The Effects of UV-C on the Total Polyphenol Contents and Antioxidant Activity of New Zealand Grown Green Asparagus

A thesis presented in partial fulfillment of the requirements for the degree of Master of Science in Human Nutrition at Massey University, Manawatū, New Zealand.

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2022

Abstract

Background: Asparagus is favoured by global consumers for its exceptional nutritional value. New Zealand has a long history of asparagus cultivation and a considerable domestic market. Recently, several technologies such as UV and gamma irradiation have been used to increase the bioactive compounds in plants, resulting in improved plant quality and human health. The main aim of the present study was to study the hoemetic effects of UV-C (0.45- 2.27 kJ/m^2) on the total polyphenol contents and antioxidant activity of New Zealand grown asparagus.

Methodology: A three-factor-three-level Box Behnken Design (BBD) was used to determine the optimum condition for ultrasonication-assisted extraction (UAE). Three doses of UV-C radiation (0.45, 1.13, and 2.27 kJ/m^2) were applied to study the effects of UV-C light on the antioxidant activity of asparagus. The antioxidant assays were evaluated by total polyphenol contents (TPC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging ability, and peroxidase (POD) activity. The leading flavonoid of asparagus extracts was analyzed by High-Performance Chromatography (liquid chromatography and mass spectrometry; HPLC-MS).

Results: The optimum conditions for extracting asparagus TPC were 60% ethanol (v/v), 20 min extraction time, and a solid to liquid ratio of 1:30 (w/v). Overall, the UV-C irradiated samples showed significantly higher TPC than unirradiated samples ($p< 0.05$). The highest TPC value (131.37 ± 2.05 mg GAE/g; dry weight) was obtained at 1.13 kJ/m^2 UV-C exposure, and the TPC started to decrease as UV-C irradiation surpassed 1.13 kJ/m^2. The DPPH activity showed a similar trend as the TPC assay. The lowest IC50 of DPPH assay was found in the 1.13 kJ/m^2 group (36.84 µM), followed by the 0.45 kJ/m^2 group (49.20 µM), 2.27 kJ/m^2 group (99.21 µM), and unirradiated group (115.01 µM). The effects of UV-C irradiation on POD activity exhibited an opposite trend as that of TPC. The HPLC-MS results showed that rutin was the main flavonoid in asparagus with a content of 14.095 mg/100 g (dry weight).

Conclusion: Ultrasonication-assisted extraction (UAE) was more effective than traditional maceration on extracting asparagus TPC. The hormetic effects of UV-C were seen in this study as promoting the plant bioactivity at an optimum UV dose and decreasing such activity when the dose continued to increase. Rutin contains *o*-diphenol, benzene ring, and keto functional group (-C=O) and might be the reason for the high antioxidant activity of asparagus.

***Keywords**:* New Zealand green asparagus; UV-C; Total Polyphenols; Antioxidant Activity; UAE; RSM.

Acknowledgement

I want to give immense gratitude to everyone who helped me walk through this critical time of my life. First and foremost, I would like to give sincere thanks to my supervisor Dr David Popovich for his profound knowledge and assiduous guidance. He endeavoured to provide timely and insightful feedback whenever needed. I appreciated his valuable assistance.

My thanks also go to all lecturers dedicated to imparting knowledge when I studied offshore. I am especially thankful to Professor Jane Coad and Associate Professor Kathryn Beck. They took the initiative to care for my study and life during the most challenging time in this COVID-19 pandemic.

In addition, I would like to express thanks to Mr John Pedley, Professor Andrew Shilton, and Dr Emmanuel Kyere, who provided technical assistance on UV-C. My thanks also go to all my seniors, including Wenliang Xu, who offered valuable guidance on HPLC operation (liquid chromatography-mass spectrometry). Kuan-Min (Kent) Chen, who shared insights on the Design of Experiments and provided essential experimental appliances and help. Thanks also go to Danxia Shi and Iqra Zulfiqar, who helped me arrange experimental chemicals and provided genuine supports. None of my experiments would have been successful without their help.

I wish to thank technicians Ms Ann-Marie, Dr Inge Merts, Ms Kylie Evans, Mr Steve Glasgow, and Mr Raoul Solomon, who provided genuine support for my laboratory work. I am also grateful to Ms Karen Pickering for providing practical assistance on student affairs.

Next, I would like to thank my family and my friends. They provided tremendous emotional and mental support when I experienced the record floods in my hometown in 2020 (the worst flooding in 70 years), Managed Isolation and Quarantine (MIQ), and New Zealand's national lockdown. Lastly, I would like to acknowledge my roommate Sruthi, who became my first friend in New Zealand. She has been very supportive and helped me get familiarised with lives in Palmerston North.

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Chapter I Introduction

1.1 Background

Food plays a role in both satiation and the prevention of nutritional-associated illnesses. Numerous in-*vivo* and ex-*vivo* studies have proven that consumption of plant is effective in mitigating oxidative stress-induced disorders such as impaired glucose tolerance, colonic cancer, neurogenetic diseases and coronary heart diseases [1]. The disease-preventing functions of plant-based foods are related to their high content of polyphenols, which are also known as the secondary metabolites of plants. They are produced by plants after a chronic exposure of environmental stresses such as dry weather, extreme temperatures, air pollution, light irradiation (ultraviolet light exposure), low oxygen levels, parasites, and bacterial invasion [2]. It is believed that technologies that enhance the concentration of health-beneficial elements in plant commodities could add value and provide new perspectives, even in the context of current financial crisis and recession [1].

Solar rays consist of light spectrums of various wavelengths, including ultraviolet lights (UV; i.e., UV-A, UVB and UV-C). Globally, more than 50% of countries receive higher UV radiation than New Zealand, including its neighbouring country Australia. In New Zealand, the UV level is the highest during summer and the lowest during winter [3]. The A band of UV light (UV-A) is mainly responsible for dermal radiation damage (e.g., tanning effects) [4], while UV-B and UV-C are in charge of germicidal properties and the production of plant secondary metabolites [5]. Theoretically, UV-C is scarcely detected on earth, exclusive of upland habitats, due to its absorption by the stratosphere of the ozone layer. However, due to previous extensive usage of chlorofluorocarbons (CFC)-containing agents such as freezer, air conditioning and aerosol nebulizer, there led to UV-C leakage on earth's surface [6]. A low dose of UV-C is assumed to be beneficial for plant growth and could promote phenolic-antioxidants in plants [5]. Mercury-based UV-C lamp that emits 254nm wavelength is most frequently used to study the polyphenol-promoting effects of UV-C light [7]. Besides, introduced plants that used to be grown in low UV environments and were brought to high UV continents during European colonisation are thought to be good samples to study the physiological effects of UV-C light [8].

Asparagus *officinalis* is a nutritious perennial vegetable [9]. It was found to be the most antioxidant-rich vegetable out of 23 commonly eaten vegetables in America [10]. The major forms of asparagus consumption during 2016-2020 are shown in Figure 1.1 below, with approximately one quarter being developed as asparagus juice drinks and 14.3% being used by meal organizations. Though multiple working processes are involved in the production of preserved asparagus, on averagely, they are sold at a much cheaper price than fresh produce ($2.78/kg vs $3.56/kg). The higher market value demonstrates that consumers are willing to purchase fresh products at a higher freight and cooling costs [11].

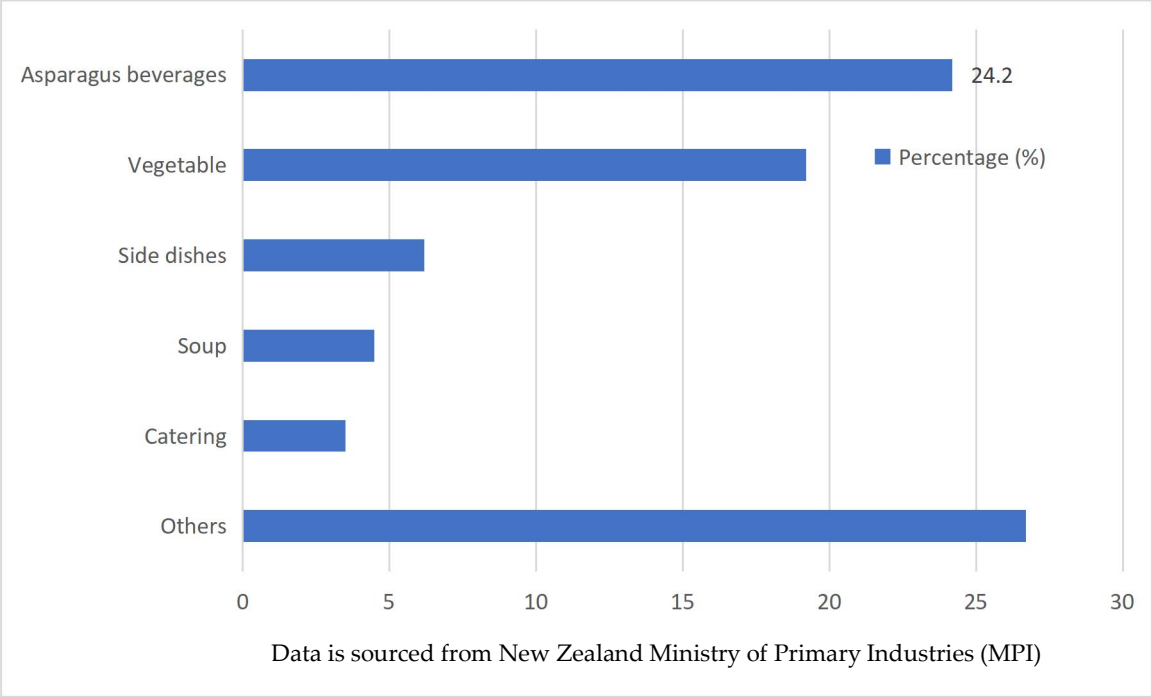


Figure 1.1 Main ways of global asparagus consumption during 2016-2020 [11].

New Zealand has a long-standing history of cultivating asparagus and a considerable internal demand. Due to the northern hemisphere and southern hemisphere experiencing opposite seasons, asparagus is available throughout the whole year. The typical growing and harvest season is between February and June in the temperate regions of the northern hemisphere [11], and according to New Zealand Asparagus Council, is between September to December in the southern hemisphere [12]. The seasonal differences between the north and south provide New Zealand with an export window for the crop. Japan and Singapore are the biggest international partners of New

Zealand regarding the amount of asparagus import and purchase price. When importing asparagus, Japan mainly evaluates cost and quality. Top-grade asparagus ought to be fresh, green, and intact, with straight spears and satisfying spear size. China only imports asparagus during the export window if premium quality is guaranteed [11].

In the early 1980s, New Zealand once was the biggest asparagus producer south of the equator, with a total planting area larger than 3000 hectares [13]. The geographical advantages offer New Zealand an opportunity to counter-seasonal supply asparagus to the northern hemisphere. However, the country's export market has been defeated over the years by other countries. Figure 1.2 below illustrates the SWOT analysis of New Zealand Asparagus Export Market as summarized by New Zealand Ministry of Primary Industries (MPI) 2021 [11]. From Figure 1.2, we can see that the competing countries, such as Mexico, Peru, and Australia, are located at a similar latitude to New Zealand. They possess a higher workforce, more abundant UV irradiation, larger production volume, and cheaper selling price. Australia imposes the biggest threat among all asparagus competitors because they share similar product reputation with New Zealand.

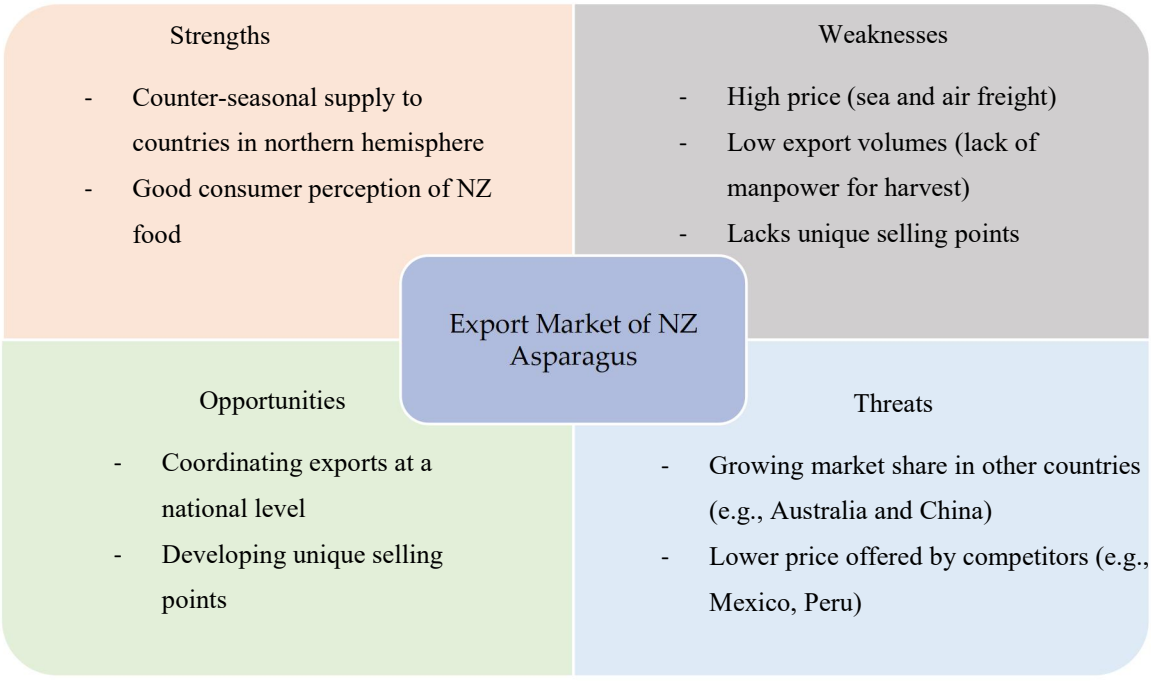


Figure 1.2 The SWOT analysis of the export market of New Zealand asparagus [11].

1.2 Research Objectives

a) To find out the optimum ultrasonication-assisted extraction (UAE) for the total polyphenol contents from New Zealand grown green asparagus.

b) To investigate the promoting effects of UV-C on the total polyphenol contents in green asparagus.

c) To discover the changes of antioxidant activity of asparagus after UV-C treatments.

1.2 Hypothesis

a) Introduced plants that are originally from the northern hemisphere might have evolved UV adaptation. New Zealand-grown asparagus, as an introduced plant, might be a good sample to study UV-C hormesis.

b) UV-C can promote phenolic-antioxidants in New Zealand-grown green asparagus.

c) UV-C in combination with ultrasonication-assisted extraction (UAE) is cheap, fast and efficient method that can enhance the extraction efficiency of polyphenol and improve the total antioxidant activities of asparagus. Thus, providing insights to industries that are interested in seeking time-saving and energy-saving approaches to increase bioactivities in asparagus, and add value to relevant products.

1.3 Significance of Research

Specifically, the findings of the study will benefit the following:

The researchers- This study intended to discover if New Zealand green asparagus, as an introduced plant originally from the low-UV-continent, would be sensitive to the physiologic effects of UV-C light. Up to now, scarce studies have explored the bioactivity of New Zealand-grown asparagus and the impacts of UV-C and ultrasonication-assisted extraction (UAE) on the phytoconstituents of this plant. This study is the first to provide information on the hormetic effects of UV-C irradiation on green asparagus. Besides, the current study revealed the leading polyphenol of the spears of New Zealand-grown green asparagus. Therefore, researchers who major in

analytical chemistry, nutrition, and food science and are interested in asparagus might find this study useful.

The entrepreneurs, quality assurance (QA) staff and Research& Development teams in asparagus industry- Research findings of the study suggested that UV-C technology could be a novel method to endow New Zealand asparagus with premium quality and unique selling points. In addition, this study implicated that compared with the traditional extraction method, the combination of UV-C with ultrasonic extraction on asparagus saved more time, produced higher total polyphenol contents, improved total antioxidant activity, and decreased peroxidase activity; offering new insights into the application of these two technologies in the innovation of commercial asparagus products (e.g., novel asparagus juice, asparagus condiments).

Chapter II Literature Review

2.1 Ultraviolet Light

2.1.1 Classification of Ultraviolet Light

Solar radiation is one of the determining factors that controls the growth and biochemical mechanism of vegetal creatures. Due to the differences in wavelengths, the light spectrum is divided into three sections, namely ultraviolet (UV light, less than 380 nm), visible light (380 - 780 nm) and near-infrared light (780- 3000 nm) [14]. Traits such as light absorption are the consequence of the evolution of higher plants to help them thrive in harsh environments. The specialized terminology for such protective behaviour is called “natural defence mechanism”; to interpret more simply, it means the natural ability of plants against herbivorous creatures, disease-causing micro-organisms, invasive neighbouring vegetation, and environmental stresses [1]. Among the light bands beyond the photosynthetically active radiation (PAR range; 400-700nm), UV light performs the most excellent effects on promoting the synthesis of bioactive compounds in plants. The increased congregation of plant chemicals helps preserve plants from solar damage [15].

The UV light is further classified as UV-C (wavelength 200 - 280 nm), UV-B (wavelength 280 - 320 nm) and UV-A (320 - 400 nm) [2]. Among these three unique UV bands, UV-A and UV-B influence plants' morphogenesis and growth orientation, whereas UV-B and UV-C stimulate phytochemical synthesis [3]. UV radiation is chiefly used as a productive technology to sanitise surfaces [4]. The proton released from the UV beam generates reactive oxidative species (ROS) from water, replacing a hydrogen atom in the nucleotide chain of microbial DNA helix, eventually leading to microbial death [16]. Food industries now favour artificial UV lights because this technology does not leave harmful residue on food products and requires less expenditure on purchasing auxiliary safety devices [17]. The US Food and Drug Administration (FDA 2000) has also authorized this technology to control microbiological contamination in fruit juice processing [18]. The artificial UV light tubes are demonstrated in Figure 2.1.

Figure 2.1. UV-A light tube (top; coated with black coloured europium-doped strontium borate) and UV-C light tube (bottom; transparent)

(Credit Fabexplosive, 2007; see origin: https://creativecommons.org/licenses/by-sa/2.5>, via Wikimedia Commons)

By far on the market, xenon-based UV lamps and mercury-based lamps are most frequently used. The former generates light at wavelengths ranging between 200 to 300 nm, mainly aiming to inhibit microbial proliferation and spore vitality, while the latter emits light at 254 nm wavelength. Light at 254 nm is typically used to study the phytoconstituents-promoting effects of UV light [19]. The commercially available mercury-based UV lamp is further divided into three categories based on tube pressure and light output, and they are separately [20]:

·Low pressure

·Low pressure high output

·Medium pressure

The amount of UV radiation applied to the sample depends on the intensity of UV light and the treatment duration and is often expressed as "microjoules/ square centimetre" ($\mu J/cm^2$) or "microwatt seconds/ square centimetre" ($\mu W \cdot s/cm^2$) [21].

2.1.2 Composition of Ozone Layer and UV Light Penetration

The composition of the earth's atmosphere is consisted of 5 layers, from the inner layer to the outer layer are separately the troposphere, stratosphere, mesosphere, thermosphere, and exosphere (shown in Figure 2.2).

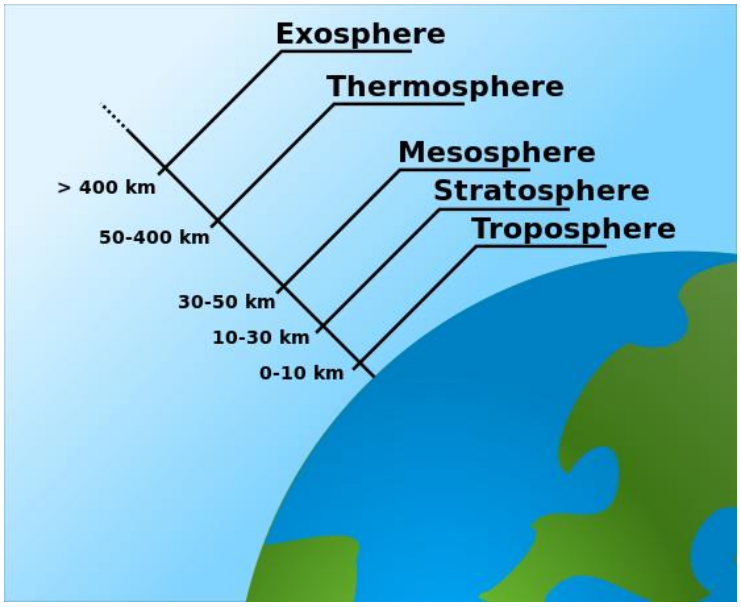


Figure 2.2. The composition of the atmosphere

(Credit Bredk, 2007; see origin: https://commons.wikimedia.org/wiki/File:Atmospheric_Layers.svg#)

According to the report, roughly 95% of UV light that reaches the earth's surface is UV-A, and the remaining 5% is UV-B [21]. The UV-C light was supposed to be fully absorbed in the top section of the stratospheric atmosphere or by gases such as oxygen and nitrogen [17, 22]. However, with the increasing human activities and massive discharge of chlorofluorocarbon (CFC), a change in the integrity of the ozone layer has occurred, which subsequently caused UV-C leakage onto earth's surface [23]. Of the three bands of the UV spectrum, UV-A carries the lowest photon energy and is responsible for the tanning effects, UV-B has higher power than UV-A, and UV-C is the most harmful band. Exposure to UV-C can induce genetic transformation or even lethal effects [24, 25]. However, a small dose of UV-C is necessary for plant growth, and the accessibility of UV-C offers extra application of this light source [4].

2.1.3 UV-C Hormesis

The word "hormesis" originated from the Greek term "hórmēsis", which stands for "swift move or keenness". In 1934, Southam and Ehrlich first recorded the word hormesis in their research paper [26]. The hormesis phenomenon in the plant is described as a dose-dependent behaviour

linked with environmental stress factors such as extreme temperature, heavy metal, solar radiation, salinity, drought, etc. In hormetic reaction, mild stress stimulation is observed to have beneficial effects on plants, while an intense stimulation triggers opposite consequences. When receiving hormesis, plants show changes in colour, bio-constituents profile, freshness, scents and productivity in response [26, 27].

As far back as thirty years ago, there already have written documents reporting UV-C hormesis in the production of crops. Most researchers investigating UV-C hormesis have used mercury-based UV lamps (which emit a wavelength at 254 nm) as the light source [7]. A UV-C dose of 1.2 or 3.0 kJ/m^2 significantly improved the hydroxycinnamic acid contents in broccoli [26]. When a UV-C does of 0.80 kJ/m^2 was applied to lettuce leaves four times, with each being two days apart, significant rises in the total polyphenol content (TPC), catalase (CAT) content and monodehydroascorbate reductase (MDAHR) content were observed [28]. Similar hormetic effects were also observed in *Lactuca sativa* leaves. The *Lactuca sativa* samples, which received 0.80 kJ/m^2 UV-C exposure showed significant increases in TPC and antioxidant activities than the controls [27]. Besides, in a study conducted by Katerova et al., (2012) [5], the authors noticed that after 30 min UV-C irradiation, both guava and banana showed an escalation in phenols and flavonoids contents at the end of post-harvest storage, along with increased antioxidant efficiencies [6].

However, there are also controversies over UV-C treatment claiming that the light's hormetic effect is inconsistent in different experiments. Some plants did not show changes after UV-C treatment. For example, in Forges et al. (2020)'s study [29], the authors reported that a UV-C dose of 12.36 J/m^2 did not impose phytochemical contents on strawberry. Also, in Gosisetty et al. (2018)'s study [30], the authors applied 0, 15, 30, 60, 120 and 240 mJ/cm^2 UV-C doses on cranberry-flavoured water (organic cranberry:water, 1:10 v/v). They observed a minor reduction in anthocyanins (cyanidin-3-galactoside (Cy3Ga), peonidin-3-arabinoside (Pe3Ar) and peonidin3-galactoside (Pe3Ga)) after subjecting samples to a UV-C dose of 240 mJ/cm^2. Subsequently, they concluded that long duration of UV-C irradiation (at 254 nm) might cause fractures in cranberry phytochemicals' conjugated bounds (e.g., aromatic rings and disulfides). However, in their research, the reductions of anthocyanins were not significant across the six doses of UV-C light, indicating

this UV spectrum has neither advantageous nor disadvantageous results on cranberry-flavoured water

Some researchers also tried to combine UV-C with UV-B to determine UV hormesis. In Formica-Oliveria et al. (2017)'s study [31], the authors used three different doses of UV-B (5, 10, 15 kJ/m^2), individually or in combination with UV-C (9 kJ/m^2) to examine UV hormesis on broccoli by-products (leaves and stalks). Their results showed that UV irradiation helped increase TPC and total antioxidant capacity (TAC) in broccoli by-products to as high as in florets. However, combined UV light does not show superior effects to single light. Further, in Urban et al. (2018)'s study [32], the authors complained about the time-consuming nature of UV-B technology. UV-B often takes several hours to several weeks to achieve similar hormetic effects as UV-C light, making the latter a recommended light source to promote phytonutrient synthesis [33-36].

2.1.4 The UV Conditions in New Zealand

Internationally, the amount of UV radiation that reaches the earth's surface is quantified as UV Index (UVI)- a measurement first coined in Canada and originally expressed on a scale ranging from 1-10. In New Zealand and Australia, the harmful level of UVI is classified as 1-2 (Low; L), 3-5 (Moderate), 6-7 (High), 8-10 (Very High), and >11 (Extremely High) [37, 38]. Two major factors are responsible for the increase of UV light penetration: 1. changes in the ozone layer thickness; and 2. the solar elevation angle [39]. In New Zealand, the stratospheric ozone is the thinnest during the autumn season and the thickest during spring [38].

Half of the world is scorched under a more intense UV radiation than New Zealand, including countries like Australia, Peru, and Mexico. The peak UVI can reach up to 25 in some South American countries during the summer, which is almost twice as much as that in New Zealand [38]. Nonetheless, due to some environmental reasons such as air pollution, destruction of the ozone, sun elevation, and the distance between the sun and earth, the peak UVI in New Zealand is almost 0.4 times higher than at equivalent latitudes in the northern region [37]. Due to the fact mentioned above, scientists infer that some vegetables (e.g., potatoes, squash, maise and asparagus), originally cultivated in the northern hemisphere and introduced to New Zealand after European

colonisation [40], might have already evolved UV adaptation, thus are ideal candidates to study UV hormesis [8].

2.2 Asparagus *Officinalis*

Asparagus *officinalis*, also known as crop asparagus, garden asparagus and edible asparagus, is a perennial plant native to the Mediterranean and Asia Minor [41]. To date, over 200 species of asparagus are recorded in *Asparagaceae* family, among which asparagus *officinalis* is the only marketable species that has been widely cultivated around the world [7]. It is a herbaceous rootstock plant which can grow up to 0.6-1.5 meters long. The plant is characterized by needle-shaped roots in a spiral pattern, whitish-greenish yellow flowers, and branch-laden stems (see Figure 2.3) [42]. From the edibility point of view, the plant consists of three sections- the edible spear, which accounts for a quarter of the whole plant, the stem, and the roots [43]. The lower part of the plant (crown and below) is buried under the earth and comprises fibrous roots, tender roots, and rhizomes. The tender roots develop for a couple of years before withering into fibrous roots. The function of fibrous roots is to transport water and nutrient to the whole plant, while the rhizome helps to convey carbohydrates from tender roots to the upper parts of the plant (viz. stem and spears) [44].

Figure 2.3 Asparagus *officinalis*

(Elizabeth Blackwell, Public domain, via Wikimedia Commons).

The buds that sprout from the rhizome are where the edible spears generate, and the spear size is positively associated with bud size. Besides, female asparagus yields thicker spears than male plants. If letting the spears grow without harvesting, they will grow uncontrollably into shrubs [44]. The general life span of green asparagus can last for a decade; therefore, it is suggested to sow young seedlings every 10-15 years to maintain a desirable output [45]. In keeping with the European Union grading standards (Commission of the European Communities, 1999), asparagus is grouped into four types in accordance with spear pigments. These are white, purple, purple-green, and green, respectively [46]. The pigmentation of anthocyanin results in a violet colour of purple asparagus. Green asparagus and white asparagus could develop from seedlings of the same variety, and what makes them differentiated in colour may credit to the degree of UV exposure during growth [44].

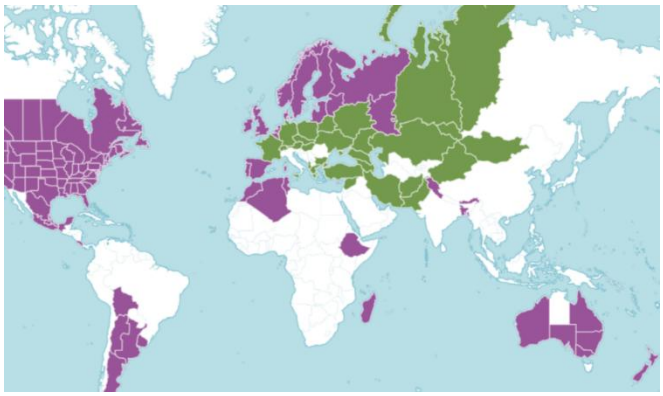
White asparagus is cultivated in the absence of sunlight, while green asparagus is produced in an open area where solar radiation is available [47]. When green asparagus spears are under direct contact with sunlight, they will first form a pinkish outlook and then gradually transit into the iconic green colour [44]. In the market, the longest spear length acceptable for white and purple asparagus is 22 cm and 27 cm for purple-green and green asparagus [46].

2.2.1 Global Distribution

Asparagus cultivation has started a few thousand years ago in some Asian, North African, and Mediterranean countries [48]. The usage of asparagus as an anti-diuretic folk remedy was well-recorded in a classic Chinese medical biology "Compendium of Materia Medica" in 1578 [41]. Evidence shows that asparagus once was used as both food and medicinal plant during the Greek and Roman periods [48]. Scientists used simple sequence repeats (SSR) as a marker to determine how two or more asparagus species are genetically close to each other. SSR genotyping showed that all varieties of asparagus are diploid organisms that evolved from the "Violet Dutch" cultivar [49]. Due to its delicious taste, the crop is now being cultivated in all five continents and produced in various kinds of commodities such as fresh produce, canned asparagus and frozen asparagus [50]

According to the map of asparagus distribution, the crop is native to (but not limited to) regions such as Afghanistan, Austria, France, Germany, Greece, Iran, Mongolia, West Siberia, and

the Xinjiang autonomous region of China. Later in days, it was introduced to countries such as New Zealand, Australia, Peru, Mexico, and North America (see Figure 2.4). According to the results of the world market, the total planting area of asparagus is over 1.5 million hectares, with an annual spear output surpassing 8.9 million tons [51].



Native (green); introduced (purple)

Figure 2.4 The origin and global distribution of asparagus.

<Available online: http://www.plantsoftheworldonline.org/taxon/urn:lsid:ipni.org:names:531229-1 (accessed on 15 April 2022).

On a global scale, Mexico, Peru, China and USA are the top four countries that dominate the asparagus exporting market. According to the asparagus export market share in 2019 (illustrated in Figure 2.5), Mexico and Peru accounted for two-thirds of the total export amount, followed closely by China (10%), the USA (9%), Spain (5%) and Netherlands (4%). The rest of the world only took up a tiny fraction at 8%. The harvest season of asparagus starts from February to June in countries north of the equator and from October to December in the southern hemisphere. This phenomenon subsequently results in an all-year-round supply of the crop. New Zealand has a long-standing record of cultivating asparagus. The domestic demand for asparagus is steady, and the planting sites are scattered all over the country. However, New Zealand's asparagus export market has always been defeated by countries at similar latitudes (i.e., Mexico, Peru, and Australia) due to expensive freight costs and inconsistent production volume. Of all the competitors, Australia throws the biggest threat to market competitiveness because they produce higher annual outputs and enjoy a similar product reputation to New Zealand [52].

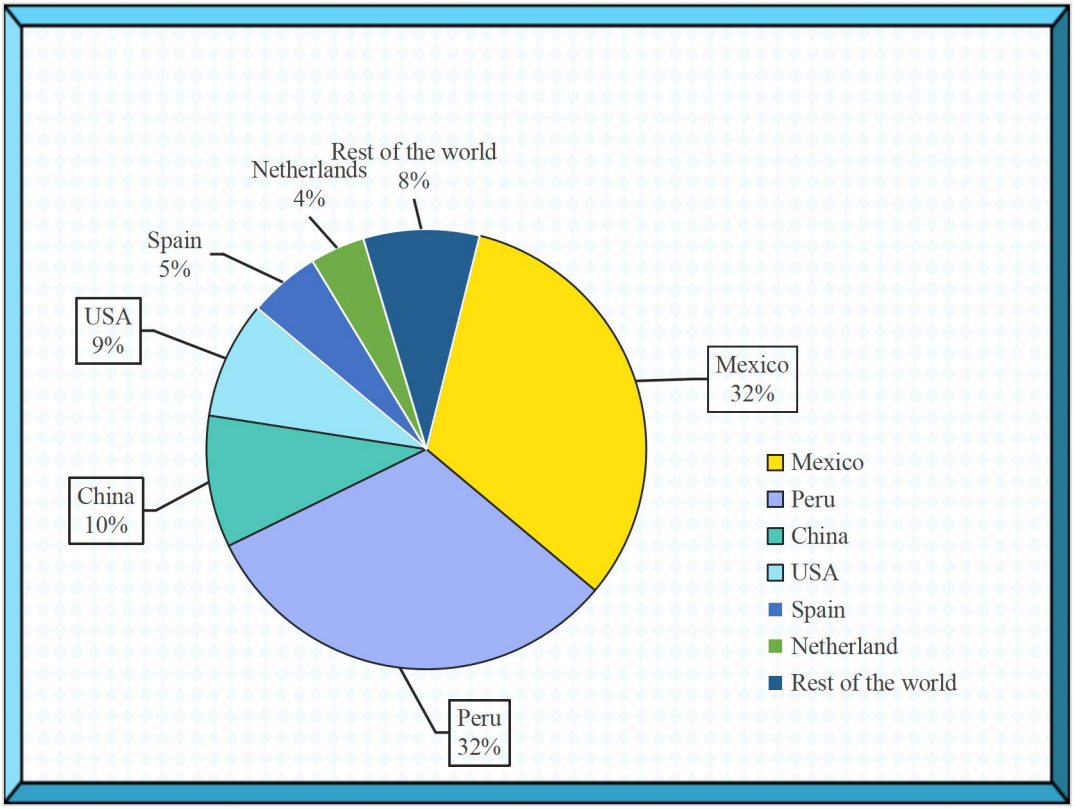


Figure 2.5 The global asparagus export market share of 2019

(from New Zealand MPI 2021 Analysis[11]).

2.2.2 Nutritional and Polyphenol Compositions

With the development of socio-economy, food nowadays provides satiety and helps prevent the occurrence of civilization-, ageing- and nutritional-related illnesses, and prolongs the lifespan of all humankind [53]. Epidemiological studies suggest that plant-based foods are an excellent source of micronutrients, dietary fibre and phytochemicals. Regular consumption of this food group is negatively correlated with chronic diseases such as obesity, diabetes, cancer and heart diseases [54]. Credit to its high contents of bioactive constituents, asparagus is renowned as “the king of vegetables” and is favoured by consumers worldwide [55]. According to the historical records, asparagus is consumed as a vegetable and a traditional herbal remedy [56]. Many countries employ asparagus to mitigate the development of sickness. For instance, traditional Chinese medicine prescribes asparagus as a potent herb to fight against cough, inflammatory symptoms, skin infection and joint pain [57]. In Ayurveda (a traditional Hindu medical system) and ancient Greek medicine,

asparagus has been treated as a restorative drug to cure kidney diseases, hepatic dysfunction, respiratory diseases and cancer [48, 58].

From the perspective of nutritional values, consumers favour asparagus due to its low calorie (approximately 22 Kcal/100g) and high nutritional contents [59]. Normal-sized asparagus holds 90% of water (by fresh weight) [60]. The nutritional profile of asparagus may vary in variety, but it generally contains 32.69% of crude protein [61], 0.16% of fat, 2.04% of carbohydrates, 1.31% of dietary fibre, and 0.31% of total nitrogen [42]. Major micronutrients found in asparagus are potassium (45 µg/100 mg), calcium (26 µg/100 mg), iron (648 µg/100 g), zinc (397 µg/100g) [62], sodium (1.84 µg/100 mg [61], and vitamins such as carotene, niacin, and Vitamin [60]. Every 180 g of cooked asparagus (one serving) fulfills 101% of the Daily Recommended Intake (DRI) of vitamin K, 67% DRI of vitamin B9, 33% DRI of copper, and approximately 20% DRI of thiamine, riboflavin, vitamin C and selenium [59]. Apart from micronutrients, other useful compounds detected in asparagus are phytochemicals such as flavonoids, phenolic acids, and essential oil [63].

Throughout the years, extensive studies have investigated the preventive effects of plant-based foods on non-communicable chronic diseases. Scientists found that the polyphenol in plants contributes to mitigating inflammation, viral infection, allergic reactions, oxidative stress, and cancer development [64]. According to the definition, polyphenols or phenolic compounds are described as non-nutritive metabolic intermediates formed during plants' pentose phosphate, shikimate or phenylpropanoid pathways [2, 106]. Vinson and colleagues (1998) [10] used Folin-Ciocalteu method to compare the polyphenol content in 23 most eaten vegetables by USA households. Their results showed that the free phenols content (based on dry weight) in asparagus (25.8 ± 12.5 µmol/g) was two times higher than that in spinach (13.4 ± 9.8 µmol/g) and five times higher than that in garlic (5.2 ± 3.1 µmol/g). In addition, asparagus also has higher free phenols than broccoli (17.5 ± 3.8 µmol/g) and bell pepper (16.4 ± 1.2 µmol/g).

Flavonoids are the dominant polyphenols in vegetables. The contents of flavonoids in asparagus vary depending on the asparagus genotype [65, 66]. Studies have proven that rutin is the most abundant and beneficial flavonoid in asparagus, with one kilogram of asparagus (fresh weight) containing 286.5 mg of rutin [67, 68]. In recent decades, numerous in-vitro and in-vivo studies have indicated rutin’s ability to reduce Aβ peptide (a hallmark for Alzheimer's disease) in

APPswe/PS1dE9 transgenic mice [69], decrease fasting glucose and HbA1c levels in type I diabetic rats [70], increase high-density lipoprotein (HDL) in diabetic patients, and retard tumour growth in human leukaemia HL-60 cells injected mice [71].

2.3 Antioxidant Activity

2.3.1 Free Radicals and Oxidative Stress

Oxygen participates in the aerobic respiration of living organisms. In such a process, the organic substances are decomposed into carbon dioxide and water in the mitochondria, with a large amount of energy released, especially ATP. Also, in this process, electrons or hydrogen are removed from organic substances to an oxidiser, resulting in the generation of free radicals (e.g., reactive oxygen species (ROS), reactive nitrogen species (RNS)) [72, 73]. Metabolically, most of the free radicals generated in *vivo* belong to ROS. In cells, mitochondria are the main places for gas exchange, therefore, are considered the main sites for intracellular generation of oxygen free radicals. Other organelles that produce ROS are phagocytes, chloroplast, plasma membrane, cytochrome P450 enzymes, peroxisomes, and endoplasmic reticulum [74-76]. A woman weighing 60 kg can produce 160-320 mmol of intracellular free radicals per day, and a man weighing 80 kg can make 215-430 mmol per day [77, 78]. Free radicals can be generated from exogenous environmental factors or through endogenous metabolism. External factors include air pollution, water contamination, tobacco addiction, alcohol abuse, heavy metals, industrial excretion, cooking oil fume inhaling, and radiation exposure. In contrast, internal factors include psychological distress, excessive aerobic training, hypoxia, immune disorders, inflammatory response, cancer, and senility [79].

From the perspective of chemical structures, free radicals are highly unstable due to a lack of fully paired electrons in the valence shell or orbit around the atomic nucleus [74]. A proper amount of intracellular ROS is beneficial for health maintenance [80]. A low or a moderate concentration of ROS positively regulates cellular response, promotes immune homeostasis, and protects against microbial infections. In contrast, a high concentration of ROS could have a detrimental effect on health. Scientists have found that too much ROS is one of the driving factors for cell membrane damage and cell apoptosis [79, 81]. If the chronic accumulation of free radicals

remains uncleaned would induce oxidative stress or nitrosative stress on the host [82]. The ROS are generally grouped into two types, namely radical species and non-radical species. Typically, non-radical species are oxidizing agents that are more stable than radical species. Under certain conditions, they can easily convert into radical species and cause oxidative stress in living creatures [74]. Some of the reactive species are listed in Table 2.1.

Table 2.1 Some of the common reactive species.

Radical species	Superoxide ($O^{\cdot -2}$), Oxygen radical ($O^{\cdot\cdot 2}$), Hydroxyl (OH·), Alkoxyradical (RO·), Peroxyl radical (ROO·), Nitric oxide (nitrogen monoxide) (NO·), nitrogen dioxide ($NO^{\cdot 2}$)
Non-radical species	Hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hypobromous acid (HOBr), ozone (O_3), singlet oxygen ($^1O_2$), nitrous acid (HNO_2), nitrosyl cation (NO+), nitroxyl anion (NO−), dinitrogen trioxide (N_2O_3), dinitrogen tetraoxide (N_2O_4), nitronium (nitryl) cation (NO^{2+}), organic peroxides (ROOH), aldehydes (HCOR), peroxynitrite (ONOOH)

2.3.1.1 Pathological Role of ROS

Numerous studies have indicated that ROS-induced oxidative stress does not directly impact human disease but indirectly involves the signaling pathways of redox reactions that are highly related to degenerative illnesses [83]. Common conditions associated with oxidative stress include central nervous impairments (such as multiple sclerosis, dementia, Parkinson's disease), cancer, cerebrovascular disease, rheumatic heart disease, kidney disease, and metabolic and hormonal diseases (such as osteoporosis, cystic fibrosis, hypothyroidism, obesity, and diabetic mellitus) [84-86].

ROS and Diabetic Mellitus

In diabetic patients, the abnormal escalation of blood glucose levels leads to the production of free radicals through four approaches [74]:

1. Increasing glycolysis: Glycolysis is a metabolic pathway during which the glucose is broken down into pyruvate with the release of energy. In patients with diabetes mellitus, the increased glycolysis causes changes in NADH/NAD+ ratio and thus results in redox imbalance. With the exaggerated process of glycolysis, pyruvate is overproduced, leading to potential upwards at the inner membrane of mitochondria, which then causes impairments in mitochondrial function and boots ROS generation at electron transport chain complex II.

2. Activating sorbitol/polyol pathways: Elevated blood sugar level facilitates the aggregation of sorbitol and fructose, causing a decrease in glutathione (GSH, a free radical scavenger) and a fluctuation in NADH/NAD+ ratio.

3. Autoxidation of glucose: The autoxidation of glucose gives rise to excessive production of free radicals, e.g., H_2O_2, $OH^{\bullet}$, $O2^{\bullet-}$ and ketoaldehydes.

4. Glycation of non-enzymatic proteins: The increased blood glucose level accelerates the covalent attachment of sugar molecules to a non-enzymatic protein, leading to the accumulation of advanced glycation end products (AGEs). The AGEs formed during the glycation combines with AGEs receptors which then cause oxidative stress in the body.

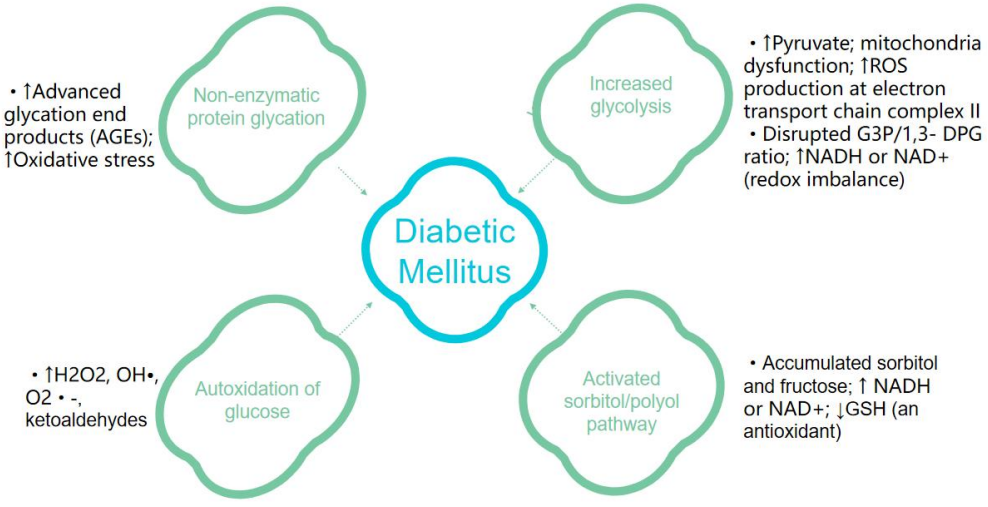


Figure 2.6 The pathogenesis of diabetic mellitus.

ROS and Multiple Sclerosis

Multiple sclerosis (MS) is an autoimmune neurological disease. Typical symptoms of MS are: increases in thiobarbituric acid reactive substances levels, decreases in -SH groups, increases in protein oxidation, and minor reductions in superoxide dismutase (SOD, an enzymatic antioxidant). All these changes are initiated by the activation of microglia or macrophages, which promote ROS production that leads to lipid peroxidation and eventually destroys the integrity of the myelin of the central nervous system [74].

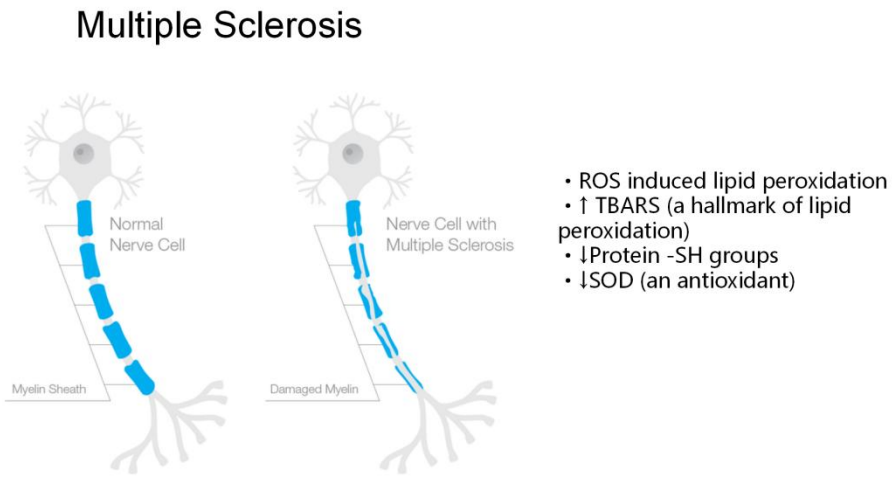


Figure 2.7 The pathogenesis of multiple sclerosis.

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ROS and Colorectal Cancer

Compared with healthy cells, higher concentrations of nitric oxide (NO), 8-Oxo-2'-deoxyguanosine (one of the primary metabolites of DNA oxidation) and lipid peroxides. These oxidative stressors are produced by daily exposure of colorectal epithelial cells to food, bacterial-secreted oxidants, and environmental carcinogens. The day-to-day contact with oxidative stressor further causes accumulation of ROS, interference of redox balance, and even damages in the DNA, eventually contributing to the development of colorectal cancer.

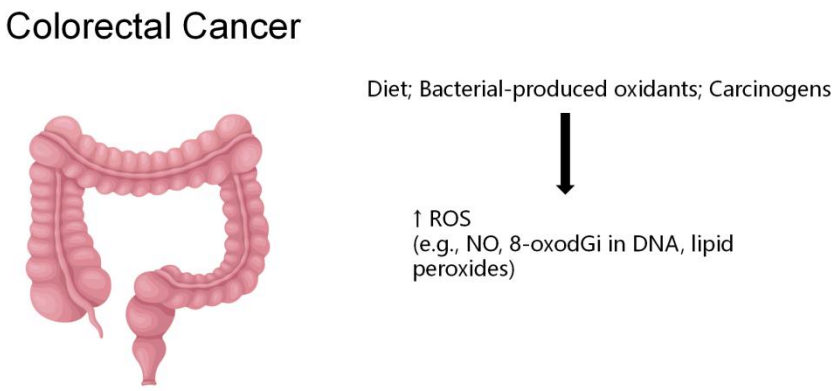

Figure 2.8 The pathogenesis of colorectal cancer.

2.3.2 Antioxidants

Antioxidants refer to natural or synthetic molecules that can slow or cease oxygen reactions. In broad terms, antioxidants exert their effects by removing free radicals, thus terminating the knock-on effects of oxidation [81, 87]. Natural antioxidants are extracted from natural plant origins, whereas artificial antioxidants are synthesized from chemical reactions [88]. Synthetic antioxidants have been frequently applied in the food and cosmetic industries for several decades due to their high stability and low price. Examples of the commonly used artificial antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), tert-butyl hydroquinone (TBHQ), 2-naphthol (2NL), 4-phenylphenol (OPP) and 2,4-dichlorophenoxyacetic acid (2,4-DA) [89]. However, with more and more health issues being reported to have a relationship with synthetic antioxidants (such as dermal allergies, digestive disorders, liver diseases, and carcinogenesis), some of these antioxidants are now being withdrawn by laws. For instance, the usage of TBHQ has been forbidden in Japan and some nations in Europe [90].

According to the catalytic reaction, antioxidants are divided into two main groups: enzymatic and non-enzymatic antioxidants [91]. The enzymatic antioxidants are represented by superoxide dismutase (SOD), glutathione reductase (GR), catalase (CAT), ascorbate peroxidase

(APX) and peroxidase (POD) [92]. The cofactors attached to the chemical structure of enzymatic antioxidants make them highly affiliated with particular protein-based ROS [93]. Catalase, an enzyme that contains four heme groups, can catalyse the dismutation of H_2O_2 in the presence of water and oxygen. Peroxidase, another heme-possessing enzyme, is capable of oxidising both organic and inorganic chemicals, with H_2O_2 being the co-substrate. Ascorbate peroxidase uses ascorbate as an electron-donating compound to scavenge H_2O_2 generated by SOD [81]. Non-enzymatic antioxidants, on the other hand, do not have specificity and can inactivate radical species at remarkable speeds. The typical non-enzymatic antioxidants are vitamins (vitamin E and C), polyphenols, GSH and carotene [94].

The specific mechanism of scavenging free radicals involves three lines of defence (see Figure 2.9). These include 1) Prevention: in the first line of antioxidant defence, bioactive compounds such as SOD, CAT, GPx, GR and a few inorganic nutrients (selenium (Se), manganese (Mn), copper (Cu), and iron (Fe)) are participated in inhibiting the generation of free radicals from the origins; 2) Eradication: the second line of antioxidant defence involves some radical scavengers such as vitamins E and C, flavonoids, and uric acid which act to eliminating oxidants, transforming the oxidant to a less deleterious form, and obstructing the secondary generation of harmful intermediates and inflammatory markers, and; 3) Repair: antioxidants take part in the third line of defence in recovering the oxidative damages caused by free radicals attacks, or strengthening the antioxidant capacity of the host [8].

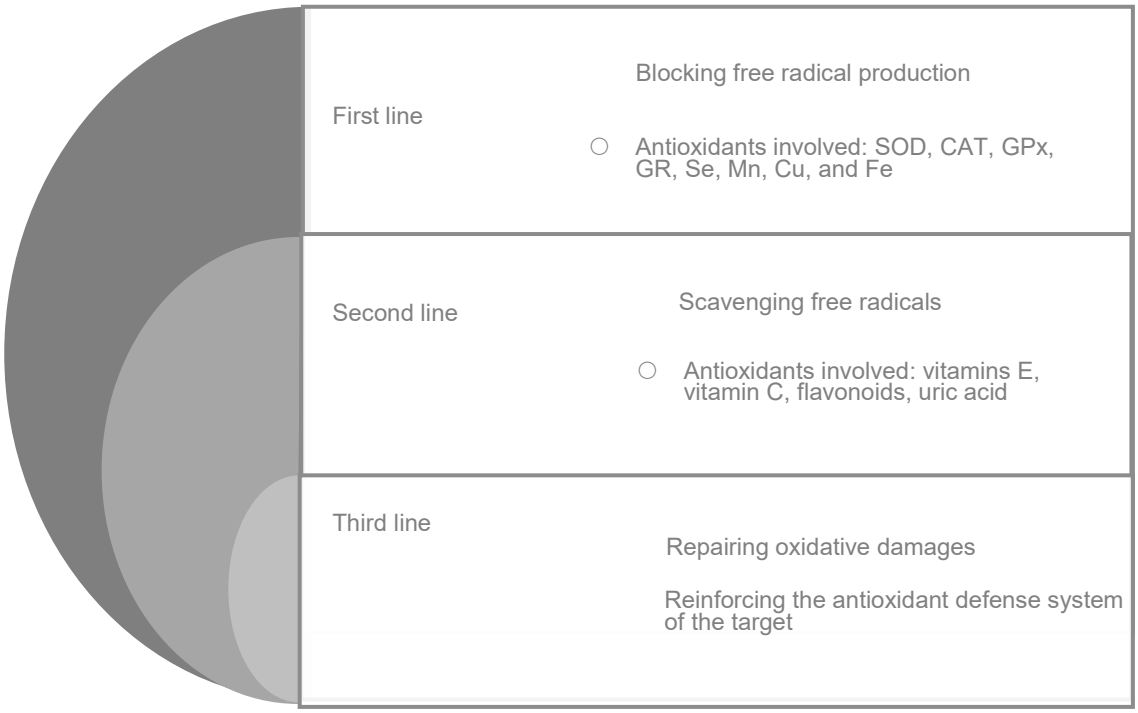

Figure 2.9 The three lines of antioxidant defence mechanism[8].

The performance of antioxidants in preventing oxidative stress is highly related to the degree of activation of antioxidants at therapeutic sites. And the endogenous concentration of antioxidants is decided by various environmental and physiological conditions such as the plant's growth stage, illumination time, radiation intensity, temperature, and soil composition [95].

2.3.3 Detection Methods

For the past few decades, techniques and tools for assessing antioxidant activity have been increasingly improved. Previous methods mainly adopt lipid oxidation measurement to gauge antioxidant efficiency, which are based on the principle of blocking the generation of certain species of oxidation products. Up to now, numerous chemical analyses incorporated with techniques of high sensitivity and automatic measurements are developed to appraise antioxidant activity via specific mechanisms, for instance, scavenging capacity on certain categories of reactive species, reduction activity, metal chelation, and so forth.

These quantification methods help researchers learn more in-depth about the antioxidant mechanism of certain compounds. In some literature, the "antioxidant activity" is also referred to as "antioxidant power", "antioxidant efficiency", and "antioxidant performance" [87]. To reduce the

fluctuations in testing, the antioxidant assay for enzymatic antioxidants needs to be conducted at a refrigeration temperature of roughly 4 °C [91, 96]. Various techniques have been invented to measure antioxidant activity, including TBARS, DPPH free radical scavenging method, oxygen radical absorbance capacity (ORAC), and Folin-Ciocalteu reaction [97]. UV-vis spectrophotometry has been the most frequently adopted method among all antioxidant assays due to its low cost, good feasibility, and efficiency. Some of the often used UV-vis spectrophotometry methods are listed in Table 2.2 below [91].

Table 2.2 The common UV- vis Spectrophotometry methods for detecting antioxidant activity.

Type of tests	Detecting methods
Enzymatic antioxidant assay	Superoxide dismutase assay
	Catalase assay
	Peroxidase assay
	Ascorbate peroxidase assay
	Ascorbate oxidase assay
	Guaiacol peroxidase assay
	Glutathione reductase assay
Non-enzymatic assay	Total flavonoids
	DPPH scavenging activity
	Ferric reducing-antioxidant power (FRAP) assay
	Hydrogen peroxide scavenging activity
	Nitric oxide scavenging activity
	Superoxide radical scavenging activity
	Hydroxyl radical scavenging activity
	Phosphomolybdate assay (total antioxidant capacity)
	Reducing power
	Metal ion chelating activity
	β-carotene/linoleic acid bleaching

In addition to spectrophotometry approaches, other techniques used to detect antioxidant activity are the cyclic voltammetry method, superwave voltammetry method, biamperometry method, thin layer chromatography (TLC), high-performance liquid chromatography (HPLC), gas chromatography, and gel-permission chromatography [91]. These methods offer scholars a better insight into the effectiveness of the antioxidants they study [98].

2.3.4 Phytonutrients

Plants utilize trace amounts of organic or inorganic substrates such as water, carbon dioxide, nitrogen and salts to produce primary metabolites (i.e., carbohydrates, proteins, fats and nucleic acids). These primary metabolites are further converted into secondary metabolites or phytoalexins such as phenolic acids, flavonoids, saponins, tannins, alkaloids and phytosterols to serve various purposes (e.g., pigment formation, pollination, seed germination, protecting against oxidative stress and UV exposure) [45, 99-101]. The primary and secondary metabolites are collectively known as phytonutrients. The prefix “Phyto” is from a Greek word meaning “plant” [102]. In nature, phytonutrients that possess defensive properties can be visually spotted, tasted, or sensed by sensorium. Some of these bioactive constitutes have distinctive colours. For example, carotenoids are often red, yellow, or orange, flavonoids are yellow, and chlorophylls are green. At the same time, others may have a unique taste or fragrance (especially those belonging to volatile compounds) [103].

Due to variations in plant origins, structural attributes, and bioactive functions, phytonutrients are categorized into polyphenols, alkaloids, polysaccharides, terpenes, glucosinolates, carotenoids, lectins, polyacetylenes, capsaicinoids, betalains, chlorophyll, and allium compounds [104]. All phytochemicals share basic structures containing acetate, isoprenoid and phenylpropanoid [105]. Phytonutrients are described as “non-nutritive compounds in plants, the consumption of which has been associated with reduced risks of chronic illness” [106]. A significant number of phytonutrients have been observed to have the ability to participate in cellular modulation, modify the actuation of transcription factors that govern gene expressions, and alter the metabolism of cells [107].

2.3.4.1 Polyphenols

Scientists have identified more than 12,000 phytonutrients, and over 66% of these compounds are polyphenols [104]. This group of phytonutrients possesses a vast array of biological activities such as scavenging oxygen free radicals, anti-tumour activity, rejuvenating power and antiallergic function [108]. Polyphenols' diverse and effective bioactivity results mainly from their chemical structures, which vary from simple to highly polymerised molecules [109]. Typically, the structure of polyphenols comprises at least one hydroxyl group (-$OH^{\cdot}$) attached directly to aromatic hydrocarbons [108], and this structural characteristic of polyphenol is the chief factor for its high scavenging ability [109].

For the convenience of our categorisation, polyphenols are consequently grouped into two main classes- flavonoids and non-flavonoids [110]. Flavonoids include flavones, flavanones, flavonols, dihydroflavonols, flavan-3-ols, flavan-3,4-diols, chalcones, dihydrocalchones and aurones; they all share the basic structure of C6-C3-C6 carbon-skeleton (diphenyl propane structure). Phenolic acids are the representatives of non-flavonoids (e.g., gallic acid, salicylic acid, and protocatechalic acid) and other phenolics (e.g., stilbenes, lignans, tannins, lignins. Xanthones) [79, 111-113]. According to the National Health and Nutrition Examination Survey (NHANES) data, the average daily consumption of flavonoids is 200-250 mg among US citizens. On condition of a suitable dose range of 1-100 μg/mL, flavonoids are shown to have clinical efficiency in treating hepatic dysfunction and digestive problems [114].

Besides, results of epidemiological research showed that regular consumption of diet riches in polyphenols could help reduce the risks of non-communicable illnesses [106, 115]. For example, one of the tea phenolic components- epigallocatechin, is proven to have potency in reducing the activity of transcription factors (e.g., AP-1 or NF-$_k$B) that accelerate the bioactivity of transforming growth factors TGF-α and TGF-β. Thus it is suggested that a periodical tea consumption would help prevent tumour growth [116]. Another example is saponarin, a flavone glucoside extracted from the new barley leaf. It has been proven to have the potential to regulate blood glucose levels by modulating glucogenesis and activating AMP-activated protein kinase (AMPK) in a calcium-relied pattern to promote muscular glucose uptakes [117].

However, controversies exist over the blood pressure lowering effects of plant polyphenols. Clifton and colleagues (2004) [118] recruited 36 patients (males and females) who had higher than average risks of vascular diseases. Subjects were randomly assigned to the treatment group (receiving oral consumption of grade seed extracts) and the placebo group in their research. The study was a 12-week double-blinded randomized design. The results showed that male subjects had an average reduction in systolic blood pressure (SBP) of -11 mm Hg in the treatment group and female subjects had a -3 mm Hg reduction in the treatment group. Whereas in Ras et al. 2013's study [119], the researchers conducted an 8-week double-blinded randomised clinical test (RCT) on pre- and stage 1 hypertension patients. In their study, patients were assigned to the treatment group (receive 300 mg/day of grape seed extracts) and the control group (receive placebo). Their results indicated that at the end of the intervention, both the treatment group (SBP -5.2 mm Hg; DBP -2.5 mm Hg) and control group (SBP 2.2 mm Hg; DBP -1.1 mm Hg) showed a reduction in blood pressures. However, the authors suggested that more RCTs with a larger sample size are needed to verify the therapeutic effects of grape seed polyphenols [120].

2.3.4.2 Rutin- the Major Antioxidant in Asparagus Extracts

Asparagus extracts contain high contents of polyphenols and thus are potential nutraceutical supplements [45]. Previous chemical analysis revealed that hydroxycinnamic acid and its derivatives are the main phenolics in white asparagus [121], whereas flavonoids are the leading phenolics in green varieties [122]. Using the LC-MS instrument of HPLC, scientists have found that the significant flavonoids in green asparagus are rutin, quercetin, isoquercetin and kaempferol [123]. Among all these compounds, rutin accounts for 60-90% of the total flavonoids making it the largest polyphenol in green asparagus [124-126]. Numerous studies have indicated that asparagus riches in rutin can exhibit potent antioxidant activity, especially non-enzymatic DPPH and ABTS activities [67, 127-129]. Figure 2.10 below displays the structural formula of rutin.

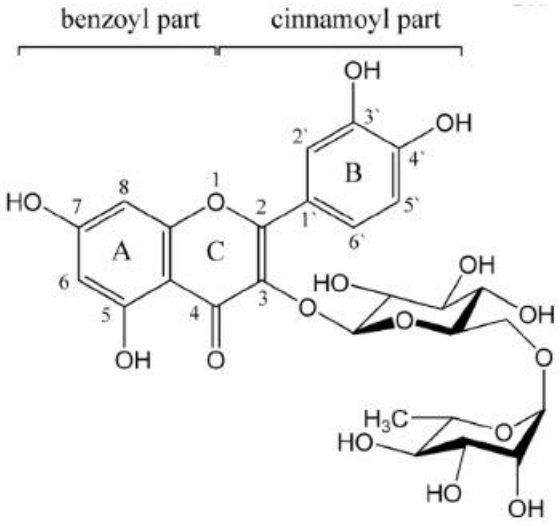

Figure 2.10. The 2D structure of rutin. From Živanović et al., (2016) [234].

Rutin, Chemical Abstracts Service (C.A.S.) number 153-18-4, is a flavonol glycoside that widely exists in fruits and vegetables [65, 121]. In clinical trials, scientists often introduce trinitrobenzene sulphonic acid (TNBS) on the intestinal linings of experimental animals to study the pathogenesis of irritable bowel syndrome (IBS) [130]. A study implemented in 2015 showed that three days of intragastric or intraperitoneal injection of rutin could reduce the concentration of pro-inflammatory cytokines in the intestinal mucosa of female Wistar rats with TNBS-induced ileitis or colitis. Thus indicating the protective effects of rutin against oxidative stress-related disorders [131]. The excellent radical scavenging effects of rutin are due to the hydroxyl groups attached to the aromatic rings [132].

2.4 Extraction Techniques for Phytonutrients

Extraction is a process which involves the separation of bioactive components from the plant- or animal sources and is often coupled with extraction solvents of different compositions and concentrations. One can also depict extraction as a procedure that uses solvents to keep targeted solid components while screening out those unwanted ones [133]. Particle size, extracting solvents, filtration methods, and drying techniques are often carefully selected to perform the best possible extractability [134]. The sample of interest can be either fresh or dried in medicinal plant research.

However, fresh samples have higher water activity and may quickly go rancid [135]. Therefore, dried subjects are more commonly used due to their longer storage time If fresh samples are the only option, they shall be quickly processed within 3 hours to avoid chemical degradation [133]. Extracting process can be both traditional and non-traditional [136]. The following content focuses on elaborating on traditional and non-traditional ways of extracting.

2.4.1 Traditional Extraction Methods

Traditional methods such as maceration, percolation, decoction, reflux extraction and hydrodistillation have been utilized for many decades now [136, 137]. According to a narrative review done by [137], the top three most popular conventional extraction methods are soxhlet extraction (22%), infusion (22%), and maceration (20%), then followed by solvent extraction (10%), decoction (8%), percolation (2%) and steam distillation (1%).

2.4.1.1 Acidified Ethanol Extraction

Based on the general principle of “like dissolves like”, the first thing to consider when selecting extraction solvents is the polarity of extractants and extracts. That is to say, molecules of higher polarity shall be extracted by water, ethanol and acetic acids [138]. Some others have encouraged us to consider environmental sustainability when selecting extraction solvents. The proposed solvents should possess minimum hazards and be manageable. They should not cause exorbitant energy expenditure [139] and are expected to be highly selective for targeted compounds. Good extractants can be easily purchased at an affordable price [140].

Common extractants used for polyphenol are ethanol and methanol (with or without the addition of acidic solvent) [141]. The addition of acid can destroy the cell membranes of the plant, thus promoting the release of phytochemicals into extractants. Researchers should remember that an extreme pH environment may distort bioactive compounds' chemical structure. Therefore, when adding acids into a solution, weak acids (e.g., formic acid or acetic acid) are preferred over inorganic acids such as 0.1% sulfuric acid [142].

Ethanol is a volatile liquid which is naturally detected in vegetal organisms [143]. It can be produced by chemical reaction or as an end product of the fermentation process [144]. This

extracting solvent is widely recognized by researchers for its non-poisonous characteristics and high extraction capacity. It is viewed as a Generally Recognised as Safe ("GRAS") small molecule solvent [145]. In the study conducted by [146], the authors used Box Behnken Design to optimize four variables (ultrasonic amplitude, ethanol concentrations, treatment temperature, and treatment duration) for propolis extraction. The results of their study showed that using 100% ultrasonic amplitude with 71.2% ethanol (v/v) at 57.2 °C for 29.25 min yielded the highest amount of total polyphenol contents of 458.23 mg (Gallic Acid Equivalence)/g, and 222.58 mg (Quercetin Equivalence)/g of total flavonoid contents.

2.4.1.2 Maceration

Maceration was initially used in wine production. Later, more and more pharmaceutical industries accepted it, especially in the extraction of phytochemical metabolites [133]. This method requires immersing plant samples (coarse texture or fine powder) in a well-sealed vessel with a selected solvent medium for more than three consecutive days. The whole process should be conducted in dim lights at room temperature [147]. Up to now, maceration is still valued as one of the most potent extraction methods. It is an uncomplicated and affordable approach especially suitable for heat-sensitive compounds. It allows researchers to study medicinal plants from various sources, and it has no special requirements on solvent constitutions and solvent pH [148]. It has received many complaints regarding its lengthy and obsolete process [136]. Moreover, maceration is often combined with shake extraction, which works by setting a sample on a shaking apparatus to increase the solid-liquid interface [149].

2.4.1.3 Cold Extraction and Hot Percolation

The plant material is extracted in a solvent of differing polarity at room temperature. It allows for maximum extraction of most components. The plant is heated in the solvent, usually under reflux. This extraction method allows for the extraction of many metabolites, from the most insoluble material like the waxes to the lipophilic natural products [149].

2.4.2 Modern Extraction Methods - Ultrasonic-Assisted Extraction (UAE)

Different from traditional ways of extracting, novel extracting technology should include merits such as high extraction capacity, uncomplicated manipulation, and solvent-saving. Ultrasound was firstly introduced in 1956 in Glasgow as medical assistance. Since that time, it has been widely applied in surface sanitation, plastic welding, and food preservation. According to the definition, ultrasound is acoustic energy generated by sound waves of different frequencies beyond the human hearing threshold (i.e.> 16 kHz). Further, based on the sound frequency and power, ultrasound is categorized as low-energy and high-energy ultrasounds. Low-energy ultrasound has a high frequency of more than 100 kHz but with a low intensity of less than 1 W/cm^2), whereas high-energy ultrasound is characterized by a low frequency ranging from 20-100 kHz and high intensity of more than $1W/cm^2$ [150]. Currently, there are three types of ultrasonication devices available in the market: the first one allows direct contact between ultrasound waves and sample matrix (ultrasonication probe), the second one attaches the reaction chamber with the equipment (circulating ultrasonication equipment), and the third one presents in the form of ultrasonic bath [151].

In recent years, it has been used as one of the cutting-edge technologies for polyphenol extraction and is especially favoured by small-to-large scale industries [152]. It is efficient in improving the recovery of bioactive compounds, including polysaccharides, phenolics and alkaloids [153]. Several factors should be evaluated when operating ultrasonication-assisted extraction (UAE), such as solvent composition, particle size, ultrasound frequency, extraction time, solvent pH, and temperature [149]. Nearly all solvents are suitable for this method (as long as the selected solvents have a high affinity with targeted compounds) [154].

The operation principle of ultrasonication is based on acoustic cavitation [155]. When the machine starts, it releases ultrasonic waves. These waves interact with each other and with solvents and dissolved gases, resulting in the formation of gas bubbles. As the cavitation process continues, the gas bubbles oscillate and expand in size, creating holes in the plant matrix [156]. Afterwards, the mechanical destruction caused by acoustic cavitation indirectly promotes the surface contact between plant tissue and extraction solvent, giving rise to the rapid diffusion of intracellular phytoconstituents into the medium [155].

2.5 Response Surface Methodology (RSM)

Design of Experiment (DoE) is a helpful statistics-mathematical practice for optimizing the research procedure. It only needs a small number of factors of inclusive combinations, and it can help to achieve optimum response by simultaneously alternating all factor combinations. DoE is especially useful when developing a new prototype or process, or optimizing existed output or process where a minimum of two variables are needed [157]. Throughout the years, analysts have employed various sorts of DoE techniques, including response surface methodology (RSM), artificial neural network (ANN), and extreme learning machine (ELM), to verify the hypothesis, as well as explore the relationship between input and response (also known as the output) [158]. Among the lists of DoE, RSM is the most widely used method and was first introduced in 1951 by Box and Wilson. It has helped researchers from various fields to determine empirical models and the optimum experimental conditions. RSM can process several variables at one time and clearly describe how much the independent factors influence the dependent variable and the degree of interactions between independent variables [159]. In most cases, the RSM quadratic model can provide a 95% accurate input prediction [160]. Figure 2.11 below demonstrates the general process of establishing an RSM.

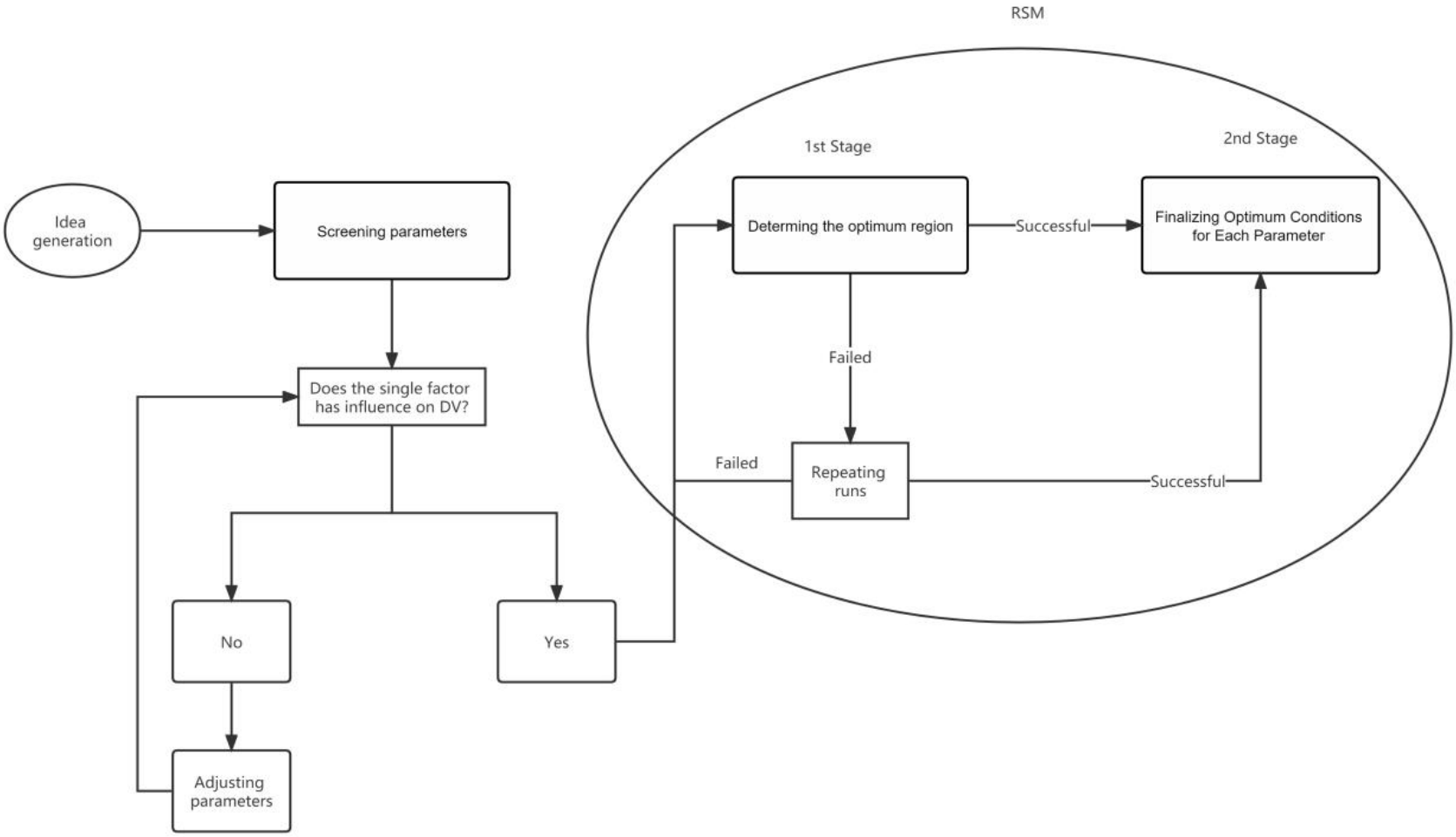

Figure 2.11 The steps of developing response surface methodology (RSM) (adapted from [161]). *DV- dependent variable.

Generally, developing an RSM consists of two stages, all starting with generating ideas based on a literature review regarding what possible factors would cause changes in research objectives. As shown in Figure 2.11, the initial step before RSM (more resemble a preliminary study) is called the screening process, in which one-way ANOVA is adopted to decide whether a single factor can be used to build an experimental model. If the statistical results indicate that the particular factor significantly impacts the dependent variable at a 95% confidential interval, this factor can be carried on for the following analysis. The screening step effectively helps narrow down the range of possible parameters to save time spent on repeating runs in the subsequent tests.

The first stage of RSM is an adjusting procedure that determines if the factors selected during the screening process could produce an outcome near the optimum conditions. In other words, if the selected independent variables failed to lead the response value to an optimum consequence, repetitions or adjustments will need to be taken place to drag the results closer to the optimum. The results of the first stage of RSM should follow a first-order model as shown in Equation (1) [162], meaning the data shall demonstrate a linear pattern in either a steady increase or decrease trend [161].

$$Y= \beta_0 + \beta_1X_1+ \beta_2X_2+...+\beta_kX_k + \varepsilon \quad (1)$$

In the equation above, β_0 represents the intercept (also known as the mean of all factors); β_1, β_2,...β_k represent the coefficient of each factor (i.e., X_1, X_2, X_3,....X_k); ε represents the random error of the model, which, along with mean and standard deviation, is supposed to follow normal distribution [162]. In addition, ε also makes known the disparity from other sources that cannot be specified by mathematical equation [163].

When researchers confirm that the response values are within optimum conditions, they can continue to conduct the second stage of RSM. Upon the second stage, the optimum region presumably has been found, and researchers are expecting to get a quadratic model (a second-order model) which can correctly generate an actual function for the response [161]. The purpose of using the second-order model is that sometimes exclusively using basic linear or interaction models cannot fully interpret the dynamics of optimizing procedure. Besides that, when researchers investigate the consequence of multitudinous factors, it is suggested to study not just one-factor-at-

a-time to help reduce the cost and time of repeating tests. Assuming the objectives of a project are money expenditure and working efficiency. The data reports are expected to be shown in curvature so that analysts can directly tell from the output under what condition the money expenditure hit the lowest point while the working efficiency hit the highest [163].

Equation (2) below gives an example of the simplest version of the quadratic model. It includes linear and squared terms of all independent variables and all factors in pairs. The regression coefficient of each factor (i.e., β_1, β_2,...β_k) reflects the influence of the corresponding factor on the outcome [161]. Lastly, the concluding step of RSM is to verify the model's fitness by viewing the model F-value, lack of fit, and the value of R-squared [164].

$$Y = \beta_0 + \beta_i X_i + \beta_{11} X_1^2 \quad (2)$$

To better explain the curvature in the data, experimenters use statistical software such as Minitab, Design Expert and SAS to display RSM results in visual pictures (e.g., three-dimensional (3D) plot and contour plot) to provide a much clearer view of the intricate relationships between variables. The generation of 3D images of RSM is completed by holding one single factor at its optimum performance and scheming the remaining two variables along with the output [158]. Therefore, the 3D models of RSM can be presented in various forms, such as bell-shaped, twisted, sloping, and crooked patterns, relying on the interaction of variables [162]. Table 2.3 lists the examples of commonly used RSM during the past ten years (from 2012 to 2022).

Table 2.3 Examples of different response surface methodology (RSM) used in plant extraction from 2012 to 2022.

Plant Source	RSM	Factors	Response	References
Cherry laurel fruit	TFFD	Microwave power (X_1); Solid-Liquid ratio (X_2); Extraction time (X_3)	Extraction yield	[165]
Taro (*Colocasia esculenta*)	CCD	Solvent Concentration (X_1); Extraction temperature (X_2); Extraction time (X_3)	TPC; TFC; TA; Antioxidant activity (FRAP and DPPH)	[166]
Mulberry Leaves	BBD	Solvent concentration (X_1); Temperature (X_2); Liquid-Solid ratio (X_3)	TPC; TFC; Antioxidant activity	[167]

*TFFD-three-level complete factorial design; CCD- central composite design; BBD-Box-Behnken design; TPC- Total Polyphenol contents; TFC- Total flavonoid content; TA- Total anthocyaninsl; FRAP-Ferric Reducing Antioxidant Power Assay; ABTS-(2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)); DPPH-(2,2-diphenyl-1-picrylhydrazyl).

Table 2.3 Examples of different response surface methodology (RSM) used in plant extraction during the year 2012-2022 (*continued*).

Plant Source	RSM	Factors	Response	References
Rheum moorcroftianum rhizomes	BBD	Vessel diameter (X_1); Solid-Liquid ratio (X_2); Extraction temperature (X_3); Sonication time (X_4)	TPC, TFC, TTC and anti -oxidant activity (ABTS and DPPH)	[164]
Moroccan *Acacia mollissima* barks	CCD	Extraction time (X_1); Solvent proportion (X_2); Microwave power (X_3)	Polyphenol contents; Condensed tannins content	[168]
Schinus molle L. Peel	CCD	Extraction temperature (X_1); Extraction time (X_2); Solvent composition (X_3); Solid-Liquid ratio (X_4)	TPC	[169]
Soghum flour	BBD	Fermentation time (X_1); Solid to liquid ratio (X_2); Flow rate (X_3); Ultrasonication Intensity (X_4)	TPC; Antioxidant activity	[170]
Gentian root	CCD	Extraction time (X_1); Ethanol Concentration (X_2); Solid-Liquid ratio (X_3); Extraction temperature (X_4)	TPC; Isogentisin; Gentiopicroside	[171]

*TTC- Total tannin content; TFFD-three-level complete factorial design; CCD- central composite design; BBD-Box-Behnken design; TPC- Total Polyphenol contents; TFC- Total flavonoid content; TA- Total anthocyaninsl; ABTS-(2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)); DPPH-(2,2-diphenyl-1-picrylhydrazyl).

As we can see from Table 2.3 that the most common RSM manipulated by analysts are Box-Behnken design (BBD), central composite design (CCD) and three-level complete factorial design (TFFD). However, in most scientific scenarios, TFFD is comparatively rarely used because it requires many experimental runs to fulfil the model. In contrast, other designs such as CCD and BBD do not need to test the combination of all scales to produce results (typically, 3-5 different levels of factors are considered sufficient) [172].

2.5.1 Central Composite Design

As aforementioned, CCD belongs to one category of RSM, which can fulfil a full quadratic model. The CCD configuration incorporates either a full factorial or fractional factorial design that is magnified with a set of star points/axial points, which help define the coefficients of a second-order model and predict the trend of curvature. Three distinct points can be found in the coordinate system of CCD. They are respectively factorial points, centroid and star points. The factorial points are located at the corners of the CCD cube, denoted with the symbols -1 and +1. The centroid is located at the centre of the design, denoted as (0,0,0). Star points are scattered at the centre of six sides, and each is at an equal distance of α from the centroid [166]. There are two major CCD forms (viz., face-centred CCD and rotatable CCD). Among these two forms, the rotatable CCD (an example is shown in Figure 2.12) represents the most popular design that befits second-order polynomials to response surface methodology [173].

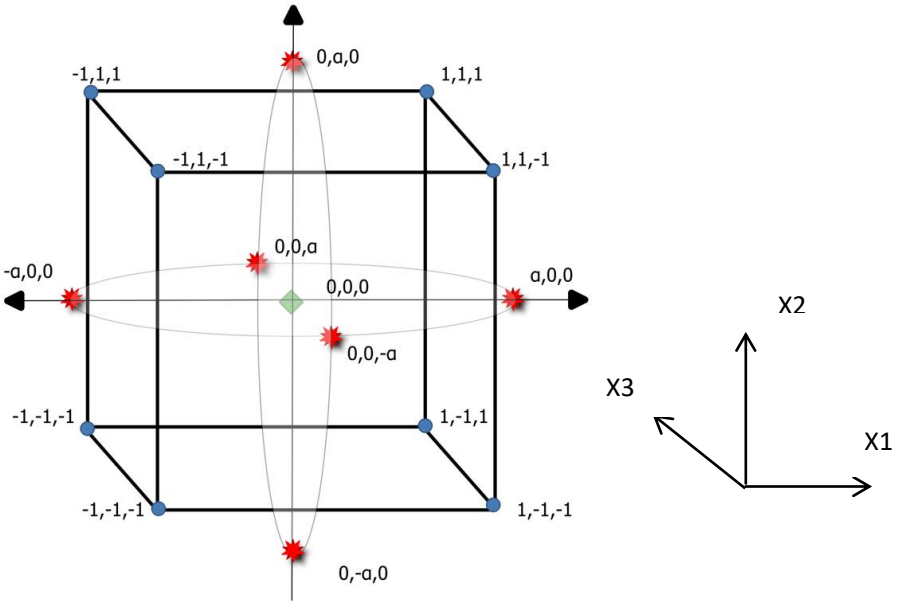

Figure 2.12 Rotatable central composite design with three factors.

The fact that the model's standard error maintains constant at a radius of +1 from the centroid makes the configuration of this design rotatable [162]. Suppose the quantity of factors of an experiment is fixed. In order to establish a rotatable CCD, the α of the design ought to be set as α= (nf)1/4, where nf represents the number of factorial points in the 2k factorial design. As it has been mentioned in the previous paragraph that CCD includes three different points (i.e., factorial points, centroid and star points); the rotatable CCD also includes three various points, namely 2k fractional factorial points, 2k star points [(±α, 0,. . .0),(0, ±α,. . .0), (0, 0, . . .±α)] and nc centroid (0,0,0,0...0) [161].

2.5.2 Box-Behnken Design

Sharing similar traits with CCD, the BBD also can adjust well to the full quadratic model of RSM [174]. However, unlike CCD, BBD does not need to include the cautiously selected subset (known as fractions) of complete factorial design to fit

the model. The combinations of experimental parameters are located in the middle of each edge of the 3D cube and at the centre (as illustrated in Figure 2.13) [161]. Box-Behnken Design is sufficient to support 3-7 factors, with each factor varying at three different levels [175, 176]. Figure 2.13 shows an example of "three factors three level" BBD below.

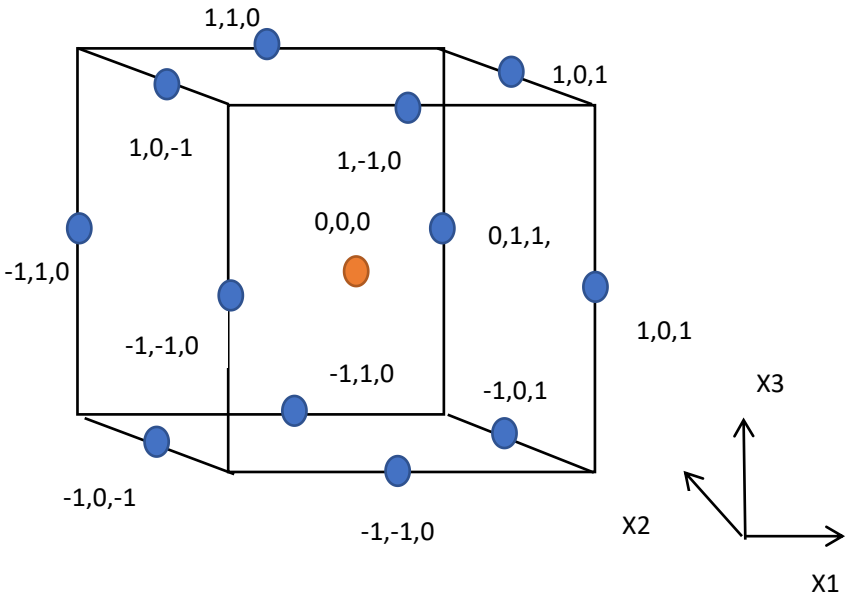

Figure 2.13. A graphic representation of Box-Bekhen design(BBD).

The independent variables of BBD are expected to be set at three levels coded as -1, 0 and 1 (representing low, medium, and high values, respectively). What is more, theoretically speaking, the factors that are coded as 0 are often the presumable optimum conditions. This model involves fewer experimental repetitions than other types of RSM, such as CCD. Besides, using BBD can reduce independent variables to a minimum of one at the central point (0) [164] meanwhile fitting the quadratic model just like other DoE [174]. Some researchers would choose BBD as a preferential alternative to other models when designing an experiment due to higher efficiency, more straightforward operation, and more accessible data interpretation [177]. Based on the statement above, BBD is a valuable tool for estimating the extraction effectiveness of modern technologies.

Chapter III Methodology

3.1 Experimental Design

Response surface methodology (RSM) is one of the popular statistical tools used in deciding the optimum parameters for extracting polyphenols. It can process multiple inputs simultaneously and generate a quadratic relationship between the independent and dependent variables. However, prior to implementing RSM, preliminary studies based on the principle of "one-factor-at-a-time" are needed to determine the possible factors that could significantly impact the outcome. In this study, a three-level-three-factor Box-Behnken design (BBD) was used for modelling and optimising UAE conditions. This study selected three independent factors (solid-liquid ratio, solvent composition, and ultrasonic time). The response determined was total polyphenol contents (TPC). When one independent factor was examined, the rest of the factors remained unchanged [188].

Coded and uncoded independent factors are demonstrated in Table 3.1. The coded factors were purposively labelled in a range from -1 to 1 so as to normalize parameters and make each of them produce equal influences on the response [189]. In total, 17 experimental runs were carried out, with five replicates at the central point for evaluating pure error. The model built up by BBD followed a second-order polynomial. The statistical software SAS ® OnDemand for Academics was employed to investigate experimental results.

Table 3.1 Coded and uncoded factors in Box-Behnken design (BBD).

Run	Design Matrix					
	Coded Factors			Uncoded Factors		
	Factor A (X1)	Factor B (X2)	Factor C (X3)	Ethanol Concentration, % (X1)	Time, min (X2)	Solid-liquid Ratio (X3)
1	-1	1	0	60	30	1:20
2	1	1	0	80	30	1:20
3	-1	-1	0	60	10	1:20
4	1	-1	0	80	10	1:20
5	-1	0	-1	60	20	1:10
6	1	0	-1	80	20	1:10
7	-1	0	1	60	20	1:30
8	1	0	1	80	20	1:30
9	0	-1	-1	70	10	1:10
10	0	1	-1	70	30	1:10
11	0	-1	1	70	10	1:30
12	0	1	1	70	30	1:30
13	0	0	0	70	20	1:20
14	0	0	0	70	20	1:20
15	0	0	0	70	20	1:20
16	0	0	0	70	20	1:20
17	0	0	0	70	20	1:20

3.2 Chemicals and Reagents

Ethanol; acetic acid; methanol; Folin−Ciocalteu reagent; sodium carbonate; gallic acid; ascorbic acid; 2,2-diphenyl-1-picrylhydrazyl (DPPH); rutin (≥94%; HPLC; powder); formic acid; acetonitrile; potassium hydrogen phosphate (K_2HPO_4); potassium hydrogen phosphate trihydrate ($K_2HPO_4 \cdot 3H_2O$); guaiacol. Unless stated, all assay materials were purchased from Sigma Aldrich.

3.3 Sample Preparation

New Zealand grown asparagus were purchased from local supermarkets from September 2021 to January 2022. All the fresh spears were harvested and packed by LeaderBrand Produce Limited in Gisborne, New Zealand (38°40'0.664"S; 177°59'38.302"E). Sample procurement and sample preparation were carried out on the same day. Attributes of the purchased asparagus were green and without visible injuries. The average length of green asparagus ranged between 21.40- 24.30 cm. Freshly collected samples were cleaned with a cotton ball under running tap water to eliminate contaminants. After that, they were sent for drying at 60 ℃ (a mild temperature to avoid colour degeneration and caramelization) in a circulating-air oven (Contherm, New Zealand) for 48 h [178]. Next, dried samples were ground into fine powders using a stainless steel blender and then passed through a 400 mesh sieve. After that, the powdered samples were sealed in a well labeled plastic bag and stored at -20 ℃ for no more than three months.

3.4 Total Polyphenol Contents

The methods of adjusting optimum conditions for detecting total polyphenol contents (TPC) were adapted from [179, 180] with modifications. Detailed instructions for TPC assessment can refer to in Appendix A1. Blue chromophore forms when polyphenols react with the Folin-Ciocolteu reagent in an alkaline environment [181]. The absorbance was read at 760 nm using Genesys 10S UV-vis spectrophotometer. All operations in this section were carried out in triplicate. The results of TPC were calculated using Equation (3) and shown as gallic acid equivalence (y= 0.0014x 0.061, R^2 = 0.9953; mg GAE/g dry weight).

$$\frac{(\text{Abs Asparagus extracts} - \text{Intercept}) \times \text{Dilution factor} \times 1000}{\text{Slope} \times \text{Sample dried weight}} \quad (3)$$

*Abs- UV-Vis absorbance

3.5 Traditional Maceration

Previously, Eun et al., (2010) [182] investigated the optimum conditions for extracting protodioscin and rutin from white and green asparagus. Their results showed that an ethanol concentration of at least 70% (v/v) is needed to extract these two compounds. Common extractants used for polyphenol extraction are ethanol and methanol (with or without the addition of acidic solvent) [183]. Among various acidic solvents, acetic acid belongs to Generally as Safe (GRAS) chemical by the Food and Drug Administration (FDA) [184]. To start the process of maceration, 1 g of asparagus spear (AS) powder was incorporated into 10, 15, and 20 mL of three different types of extraction solvents (ethanol:water:acetic acid = 70:29.5:0.5; v/v/v; 70% EtOH v/v; or deionised water), respectively, in a flask. The mixtures were then placed on an orbital shaker at 240 rpm for 2 h, 4 h and overnight. Each experimental parameter was examined three times. After maceration, mixtures were sent for centrifugation at 6535 x g for 5 min in an IEC centra CL3R centrifuge (Thermo Scientific, New Zealand). Supernatants were collected, and samples that contained organic solvents were subjected to a rotary evaporator (BÜCHI Labortechnik) at 40 ℃ to expel alcoholic residues [66]. The condensed extracts were then topped with deionized water to 200 mL in a volumetric flask, making the extracts 1g of spear/mL per dry matter (dw) [185].

3.6 Ultrasound-Assisted Extraction (UAE)

Applying the direct ultrasonic probe might produce heat to distort the chemical structure of bioactive compounds [186]; thus, in this study, an ultrasonic bath (SONOREX DigiTEC DT-52, Bandelin Electronic, Berlin, Germany) was used. The device has a 1.8 L capacity and provides an ultrasonic power of 240 W. To conduct UAE, 1.2 L of deionized water was added to the ultrasonication bath to generate an acoustic power of 200 W/L. The independent variables selected in this study were UAE time (10, 20, and 30 min), solid-liquid ratio (1:10, 1:20, and 1:30; w/v), and

ethanol composition (60, 70, and 80%; v/v). After being treated with ultrasonication, samples were sent for centrifugation at 6535 x g for 5 min. The supernatants were collected and subjected to rotary evaporation at 40 °C [187].

3.7 The Effects of UV-C Hormesis

The UV-C effects were examined following methods described by Gamage (2015) [190] with slight modification as considering the differences in sample population. The UV-C lamps (Philips ultraviolet TUV 30w/g30t8, Holland) used in this study were mercury-based, which emitted light at 254 nm. Earlier in 2014, the bottom surface of the UV-C light box was divided by Gamage (2015) [190] into 25 blocks of the same size. According to the Inverse Square Law, the intensity of UV-C light weakens from the centre to the edges. On this account, fresh asparagus samples were placed at only the central 9 blocks of the bottom surface and were 50 cm vertically apart from the light sources. Before the experiment, a UV-C meter was placed in the centre of each of the 9 blocks, to record light intensity. It took approximately 12 min for this UV-C device to warm up (as shown by a stable reading of UV intensity). After warming up for 12 min, the UV lights were switched off swiftly before placing asparagus samples.

In this study, asparagus samples were treated by UV-C light for 2 min, 5 min and 10 min, separately, which equivalent to 0.45 kJ/m^2, 1.13 kJ/m^2, and 2.27 kJ/m^2. Each light treatment duration was divided into four equal lengths (i.e., 4 x 30 s, 4 x 75 s, and 4 x 150 s). Asparagus samples were rotated 90 degrees at each time interval to ensure each side of the samples received an equal amount of UV-C irradiation. Then, the samples were stored in a well-ventilated darkroom at ambient temperature for 22 h before sending them to scanning electron microscopy (SEM) to check if there were a symptom of damage on sample surface structures [191]. Samples without UV-C treatment (recorded as 0 kJ/m^2 UV-C irradiation) were served as ‘controls’. The SEM assessment was completed at Manawatu Microscopy and Imaging Center, Massey

University, Palmerston North. After UV-C treatment, samples were oven dried, pulverized and then subjected to UAE by using conditions optimized in the BBD experiment.

3.7.1 Determination of DPPH

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) is one of the stable commercial grade reactive nitrogen species [192]. In normal conditions, DPPH free radical has a single electron and a strong absorption at 517 nm, and its alcohol solution looks purple [193]. In this study, the conduction of DPPH assay was carried out by following Danxia et al.'s (2021) and Truong et al.'s (2019) [194, 195] instructions with slight modifications. The DPPH stock solution was prepared by dissolving 20 g of DPPH into 100mL of MeOH. It was then kept in the freezer at – 20 °C before the experiment. Condensed asparagus extracts were diluted with MeOH to 25, 50, 100, 200, and 500 µg/mL (w/v). One mL of DPPH was mixed with same volume of each dilution and was sent for incubation at 37 °C for half an hour. The absorbance of the reaction was read at 517 nm by a spectrophotometer (Genesys 10S UV-vis spectrophotometer). Two mL of absolute MeOH served as a negative control. The DPPH antioxidant activity was quantified by using Equation (4):

$$\left(1 - \frac{A517\ (Sample)}{A517\ (Blank)}\right) \times 100\% \qquad (4)$$

Where A517 (Sample) denotes the absorbance of extract dilutions at a UV-vis wavelength of 517 nm, and A517 (Blank) denotes the absorbance of absolute MeOH measured at 517 nm. Ascorbic acid served as a positive control. The concentration needed to exhibit 50% inhibition of DPPH activity (IC_{50}) was acquired via the plots of the percentage of residual DPPH against the concentration of extract dilutions.

3.7.2 Determination of the Antioxidant Activity of Peroxidases

The antioxidant assay of peroxidases (POD, EC 1.11.1.7) was conducted by following experimental protocol [196]. The phosphate buffer solution (PBS) preparation was done in three steps. Firstly, 13.61 g of K_2HPO_4 was weighed and dissolved in 1000 mL ultrapure water (tape water purified using a Millipore Milli-Q lab water system), secondly, 22.82 g of $K_2HPO_4 \cdot 3H_2O$ was weighed and dissolved in 1000 mL ultrapure water, after that, 39 mL K_2HPO_4 solution was mixed with 61 mL $K_2HPO_4 \cdot 3H_2O$ solution to make 100 mM PBS (pH 7.0). The buffer pH was measured by a pH meter. In this study, 20 mM guaiacol was used as substrate. It was prepared by dissolving 249 mg guaiacol in 100 mL ultrapure water. Thirty grams of fresh samples (with or without UV-C treatment) were placed in a measuring cup, then 90 mL of 100 mM phosphate buffer was added. The mixtures were ground by a homorgenizer. The grinding procedure was conducted in TCR room at 4 °C. The homogenates were sent for centrifugation at 6353 x g for 15 min. Supernatants were collected and then were sent back to the TCR room. The extractions of enzyme suspension were conducted three times to ensure reproducibility and accuracy of absorbance reading [197].

A mixture contained 50 μL of 20 mM guaiacol solution, 3 mL of PBS and 30 μL of 12.3 mM H_2O_2 was served as a reference solution. The peroxidase assay commenced after adding 100 μL asparagus supernatants to the reference solution in a glass cuvette with a light path of 1 cm. The cuvette was placed in a UV-vis spectrophotometer that was preset at a wavelength of 436 nm. The spectrophotometer was adjusted to zero by using the reference solution. The sample absorbance was recorded after the extinction reached 0.050. A stopwatch was used to record the time for the extinction to increase from 0.050 to 1.000. The antioxidant activity of POD was calculated by Equation (5) as below:

$$\text{Antioxidant activity of POD (U/L)} = Vr \times Vt \times 1000 / \varepsilon \times l \times \Delta t \times 1.000 \quad (5)$$

Where, Vr represents volume of reaction solution (mL), Vt represents the volume of asparagus supernatants (mL), Ɛ represents the extinction coefficient for guaiacol dehydrogenation product at 436 nm (6.39 cm^2/ μmol), l represents the light path of cuvette (1 cm), Δt represents the time for the extinction to increase from 0.050 to 0.100, and the constant "1.000" represents one unit of POD, which denoted as the quantity of enzyme that increases 1.000 of absorbance at 436 nm/min.

3.8 Ultra High-Performance Liquid Chromatography (UHPLC) Analysis

The preparation of rutin and caffeic acid standard solution

The stock solution of rutin was prepared by dissolving 0.1 g of HPLC grade rutin (≥ 94% purity) in 100 mL of 70% EtOH (v/v). The solution was prepared in a 80-320 μg/mL concentration to plot a 5-point rutin standard curve. The stock solution of caffeic acid was prepared by dissolving 0.1 g of HPLC grade caffeic acid (≥ 98% purity) in 100 mL of 70% EtOH (v/v). The solution was prepared in a 15-500 μg/mL concentration to plot a 5-point rutin standard curve.

The preparation of asparagus extracts for HPLC analysis

The sample solution was prepared by dissolving 0.2 g condensed asparagus extract in 100 mL of 60% EtOH under the extracting conditions optimized in Section 3.1. Standard solution and sample solution were degassed and filtered using 0.22 μm pore size filter paper. The content of rutin in asparagus extracts was quantified using ultra-high performance liquid chromatography (UHPLC) as stated by Yu et al., (2020) [198]. Quantitative HPLC analysis was carried out using UHPLC model with silica-based C18 column. Two mobile phases were used for compound separation. Mobile phase A consisted of Milli-Q water with 0.1% formic acid, and mobile phase B consisted of acetonitrile with 0.1% formic acid. The gradient elution was design as: 0-

8 min, 95% A; 8-18 min 65% A; 18-25 min 60% A; 25-27 min, 60% A; 27-29 min, 5% A. The C18 column was washed by 95% B for 2 min and then equilibrated with 95% A for another 2 min prior to the next injection, making the whole run time 35 min.

The system flow rate was maintained at 0.5 mL/min throughout the entire separation process, and the sample injection volume was 20 µL. The target compounds were monitored at three UV wavelengths (325 nm, 280 nm, and 260 nm) with diode array detectors (DAD). The retention time for caffeic acid and rutin were 20.137 min and 20.763 min, separately. The temperature of column chamber was controlled at 25 °C. The identification and quantification of the leading polyphenol (rutin) in asparagus extracts was confirmed by comparing the retention time and mAU*Area of the sample with the external rutin standards. Rutin fraction was detected by HPLC-MS equipped with electrospray ionisation (ESI) mass spectra under negative mode.

3.9 Statistics

This study used Microsoft Excel and the online software SAS ®OnDemand for Academics (SAS Inc., Cary, NC) for statistical analysis. Proc Mixed procedure was adopted to proceed ANOVAs. Statistical results were expressed as least square means with standard errors (SE; if used SAS) or means with standard deviations (SD; if used Microsoft Excel). Standard deviation provides insights into the shape of a probability distribution and reflects variances between individual results and the mean value. The SE indicates the variances between the sample mean and the true mean of the whole population [199]. Type I error was used to make a statistical conclusion, and the significant levels were set at 0.05,0.01 or 0.0001.

Chapter IV Results and Discussion

4.1 Ultrasonication-assisted Extraction

4.1.1 Model Fittings

The Box-Behnken Design (BBD) was used to predict the optimum conditions for UAE. Based on previous literature and the results from my preliminary studies (see Appendix A2.), I selected three factors, i.e. X_1 (Solvent Composition: 60%, 70% and 80% ethanol, v/v), X_2 (Extraction Time: 10, 20, and 30 min), and X_3 (Solid-liquid Ratio: 1:10, 1:20, and 1:30, w/v) and corresponding ranges to establish BBD model. Table 4.1 displays the summary of model fittings. The F-values at a probability of 0.001, 0.01 or 0.05 were used to analyse variance (ANOVA) in the RSM model.

Table 4.1 Fitness of the model.

Components	Df	Sum of Square	Mean Square	R^2	F Value	P Value
Model	9	3157.34		0.9959 (adjusted R^2 0.9950)	187.05	< 0.0001
Linear	3				471.39	< 0.0001
Quadratic	3				80.97	< 0.0001
Cross product	3				49.47	0.0090
X_1	4	1328.82	332.21		177.13	< 0.0001
X_2	4	134.97	33.74		17.99	0.0009
X_3	4	1755.08	438.77		233.95	< 0.0001
Lack of Fit	3	8.54	2.80		2.48	0.2005
Pure Error	4	4.59	1.15			
Total Error	7	13.13	1.88			

*Df-degree of freedom.

As shown in Table 4.1, the model's overall probability of $p< 0.0001$ was considered a significant outcome. It indicates that the response values offered more than 99.99% confidence that the total model coefficient did not equal to zero [200]. The R-squared stands for the correlation coefficient. From Table 4.1 we can see that the model R^2 (0.9959) and the model adjusted R^2 (0.9905) are very close to 1, and the difference between them was less than 0.2, suggesting that the model was accurate and adequate for predicting response performances [201]. Generally, the model is considered appropriate and reliable when the R-squared is higher than 0.75 [169]. Besides, the model obtained a low pure error with a sum of square of 4.59 and a mean square of 1.15, indicating that only lower chances of error are expected to occur if redone the experiment with the same experimental parameters [202]. Lastly, the model lack-of-fit was found non-significant, with an F-value larger than 0.05 ($p= 0.2005$), denoting the goodness of the proposed model.

4.1.2 Effects of Extraction Parameters on Total Polyphenol Contents (TPC)

Table 4.2 demonstrates the responses of TPC (based on dry weight; dw) computed by BBD, and Table 4.3 presents the interactive relationships between inputs and response values. As shown in Table 4.2, the highest TPC (76.25 mg GAE/g, dw) was found with experimental run number 7 (60% ethanol, v/v; 20 min extraction time and solid-liquid ratio of 1:30); while the lowest TPC (21.61 mg GAE/g, dw) was observed with experimental run number 4 (80% ethanol, v/v; 10 min extraction time and solid-liquid ratio of 1:20). The analysis of variance (ANOVA) for RSM model shows that total polyphenol contents (TPC) were significantly influenced by all factors except the quadratic effect of X_2 (extraction time) and the interactive effect of X_2X_3 (extraction time x solid-liquid ratio) ($p< 0.05$). Expelling non-significant variables, the RSM model generated a polynomial equation for TPC, which came as:

$$TPC = 42.66 – 12.32X_1 + 3.60\ X_2 + 12.91\ X_3 – 4.04\ X_{12} + 9.64\ X_{32} + 2.23\ X_1X_2 – 2.55\ X_1X_3 \quad (6)$$

Table 4.2 The three-factor-three-level Box Bekhen Design (BBD) and responses.

Run	Design Matrix						
	Coded Factors			Uncoded Factors			Response
	Factor A (X_1)	Factor B (X_2)	Factor C (X_3)	Ethanol Concentration, % (X_1)	Time, min (X_2)	Solid-liquid Ratio (X_3)	Total Polyohenol Contents (mg GAE/ g, dw)
1	-1	1	0	60	30	1:20	53.84
2	1	1	0	80	30	1:20	34.82
3	-1	-1	0	60	10	1:20	49.55
4	1	-1	0	80	10	1:20	21.61
5	-1	0	-1	60	20	1:10	46.07
6	1	0	-1	80	20	1:10	45.36
7	-1	0	1	60	20	1:30	76.25
8	1	0	1	80	20	1:30	25.36
9	0	-1	-1	70	10	1:10	63.13
10	0	1	-1	70	30	1:10	70.71
11	0	-1	1	70	10	1:30	38.48
12	0	1	1	70	30	1:30	42.23
13	0	0	0	70	20	1:20	43.21
14	0	0	0	70	20	1:20	40.80
15	0	0	0	70	20	1:20	43.48
16	0	0	0	70	20	1:20	43.04
17	0	0	0	70	20	1:20	42.77

Table 4.3 ANOVA for the RSM model.

Components	TPC (mg GAE/g; dw)			
	Regression Coefficient	Standard Error	T-value	P-value
Intercept	42.66	0.61	69.55	< 0.0001
X_1	-12.32	0.48	-25.44	< 0.0001
X_2	3.60	0.48	7.44	0.0001
X_3	12.91	0.48	26.67	< 0.0001
X_1^2	-4.04	0.67	-6.06	0.0005
X_2^2	1.34	0.67	2.00	0.0803
X_3^2	9.64	0.67	14.45	< 0.0001
X_1X_2	2.23	0.68	3.26	0.0139
X_1X_3	-2.55	0.68	-3.72	0.0075
X_2X_3	0.96	0.68	1.40	0.2047

The negative and positive signs of the regression coefficient reflect how independent variables influence the response: a negative sign indicates that the response value decreased as the independent variable increased, whereas a positive sign indicates the response value increased as the independent variable increased [203]. It can be seen from the coefficients in Table 4.3 that X_3 (solid-liquid ratio) had the most significant linear effects on TPC, followed by X_1 (ethanol concentration) and X_2 (extraction time) ($p< 0.01$). The interaction between X_1 and X_3 was negative, but the interaction between X_1 and X_2 was positive, indicating that X_3 had less effect on TPC when optimum solvent concentration was pronounced. The binary interaction coefficient between X_2 and X_3 was not significant ($p= 0.2047$), reflecting that they performed independently from one another [169]. The 2D contour plots and 3D surface plots of RSM helped visualise the research data and are displayed in Figure 4.1-2.

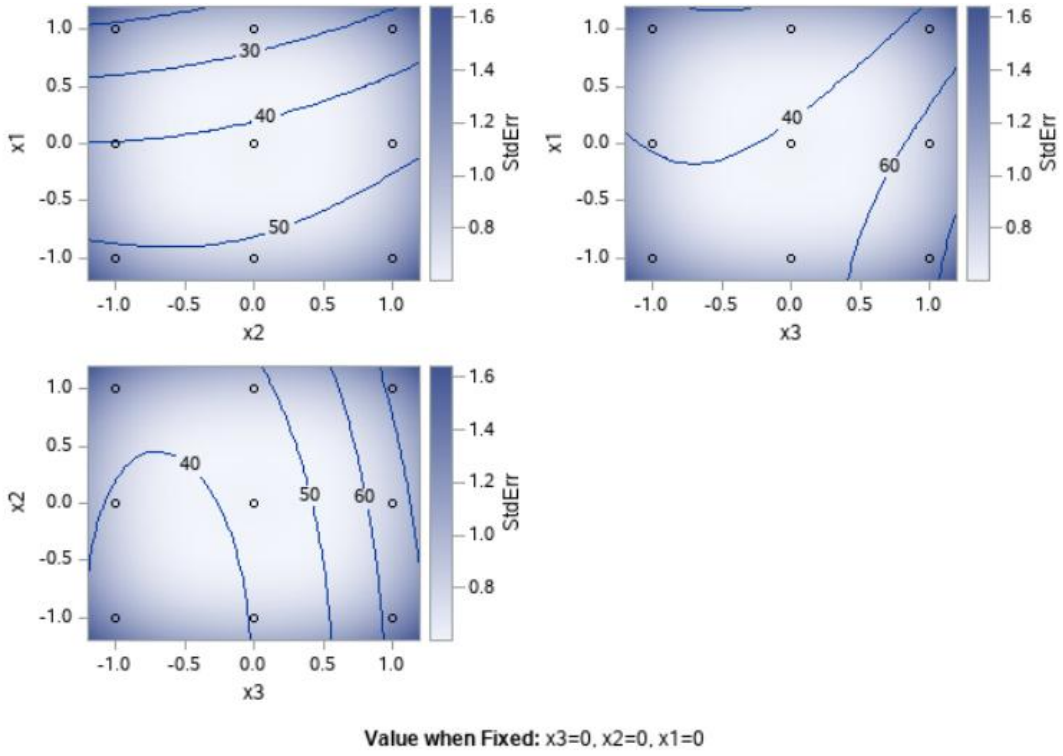

Figure 4.1 The two dimensional (2D) countour plots for total polyphenol content (TPC).

Where X_1 is ethanol concentration (60, 70, 80%, v/v), X_2 is extraction time (10, 20, 30 min), and X_3 is solid-liquid ratio (1:10, 1:20, 1:30; w/v). All independent variables were coded between $-1 \leq xi \leq 1$.

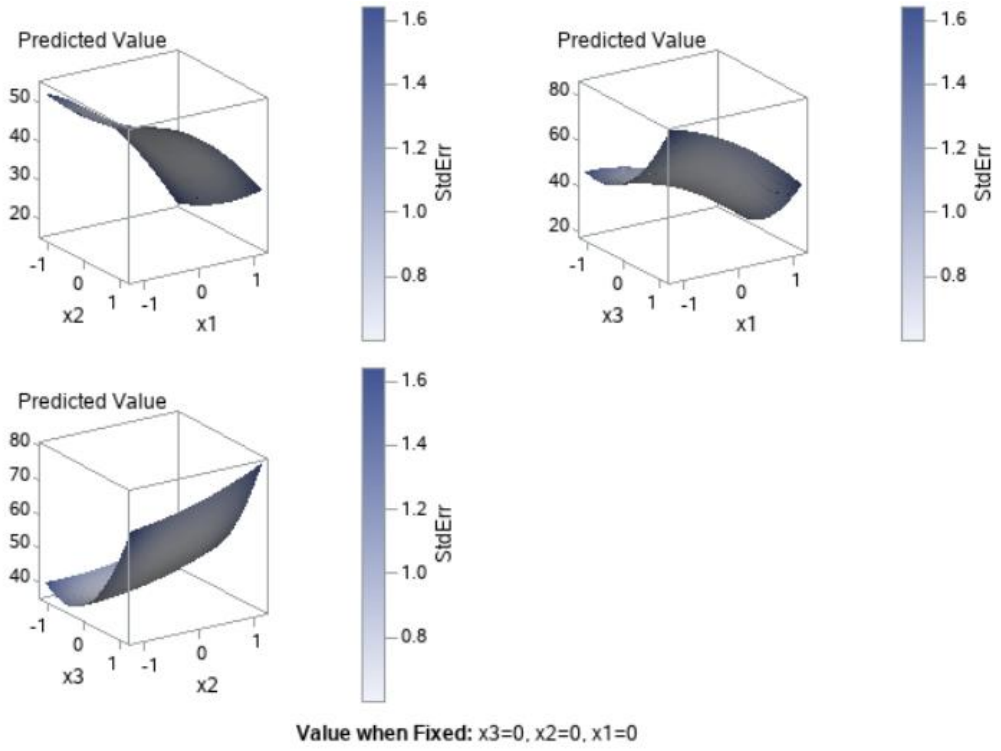

Figure 4.2 The three dimensional (3D) surface plots for TPC.

Where X_1 is ethanol concentration (60, 70, 80%, v/v), X_2 is extraction time (10, 20, 30 min), and X_3 is solid-liquid ratio (1:10, 1:20, 1:30; w/v). All independent variables were coded between $-1 \leq xi \leq 1$.

The 2D contour plots and 3D surface plots were produced based on Equation (6). Based on the graphs above, we can see that X_1 and X_3 were the main factors affecting the extraction ability of UAE on TPC. These findings were consistent with ANOVA results in Table 4.3, meaning the target TPC value can be obtained by focusing on adjusting the solvent composition and solid-solvent ratio only. In this study, the optimum extracting conditions were 60% ethanol (v/v), 20 min extraction time, and 1:30 solid-liquid ratio (w/v). Combining these three conditions yielded a 76.25 mg GAE/g (dw) TPC.

As for the effects of ethanol concentration, the TPC value increased as the ethanol concentration decreased. This finding accords with Kobus et al., (2017) [204], who investigated the influence of solvent polarity on the extracting performance of UAE on TPC from apple pomace. In their research, 60% ethanol produced the highest amount of TPC, followed by pure water and 96% ethanol ($p< 0.05$). Similar results were also reported by Arteaga-Crespo et al., (2020) [205], who studied the influences of ethanol concentration, temperature, extraction time, and solid-liquid ratio on the UAE efficiency of polyphenol contents from *I. guayusa* leaves. They found that ethanol concentration (ranged between 70-90%, v/v) was the most impactful factor on TPC. A maximum TPC (3.38 g GAE/100 g; dw) was achieved with 76.8 % ethanol (v/v), while an ethanol concentration higher than this value caused a decline in TPC. The polarity of ethanol may partly explain these findings. Ethanol is often used as extracting agent for chlorogenic acid and flavonoids [206]; for example, gallic acid (a phenolic acid) contains a carboxy group and four hydroxyl groups with high polarities, thus making it more soluble in water than in ethanol [204]. In addition, the combination of water with ethanolic solvent provides a better extraction efficiency for phenolics than the employment of these solvents alone [169].

Although the extraction time contributed the least significant effects on TPC in this study, an extraction duration of 20 min was considered sufficient for the UAE process. This result was contrary to Saifullah et al., (2020) [207], who used an ultrasonication bath (50 Hz) to treat *Leptospermum petersonii* leaves for 30, 45, and 60 minutes. They reported that there was a linear positive significant influence of ultrasonication time on polyphenol contents, and 60 min (the lonest extraction duration) produced the maximum TPC ($p< 0.05$) [207]. However, in another study conducted by Daghaghele et al., (2020) [208], the researchers used a 24 KHz ultrasonication bath to treat *Moringa Oleifera* Leaves for 5, 15, and 25 min and found that 15 min extracted the highest phenolic compounds from leave samples. The authors concluded that an extended extraction duration led to a decreased TPC level as oxidation might happen during the ultrasonication process. In addition to that, Fick's law of diffusion could

also explain the shorter extraction time of this study that a balanced concentration between solute and plant phenolics could improve the production of target compounds and reduce time and solvent usage for extraction [209].

Also, in the current study, X_3 (solid-liquid ratio) imposed both linear and quadratic significant effects on UAE efficiency ($p< 0.0001$), with a ratio of 1:30 considered as the optimum condition. This result was in agreement with Hayta et al., (2017); Mazza et al., (2019); Ryu et al., (2019) [210-212], who reported that a lower solid-liquid ratio (S/L) yielded the highest amount of polyphenols from grape skin (S/L 1:15; from a range of 1:5- 1:15. w/v), black soybeans (S/L 1:50; from a range of 1:30-1:50, w/v), and chickpea (S/L 1:40; from a range of 1:10 to 1:40, w/v), respectively. The higher solvent volume provides greater gradient concentration, thus resulting in higher diffuse rates and TPC value, which fulfills the principle of mass transfer [213]. However, it is worth noting that when the optimum solid to liquid ratio was found, there was no need to continually increase solvent volume as the target compound might have been fully extracted. Insisting on doing so will merely cause solvent waste and unnecessary financial expenditure [214]. In conclusion, the optimum UAE conditions for extracting TPC from asparagus spear were: 60 % ethanol (v/v), 20 min extraction time, and a solid to liquid ratio of 1:30 (w/v).

4.2 The Effects of UV-C Hormesis

4.2.1 Total Polyphenol Contents (TPC)

Plant polyphenols possess antioxidant properties and have been reported by numerous studies for their efficiencies in preventing oxidation and reducing free radical species [215]. The effects of UV-C treatment on total polyphenol contents (TPC) are shown in Figure 4.3. It can be seen from the bar chart that all three chosen UV-C doses (i.e. 0.45, 1.13, and 2.27 kJ/m^2) induced significantly higher TPC values than that produced by unirradiated samples (0 kJ/m^2; controls) ($p<0.05$). A minimum UV-C dose at 0.45 kJ/m^2 was needed to achieve a stimulating effect on TPC, which

resulted in a 61% increase compared with the controls. The TPC was the highest after 1.13 kJ/m^2 UV-C exposure, almost twice the amount found in unirradiated samples (131.37 ± 2.05 vs 75.39 ± 0.95 mg GAE /g; dw).

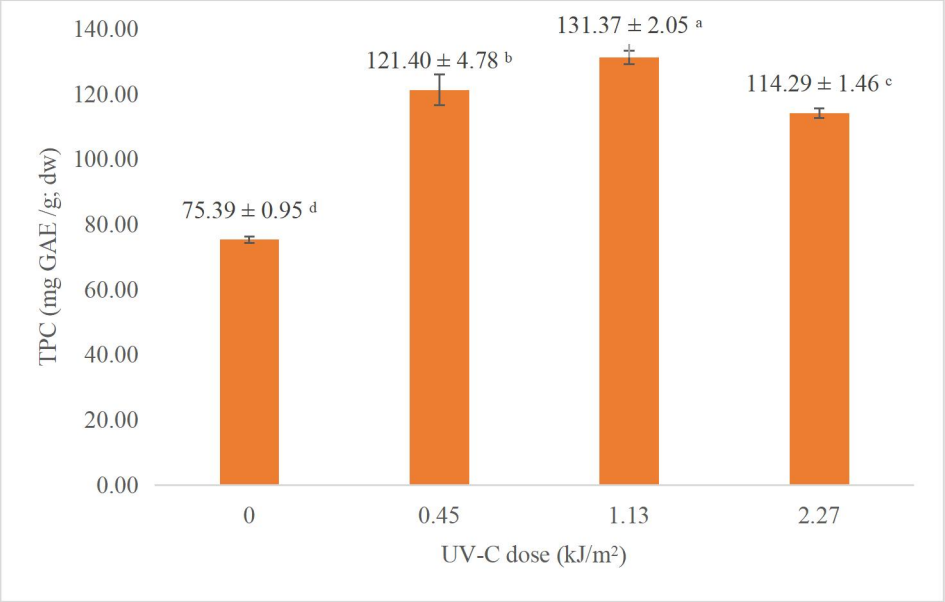

Figure 4.3 The hormetic effects of UV-C irradiation on TPC value.

Data expressed as Mean ± SD (n= 3). Different letters in superscript indicate significant differences across four UV-C treatment groups (i.e., 0, 0.45, 1.13 and 2.27 kJ/m^2) at α= 0.05.

Previously, the stimulating effects of UV-C were observed by Martinez-Sanchez et al., (2019) [216], who employed two doses of UV-C radiation (1.5 and 3.0 kJ/m^2) throughout the three different growth stages of spinach. The authors found that compared to unirradiated samples, both UV-C doses significantly increased TPC in spinach ($p \leq 0.05$). Similar findings were also reported by Maharaj et al., (2014) [217], who noticed that compared with control groups, post-harvest UV-C treatments of 0.32, 0.97, 2.56, 4.16 and 4.83 kJ/m^2 significantly improved TPC in fresh whole tomatoes at the end of 15 days of storage. The possible mechanism for the promotive effects of UV irradiation involves intricate regulations of plant proteins (e.g., heat shock proteins, proteasomes, kinase cascades, hormones) and biological nitrogen assimilation [27].

Besides, the UVR8 photoreceptor of plants also plays a role in such mechanisms. It receives UV-C and UV-B lights and then engages with constitutive photomorphogenic 1 (COP1; a ubiquitin ligase of E3s in charge of regulating plant morphogenesis) to mediate HY5 gene expression; this chain reaction then further induces the synthesis of polyphenols [14, 27].

Plant morphology and the synthesis of secondary metabolites are highly related to the quality (wavelength), lighting direction, intensity, and exposure duration of the light source [218]. A low to moderate UV-C dose (ranging between 0.5-9.0 kJ/m^2) could elicit the accumulation or production of plant bioactive compounds [219], whereas a high dose of UV irradiation may have reverse impacts [218]. In this study, I found that when applied 0.45 kJ/m^2 and 1.13 kJ/m^2 doses of UV-C on fresh asparagus, the amounts of TPC were positively correlated with UV irradiation; and the value started to decrease when magnified UV-C dose to 2.27 kJ/m^2. These findings may be credited to the hormetic effects of UV-C light. Previously, Ranjbaran and colleagues [220] used three doses of UV-C lights (0.8, 1.2 and 4.2 kJ/m^2) on grapes and found that low-to-moderate UV treatments provided the highest TPC value than the high dose (0.8, 1.2 > 4.2 > 0 kJ/m^2, $p< 0.05$). Besides that, Bobkova et al., (2020); Boeing et al., (2014); Li&Wang (2019) [179-181] have also observed UV-C hormesis on the TPC from red cabbage sprouts, fresh-cut Chinese yam, and fruit juices. Therefore, the results of this study corroborated with the dose-dependent phenomenon of UV-C hormesis and were in alignment with previous studies.

4.2.2 Free Radical Scavenging Ability (DPPH)

In recent years, several technologies have been utilized as post-harvest treatments for improving or changing the bioactivity of crops. These technologies include non-thermal treatments, UV and gamma exposure, and modified atmosphere packaging [221]. Table 4.2.1. demonstrates the effects of UV-C irradiation on the DPPH scavenging ability of New Zealand grown green asparagus. Theoretically, an

RSA% ranging between 15-75% indicates a good linear correlation between the value and sample concentration [222]. As can be seen from Table 4.2.1, the radical scavenging ability (RSA%) varied between 36.67 ± 9.17 to 74.00 ± 3.71 in the unirradiated group and 37.33 ± 2.67 to 82.22 ± 2.69 in UV-C treated groups, which reflected the validity of the results. In addition, the lowest IC50 was observed in samples that received 1.13 kJ/m^2 UV-C treatment (IC50= 36.84 μM). Scientists often use IC50 value as an indicator of antioxidant ability. The smaller IC50 result indicates more potent antioxidant activity [222]. The DPPH results of the current study correlated with that obtained in Section 4.2.1 that the optimum UV-C doses (especially at 1.13 kJ/m^2) strengthened asparagus antioxidant ability and were presumably associated with the increase of TPC (shown as Figure 4.4) [223].

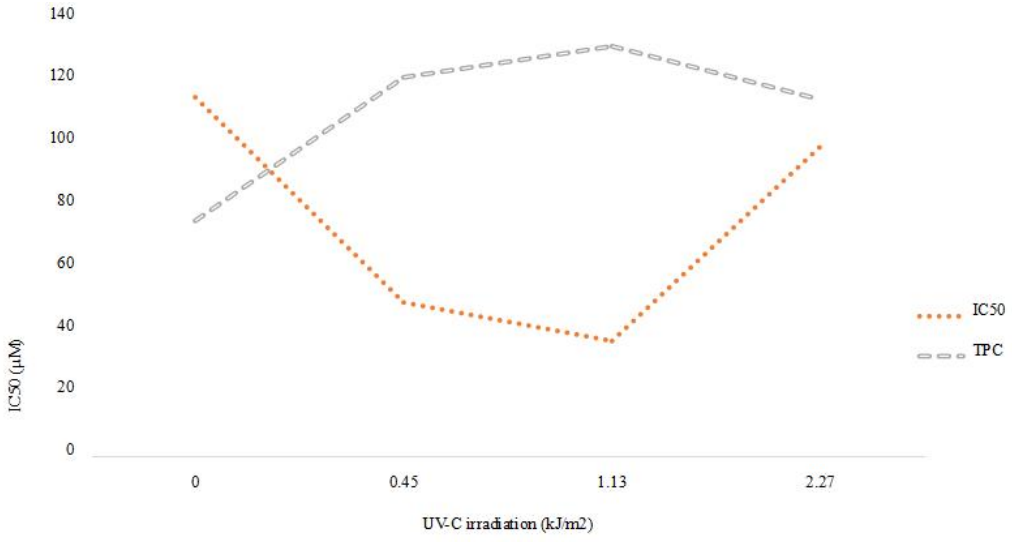

Figure 4.4 Correlation between the half maximal inhibitory concentration (IC50) and total polyphenol contents.

Table 4.4 The Effects of UV-C on free radical scavenging ability (DPPH).

UV-C treatment (kJ/m2)	Concentration	RSA (%)	Equation	R^2	IC50 (µM)
0	25	36.67 ± 9.17	y= 0.0719x + 41.731	0.838	115.01
	50	44.22 ± 2.04			
	100	51.56 ± 4.54			
	200	65.11 ± 6.48			
	500	74.00 ± 3.71			
0.45	25	41.33 ± 3.53	y= 0.0611x + 46.994	0.781	49.20
	50	47.78 ± 3.01			
	100	58.44 ± 2.69			
	200	66.59 ± 1.54			
	500	74.00 ± 2.40			
1.13	25	43.33 ± 1.76	y= 0.074x + 47.272	0.9276	36.84
	50	52.44 ± 1.92			
	100	56.44 ± 0.77			
	200	66.67 ± 2.40			
	500	82.22 ± 2.69			
2.27	25	37.33 ± 2.67	y= 0.0634x +43.71	0.766	99.21
	50	44.67 ± 1.76			
	100	55.78 ± 5.18			
	200	64.67 ± 1.33			
	500	71.56 ± 1.02			

*RSA- Radical scavenging ability. Data were expressed as Mean ± SD (n=3).

4.2.3 Peroxidase Activity

Peroxidase (POD, EC 1.11.1.7) is an enzyme that widely exists in fruits and vegetables. It catalyses the oxidation of polyphenols. The increased POD concentration in food can reduce antioxidant capacity and accelerate the accumulation of dark pigments (browning), resulting in decreased consumers' buying intentions [224]. In this study, freshly purchased green asparagus samples were subjected to UV-C irradiation for 0, 2, 5, and 10 min (equivalent to 0, 0.45, 1.13 and 2.27 kJ/m^2). Then, separately, after 2 h and 22 h of UV-C adaptation, the antioxidant activities of POD were examined (shown in Figure 4.5). Overall, the 22 h UV-C adaptation group showed significantly higher POD values than the 2 h adaptation group ($p< 0.05$). The POD contents of both groups reached the bottom at 1.13 kJ/m^2 UV-C irradiation. The POD value peaked at 2.27 kJ/m^2 UV-C irradiation in the 2 h adaptation group. Regarding the changes in POD in the 2 h UV-C adaptation group, 0.45 kJ/m^2 and 1.13 kJ/m^2 UV-C exposure significantly decreased POD in asparagus by 22.78% and 43.26%, respectively. These findings confirm that low doses of UV-C could induce adaptation reactions in herbal organisms, including provocation of enzymatic and non-enzymatic antioxidant activities [4]. Results of this study were also in line with those of Maharaj et al., (2014) [217], who reported that a UV-C dose higher than the hormetic range exaggerated maturation and resulted in unwanted browning in tomatoes.

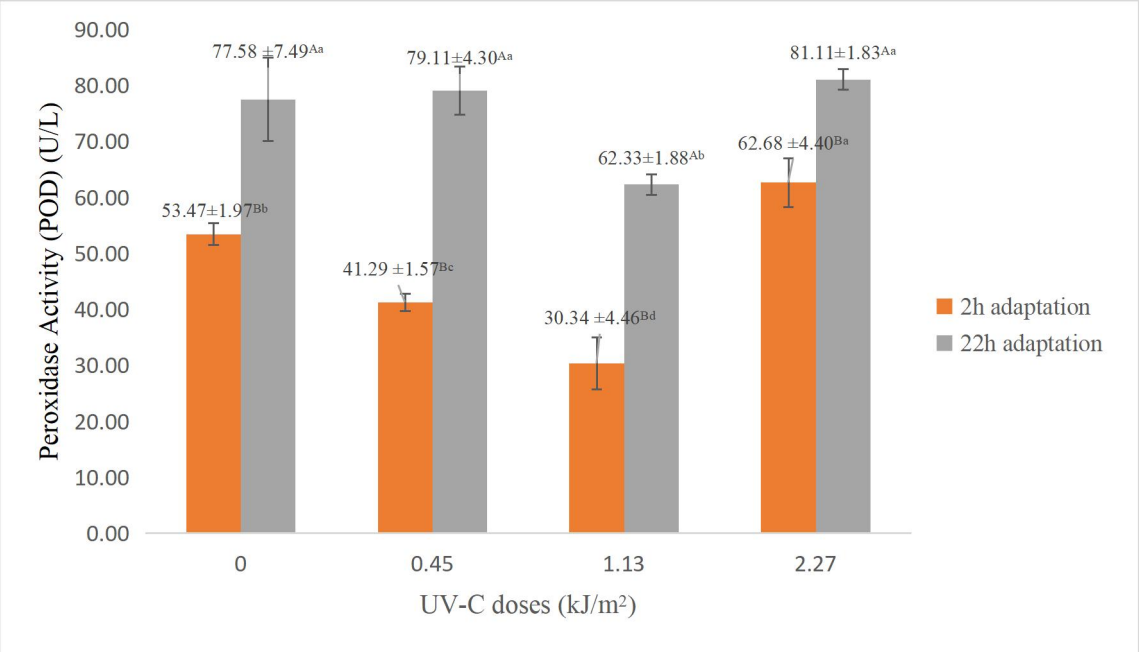

Figure 4.5 Changes in peroxidase (POD) activity in four UV-C treatment groups after 2 h and 22 h adaptation.

- Orange columns demonstrate the changes in POD in four UV-C treatment groups (viz. 0, 0.45, 1.13 and 2.27 kJ/m^2) after 2 h UV adaptation. Gray columns demonstrate the changes in POD in four UV-C treatment groups (viz. 0, 0.45, 1.13 and 2.27 kJ/m^2) after 22 h UV adaptation.

- Data expressed as Mean ± SD (n= 3). Different capital letters indicate significant differences in POD value between '2 h UV-C adaptation group' and '22 h UV-C adaptation group' ($p< 0.05$). Different letters in lower case indicate significant differences in POD value between four UV-C doses (viz. 0, 0.45, 1.13 and 2.27 kJ/m^2) ($p< 0.05$).

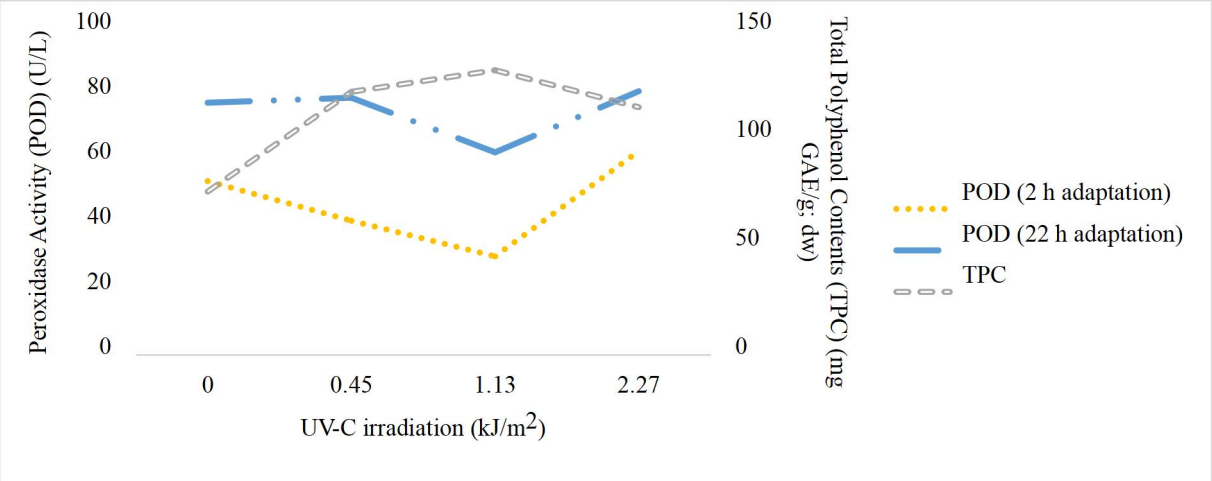

Figure 4.6 Correlations between total polyphenol contents and peroxidase (POD) activities.

When plants encounter environmental stimuli, they generate oxygen radicals such as H_2O_2, which accept electrons from polyphenols (such as catechin, quercetin and its ramifications) [224]. Peroxidases (POD) play a role in catalysing the conversion of polyphenols into lignin, resulting in rapid senescence; however, interestingly, a higher concentration of total polyphenols can inhibit the POD activity [225]. This phenomenon explained why the current study saw an opposite effect of UV-C on POD than that in DPPH assay (as shown in Figure 4.6).

4.2.4 Scanning Electron Micropscope (SEM) Results

A previous study on New Zealand grown asparagus reported that the tip section contains higher amounts of meristematic cells, thus making it more perishable and vulnerable than other spear parts [226]. My initial intention for SEM imaging was to explore if UV-C treatments would cause damage on spear surfaces that naked eyes cannot spot. Results from SEM showed that both UV-treated and unirradiated samples demonstrated no changes on the surface but differences in stomatal behaviours (see Appendix A4). The stomata were widely open in unirradiated samples and were close in irradiated samples. Previous studies stated that the changes in stomatal conductances are consequences of plants' adaptation to environmental factors (e.g., carbon dioxide and oxygen level, solar radiation, temperature and humidity). The A band of UV light triggers stomatal opening, whereas the C band causes stomatal closure [227]. Based on this fact, future studies are warranted to measure the size of stomatal apertures after UV-C irradiation to better understand the relationship between UV light and asparagus quality and shelf-life.

4.3 Quali-Quantitative Analysis of Rutin by Using UHPLC and LC-MS

Chromatography technologies are widely applied for fragmentation, identification and quantification of flavonoid compounds. Among the chromatography

methods, HPLC is the most frequently used. It provides fast and accurate assessments for various chemical compounds, including rutin [228]. This study obtained the asparagus crude extracts (ACE) using the optimum UAE conditions determined in Section 4.1 (60% ethanol, v/v; 20 min extraction time; solid-liquid ratio 1:30, w/v). For HPLC analysis, the asparagus extracts were properly diluted to 2 mg/mL. The representative asparagus phenolic compounds (caffeic acid and rutin) were detected at 260 nm (shown in Figure 4.7). Table 4.5 displays the identification of the representative leading polyphenols in asparagus extracts. The retention time of caffeic acid and rutin were observed at 20.137 min and 20.763 min, respectively.

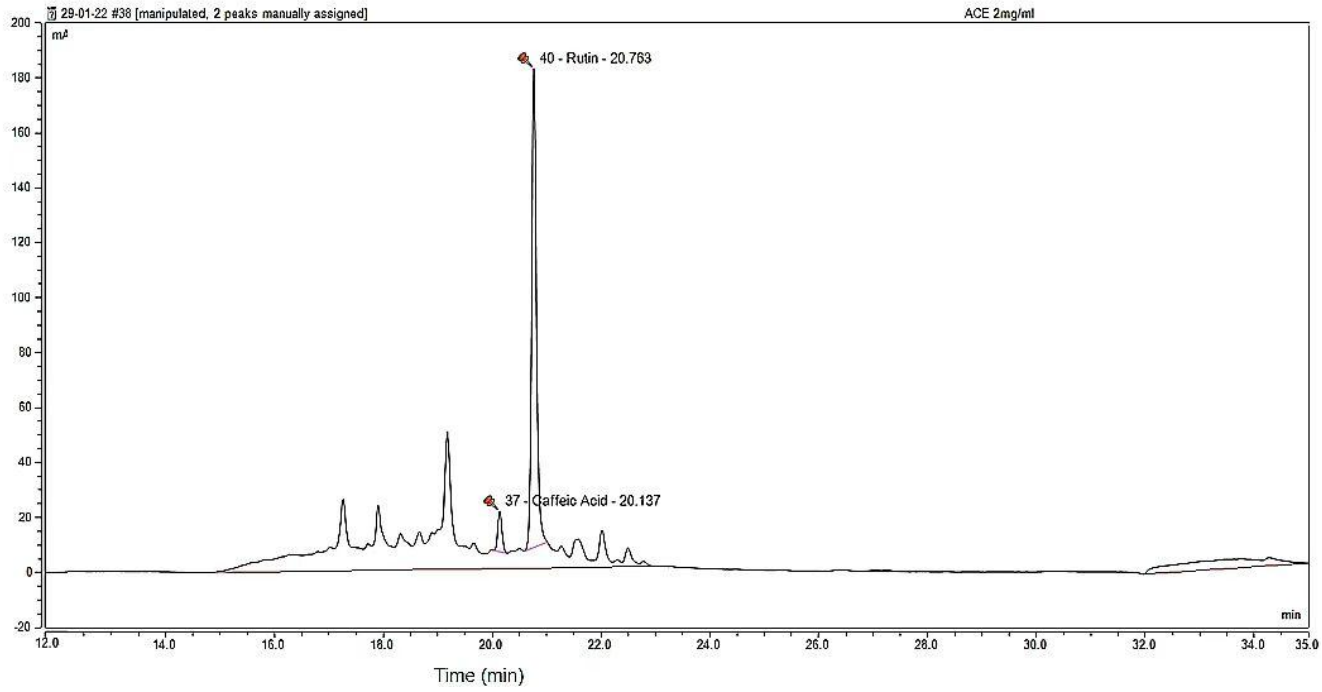

Figure 4.7 The HPLC DAD profile (260 nm) of the representative polyphenols of asparagus extracts.

*The identified polyphenolic compounds are labeled as Peak Number- Compound Name- Retention Time (RT).

Table 4.5 Identification of the representative flavonoids (rutin) and phenolic acid (caffeic acic) by using HPLC method.

Parameters	Rutin	Caffeic acid
DAD wavelength (nm)	260	260
Retention time (min)	20.763	20.137
Calibration curve equation	y= 0.7203x +7.9880	y= 118.4x - 80.486
R^2	0.9997	0.9729
Peak area (m*AU)	28.1856	nr

nr- not recorded

As it is demonstrated in Figure 4.7 that rutin the the major polyphenol in New Zealand-grown green asparagus. The rutin content in asparagus was quantified by comparing corresponding retention time and absorbance area with external rutin standards (y= 0.7203x + 7.9880; R^2= 0.9997). According to the data shown in Table 4.5, every 100 g of asparagus contained 14.095 mg rutin (based on dried matter). The detailed UHPLC profile can be found in Appendix A5. This study showed that rutin was the main flavonoid in New Zealand grown green asparagus extracts. This finding was in line with previous asparagus studies. For example, Kobus-Cisowska et al., (2019) [68] used HPLC with diode array detection (DAD) to determine the polyphenol profiles in green asparagus spears of five different genotypes, i.e., Schwetzinger Meisterschuss, Huchel's Alpha, Gijnlim, Grolim and Eposs. They found that rutin was the most abundant polyphenol, with contents ranging from 1818.70 μg/100 g (dry weight) in the Gijnlim cultivar to 16318.67 μg/100 g (dry weight) in the Grolim cultivar.

Furthermore, the presence of rutin was confirmed via ESI-MS and ESI-MS/MS (negative mode) by comparing retention time, molecular formula and ion fragments. Figure 4.8 shows that the ion peak of rutin was monitored at m/z 609.1454 ($C_{27}H_{19}O_6$) with a main fragment detected at m/z 301.0350 (shown as Figure 4.9). Results of UHPLC-MS were in agreement with Wang et al., (2003) [229] who stated that under an appropriate research condition, rutin can be monitored at m/z 611 (positive mode)

or m/z 609 (negative mode). The main fragment detected at m/z 301.0350 suggesting the loss of rutinoside (rhamnoglucoside; molecular formula $C_{12}H_{20}O_9$; 308 Da) [230]. Besides, the chemical structure of rutin contains o-diphenol, benzene ring, and keto functional group (-C=O), which might partly explain the high antioxidant activity of asparagus extracts [224]. Earlier studies conducted by Laughton et al., (1991); Morel et al., (1993) [231, 232] showed that the o-diphenol structure of phenols helped improve their efficiency on inhibiting iron and copper-catalysed reactions, resulting in decreased generation of free radical species. In addition, Cervantes-Laurean et al., (2006) [233] also reported in their study that the hydroxyls contained in rutin structure were potent in inhibiting glucose autoxidation.

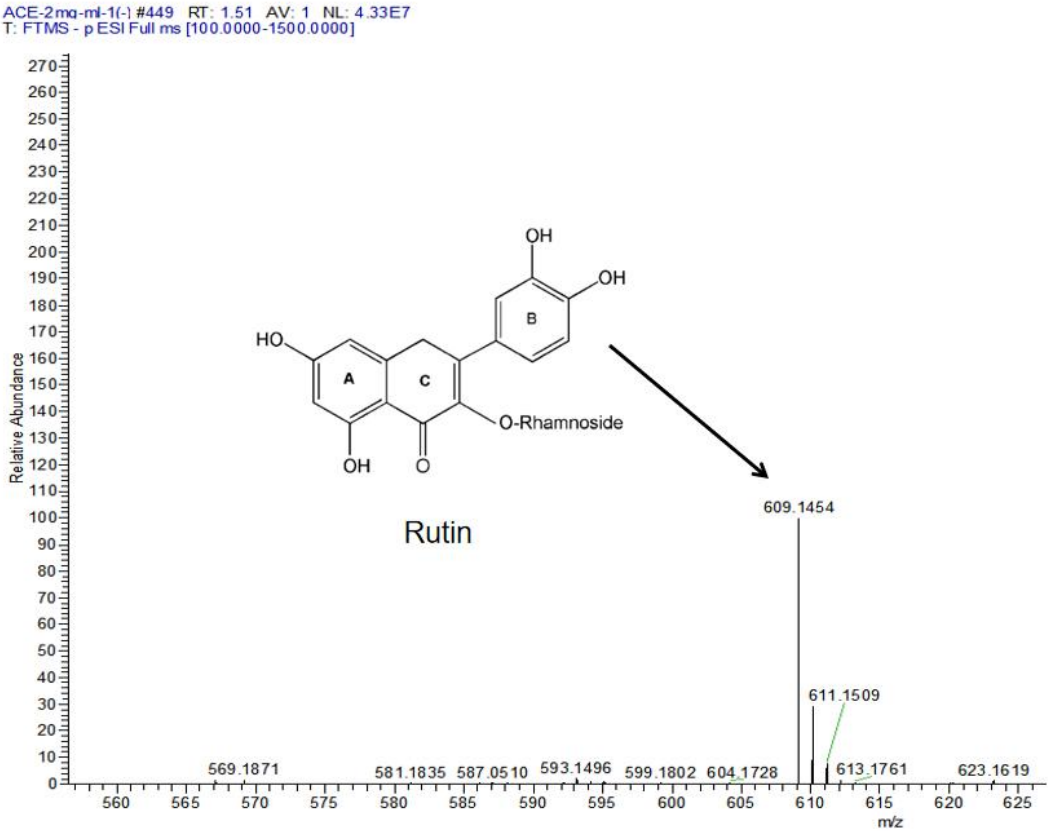

Figure 4.8 ESI-MS (-) of rutin from asparagus extracts.

*The chemical structure of rutin is adapted from [233].

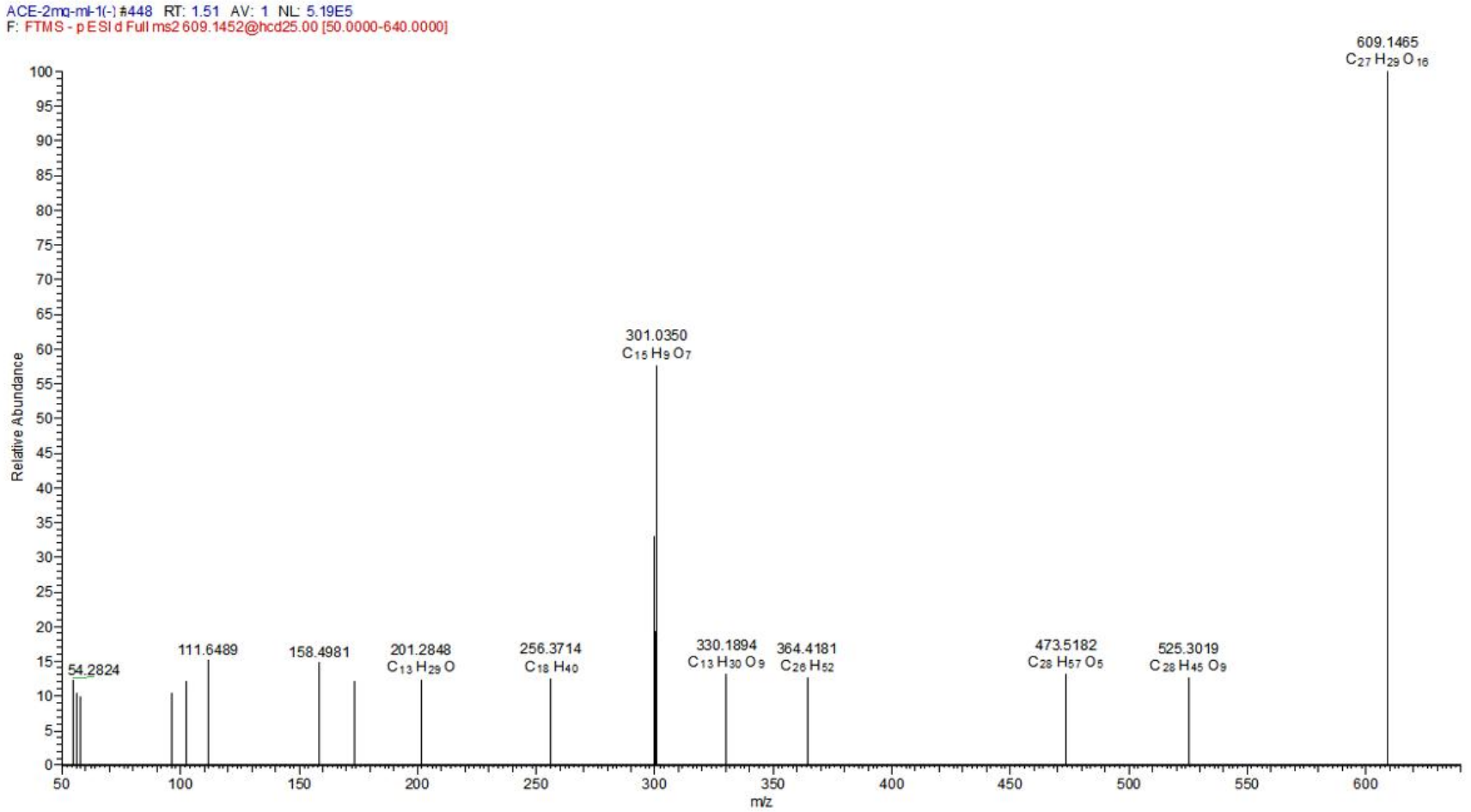

Figure 4.9 ESI-MS^2 of rutin from asparagus extract.

Chapter V Conclusion and Future Indications

5.1 Conclusions

Ultrasonication-assisted extraction (UAE) has been used as one of the cutting-edge technologies for polyphenol extraction and is especially favoured by small-to-large scale industries. The UAE approach provided 70.46% higher polyphenol yields and saved extraction time to 20 min compared with traditional maceration. Ultrasound induces acoustic cavitation on plant tissue and indirectly promotes the surface contact between plant matrix and extraction solvent, giving rise to the rapid diffusion of intracellular phytoconstituents into the medium [9]. Several factors should be considered when operating UAE, such as solvent composition, particle size, ultrasound frequency, extraction time, solvent pH, and temperature. In this study, three extraction parameters were selected, ie. X1 (solvent composition), X2 (extraction duration), and X3 (solid to liquid ratio). The optimum UAE condition was computed using the response surface method (RSM) and was shown as 60% ethanol (v/v), 20 min extraction time, and a solid-liquid ratio of 1:30.

This study reports for the first time the hormetic effects of UV-C on the antioxidant capacity of green asparagus. The phenomenon of hormesis is described as a dose-dependent behaviour of plants linked with environmental stressors such as extreme temperature, heavy metals, solar radiation, salinity, and drought. When receiving hormesis, plants show responses in colour, biochemical constitutions, freshness, scents, and productivity. An optimum dose of hormesis is beneficial for plants, whereas a too low or too high dose causes no change or reverse effects in the bioactivity of plants. This study imposed three doses of UV-C irradiation (0.45, 1.13, and 2.27 kJ/m$^{2)}$ on fresh asparagus spears, among which, 1.13 kJ/m^2 was determined as the hormetic dose, which resulted in a TPC of 131.37 ± 2.05 mg (gallic acid equivalent; GAE)/g (dried weight). In addition, samples that received the hormetic dose of UV-C (1.13 kJ/m^2) showed the highest radical scavenging ability (RSA%, 82.22 ± 2.69), and the lowest IC_{50} (36.84 μM) and peroxidase (POD) activity. Results from High-Performance Chromatography (liquid chromatography and mass spectrometry; HPLC-MS) showed that rutin was the main flavonoid in

asparagus extracts, with a content of 14.02 mg/100g (dry wright). The excellent radical scavenging effects of asparagus extracts might accredit to the hydroxyl groups attached to the chemical structure of rutin.

5.2 Future Indications

⇨In this study, samples were sent for SEM imaging 22 h after UV-C irradiation to explore if UV-C treatments would cause damage on spear surfaces. However, no changes were spotted on the surface but differences in stomatal behaviours. Considering that: 1. stomatal closure is linked with reduced gas exchange [225]; 2. radiations ranging between 100-400 nm control the stomatal closure in plants [218]. Therefore, the present study raises the possibility that an optimum UV-C dose could reduce the respiration rate and extend the product shelf-life of New Zealand green asparagus.

⇨This study is the first to reveal the phytochemical profile in the spears of New Zealand-grown green asparagus. Two of the representative polyphenols were identified through ultra-high performance liquid chromatography detection, they are rutin (representative of asparagus flavonoids) and caffeic acid (representative of phenolic acid), respectively. However, future extensive studies are needed to characterize and quantify all the other major polyphenols in New Zealand green asparagus.

⇨Comparative studies by using HPLC method are suggested to be carried out to determine to what extend does individual polyphenolic compound change before and after UV-C irradiation; So as to provide valuable information to both pharmaceutical and food industries to launch new products high in particular compounds.

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Appendices

A.1 The optimization of Folin-Ciocalteu methods

The conduction of A1 was followed [179] with modification.

1. Preparation of crude extracts and gallic acid standard solution

Two grams of asparagus powders were weighed and placed in a reagent bottle to prepare asparagus crude extracts. After that, 20 mL 70% EtOH was added. Then, the samples were placed on an orbital shaker at 240 rpm for maceration for 2h before adding another 30 mL of 70% EtOH. Followed by that, the sample mixtures were centrifuged at a speed of 6536 g for 5 min, then were filtered through Whatman No. 1 paper under vacuum. Supernatants were collected and topped up using deionised water to a volume of 200 mL, making a final concentration of 1g/mL (dry weight). Regarding the preparation of the gallic acid standard solution, a total of 0.25 g of gallic acid was weighed. The chemical was dissolved in deionised water and then topped up using deionised water to a volume of 500 mL, making the final concentration of 500 ug/mL.

2. Determination of optimum UV-Vis wavelength

To decide the optimum wavelength for examining total polyphenol contents, 250 µL gallic acid solution was added to a test tube, followed by adding 250 µL Folin-Ciocaultea reagent (1:1 v/v in deionised water). The mixture was stood still for 30s before adding 500 µL Na_2CO_3 and 4 mL of deionised water. After that, the mixture was put in a dark cabin for 2h followed by an absorbance reading at 400-900nm (60nm/interval) in a UV-VIS spectrophotometer (Massey Brand needed). Identical processes were conducted to determine the optimum absorbance wavelength for the TPC of asparagus crude extracts. A blank sample was also prepared in the same way, excluding the addition of gallic acid/asparagus extracts.

Table A1. Optimum Absorbance Wavelength

Wavelength (nm)	**Gallic acid standard solution (Abs)**	**Asparagus crude extracts (Abs)**
400	0.46	0.14
460	0.44	0.18
520	0.47	0.187
580	0.5	0.28
640	0.55	0.33
700	0.6	0.40
760	**0.64**	**0.42**
820	0.52	0.27
880	0.4	0.24

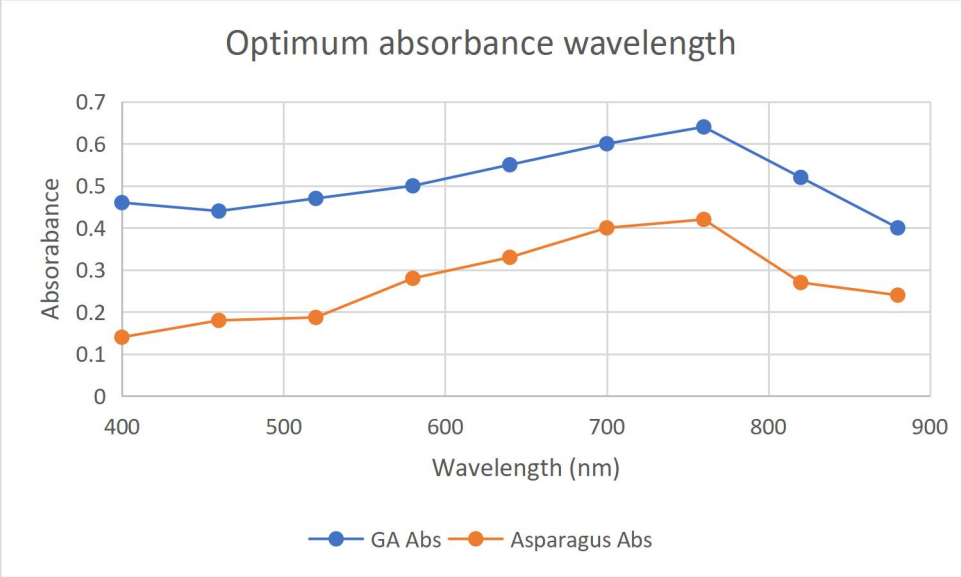


Figure A1. The optimum UV-vis absorbance wavelength for asparagus extracts and gallic acid standard solution.

*GA- gallic acid standards. Abs- absorbance.

Table A1 and Figure A1 concluded that the optimum UV-Vis wavelength for asparagus extracts and the gallic acid solution was 760nm.

3. Determination of optimum colour development time

As for the determination of optimum reaction time, 6 test tubes were used in total. To start the assay, 250 µL of asparagus crude extract was added to each test tube, followed by the addition of 250 µL diluted Folin-Ciocalteu reagent (1:1 v/v in deionised water). After the mixture reacted for 30 s, 500 µL sodium carbonate (Na_2CO_3) and 4 mL deionised water were added to the tubes in sequential order. The test tubes then were put in a dark cabin for 0 h, 0.5 h, 1 h, 2h, 3h and 4h, separately, before being sent for absorbance reading at the optimum wavelength determined in Section 3.2.2.

Table A2. Optimum color development time

Time (h)	**Absorbance**
0.5	0.72
1	0.74
2	0.76
3	**0.77**
4	0.75

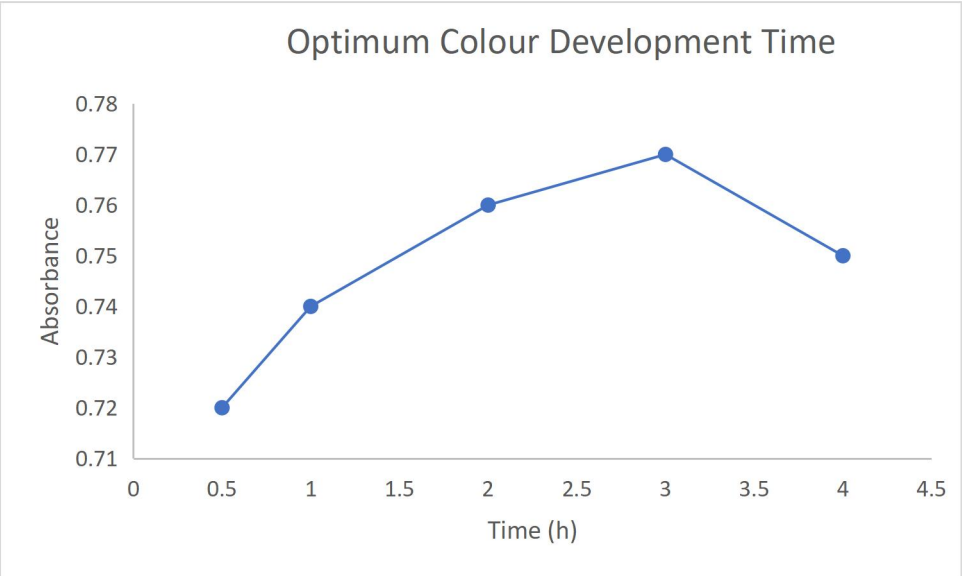


Figure A2. The Optimum Development Time for Gallic Acid Stock Solution.

Table A2 and Figure A2 concluded that the optimum colourimetric development time for asparagus extracts and the gallic acid stock solution was 3h.

4. Plotting gallic acid standard curve

One millilitre of gallic acid was taken out from the stock solution (500 µg/mL), and then it was serially diluted into a concentration of 250, 125, 62.5, 31.25 and 15.63 µg/mL. After that, 250 µL diluent, 250 µL Folin-Ciocalteu, 500 µL Na_2CO_3 and 4 mL deionised water were sequentially added to each tube. The blank tube contained all chemicals, excluding the diluent. Each concentration's absorbance reading was recorded, and the standard curve of gallic acid was plotted in a linear regression model (y= 0.0014x + 0.016; R^2= 0.995) shown as absorbance versus concentrations.

Table A3. Gallic Acid Standard Curve

Gallic Acid Concentration (ug/mL)	Absorbance
31.25	0.10448
62.5	0.166
125	0.2276
250	0.377
500	0.76

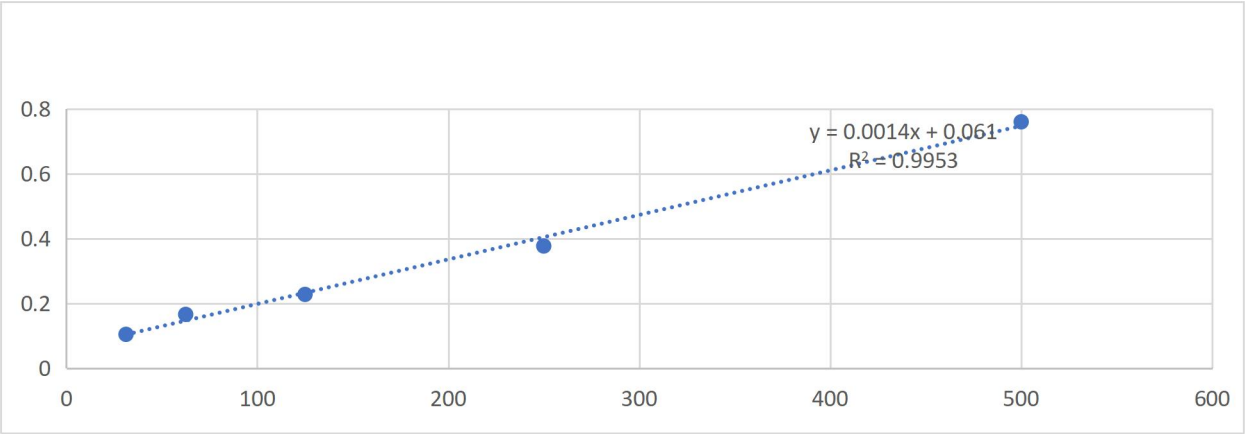


Figure A3. Gallic Acid Standard Curve.

Equation A(1). Calculation for TPC

$$TPC = \frac{(\text{Abs Asparagus extracts} - \text{Intercept}) \times \text{Dilution factor} \times 1000}{\text{Slope} \times \text{Sample dried weight}} \quad A\ (1)$$

A.2 Preliminary study- traditional maceration (selecting extraction factors for UAE)

Table A4. The ANOVA for traditional maceration.

SC	Time	SL	TPC LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
AE	2h	1:10	21.0720000	2.1989825	<.0001	1
AE	2h	1:15	26.1313333	2.1989825	<.0001	2
AE	2h	1:20	28.0656667	2.1989825	<.0001	3
AE	4h	1:10	28.5416667	2.1989825	<.0001	4
AE	4h	1:15	38.9286667	2.1989825	<.0001	5
AE	4h	1:20	36.9050000	2.1989825	<.0001	6
AE	Overnigh	1:10	26.5773333	2.1989825	<.0001	7
AE	Overnigh	1:15	35.7740000	2.1989825	<.0001	8
AE	Overnigh	1:20	37.2323333	2.1989825	<.0001	9
D	2h	1:10	17.6486667	2.1989825	<.0001	10

SC	Time	SL	TPC LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
D	**2h**	**1:15**	13.2143333	2.1989825	<.0001	11
D	**2h**	**1:20**	23.8393333	2.1989825	<.0001	12
D	**4h**	**1:10**	18.6310000	2.1989825	<.0001	13
D	**4h**	**1:15**	28.3036667	2.1989825	<.0001	14
D	**4h**	**1:20**	31.3393333	2.1989825	<.0001	15
D	**O**	**1:10**	23.6306667	2.1989825	<.0001	16
D	**O**	**1:15**	30.5653333	2.1989825	<.0001	17
D	**O**	**1:20**	32.5296667	2.1989825	<.0001	18
E	**2h**	**1:10**	26.6663333	2.1989825	<.0001	19
E	**2h**	**1:15**	33.2736667	2.1989825	<.0001	20
E	**2h**	**1:20**	41.9046667	2.1989825	<.0001	21
E	**4h**	**1:10**	21.9940000	2.1989825	<.0001	22
E	**4h**	**1:15**	24.3746667	2.1989825	<.0001	23

SC	Time	SL	TPC LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
E	**4h**	**1:20**	27.9463333	2.1989825	<.0001	24
E	**O**	**1:10**	30.0296667	2.1989825	<.0001	25
E	**O**	**1:15**	40.1786667	2.1989825	<.0001	26
E	**O**	**1:20**	44.7313333	2.1989825	<.0001	27

*SC- Solvent composition; Time- extraction time; SL- Solid to liquid ratio; 2h- Two hours extraction; 4h- Four hours extraction; O- Overnight extraction; LSMEAN- least-square means.

* Multiple comparisons was conducted according to Tukey's test; the significance level was set as 0.0001 by SAS statistical package. All tests were done in triplicates.

From the table above (Table A4), we can see that the most optimum condition for maceration was 70% ethanol (v/v); Solid to liquid ratio of 1:20; Overnight extraction.

A.3 SAS code for RSM

```
Option is = 100;

data predict;

input x1 x2 x3 TPC;

cards;

-1 1 0 53.84

1 1 0 34.82

-1 -1 0 49.55

1 -1 0 21.61

-1 0 -1 46.07

1 0 -1 25.36
```

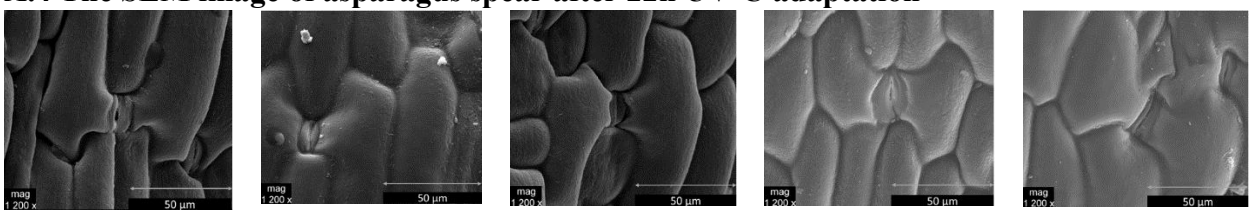
```
-1 0 1 76.25
1 0 1 45.36
0 -1 -1 38.48
0 1 -1 42.23
0 -1 1 63.13
0 1 1 70.71
0 0 0 43.21
0 0 0 40.80
0 0 0 43.48
0 0 0 43.04
0 0 0 42.77
;
proc print;run;
proc glm;
model TPC=x1 x2 x3;run;

/*plots=(surface)*/
ods graphics on;
proc rsreg data=predict plots=(surface);
model TPC=x1 x2 x3/lackfit;
run;
ods graphics off;

/*surface(3D)*/
ods graphics on;
proc rsreg data=predict plots=surface(3D);
model TPC=x1 x2 x3/lackfit;
run;
ods graphics off;
```

```
/*plot all*/
ods graphics on;
proc rsreg data=predict plots=all;
model TPC=x1 x2 x3/lackfit;
run;
ods graphics off;
```

A.4 The SEM image of asparagus spear after 22h UV-C adaptation



Control-freshly purchased | 0.45 kJ/m^2 UV-C | 1.13 kJ/m^2 UV-C | 2.27 kJ/m^2 UV-C | Control- 22h after purchase

*mag- magnification 1200x; Size 50 µm.

A.5 UHPLC analysis for asparagus extracts

UHPLC profile of asparagus crude extracts (2mg/mL). The leading flavonoid (rutin) is expressed as Peak Number-Compound Name-Retention Time (min).

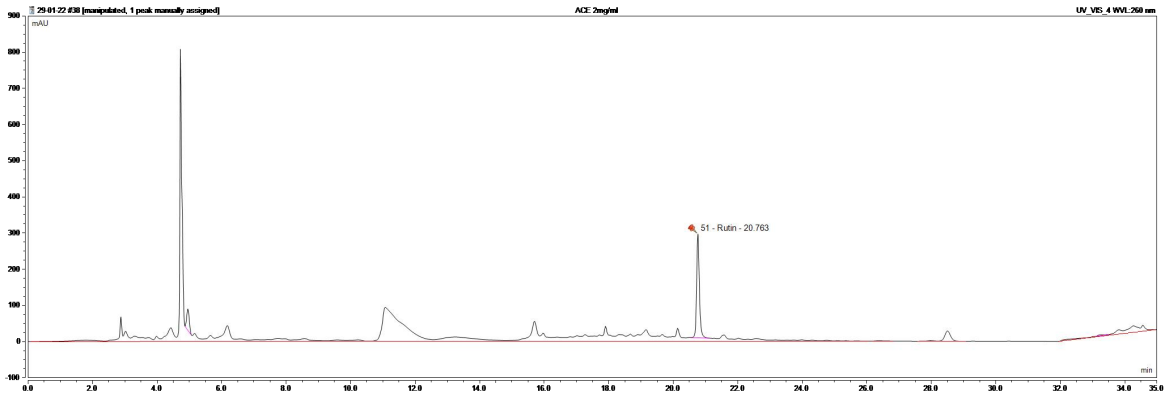


Figure A5. HPLC-DAD chromatogram of asparagus extracts recorded at 260nm.

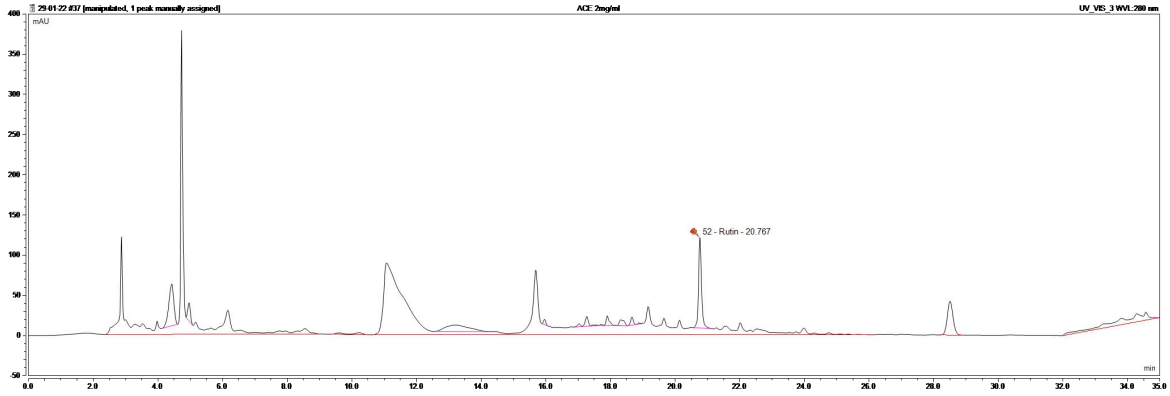


Figure A6. HPLC-DAD chromatogram of asparagus extracts recorded at 280nm.

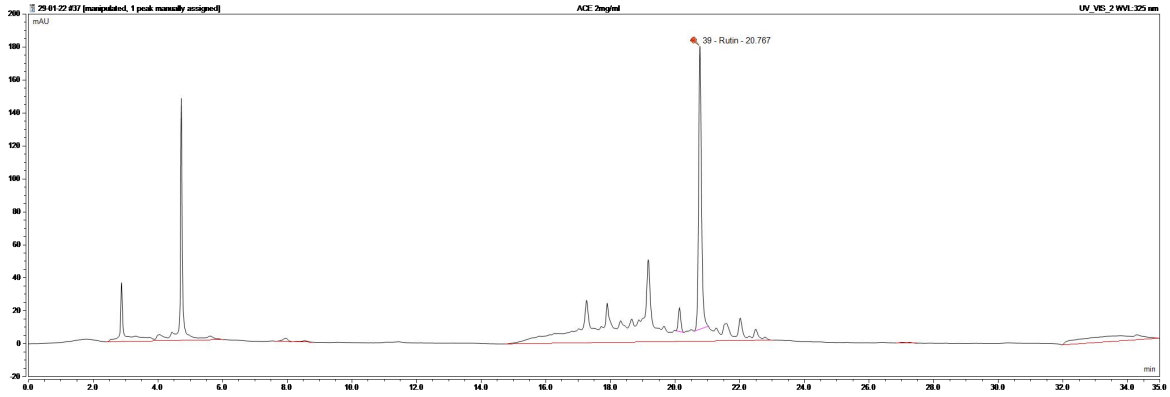


Figure A7. HPLC-DAD chromatogram of asparagus extracts recorded at 325nm.

Table A5 HPLC detection of rutin standard (260nm)

Rutin concentration (ug/mL)	**mAU*Area**
80	29.0964
120	51.0393
160	100.5766
200	190.3122
320	366.7132

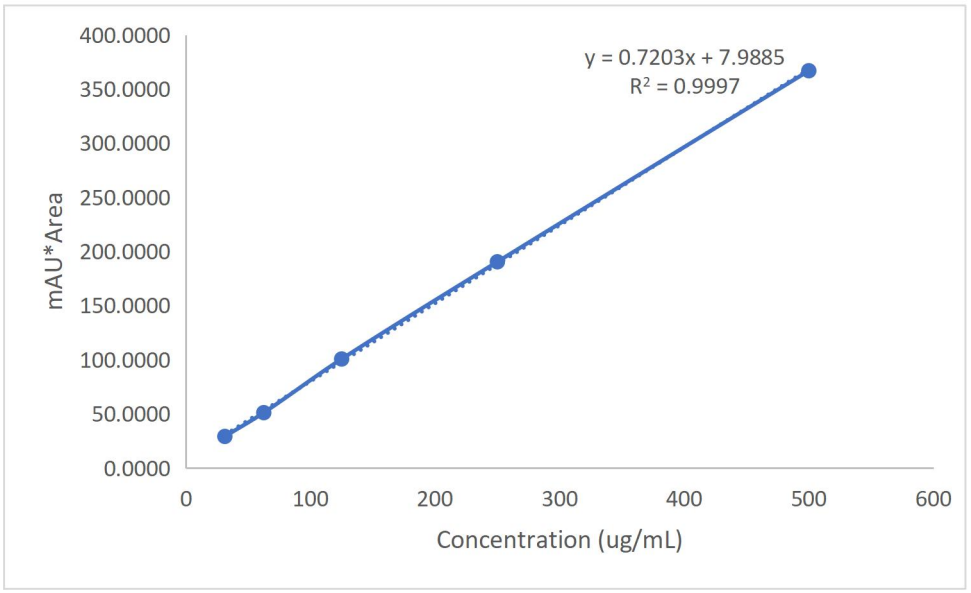


Figure A8. The standard curve of rutin solution (HPLC grade).

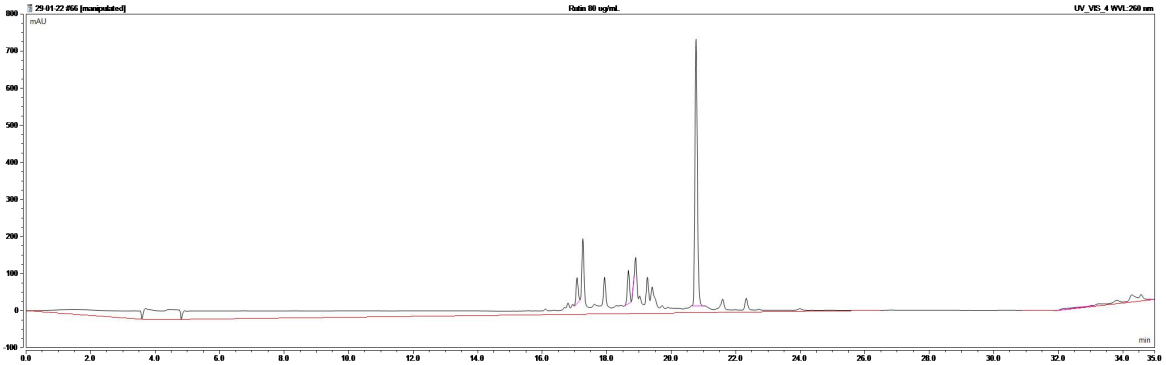


Figure A9. HPLC-DAD chromatogram of rutin standards (80 µg/mL) recorded at 260nm.

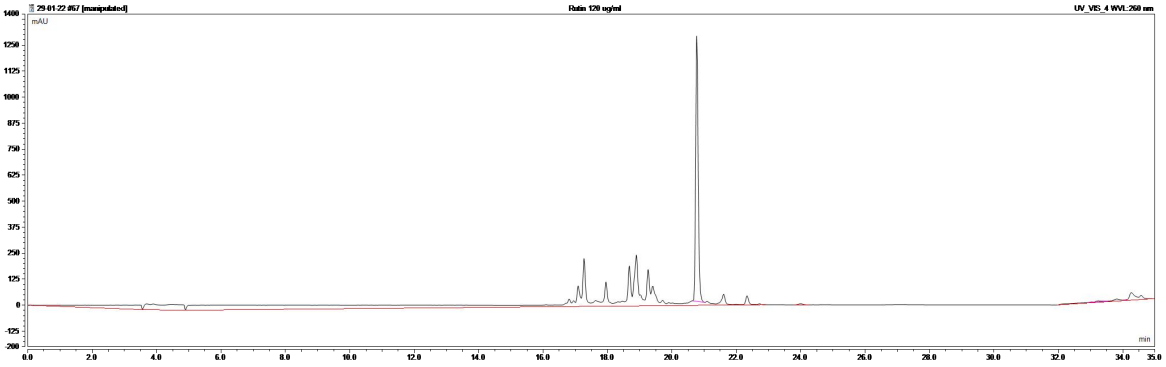


Figure A10. HPLC-DAD chromatogram of rutin standards (120 µg/mL) recorded at 260nm.

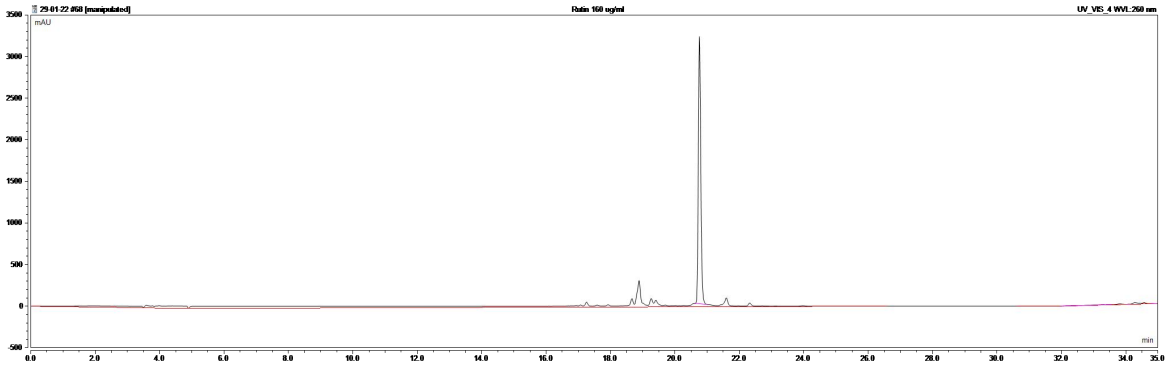


Figure A11. HPLC-DAD chromatogram of rutin standards (160 µg/mL) recorded at 260nm.

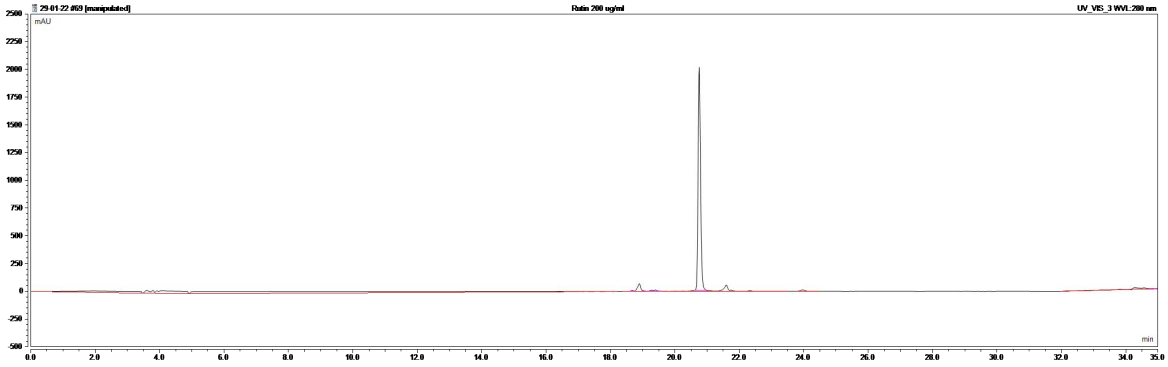


Figure A12. HPLC-DAD chromatogram of rutin standards (200 µg/mL) recorded at 260nm.

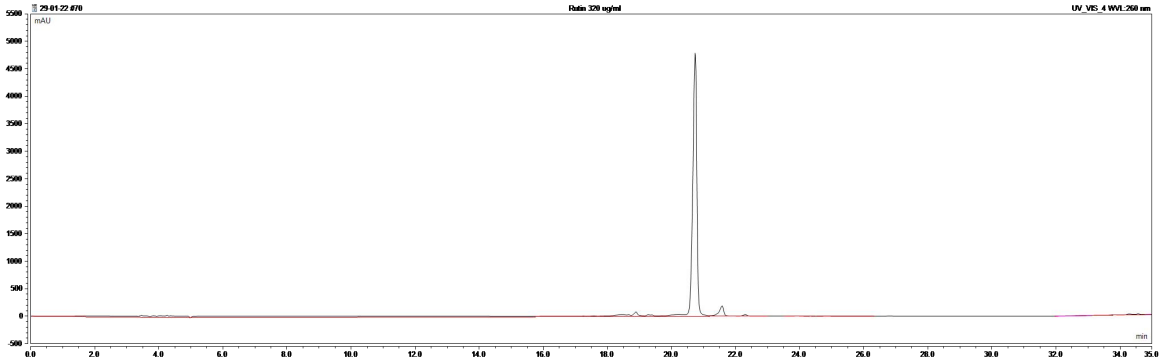


Figure A13. HPLC-DAD chromatogram of rutin standards (320 µg/mL) recorded at 260nm.

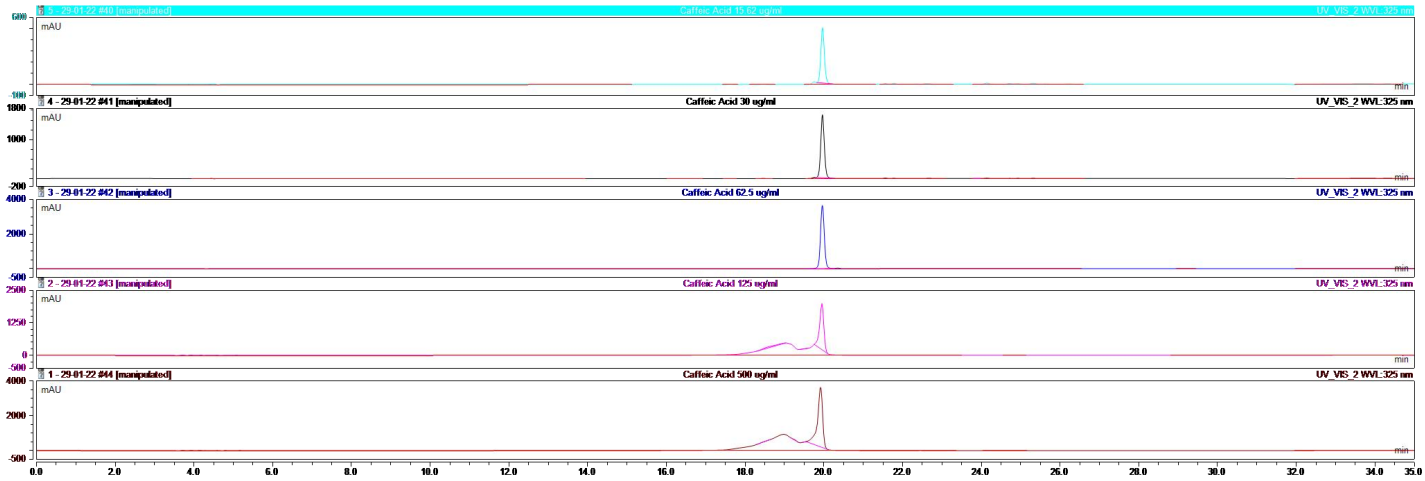


Figure A14. HPLC-DAD chromatogram of caffeic acid standards (from top to bottom are separately 15.625, 31.25, 62.5, 125, 250, and 500 µg/mL) recorded at 260 nm.