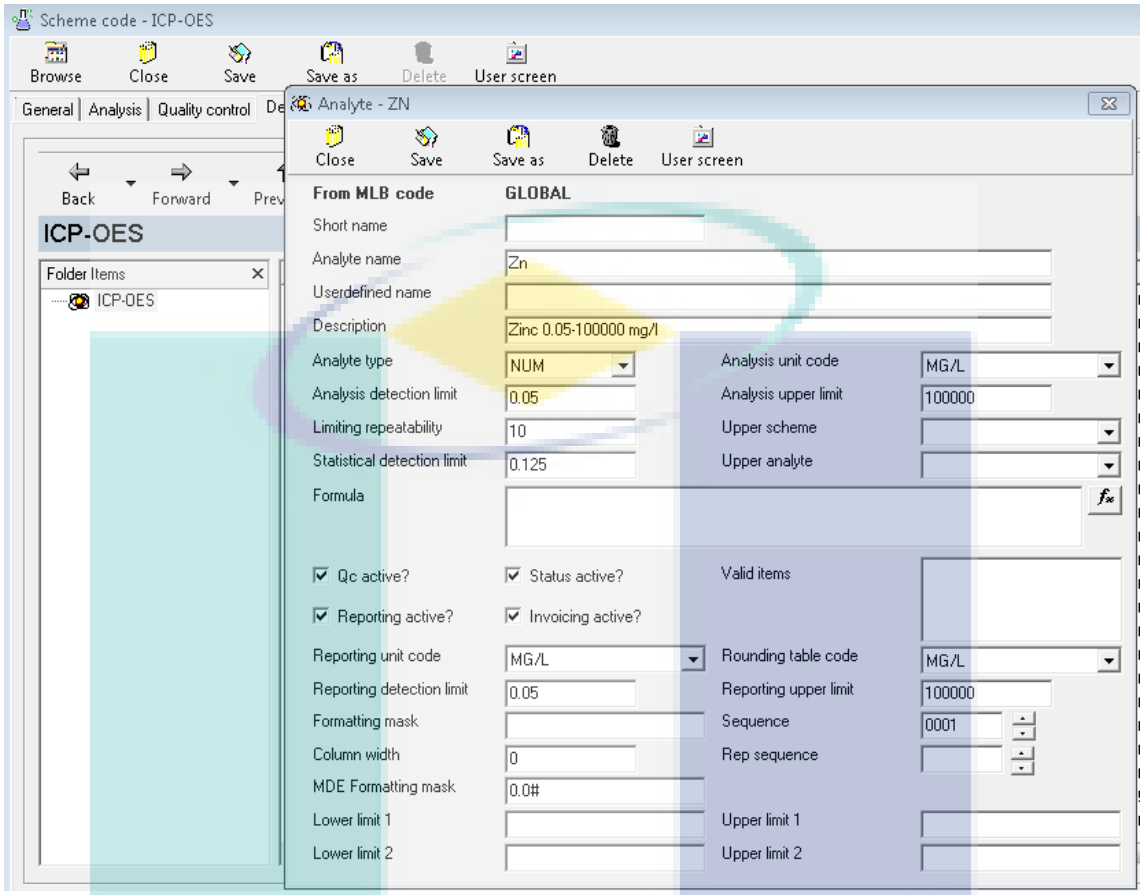
Tolerance % = $\frac{0.05}{10}\ x100 + 10 = 10.5$

Tolerance Value Yittrium (Y)



Tolerance % = $\frac{0.05}{10}\ x100 + 10 = 10.5$

Tolerance Value Zinc (Zn)



Tolerance % = $\frac{0.125}{10}\ x100 + 10 = 11.25$

APPENDIX E

MS/MS FRAGMENTATION OF PHENOLIC COMPOUNDS

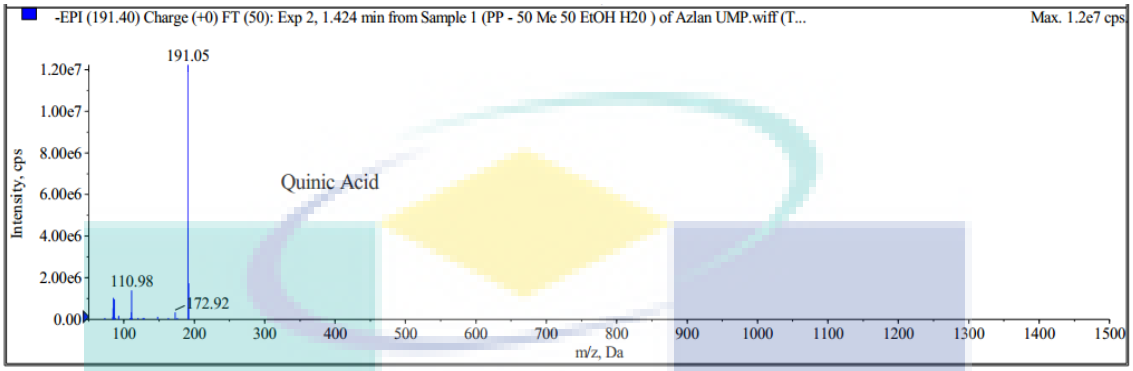


Figure 6.3a The MS/MS fragmentation of Quinic acid from pitaya peel extract

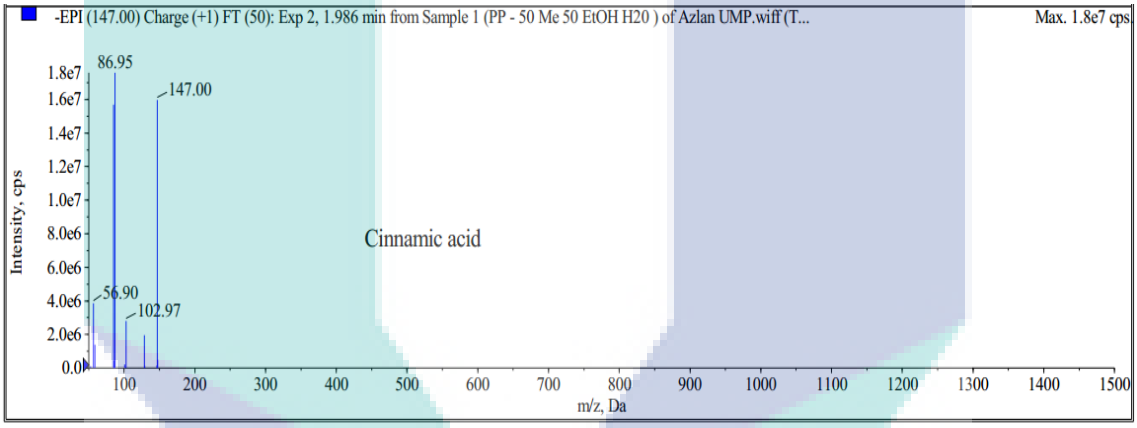


Figure 6.3b The MS/MS fragmentation of Cinnamic acid from pitaya peel extract

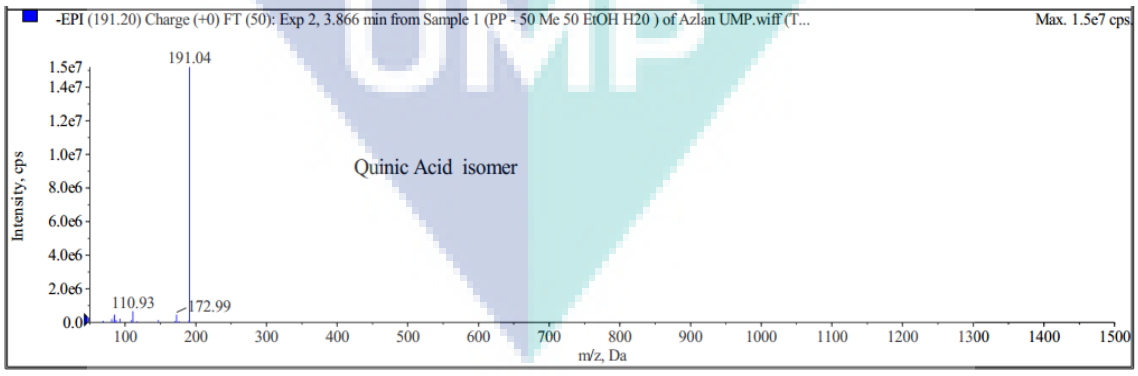


Figure 6.3c The MS/MS fragmentation of Quinic acid isomer from pitaya peel extract

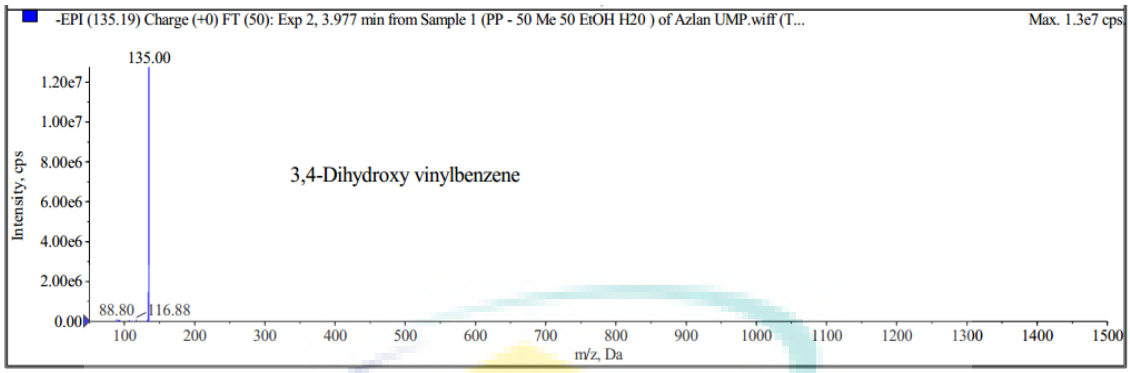


Figure 6.3d The MS/MS fragmentation of 3,4-Dihydroxy vinylbenzene acid isomer from pitaya peel extract

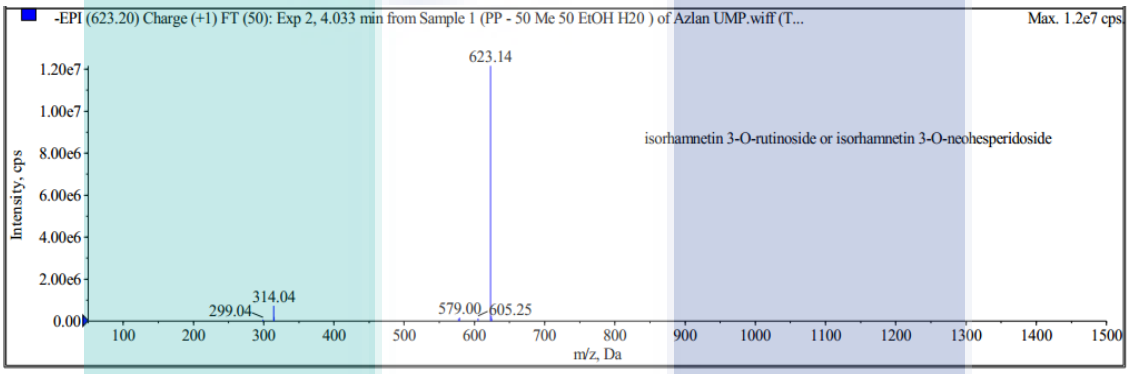


Figure 6.3e The MS/MS fragmentation of Isorhamnetin 3-O-rutinoside acid from pitaya peel extract

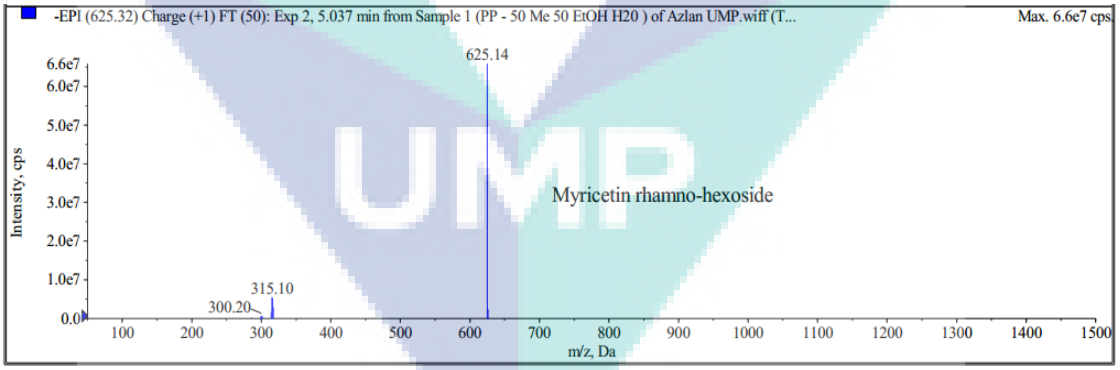


Figure 6.3f The MS/MS fragmentation of Myricetin rhamno-hexoside acid from pitaya peel extract

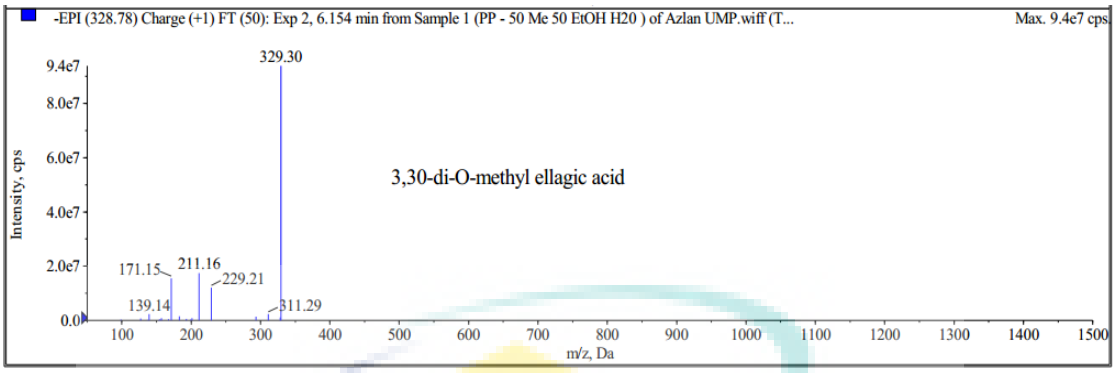


Figure 6.3g The MS/MS fragmentation of 3,30-di-O-methyl ellagic acid from pitaya peel extract.

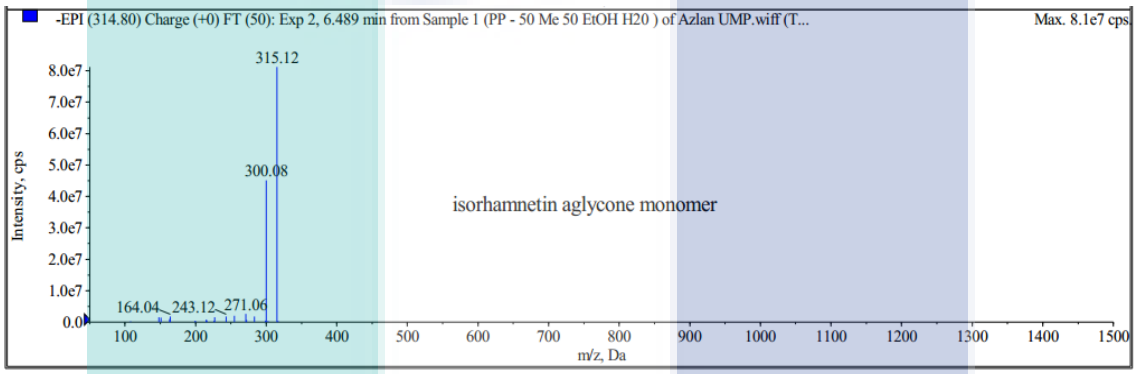


Figure 6.3h The MS/MS fragmentation of Isorhamnetin aglycone monomer from pitaya peel extract.

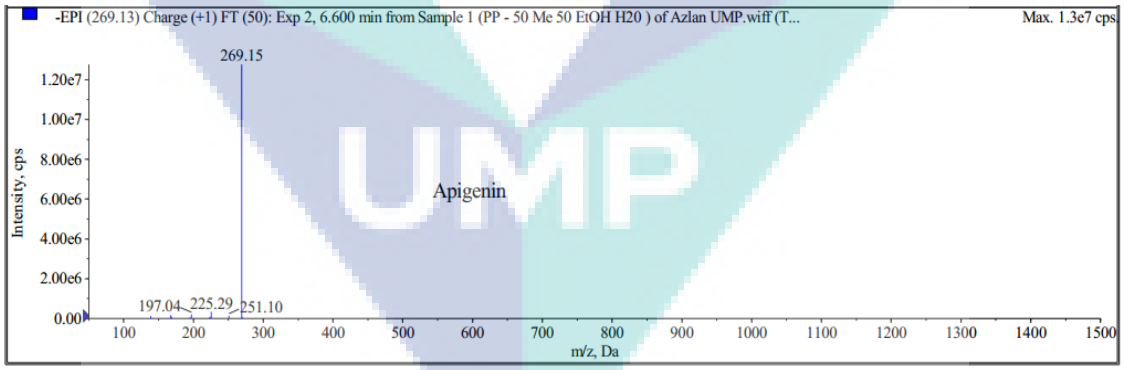


Figure 6.3i The MS/MS fragmentation of Apigenin from pitaya peel extract.

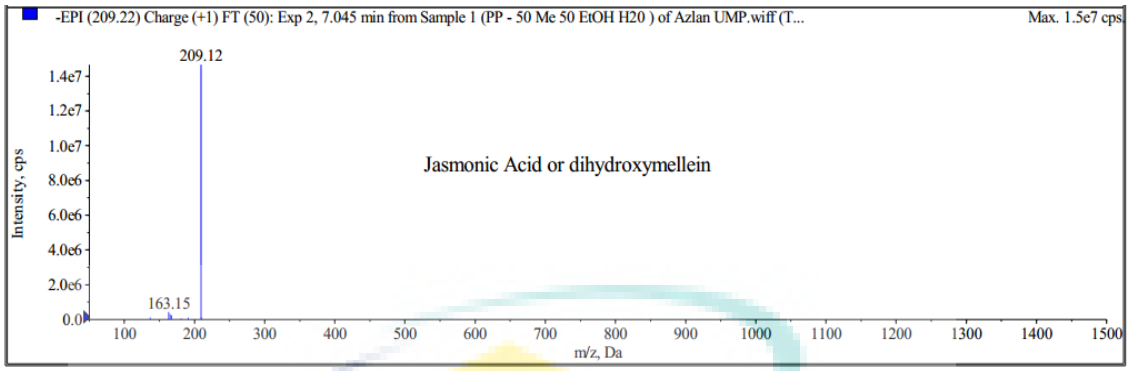


Figure 6.3j The MS/MS fragmentation of Jasmonic acid from pitaya peel extract.

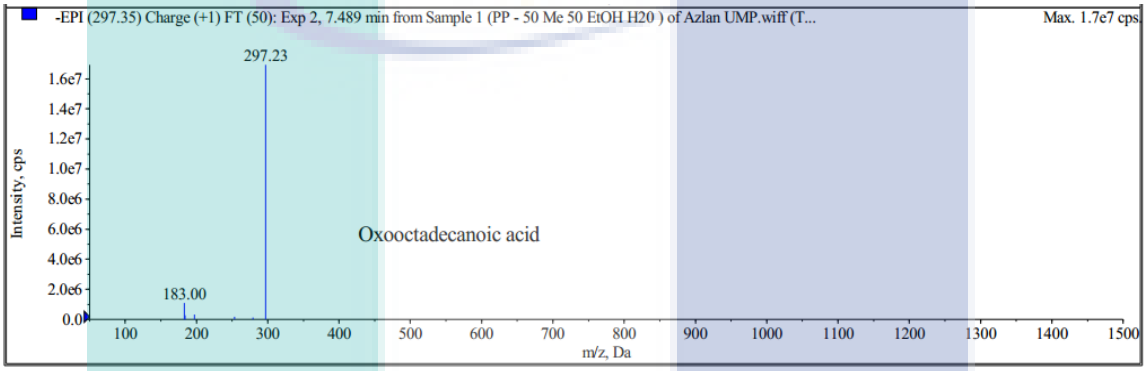


Figure 6.3k The MS/MS fragmentation of Oxooctadecanoic acid from pitaya peel extract

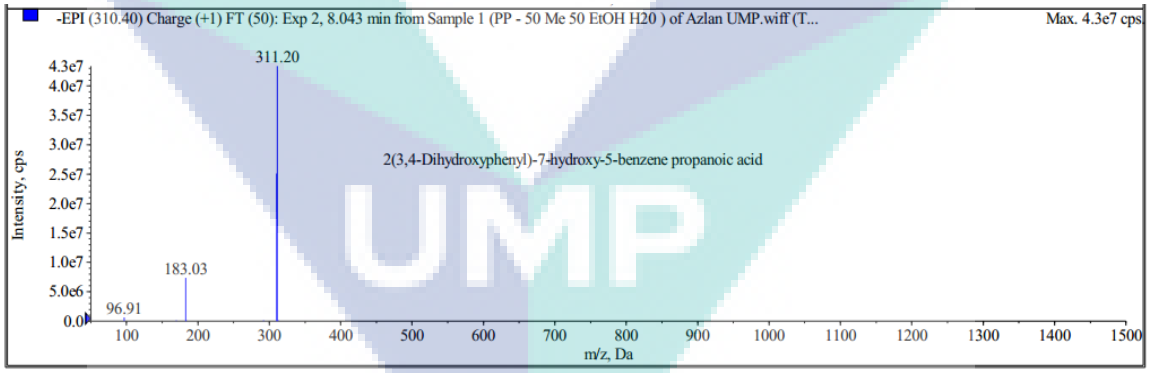


Figure 6.3l The MS/MS fragmentation of 2 (3,4-Dihydroxyphenyl)-7-hydroxy-5-benzene propanoic acid from pitaya peel extract

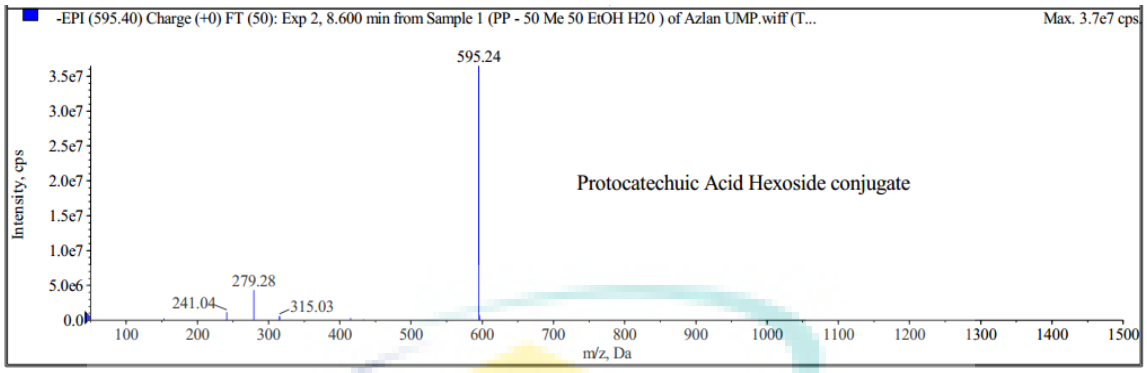


Figure 6.3m The MS/MS fragmentation of Protocatechuic Hexoside conjugate from pitaya peel extract

FULL CHROMATOGRAM FOR POLYPHENOLIC COMPOUNDS

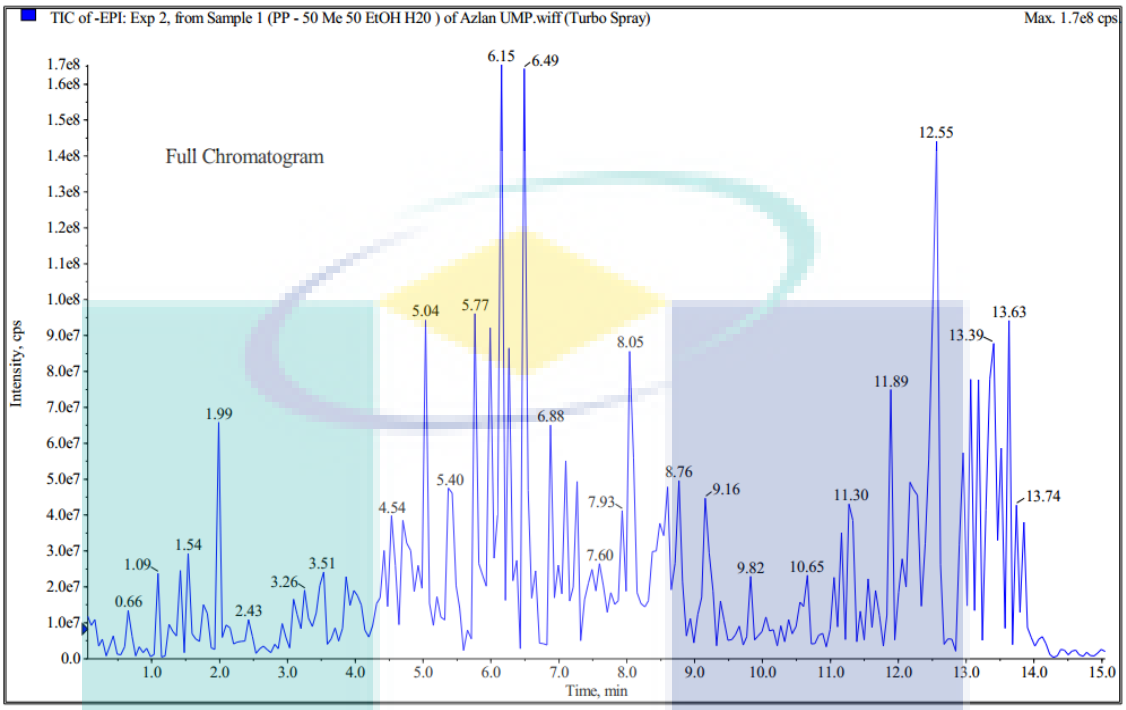


Figure 6.4 The UHPLC-ESI-MS TIC (negative mode) of the extracts of pitaya peel.

Table 6.0 Unknown Compounds identified in the pitaya peel extract based on UHPLC-ESI-QTRAP-MSMS

Retention time (min)	**[M-H]$^-$**	**Mass fragment, MS/MS (m/z)**	**Compound identified**
0.66	198.02	160.80	Unknown
1.09	219.77	201.84, 184.91, 166.86, 147.84	Unknown
7.1	221.34	221.16, 207.19	Unknown
1.54	269.10	195.0, 184.98, 166.95, 86.96	Unknown
6.88	293.35	249.19, 221.18, 205.14, 177.12	Unknown
7.6	307.46	289.44, 265.20, 223.14, 185.04, 137.04, 125.04	Unknown
7.93	309.34	291.24, 209.13, 185.15	Unknown
7.267	310.42	292.24, 223.23, 210.12, 186.12, 99	Unknown
3.699	315.59	285.96, 243.20, 199.32, 129.96	Unknown
8.76	340.34	183.02	Unknown
2.43	365.18	275.13, 203.09, 159.24	Unknown
3.478	403.41	385.20, 343.16, 313.20, 241.15, 218.04, 197.16, 181.92	Unknown
2.923	479.21	389.10, 349.11, 147.16	Unknown
10.661	482.50	410.34, 353.52, 338.28, 256.20	Unknown
3.089	515.25	395.16, 305.16, 275.16	Unknown
3.26	516.21	426.16, 396.20, 276.17	Unknown
5.204	521.51	503.04, 315, 191.04, 153	Unknown
9.16	571.37	315.04, 255.26, 241, 152.95	Unknown

Table 6.0 Continued

Retention time (min)	**$[M-H]^-$**	**Mass fragment, MS/MS (m/z)**	**Compound identified**
4.423	581.42	566.40, 535.23, 419.21, 401.16, 202.20	Unknown
4.54	583.58	568.28, 537.31, 421.13, 406.16, 195.15	Unknown
4.814	633.56	573.26, 335.14, 317.11, 273.09	Unknown
12.55	641.59	625.62, 387.37	Unknown
11.89	642.66	626.52, 388.32	Unknown
13.39	688.36	642.58	Unknown
5.77	724.81	678.51, 660.36	Unknown
5.986	826.85	790.71	Unknown
9.82	828.26	768.57, 279.21	Unknown
11.30	828.64	768.56, 702.56, 279.25	Unknown

APPENDIX F

LIST OF PUBLICATIONS

N.M.Zain and M.A.Nazeri. 2016. Antioxidant And Mineral Content of Pitaya Peel Extract Obtained Using Microwave Assisted Extraction (MAE). *Australian Journal of Basic Applied Science*. 10(17):63-68

Muhd Azlan Nazeri and Norashikin Mat Zain. 2018. Effect of Different Operating Parameters on Extraction of Active Compounds from Pitaya Peel by Microwave Assisted Extraction (MAE). *Journal Teknologi*. 80(2):1-8

Assessment on Phenolic Compounds and The Effect of Microwave on Pitaya Peel. *IIUM Engineering Journal* - (**Submitted: In review**)