A STUDY OF FLAVONOLS IN BOK CHOY AND THEIR ANTI-CANCER PROPERTIES

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ABSTRACT

Phytochemicals are defined as biologically active, non-nutrient (i.e. not essential for the maintenance of life), plant compounds. They are present in all fruits, vegetables, grains, and other plant foods, and have been shown to be beneficial to human health. Flavonols are a group of phytochemicals present in a variety of different vegetables including broccoli, Brussels sprouts, cabbage, cauliflower, kale and bok choy. Flavonols have been shown to exhibit powerful antioxidant activity as well as possess potential protective properties against certain cancers. The aim of this research was to measure the levels of the major flavonols in bok choy and to assess the antiproliferative activity of crude bok choy extracts, selected fractions, and individual flavonol compounds on human colon cancer cells (HT-29) *in vitro*.

The flavonol composition of three bok choy cultivars (Sumo, Karate, and Miyako) was determined after acid hydrolysis by HPLC-PDA/ESI-MS^n. Kaempferol (85.5 – 122 mg/100 g DW), isorhamnetin (38.3 – 66.7 mg/100 g DW), and quercetin (10 – 20.6 mg/100 g DW) were the main flavonols present. The Miyako variety contained the highest levels of both quercetin and isorhamnetin, however, the levels of kaempferol were comparable in all three cultivars. The total flavonol aglycone content in the three bok choy cultivars did not vary significantly (183.3 mg/100 g DW, 159.9 mg/100 g DW, and 197.3 mg/100 g DW for Sumo, Karate, and Miyako respectively), therefore, no conclusions were made as to whether one bok choy cultivar may contain more health-promoting flavonols than another.

Several glycoside and hydroxycinnamic acid derivatives of quercetin, isorhamnetin, and kaempferol were identified in the alkaline and hydroalcoholic bok choy extracts by HPLC-PDA/ESI-MS^n. Two flavonol-3-sophoroside-7-glucosides and three flavonol-3,7-diglucosides were identified in the bok choy after alkaline hydrolysis, and six complex flavonol glycoside-hydroxycinnamic acid derivatives, two flavonol di-glycosides, and one flavonol mono-glucoside were identified in the hydroalcoholic extracts. Kaempferol-3-*O*-sophoroside-7-*O*-glucoside was purified by preparative HPLC and SPE and fully characterised by NMR, UV and MS^n.

The ability of crude bok choy extracts, selected fractions, and individual flavonol compounds to inhibit cell proliferation of the human colon adenocarcinoma cell line, HT-29, *in vitro* was assessed using the MTT assay. The antiproliferative effects on HT-29 cells was evident for all extracts/fractions/compounds examined, however, the crude bok choy extracts were found to be the most potent. The IC_{50} values for the three bok choy cultivars ranged between 1.58 – 4.01 mg/L after 72 hours of exposure. Preliminary results suggested that bok choy has the potential to positively influence human health, both from a chemopreventive perspective as well as a chemotherapeutic.

DECLARATION

I, Aida Isić, declare that the PhD thesis entitled "A study of flavonols in bok choy and their anti-cancer properties" is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Signature:  Date: 31/08/2017

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LIST OF ABBREVIATIONS AND ACRONYMS

1D	One-Dimensional
2D	Two-Dimensional
3Q	Triple Quadrupole Mass Spectrometer
APCI	Atmospheric Pressure Chemical Ionisation
API	Atmospheric Pressure Ionisation
APPI	Atmospheric Pressure Photo Ionisation
ATCC	American Type Culture Collection
BHT	Butylated Hydroxytoluene
BrdU	5'-bromo-2'-deoxy-uridine
CC	Column Chromatography
CCC	Countercurrent Chromatography
CE	Capillary Electrophoresis
C-H HECTOR	Heteronuclear Chemical Shift Correlation
CID	Collision Induced Dissociation
COSY	Correlation Spectroscopy
CPC	Centrifugal Partition Chromatography
CRMs	Certified Reference Materials
% CV	Percent Coefficient of Variation
CZE	Capillary Zone Electrophoresis
DMSO	Dimethyl Sulfoxide
DMSO-d_6	Deuterated Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPI	Department of Primary Industries, Australia (now DEPI and DEDJTR)
DW	Dry Weight
ECD	Electrochemical Detector
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ESI	Electrospray Ionisation
FAB	Fast Atom Bombardment
FBS	Fetal Bovine Serum
FID	Flame Ionisation Detector
FIDs	Free Induction Decays
FL	Fluorescence Detector
FW	Fresh Weight

GC	Gas Chromatography
HMBC	Heteronuclear Multiple-Bond Correlation
HMQC	Heteronuclear Multiple-Quantum Coherence
HPLC	High Performance Liquid Chromatography
HSQC	Heteronuclear Single-Quantum Correlation
HT-29	Human Colorectal Adenocarcinoma Cell Line
IC_{50}	Inhibitory Concentration (at which 50% inhibition is observed)
IT	Ion Trap Mass Spectrometer
LLE	Liquid-Liquid Extraction
LOD	Limits of Detection
LOQ	Limits of Quantification
LPD	Lactase Phlorizin Hydrolase
MAE	Microwave Assisted Extraction
MALDI	Matrix Assisted Laser Desorption/Ionisation
MAP	Modified Atmosphere Packaging
MDLC	Multidimensional Liquid Chromatography
MEKCC	Micellar Electrokinetic Capillary Chromatography
MeOH-d_4	Deuterated Methanol
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometer
MS^n	Multiple fragmentation mass spectrometer (refers to an IT MS in this study)
MSPD	Matrix Solid Phase Dispersion
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
MU	Measurement Uncertainty
m/z	Mass to Charge Ratio
NATA	National Association of Testing Authorities, Australia
NL	Normalised Level
NMR	Nuclear Magnetic Resonance Spectroscopy
NOESY	Nuclear Overhauser Effect Spectroscopy
PBS	Phosphate Buffered Saline
PDA	Photodiode Array Detector
PFE	Pressurised Fluid Extraction
PTFE	Polytetrafluoroethylene
QC	Quality Control
QqQ	Triple Quadrupole Mass Spectrometer
Q-TOF	Hybrid triple quadrupole and ion trap mass spectrometer
Q-TRAP	Hybrid triple quadrupole and time of flight mass spectrometer

RMs	Reference Materials
ROESY	Rotating Frame Overhauser Effect Spectroscopy
SCF	Supercritical Fluid Extraction
SD	Standard Deviation
SIM	Selected Ion Monitoring
S/N	Signal to Noise Ratio
SPE	Solid Phase Extraction
SRM	Selected Reaction Monitoring
T-25	25 cm^2 Cell Culture Flask
T-75	75 cm^2 Cell Culture Flask
TBHQ	*tert*-butylhydroquinone
TCD	Thermal Conductivity Detector
TIC	Total Ion Chromatogram
TMS	Tetramethylsilane
TOCSY	Total Correlation Spectroscopy
TOF	Time of Flight Mass Spectrometer
t_R	Retention Time
UAE	Ultrasound Assisted Extraction
UHPLC	Ultra-High Performance Liquid Chromatography
USDA	United States Department of Agriculture
USEPA	United States Environmental Protection Agency
UV	Ultraviolet
UV-Vis	Ultraviolet Visible

CHAPTER I
PREFACE

1.1 BACKGROUND

Traditionally, the benefits of grains, vegetables, and fruit have been associated with their nutrients, for example: proteins, lipids, carbohydrates, and vitamins. However, recent research suggests that the naturally occurring phytochemicals in these foods may also offer benefits to human health. Flavonoids are a particular group of phytochemicals which exhibit powerful antioxidant activity [1-4]. In addition to their antioxidant properties, flavonoids have displayed several interesting effects in animal models and *in vitro* systems. For example: they scavenge free radicals, regulate nitric oxide, decrease leukocyte immobilisation, induce apoptosis, inhibit cell proliferation and angiogenesis, and exhibit phytoestrogenic activity. These effects may contribute to their potentially protective role in certain degenerative diseases such as cancer [5-10].

Further work is necessary to discover new phytochemicals that are effective, not only as antioxidants, but also as anti-cancer agents. Comprehensive studies are also necessary to understand the biochemical pathways associated with these effects. These studies would provide invaluable information for consumers who recognise the importance of diet as a means of promoting health. In turn, this would increase economic benefits for producers and manufacturers. Similarly, continued research into the anti-cancer properties of these naturally occurring compounds would identify health promoting plant foods that could possibly contribute to the prevention of certain types of cancers, as well as offering the possibility of designing novel chemotherapy drugs that are derived from a natural source.

Flavonoids have been found in a variety of different plant foods and are prominent within the *Brassicaceae* plant family, including broccoli, Brussels sprouts, cabbage, cauliflower, kale and bok choy. Flavonoids are further classified into flavones, flavanols (catechins), isoflavones, flavonols, flavanones, and anthocyanins. The need to correctly identify and determine the levels of flavonoids and their glycosylated and acylated derivatives in various fruits and vegetables is crucial in understanding the health benefits of plants containing these compounds. Different varieties of the same vegetable, or fruit, can contain varying levels of flavonoids, implying that certain varieties of a fruit or vegetable may be healthier, as they may contain higher amounts of bioactive compounds. Bok choy (*Brassica rapa* L. ssp. *chinensis*), also commonly known as pak choi, contains a wide variety of bioactive compounds including relatively high levels of the flavonols quercetin, isorhamnetin and kaempferol, however, little research has been conducted on the flavonol profile of different varieties of this vegetable.

The most frequently used analytical technique for flavonol analysis is HPLC-PDA/ESI-MS^n [11]. HPLC-PDA/ESI-MS^n has been used extensively to determine the total aglycone levels of flavonols in plants after hydrolysis of their respective glycosides, as well as identifying the complex sugar and acyl moieties attached to the aglycone without the need to isolate the compounds [12-15]. Nuclear magnetic resonance spectroscopy (NMR) is also used in conjunction with HPLC-PDA/ESI-MS^n for structure elucidation.

Biological activities, such as antiproliferative effects of flavonols, are often assessed by using cultured cells as tissue models. Human colorectal carcinomas have been used in many studies to assess such effects, including the popular cell line HT-29, due to the direct exposure of the intestinal tract to dietary components. Colorectal cancer is also the second most commonly diagnosed cancer in both men and women in Australia, hence any advancements towards the prevention and/or treatment of this type of cancer would be invaluable. In this context, this study aimed to explore the antiproliferative effects of crude bok choy extracts, selected fractions, and various flavonol compounds on HT-29 cells *in vitro*.

A further detailed review of the research conducted within this area will be presented and referenced in the literature review in the following chapter.

1.2 AIMS AND OBJECTIVES

Bok choy is becoming increasingly popular in the Australian diet. Characterising the flavonols present in different varieties of bok choy and determining which of these flavonol compounds may be efficacious would provide more reliable data on the potential health benefits of bok choy. This represents a pro-active or preventative approach to healthcare. If certain varieties of bok choy are more beneficial to human health than others, consumers can be educated to make more informed choices about which varieties to include in their diet.

The main aims of this research were:

1. To measure the levels of the major flavonols present in three bok choy cultivars and to identify the naturally occurring glycosylated and acylated flavonol derivatives by HPLC-PDA/ESI-MS^n and NMR, and

2. To assess the anti-cancer properties of these compounds, as well as hydroalcoholic extracts of bok choy, by investigating their antiproliferative activity on a human colon cancer cell line *in vitro*.

The specific objectives of this work were:

- To determine the total flavonol aglycone content of three different cultivars of bok choy, after acid hydrolysis, using HPLC-PDA/ESI-MS^n.
- To identify and characterise the flavonol glycosides and the flavonol glycoside-hydroxycinnamic acid derivatives (as well as any other naturally occurring flavonol conjugates) in three different cultivars of bok choy, after alkaline hydrolysis and hydroalcoholic extraction respectively, using HPLC-PDA/ESI-MS^n.
- To isolate and purify the major flavonol glycosides from an alkaline hydrolysate and the major flavonol glycoside-hydroxycinnamic acid derivatives from a hydroalcoholic extract, using preparative HPLC, and confirm the structures of any purified compounds by NMR spectroscopy.
- To assess the antiproliferative effects of three cultivars of crude bok choy extracts on HT-29 cells *in vitro.*
- To assess the antiproliferative effects of quercetin, kaempferol and isorhamnetin aglycones and mono-glucosides, as well as any purified flavonol conjugates/fractions isolated from bok choy, on HT-29 cells *in* vitro, both individually and in various combinations.

1.3 OUTLINE OF THESIS

There are seven chapters in this thesis. Chapter I (Preface) provides a brief overview of the context of this study and the aims and objectives.

Chapter II (Literature Review) presents a review of the research on phytochemicals in fruits, vegetables, and other plant foods, with a particular focus on phenolic compounds and flavonols in *Brassica* species. The chemical analysis of these compounds in plants is summarised, including sample preparation, extraction, purification, and various instrumental analytical techniques. Previous research on the anti-cancer properties of these naturally occurring plant compounds is also presented, with a particular focus on cell culture models (especially the HT-29 cell line) and antiproliferative activities of the flavonol compounds that are of interest to this study and *Brassica* specie plant extracts.

The experimental design, and materials and methods, applied for the analyses are detailed in Chapter III (Materials and Methods).

There are three main results and discussion chapters: chapters IV, V, and VI. Method optimisation and other preliminary studies are reported and discussed in Chapter IV. Chapter V (Identification and Quantification of Flavonols in Bok Choy Cultivars) reports and discusses the results of the chemical analysis and structure elucidation of flavonol conjugates present in three bok choy cultivars. Following on from this study, Chapter VI (Cancer Cell Studies) reports and discusses the results of the antiproliferative effects of flavonol compounds, selected fractions, and bok choy extracts on HT-29 human colorectal adenocarcinoma cells *in vitro*.

In Chapter VII (General Discussion, Conclusions and Future Directions), the main results are briefly discussed, the conclusions are summarised, and future directions are suggested.

CHAPTER II
LITERATURE REVIEW

2.1 INTRODUCTION

Research on phytochemicals in fruits, vegetables, and other plant foods, can be divided into two main areas; the analytical chemistry of phytochemicals in plants, and their bioactivities both *in vitro* and *in vivo*. This chapter will review literature in both these areas.

Sections 2.2-2.4 provide some general information regarding phytochemicals, their classification, and health benefits, with a specific focus on the prevention and management of colorectal cancer. This is followed by further information on the particular phytochemicals of interest to this study; phenolic acids and flavonoids, which can be further subdivided into hydroxycinnamic acids and flavonols respectively. Section 2.4 then provides more detailed information concerning the plant family that is of interest to this study; *Brassicaceae*, and more specifically the *Brassica* species and the vegetable bok choy.

Sections 2.5 and 2.6 address the analytical chemistry of phytochemicals in plants, including methodologies used for extraction, purification, and instrumental analysis for quantification and structure elucidation.

Section 2.7 reviews the literature on the bioactivities and anti-cancer properties of flavonols such as antiproliferative effects on cell culture models. This is inclusive of a review on studies that investigated the three flavonols of interest in this study: quercetin, kaempferol, and isorhamnetin, as well as the effects of plant extracts, in particular *Brassica* species, on cancer cells *in vitro*.

Section 2.8 provides a brief summary of the information covered in this chapter.

2.2 PHYTOCHEMICALS

Phytochemicals are defined as biologically active non-nutrient plant compounds ('phyto' is from the Greek word meaning plant) and are present in all fruits, vegetables, grains, as well as other plant foods [16]. Nutrients, e.g. vitamins, minerals, proteins, carbohydrates and lipids are substances that are essential for the human body; for the maintenance of life, growth, and nourishment, however, non-nutrients are not required for the body to function. The type and quantity of phytochemicals present in plant foods varies significantly from source to source. There have been more than 5,000 different phytochemicals identified to-date in an extensive variety of plant foods and it is estimated that thousands more have yet to be discovered [17].

Phytochemicals are secondary plant metabolites and many of these are produced via shikimate and chorismate biosynthesis from the essential precursor L-phenylalanine, an amino acid with an aromatic ring [18-19]. The synthesis of these compounds involves a series of reactions including deamination, enzymatic conversion, and enzymatic hydroxylated reactions [20], which generates a variety of phenolic phytochemicals with one or more phenolic rings (Figure 2.1). Based on the number and pattern of phenolic rings, these phytochemicals may be relatively simple structures such as phenolic acids, which consist of a single phenolic ring, or more complex structures known as polyphenolics and tannins, which consist of two or more phenolic rings, respectively. These compounds can also conjugate with different moieties including sugars, long carbon chains, and phytosterols to form complex phenolic derivatives.

Within the plant kingdom, phytochemicals have been found to play important roles in plant metabolism in addition to being key players in defence mechanisms and disease resistance. Their major physiological function is to defend against oxidative and environmental stresses including protection against UV radiation, microbes, pathogens, and parasites [21]. They can also be responsible for the colours in plants; for example, anthocyanins (Figure 2.2), are plant pigments that give fruits and vegetables their black, purple, blue, red, or orange colour. In addition to colour they also contribute to the taste in fruits and vegetables, e.g. flavanols, flavanones, and flavones can be responsible for the astringency, bitterness or spicy taste of edible plants [22-23].

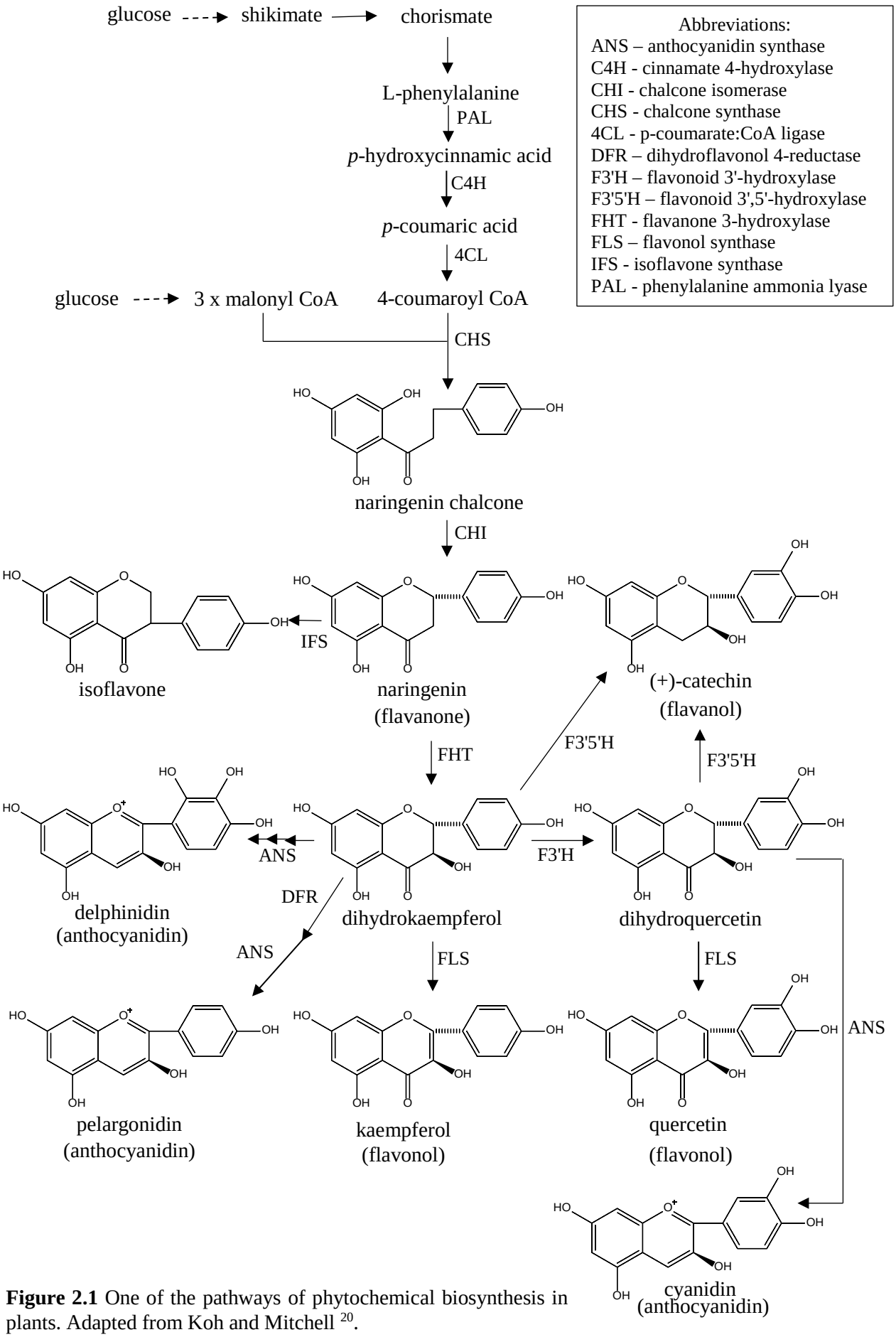


Figure 2.1 One of the pathways of phytochemical biosynthesis in plants. Adapted from Koh and Mitchell [20].

2.2.1 Classification

Phytochemicals can be classified into the following five groups; carotenoids, phenolics, alkaloids, nitrogen-containing compounds, and organosulfur compounds. Figure 2.2 depicts a flow diagram of the most common classes of dietary phytochemicals. The phenolics group are the most abundant in the human diet and consist of an aromatic ring with one or more hydroxyl moieties attached. The group can be further subdivided into several classes according to their structure, i.e. the number of phenol rings they contain, as well as the structural elements that bind these rings [24-25]. The main dietary classes of phenolics consist of the following five groups; phenolic acids (which comprises both hydroxybenzoic and hydroxycinnamic acids), flavonoids, stilbenes, coumarins, and tannins. The flavonoid group can be further subdivided into six major classes; flavonols, flavones, flavanols (also known as catechins), flavanones, anthocyanidins, and isoflavonoids. These compounds consist of a C_6–C_3–C_6 structure, whereas the classes that belong to the non-flavonoid groups are classified based on the number of carbons in their structure [24]. In general, phytochemicals occur naturally as glycosylated conjugates in plants and not in their aglycone form (an aglycone is the compound remaining after the glycosyl group is replaced by a hydrogen atom).

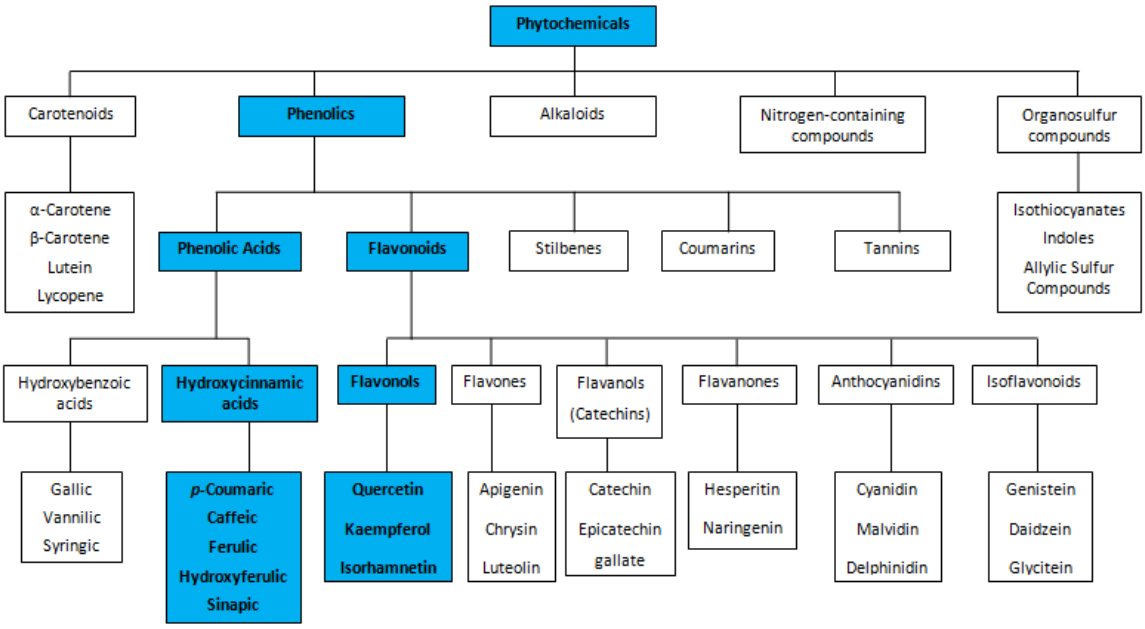


Figure 2.2 Flow diagram showing classification of dietary phytochemicals adapted from Liu [16]. Phytochemicals of interest in this study are highlighted in blue.

2.2.2 Health Benefits

Increasing evidence suggests that the phytochemicals present in edible plants may also be beneficial components for human health, either individually or in combination with essential nutrients such as proteins, lipids, carbohydrates, and vitamins [17].

Many studies support the pharmacological activities of phytochemicals in humans, including anti-inflammatory, anti-allergic, antiviral, antimicrobial, anti-hepatotoxic, anti-osteoporotic, antispasmodic, antiulcer, antidiabetic, estrogenic/antiestrogenic, anxiolytic, analgesic, vasodilating, cardioprotective, neuroprotective, and anticancer activities. The most widely studied activity, however, is their antioxidant properties [26]. Antioxidants are substances that inhibit the generation of oxidation-initiating free radicals. This inhibition helps to prevent or delay oxidation reactions such as lipid peroxidation, which is one of the most important actions of free radicals that leads to the damage of cell membranes and, ultimately, cell death [27]. The antioxidant function of phytochemicals stems from the hydroxyl groups attached to the phenolic rings, which donate electrons to free radicals in order to stabilise them in a system. The phenolic radicals that are formed are relatively stable due to resonance occurring on the phenolic ring preventing the initiation of a new free radical chain reaction. Furthermore, the phenolic radical intermediates can react with other free radicals within the system to terminate the chain reaction. Phytochemicals can also suppress reactive oxygen and nitrogen species formation by deactivating related enzymes and chelating metal ions that produce free radicals. The antioxidant activity of a phenolic compound is directly related to its structure and the number of free hydroxyl groups on its phenolic rings [17].

In addition to their antioxidant activities, many phytochemicals alter cell signalling pathways and gene expression and therefore play important roles in preventing various chronic diseases such as cancer, diabetes and obesity, cardiovascular diseases, and lowering blood cholesterol [28]. As more and more consumers become aware of the secondary health benefits of foods, it is becoming increasingly important to understand and utilise these naturally occurring, powerful antioxidant phytochemicals in health-promoting food and other products, as well as investigate the possibility of natural treatments and preventions for chronic diseases. A list of possible health benefits for some common phytochemicals is shown in Table 2.1.

Table 2.1 Potential health benefits of some phytochemical compounds and their sources in plant foods [29, 17, 30-31, 23, 32-35, 25, 36, 8, 6].

Phytochemical	Food Source	Possible Health Benefits
Isoflavones	Soy beans, soy milk, tofu and other legumes	Lower blood pressure and increase vessel dilation, reduce the formation/progression of cancer (especially estrogen-sensitive cancers, i.e. breast, ovarian, colon, prostate), decrease total cholesterol and cardiovascular disease, decrease triglycerides and thrombosis, reduce onset of Alzheimer's disease and osteoporosis
Anthocyanidins and anthocyanins (glycosylated anthocyanidins)	Berries (strawberries, blackberries, raspberries, blueberries, cranberries, bilberries), cherries, plums, pomegranates, red grapes, red wine, red apples, red potatoes, red radishes, red onions, beans and egg plants	Improve vision, lower blood pressure, protect LDL cholesterol oxidation, display anti-inflammatory and antimicrobial activity. Act as antioxidants, inhibit nitric oxide production, induce apoptosis and therefore provide protection against cancer and heart disease. Decrease platelet aggregation, display neuroprotective effects, may prevent obesity and diabetes
Flavanones	Citrus fruits (grapefruit, oranges, lemons and limes), tomatoes and mint	Provide protection against cardiovascular disease and cancer, improve bone health and lower cholesterol. Resist neurodegenerative diseases, e.g. Alzheimer's disease.
Flavanols and proanthocyanidins (oligomers of flavanols)	Red wine, grapes, cocoa, tea, cranberries, apples, plums, pears, mangoes, peaches, okra and Swiss chard	Inhibit LDL oxidation, decrease platelet aggregation, antioxidant activity, inhibit cellular oxygenases, antimutagens, decrease tumour initiation/promotion, induce apoptosis, inhibit pro-inflammatory responses in the arterial wall
Flavones	Apples, cereals, beets, peppers, Brussels sprouts, cabbage, cauliflower, celery, parsley, thyme, chives, kale, lettuces, spinach, tomatoes and watercress	Prevent coronary heart disease, anti-inflammatory, antimicrobial, anti-cancer activities
Flavonols	Onions, apples, berries, grapes, red wine, tea, broccoli, cabbages, bok choy, kale, leeks, chives, peppers, tomatoes, spinach, Swiss chard and watercress	Strong antioxidant activity, chelate free radical-producing metals, prevent coronary heart disease, decrease total cholesterol, decrease risk of dementia, protect against LDL oxidation and atherosclerosis, antimutagens, decrease tumour initiation/promotion, induce apoptosis and decrease platelet aggregation
Phenolic acids (e.g. hydroxycinnamic acids)	Kiwi fruit, blueberries, plums, cherries, apples, pears, chicory, artichokes, carrots, lettuce, eggplant, wheat, coffee and tea	Protect against oxidative damage diseases, e.g. heart disease, stroke, cancer

Phytochemical	Food Source	Possible Health Benefits
Stilbenes	Grapes, wine, peanuts, blueberries, bilberries and cranberries	Prevent damage to blood vessels, decrease platelet aggregation/thrombosis and prevent blood clots, reduce LDL cholesterol, antioxidant activity, carcinogen detoxification, antimutagen, decrease tumour initiation/promotion
Coumarins	Apricots, strawberries, cinnamon and cherries	Anticoagulant, antibacterial, anti-fungal, vasodilators, anti-tumour, anti-HIV, anti-hypertension, anti-inflammatory, anti-arrhythmia, antiseptic, anti-osteoporosis and analgesic, also used to treat asthma
Tannins	Berries, grapes, wine, persimmons, lentils, tea and chocolate	Powerful antioxidants and may reduce the risk of cancer
Carotenoids	Carrots, tomatoes, sweet potatoes, pumpkins, squash, apricots, peaches and dark leafy green vegetables	Neutralise free radicals that cause cell damage, act as antioxidants; may reduce the risk of cancer, heart disease, age-related macular degeneration. May enhance the immune system response
Glucosinolates/ Isothiocyanates	Cruciferous vegetables such as broccoli, brussels sprouts, cabbage, cauliflower, mustard greens, turnip greens, kale and horseradish	Neutralise free radicals that cause cell damage and protect against some cancers, decrease tumour initiation/promotion, act as antioxidants and may increase the activity of enzymes that function in the detoxification and elimination of toxins
Sulfides and Thiols	Garlic, onions, leeks, olives and scallions	Decrease total cholesterol and LDL cholesterol, decrease triglycerides, decrease cholesterol and fatty acid synthesis, decrease blood pressure, decrease thrombosis, antioxidant activity, carcinogen detoxification and decrease tumour promotion

2.2.2.1 Colorectal Cancer

Colorectal cancer is the fourth most common malignant tumour worldwide [37] and the second most commonly diagnosed cancer in both men and women in Australia [38]. It is estimated that one in twelve Australians will develop colorectal cancer in their lifetime, with the disease claiming the lives of almost 4,000 Australians every year [39].

Colorectal cancer, also known as bowel (colon and rectum) cancer, generally develops via a multistage process in which a series of cellular mutations occur over time. The most common type of bowel cancer is adenocarcinoma. Adenocarcinoma forms in mucus-secreting glands throughout the body, and in the case of colorectal cancer, develops in the intestinal gland cells that line the inside of the colon and/or rectum. In the early stages, the abnormal epithelial cells mutate to form benign polyps which may then undergo additional mutations and become benign adenoma and, ultimately, a malignant cancer. Later stages of colorectal cancer can spread to other sites in the body through the lymphatic or vascular system [40].

The majority of recent epidemiological, *in vitro*, and *in vivo* studies have confirmed that a diet rich in vegetables, fruits, and legumes containing naturally occurring antioxidant phytochemicals correlates with a reduced risk in cancers [17], in particular colorectal cancer. Table 2.2 lists a number of epidemiological studies on the incidence of colorectal and other related cancers and its association with the consumption of phytochemicals through fruits, vegetables, and other plant foods/beverages in the diet. Many of the *in vitro* and *in vivo* studies have shown plausible mechanisms, such as regulating specific signalling pathways and molecular markers [41], by which phytochemicals may be possible agents for the prevention and treatment of cancers. Such studies will be discussed later in this chapter and will focus primarily on phenolic compounds.

Table 2.2 Epidemiological studies that have investigated the relationship between a diet high in fruits and vegetables containing phytochemicals and the incidence of colorectal, and other related, cancer.

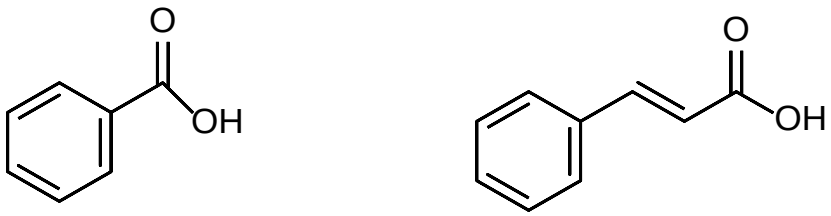
Type of Study	Cancer	Dietary Phytochemical	Reference
Case-control	Gastric	Carotenoids (α-carotene, β-carotene, lutein, lycopene) and flavonoids (quercetin, kaempferol, myricetin, luteolin)	Garcia-Closas *et al.*[42]
Case-control	Colorectal	Flavonols, procyanidins, catechins, flavanones	Kyle *et al.*[43]
Prospective cohort	Colorectal	General fruit and vegetable intake	Van Duijnhoven *et al.*[44]
Prospective cohort	Colorectal	Total flavonoids, quercetin, kaempferol, myricetin	Lin *et al.*[45]
Prospective cohort	Colorectal	Flavonols, flavones, flavanones, flavanols, anthocyanins	Nimptsch *et al.*[46]
Case-control	Colorectal	Total flavonoids, anthocyanidins, flavanols, flavanones, flavones, flavonols, isoflavones, theaflavins and thearubigins, proanthocyanidins	Xu *et al.*[47]
Meta-analysis of 23 studies (10 prospective cohort and 13 case-control)	Stomach and colorectal	Total flavonoids, flavonols, flavanones, flavanols, anthocyanins, procyanidins, isoflavones	Woo and Kim[48]
Meta-analysis of 18 studies (9 prospective cohort and 9 case-control)	Colorectal	Total flavonoids, flavones, flavonols, flavanones, flavanols, anthocyanins, isoflavones, procyanidins	He and Sun[49]

2.3 PHENOLICS

Phenolics are a large group of phytochemicals and are widespread in plants, especially in fruits, vegetables and associated beverages, and are of considerable interest due to their powerful antioxidant properties. These compounds consist of an aromatic ring with one or more hydroxyl groups, and their structures can range from a simple phenolic molecule to a complex high-molecular weight polymer [24]. Their antioxidant ability depends on their structure, in particular the number and location of the hydroxyl groups, and other groups/moieties that may be attached to the aromatic rings. As mentioned previously, phenolics are sub-divided into five different classes; phenolic acids, flavonoids, stilbenes, coumarins, and tannins. Both the phenolic acid and flavonoid groups are of particular interest to this study and will be discussed in greater detail.

2.3.1 Phenolic Acids

Phenolic acids are divided into two subgroups: hydroxybenzoic and hydroxycinnamic acids. They are found in a range of different fruits, vegetables, and grains, although the hydroxybenzoic acid content of edible plants is generally very low, except for certain red fruits, black radish, and onions. Hydroxycinnamic acids on the other hand are much more common, with the most commonly occurring compounds in edible plants being *p*-coumaric, caffeic, ferulic, and sinapic acids [24-25]. They are secondary metabolites that are synthesised in plants as a response to environmental stress as the plant grows, for example the presence of insects and pathogens, physical lesion, and UV radiation [21]. The amino acid L-phenylalanine is the pre-cursor for the synthesis of phenolic acids via the shikimate and chorismate pathway. After several reactions including deamination, enzymatic conversion, and enzymatic hydroxylation, L-phenylalanine is converted to different hydroxylbenzoic acids and other phenolic acids [17]. Their generic structure is based on either a benzoic acid or cinnamic acid core (Figure 2.3). Hydroxybenzoic and hydroxycinnamic acids are formed when one of the positions on the benzoic acid/cinnamic acid aromatic ring is occupied by a hydroxyl group. The remaining four positions on the aromatic ring can be further substituted with other groups, e.g. methoxyl or hydroxyl groups. The chemical properties of the hydroxybenzoic acid and hydroxycinnamic acid derivatives differ, for example, hydroxybenzoic acids generally have a maximum UV absorption between 240 to 280 nm whereas hydroxycinnamic acids display maxima in the UV spectrum between 300 and 330 nm. Compared to polyphenolics, such as flavonoids, phenolic acids are more water-soluble and more bioavailable in the human body. Hydroxycinnamic acids, such as caffeic and ferulic acid for example, are directly absorbed and circulated into the bloodstream [17].

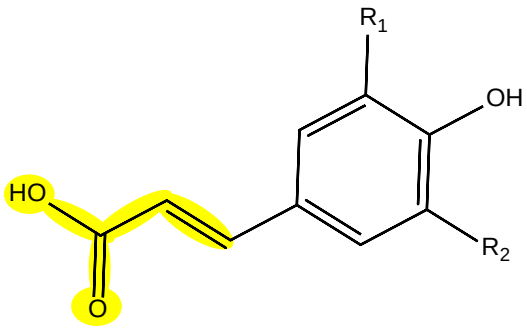


Benzoic acid Cinnamic acid

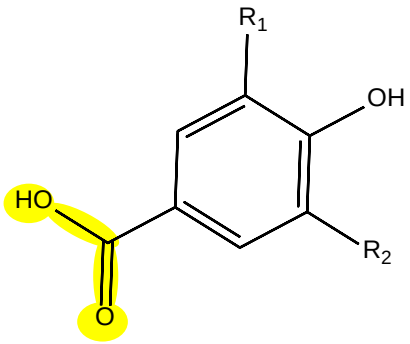
Figure 2.3 Chemical structures of benzoic acid and cinnamic acid; the primary structures of all phenolic acids.

2.3.1.1 **Hydroxycinnamic Acids**

Hydroxycinnamic acids are present in all parts of fruits and vegetables, however, the highest concentrations have been found to be present in the outer part of mature fruits. It has been reported that the total concentration of hydroxycinnamic acids increases as the size of the fruit increases (i.e. matures), although a decrease in concentration is observed as the fruit ripens [50, 24-25]. Figure 2.4 shows the generic structures of a hydroxycinnamic acid and hydroxybenzoic acid along with a list of some of the more common dietary phenolic acids and their respective R groups. Hydroxycinnamic acids exhibit higher antioxidant activity than hydroxybenzoic acids due to the greater proton donating ability and radical stabilisation of the CH=CH-COOH than the -COOH group in the corresponding hydroxybenzoic acids [51] (refer to highlighted groups in Figure 2.4).



Hydroxycinnamic Acid	R_1	R_2
p-Coumaric acid	H	H
Caffeic acid	H	OH
Ferulic acid	H	OCH_3
Hydroxyferulic acid (or Methoxycaffeic acid)	OH	OCH_3
Sinapic acid	OCH_3	OCH_3



Hydroxybenzoic Acid	R_1	R_2
Vanillic acid	OCH_3	H
Gallic acid	OH	OH
Syringic acid	OCH_3	OCH_3

Figure 2.4 Some common hydroxycinnamic and hydroxybenzoic acid structures.

In nature, hydroxycinnamic acids rarely exist in the free form, except for some processed foods that have undergone fermentation, sterilisation, or freezing. For example, if fruits are stored over a long period of time, a mass hydrolysis of hydroxycinnamic derivatives to free acids leads to advanced senescence of the fruit and the formation of malodorous compounds such as vinyl phenols [24-25]. In general, they exist naturally as glycosylated derivatives or acylated to other phytochemicals such as flavonols or anthocyanins, as well as esters of tartaric acid, quinic acid, shikimic acid, or malic acid [50]. The most abundant hydroxycinnamic acid in vegetables and fruits, both in the free form and bound to other molecules, is caffeic acid. Foods that contain the highest

quantities of caffeic acid include coffee, blueberries, blackberries, cranberries, sweet potatoes, potatoes, carrots and lettuce. Other foods such as prunes, peaches, apples, orange juice, grapes, and tomatoes contain smaller amounts. Ferulic acid is also present in some fruits and vegetables such as broccoli, eggplant, asparagus, some berries, orange juice, carrots, potatoes, beetroot, apples, and coffee, although is much less common than caffeic acid [24-25]. Ferulic acid is the most abundant hydroxycinnamic acid in cereal grains. Wheat grain can contain approximately 800-2000 mg/100 g dry weight of ferulic acid and it is found primarily in the outer parts of the grain[24].

A German study [52] estimated that the daily consumption of hydroxycinnamic acids in a Bavarian subgroup from the German National Food Consumption Survey was 211 mg/day. The primary source was from coffee, with caffeic acid being the most abundant compound; it's intake alone was 206 mg/day [52]. The intake of hydroxycinnamic acids therefore varied according to coffee consumption. Regular coffee drinkers who might drink several cups a day may ingest between 500-800 mg of hydroxycinnamic acids per day, whereas those that do not drink coffee, and who also consume small amounts of fruits and vegetables, may only ingest as little as 25 mg/day [25].

2.3.2 Flavonoids

Flavonoids are one of the most widespread groups of phytochemicals and account for approximately half of the eight thousand naturally occurring plant phenolic compounds [10]. Flavonoids contribute to the coloured pigments found in flowers but also act as enzyme inhibitors as a defence system in plants against ultraviolet radiation exposure and insects, as well as chelating agents against metals that are harmful to plants. Furthermore, they are involved in processes such as photosensitisation and energy transfer morphogenesis and sex determination, photosynthesis and regulation of plant growth hormones [27].

Flavonoids are low molecular weight compounds comprising fifteen carbon atoms that are arranged in a C_6–C_3–C_6 configuration. The C_6–C_3–C_6 structure arises from two aromatic rings, A and B, connected by a three-carbon bridge which commonly forms a third pyran ring, C [25, 36] (Figure 2.5). The synthesis of these compounds occurs via a series of condensation reactions between a hydroxycinnamic acid and malonyl residues (refer to Figure 2.1). The hydroxycinnamic acid forms the B-ring and carbon atoms 2, 3 and 4 of the C-ring, while the malonyl residues produce the A ring [11].

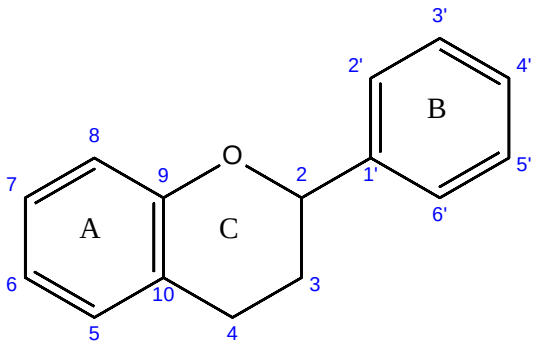
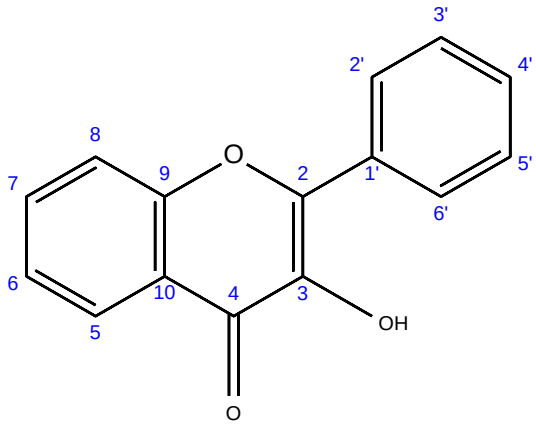


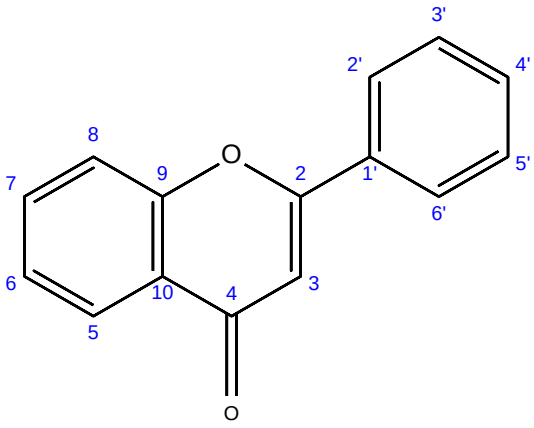
Figure 2.5 Generic structure of a flavonoid molecule

Studies on the structure-antioxidant activity of these compounds have identified several structural elements that contribute to their powerful antioxidant properties. These include the C2=C3 double bond (flavonols, flavones, and isoflavones), a keto group on the carbon in position 4 (flavonols, flavones, flavanones, and isoflavones), a hydroxyl group on the carbon in position 3 (flavonols, flavanols, and anthocyanidins), and an ortho-diphenolic structure (catechol) group in the B-ring (found in some variations of flavonoids) [53]. See Figure 2.6 for the structures of different flavonoid subclasses.

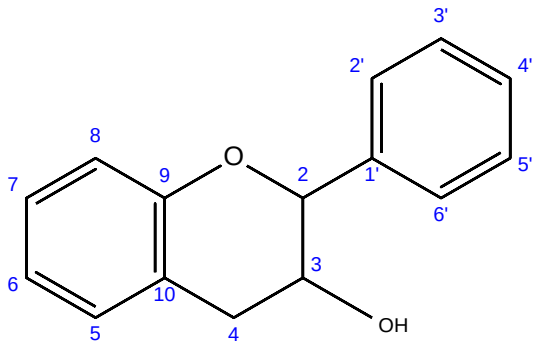
Variations in the three-carbon segment (ring C) such as cyclisation, degree of unsaturation, and oxidation leads to the classification of the different flavonoid subclasses, of which there are six major groups (shown in Figure 2.6). There are also further groups, e.g. dihydroflavonols, flavan-3,4-diols, chalcones, dihydrochalcones, and aurones that make a smaller contribution to the flavonoid component of the human diet [54]. Flavones and flavonols are the most widely occurring and structurally diverse compounds [10]. In 2012, a study reported that 523 flavone, 509 flavonol, 438 flavanone, 300 isoflavone, 292 chalcone, and 24 anthocyanidin aglycones had been identified in the plant kingdom [17].



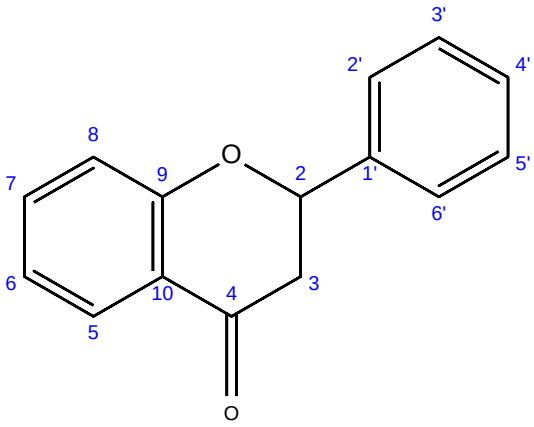
Flavonols



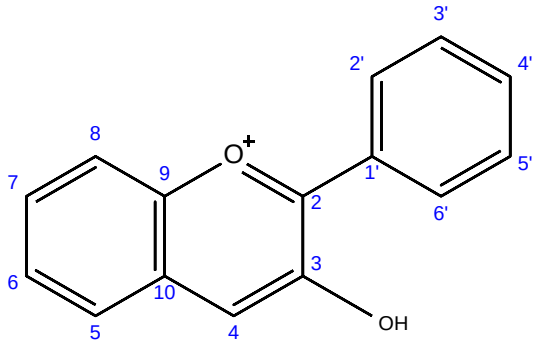
Flavones



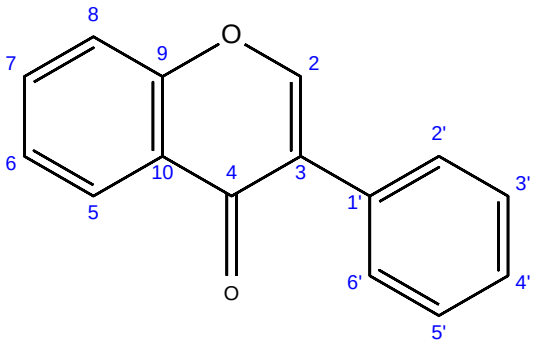
Flavanols



Flavanones



Anthocyanidins



Isoflavones

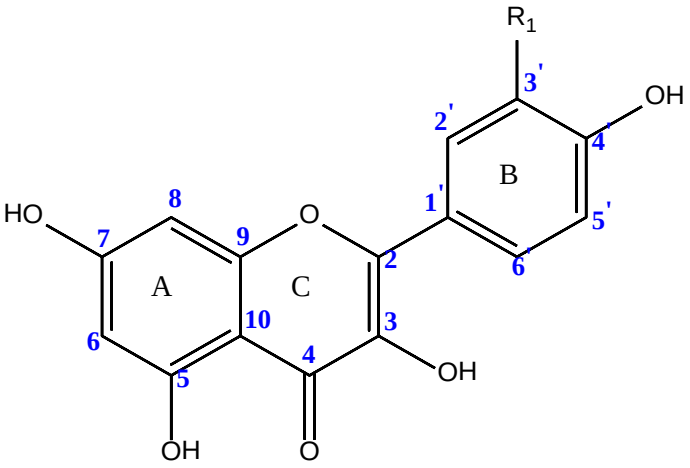
Figure 2.6 Generic structures of the six major dietary flavonoid subclasses.

Substitution on the A and B rings generate the different compounds within each subclass of flavonoids. The most common substitutions in naturally occurring flavonoids are hydroxylation, methylation and glycosylation. Others include oxygenation, alkylation, acylation, and sulfation [55-56], as well as prenyl, isoprenyl, sulfate, benzyl, methylenedioxy, aromatic and aliphatic acid groups [10]. Most flavonoids have a sugar moiety conjugated to a hydroxyl group, with glucose being the most common sugar. Other sugars include galactose, rhamnose, xylose, and arabinose [24]. Theoretically, any of the hydroxyl groups of a flavonoid compound can be glycosylated. However, certain positions are favoured in certain subclasses, e.g. flavonols and flavanols are commonly glycosylated at the C3 and C7 positions whereas the C3 and C5 positions are favoured for anthocyanidins, and the hydroxyl group on the C7 position is the most common site for glycosylation for flavones, flavanones, and isoflavones [17].

Flavonoids occur in all parts of plants. Flavonoid aglycones are generally found in substances secreted by the plant or wax on leaves, as well as barks and buds. In most cases, however, flavonoids are present as glycosides in cell vacuoles of flowers, leaves, stems, and roots [27]. The flavonoid becomes less reactive and more water-soluble when glycosylated and is generally thought to provide a protective function in plants; e.g. to protect cytoplasmic damage, and to also store the flavonoids safely in the cell vacuole [17]. Most compounds have between one and three glycosyl moieties, however, there have been some naturally occurring flavonoid compounds identified with four and five moieties [57]. Flavonoids can occur as either *O* or *C*-glycosides in nature, however, the most common dietary flavonols, kaempferol, quercetin, isorhamnetin, and myricetin, exist mainly as *O*-glycosides. *O*-glycosides have one or more hydroxyl groups attached to the aglycone core through a glycosidic O—C bond, which is an acid-labile hemiacetal bond. Whereas for *C*-glycosides, glycosylation takes place via a direct linkage of the sugar moiety and the flavonoid aglycone core structure with an acid-resistant C—C bond. Acylated glycosides, where one or more of the sugar hydroxyls are esterified with an acid, are common in nature. Although there are limited variations in flavonoid aglycone structures, the various substitutions that are possible result in a vast array of compounds. Substantial variation of flavonoid types can occur in the same foods due to the impact of environmental, seasonal, and agricultural productions as well as other factors, e.g. differences between cultivars of the same species [24].

2.3.2.1 **Flavonols**

Flavonols are polyphenolic compounds present in many food products, medicinal plants and spices. In general, their structure comprises of two aromatic rings and a double bond between C2 and C3, as well as a carbonyl group at the C4 position. Flavonols will always have a hydroxyl group at the C3 position and often have four or more phenolic hydroxyl groups [24] (Figure 2.7). They have a planar structure which allows for intermolecular interactions and the formation of stacked complexes with anthocyanins (pigments in fruits and vegetables) and are therefore considered co-pigments [25].



Flavonol	R_1
Kaempferol	H
Quercetin	OH
Isorhamnetin	OCH_3

Figure 2.7 Chemical structures of some common flavonol aglycones.

Flavonols commonly exist in nature as *O*-glycosidic combinations with mono- and disaccharides e.g. glucose, galactose, rhamnose, xylose and arabinose, although combinations with a higher number of sugars can also occur (Section 2.3.2.). The sugar moiety is most commonly conjugated at the C3 position of the unsaturated C-ring of the flavonol aglycone. Furthermore, naturally occurring flavonols are often found conjugated to uronic acids, predominantly glucuronic acid, e.g. strawberries and grapes contain quercetin and kaempferol 3-*O*-glucuronides and spinach contains flavonol-methyl ether and methylendioxy glucuronides (spinatoside and jaceidin glucuronide) [58]. Acylation of flavonol-glycosides with monocarboxylic (e.g. acetic, malic) and dicarboxylic acids (e.g. malonic, tartaric), as well as aromatic acids (primarily hydroxycinnamic acids such as caffeic, *p*-coumaric, ferulic, and sinapic) is also common. In some instances, flavonols are conjugated with sulfate e.g. palm dates [59]. Flavonol aglycones are present in low levels in some foods as they are more susceptible to oxidative degradation.

The synthesis of flavonols is stimulated by sunlight, therefore the majority of flavonols accumulate in the outer tissues (i.e. skin and leaves) of fruits and vegetables [25, 24]. The flavonol concentration within difference pieces of fruit on the same tree, and even on different sides of the same piece of fruit, can vary significantly depending on the amount of sunlight exposure. Similarly, it was reported that the flavonol glycoside concentration in leafy vegetables such as lettuce and cabbage was ten times higher in the green outer leaves of the vegetable, as compared to the light-coloured inner leaves. This phenomenon also explains why there is a higher content of flavonols in cherry tomatoes than that of standard tomatoes, due to the difference in proportions of skin to whole fruit [25]. At least 279 and 347 different glycosides have been reported for quercetin and kaempferol respectively [24].

2.3.2.1.1 Levels in Food

The dietary intake of flavonols has been estimated to be approximately 20-25 mg/day in several countries, e.g. the United States, Denmark, and Holland, whereas in Italy, the levels ranged from 5-125 mg/day with a mean of 35 mg/day [25]. Quercetin and kaempferol are the main flavonols present in commonly consumed vegetables, followed by myricetin and isorhamnetin (a methyl ether of quercetin) [60].

2.3.2.1.2 Quercetin

A wide variety of foods contain high levels of quercetin. Capers contain the highest amount of quercetin (234 mg/100 g) [29], with lovage leaves (170 mg/100 g), radish leaves (70 mg/100 g), rocket (66 mg/100 g), coriander leaves (53 mg/100 g), hot yellow peppers (51 mg/100 g), and fennel leaves (49 mg/100 g) being good sources of quercetin [29]. Other vegetables that contain a relatively high content of quercetin include onions, okra, watercress, kale, and asparagus. As for beverages, elderberry juice concentrate has been reported to contain high levels of quercetin at 108 mg/100 g.

2.3.2.1.3 Kaempferol

Capers contain the highest amount of kaempferol (260 mg/100 g, edible portion) [29]. The spice saffron (*Crocus sativus*) is also very high in kaempferol containing 205 mg/100 g, and kale (*Brassica oleracea acephala*) contains 47 mg/100 g [29]. Other vegetables containing relatively high amounts of kaempferol ranging from 22 to 38 mg/100 g include mustard greens (*Brassica juncea*), arugula (*Eruca sativa*), ginger (*Zingiber zerumbet*), beans (*Phoasealus vulgaris* cv. Zolfino), onions (*Allium fistulosum*), watercress (*Nasturtium officinale*), Chinese cabbage, and radish seeds (*Raphanus sativus*) [29]. Fruits do not contain very high levels of kaempferol.

2.3.2.1.4 Isorhamnetin

Isorhamnetin is a methoxylated derivative of quercetin, as well as the major metabolite of quercetin in plasma. It is not as ubiquitous in the plant kingdom as quercetin and kaempferol. According to the United States Department of Agriculture database for the flavonoid content of selected foods (release 3.1, 2013), Parsley (*Petroselinum crispum*) contains the most isorhamnetin (331 mg/100 g) [29]. Dill weed also contains a relatively large amount of isorhamnetin (43 mg/100 g). Sea buckthorn berry contains 38 mg/100 g, kale (24 mg/100 g), and mustard greens contain 16 mg/100 g.

2.3.2.1.5 Health Benefits of Flavonols

Quercetin, which is the main aglycone in human nutrition, is a potent free radical scavenger and antioxidant, and is thought to protect humans against several types of cancer and cardiovascular diseases [61-63, 53, 64-66]. Quercetin can also act as a pro-oxidant, and can induce apoptosis in cancerous cells, thereby preventing tumour proliferation. It has also demonstrated other important properties such as modulation of genes that are related to cell cycle, signal transduction, and xenobiotic metabolism. High intakes of kaempferol result in a lower risk of coronary heart disease, and isorhamnetin demonstrated distinct vasodilatory effects in animal models, suggesting vascular protective effects in human cardiovascular diseases [67]. Quercetin, kaempferol and isorhamnetin also exhibit anti-inflammatory effects, and quercetin and kaempferol show chemopreventive properties in various tumours and synergistically suppress cell proliferation in human gut cancer cell lines [68]. The health-promoting activity of flavonoid glycosides (e.g. antioxidant activity) and their characteristics (e.g. bioavailability) depend on their structural composition, including glycosylation and acylation [69, 25, 70].

2.4 EFFECT OF PRE AND POST-HARVEST CONDITIONS ON PHENOLIC PROFILES OF FRUITS AND VEGETABLES

Different varieties of the same fruit or vegetable can contain different concentrations of phenolic compounds. These differences can be due to the cultivar of the plant itself, as well as an array of other factors both pre-harvest, such as: genetic and environmental factors, maturity or ripeness at the time of harvest, UV light exposure, and post-harvest conditions, such as: storage and processing procedures [71]. Agricultural practices including culture in greenhouses or fields, organic vs. conventional farming, biological culture, hydroponic culture, use of insecticides [72] and fertilisers [73] and fruit yield per tree can all significantly affect the phenolic profile of a plant. Furthermore, environmental factors can also modify the phenolic content. These include geographic location, elevation, soil type [74] and mineral nutrients, sun/UV radiation exposure [75-77], temperature [78-79], rainfall, growing season, climate, and maturity. In many cases, fruits and vegetables are harvested prematurely for transporting long distances and storing for longer periods of time [71].

As mentioned previously in this chapter, the synthesis of phenolic compounds such as flavonols and phenolic acids is stimulated by sunlight, therefore the outer layers of fruits and vegetables (i.e. skins and leaves) tend to contain the highest concentration [25, 24]. This was demonstrated by Tsao *et al.* [80] on eight different cultivars of apple which showed that the peel contained higher concentrations of polyphenolic compounds compared to the flesh. The total polyphenolic content was also higher in fruits and vegetables that were grown using organic or sustainable agriculture compared to the levels in plants grown in conventional or hydroponic conditions, without the natural stresses from the environment [81]. Other studies have also examined the effects of temperature and UV irradiation on phenolic content [77 79, 76, 78, 75].

Most research on the post-harvest storage of fruits and vegetables has surprisingly found that the conditions applied do not in fact effect the phenolic content of the plant, e.g. studies on the cold storage of apples [82-84], pears [85], and onions [86] reported that there were no significant changes to phenolic profiles. Furthermore, there were no negative effects from modified atmosphere packaging (MAP) and cold storage on the total flavonoid content of Swiss chard and spinach [87-88]. In contrast, some studies did observe a negative effect on phenolic content because of cold storage. Hakkinen *et al.* [89] investigated the effects of storing five berries at -20°C for 9 months and reported a substantial decrease in quercetin content (40%) in bilberries and lingonberries, but not in red raspberries and black currants. The authors also reported that myricetin and kaempferol were more prone to losses during storage than quercetin.

A comprehensive review by Bjorkman *et al.* [90] includes sections on the influence of agro-ecosystems, climate, and light on compounds in *Brassicaceae* plants. In addition, a recent paper

by Francisco *et al.* [91] reviewed the effects of numerous pre and post-harvest conditions on the bioactive compounds found in *Brassica* foods and reported that "from seed to table, each and every factor counts."

2.5 *BRASSICACEAE* – THE MUSTARD FAMILY

The *Brassicaceae* family, also known as the mustard family and *Cruciferae*, consist of a wide range of flowering plants that are of considerable economic importance to humans. They are an important part of the human diet worldwide, especially in China, Japan, India, and European countries. The family is composed of 350 genera and about 3,500 species [92], which are characterised by simple leaves that are alternately arranged (many with a peppery flavour to them), and flowers with four petals and four sepals. The cross-shaped flowers (i.e. cruciform) is the reason they are commonly referred to as "crucifers" or "cruciferous." They feature two long and two short stamens and produce pod-like fruits known as siliques (long, thin fruits) and silicles (short, rounded fruits). Some genera that are included in the *Brassicaceae* family are *Arabis*, *Barbarea*, *Brassica*, *Camelina*, *Cardamine*, *Crambe*, *Iberis*, *Lepidium*, *Lunaria*, *Sinapis*, *Sisymbrium*, and *Thlaspi*. The genus *Brassica* is by far the most important and the popularity and consumption of *Brassica* species is increasing due to their nutritional value. A variety of *Brassica* vegetables have been found to reduce the risk of chronic diseases including cardiovascular diseases and cancer. These nutritive vegetables provide an array of health-promoting phytochemicals and nutrients such as vitamins, fiber, soluble sugars, minerals, carotenoids, glucosinolates, and phenolic compounds [93-94]. The most popular, and economically important, worldwide species within the *Brassica* genera include *Brassica oleracea* L., *Brassica rapa* L, *Brassica napus* L., and *Brassica juncea* L. (refer to Table 2.3 for a list of these species and their common names). Different parts of the same species such as their buds, inflorescences, leaves, roots, seeds, and stems, can be used depending on whether they fall into the oilseed, forage, condiment, or vegetable crop category. The main vegetable species is *Brassica oleracea* L., which consists of vegetable and forage forms such as kale, cabbage, Brussels sprouts, cauliflower, and broccoli. *Brassica rapa* L. includes vegetables such as bok choy, Chinese cabbage, and turnip, along with some forage and oilseed types. *Brassica napus* L. species are predominately used for oilseed, however, there are some forage and vegetable types such as leaf rape and nabicol. Finally, *Brassica juncea* L., along with two other species; *Brassica carinata* L. and *Brassica nigra* L., form the mustard group. These species are mainly used as a condiment, although leaves of the *Brassica juncea* L. are also consumed as vegetables [92].

The focus of this study was on the *Brassica rapa* L. species, in particular *Brassica rapa* L. ssp. *chinensis*, also known as bok choy.

Table 2.3 List of commonly consumed *Brassica* species and the plant part/organ used for consumption, adapted from Cartea *et al.* [92].

Species	Group	Common Name	Organ
Brassica oleracea	*acephala*	Kale, collards	Leaves
	capitata capitate	Cabbage	Terminal leaf buds (heads)
	capitata sabauda	Savoy cabbage	Terminal leaf buds (heads)
	costata	Tronchuda cabbage	Loose heads
	gemmifera	Brussels sprouts	Vegetative buds
	botrytis	Cauliflower	Inflorescences
	botrytis italica	Broccoli	Inflorescences
	gongylodes	Kohlrabi	Stem
	albogabra	Chinese kale	Leaves
Brassica rapa	*chinensis*	Bok choy, pak choi	Leaves
	dichotoma	Brown sarson, toria	Seeds
	narinosa	Chinese flat cabbage, wutacai	Leaves
	nipposinica	Mibuna, mizuna	Leaves
	oleifera	Turnip rape, rapeseed	Seeds
	pekinensis	Chinese cabbage, pe-tsai	Leaves
	perviridis	Komatsuna, Tendergreen	Leaves
	parachinensis	Choy sum	Leaves
	rapa	Turnip, turnip greens, turnip tops	Roots, leaves and shoots
	ruvo	Broccoleto	Shoots
	trilochularis	Yellow sarson	Seeds
Brassica napus	*pabularia*	Leaf rape, nabicol	Leaves
	napobrassica	Swede, rutabaga	Roots
Brassica juncea	*rugosa*	Mustard greens	Leaves
	capitata	Head mustard	Heads
	crispifolia	Cut leaf mustard	Leaves

2.5.1 Bok Choy – *Brassica rapa* L. ssp. *chinensis*

Brassica rapa L. ssp. *chinensis* has many English names, including non-heading Chinese cabbage, Chinese chard, Asian cabbage, celery mustard, Chinese mustard, Chinese mustard cabbage, Chinese white mustard cabbage, white cabbage, Chinese white cabbage, spoon cabbage, pak choi, baak choi, and bok choy; with pak choi and bok choy being the most common. It has been cultivated in China for centuries and has played a significant role in its cuisine, in addition to traditional Chinese medicine. Bok choy has glossy dark green leaves and thick white or green stalks in a loose head (Figure 2.8). It looks similar to cos lettuce (*Lactuca sativa* L. var. *longifolia*) on top and a large celery on the bottom, even though it's closest relative is the cabbage. The entire vegetable can be consumed and is often added raw to salads, in soups, steamed or boiled, stir fried as well as pickled. Young tender plants are harvested immaturely and often sold as "baby bok choy" and have a milder flavour. The leaves of the plant have a slight sweet flavour with a hint of mustard, whereas the stems tend to have a mild, almost bland flavour. An edible oil can also be obtained from the seed of the plant. Bok choy is a self-fertile plant, i.e. the flowers have both male and female organs, and are pollinated by bees. It is suitable for light, medium, and heavy soils and prefers well-drained, moist soil that is preferably alkaline. It can grow in semi-shade or full sun. The vegetable is gaining increasing popularity in Western diets and as a result has sparked recent interest within the scientific and agricultural fields. This study involved the analysis of the phenolic compounds present in three cultivars of bok choy grown in Victoria, Australia.

Figure 2.8 The vegetable bok choy, also commonly known as pak choi.

2.6 CHEMICAL ANALYSIS OF PHENOLICS IN PLANTS

Phenolic compounds and their chemical properties, including health benefits for humans, have been investigated extensively by scientists since the 1850's [17]. The chemical analysis of phenolics in plant products usually involves quantification of target compounds, as well as profiling and identifying complex conjugated derivatives. The analysis of these compounds generally consists of four steps; sample preparation, extraction, purification and instrumental analysis via a combination of chromatographic and spectroscopic techniques (Figure 2.9). Sample preparation includes obtaining a representative sample, some form of freeze-drying or air-drying, and particle size reduction. The analytes of interest can be extracted from the plant material using a variety of techniques and appropriate solvents or mixtures of solvents. These include pressurised fluid extraction (PFE), supercritical fluid extraction (SCF), ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), matrix solid-phase dispersion (MSPD), in addition to conventional solid-liquid extraction techniques. The purification step is performed to remove any other compounds that might interfere with the analysis, and further concentrate the key analytes. This can be achieved via several methods including liquid-liquid extraction (LLE), solid-phase extraction (SPE), column chromatography (CC), countercurrent chromatography (CCC), and semi-preparative HPLC. The sample extract is then filtered and ready for analysis. A variety of techniques are available for either qualitative or quantitative analysis including HPLC-PDA/MS, GC-MS, CE-PDA/MS and NMR, just to name a few.

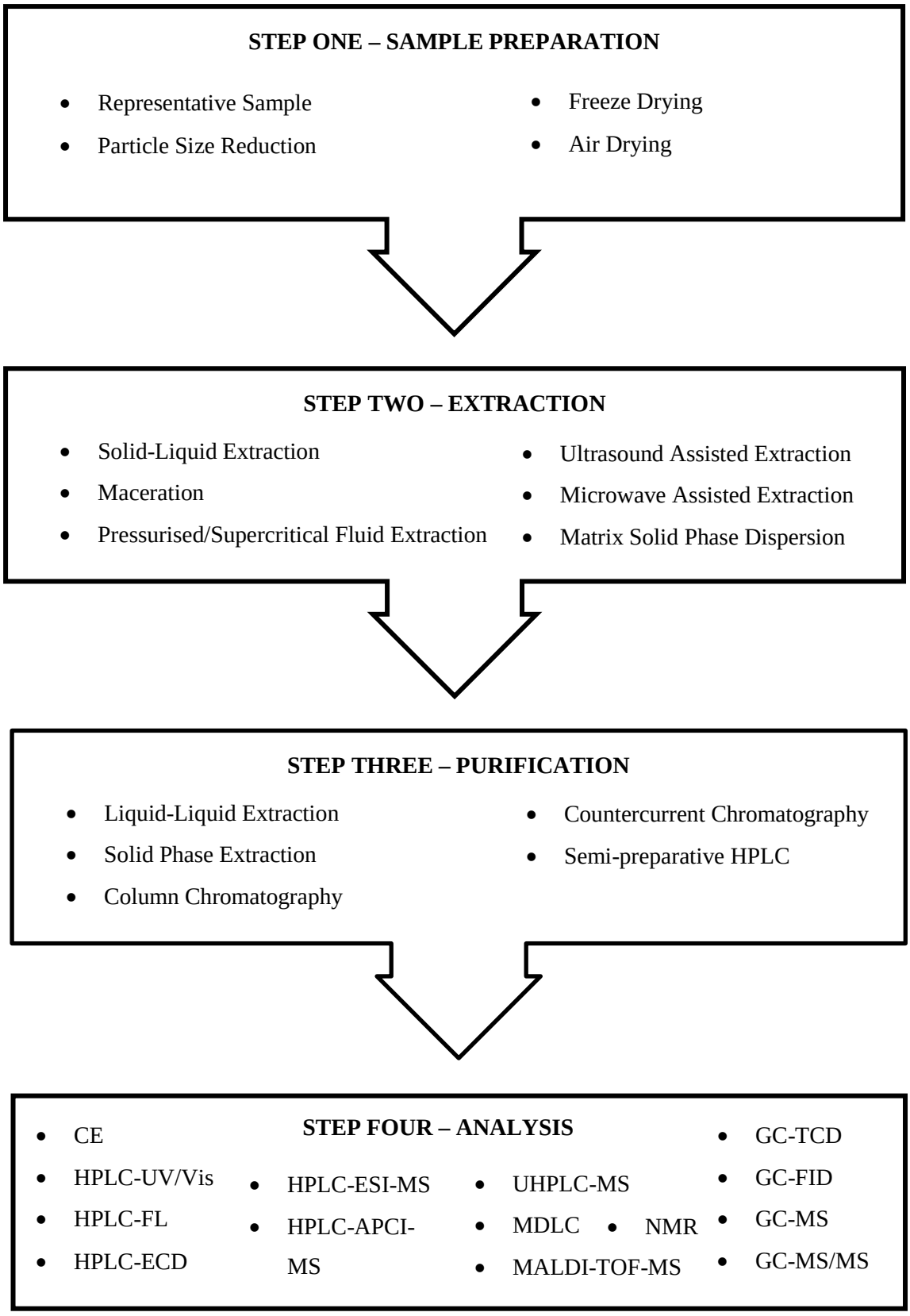


Figure 2.9 Four steps generally performed in the analysis of phenolics in plants. Adapted from Xu *et al.* [17].

2.6.1 Sample Preparation

The first step in the analysis of phenolic compounds in plant material is to select a representative sample of the fruit/vegetable/plant after harvesting. Once harvested it is necessary to consider the physical/structural complexity and the biological variability of the matrix to obtain a correct phenolic fingerprint of the fruit/vegetable/plant. Care must be taken during the collection, handling and storage of the plant material to avoid mechanical damage as this could trigger the release of endogenous enzymes, such as polyphenol oxidase or glycosidases that are stored in plastids, resulting in the degradation or alteration of the phenolic compounds into melanins, for example. [95-96].

To prevent enzymatic activity and preserve as much of the phenolic content of the plant material as possible, the samples are either air-dried or freeze-dried prior to extraction. Freeze-drying is the preferred method as it has been shown to preserve higher levels of the target compounds [97-98, 24, 81]. Newly-developed drying techniques, e.g. radiant zone drying and vacuum microwave drying, have shown promising results and similar retention of phenolic compounds as compared to freeze-drying [99-100].

Particle size reduction is another key element of sample preparation. This is usually achieved by grinding the freeze-dried sample into a fine powder. This increases the efficiency at which the target compounds are extracted by significantly reducing the distance that the compounds have to travel to reach the surface of the particle [101-102], as well as increasing the available surface area to maximise solid-liquid interaction. Furthermore, it has been shown that particle size reduction can disrupt the food matrix (plant vacuoles in which the compounds are stored) which may aid in the extraction of bound phenolic compounds [97]. Care must be taken, however, during the grinding process to minimise extreme heat on the sample and the time it is exposed to oxygen, as both factors could result in considerable losses of phenolic compounds.

The most prudent method for preserving phenolic compounds in plant tissues and increasing efficiency of extraction involves flash freezing with liquid nitrogen and lyophilisation (freeze-drying), followed by grinding to a fine powder. It is also highly recommended to store samples at sub zero temperatures and away from light, if extraction cannot be performed immediately.

2.6.2 Extraction

The second step in the analysis of phenolic compounds in plant material is the extraction of the compounds from the plant matrix. This needs to deliver an accurate fingerprint of the phenolic content of the sample. For liquid samples, extraction prior to analysis is not required, however, liquid-liquid extraction (LLE) may be performed to isolate a certain class of compounds. Aqueous mixtures of methanol, ethanol or acetone (where the organic solvent is typically present at highest percentage by volume) are commonly used to extract phenolic compounds from plant material [24, 103]. Aqueous mixtures are preferred to pure solvents because water hydrates the plant matrix and facilitates extraction of the compounds [17]. Antioxidants such as *tert*-butylhydroquinone (TBHQ), butylated hydroxytoluene (BHT), ascorbic acid, sulfites or enzyme inhibitors can also be added to a solvent extraction mixture to prevent degradation/oxidation of the compounds during extraction [96, 24].

Phenolic compounds are generally stored in the vacuoles of the plant cells, so it is therefore imperative that the extraction solvents can penetrate the cell walls to reach the target compounds. Maceration or homogenisation of the sample with a solvent is commonly used, however, newer techniques using high pressure and high temperature systems, such as pressurised fluid extraction (PFE) [104-105], supercritical fluid extraction (SCF) [106], ultrasound-assisted extraction (UAE) [107], microwave-assisted extraction (MAE) [108-109], and matrix solid-phase dispersion (MSPD) [110], are also used [17].

Maceration/homogenisation involves mixing the solid sample (e.g. freeze-dried plant material) with an aqueous mixture of solvent to facilitate the transfer of target compounds to the solvent. The resulting mixture is usually filtered or centrifuged, the supernatant containing the extracted phenolic compounds is collected, and the extraction process is repeated on the remaining residue. The choice of extraction method is highly dependent on the objective of the study; whether it is quantitative or qualitative, and what the compounds of interest are, i.e. free aglycones, glycosylated phenolic compounds, or more complex acylated phenolic glycosides.

A variety of extraction procedures are used to isolate the various conjugates of phenolic compounds in fruits/vegetables/plants; acid hydrolysis, for example, is performed to release the aglycone core from the complex glycoside/organic acid conjugates. A milder alkaline hydrolysis is performed to hydrolyse any conjugated hydroxycinnamic acids to produce the glycosylated phenolic compounds only, and a hydroalcoholic solvent extraction is conducted to obtain a full fingerprint of all the phenolic conjugates, both glycosylated and acylated, present within the plant sample.

2.6.2.1 **Acid Hydrolysis**

Acid hydrolysis of a plant sample involves refluxing the freeze-dried, powdered plant material in methanolic hydrochloric acid for a set number of hours. This cleaves the sugar and/or organic acid residues that are linked to the aglycone core for further studies, e.g. quantification or metabolite fingerprinting. There are numerous published methods that have utilised acid hydrolysis to quantify the phenolic aglycones in a variety of fruits and vegetables. Rochfort *et al.* [60] optimised an acid hydrolysis method for the extraction and quantification of flavonoid aglycones in 11 samples of freeze-dried, powdered pak choi. Sample size, acid concentration, and hydrolysis period were all optimised to maximise the optimal recovery of the compounds of interest. Red curly kale samples were hydrolysed with methanolic hydrochloric acid to obtain the flavonol and anthocyanidin aglycones for characterisation and quantification by Olsen *et al.* [111] and Lin and Harnly [112] analysed the phenolic aglycone content of 17 different *Brassica* vegetables after acid hydrolysis.

2.6.2.2 **Alkaline Hydrolysis**

Alkaline hydrolysis involves mixing the freeze-dried, powdered plant material with methanolic sodium hydroxide at room temperature overnight. The organic acid esters (e.g. hydroxycinnamic acids) are cleaved from the glycosylated phenolic compounds, therefore enabling the organic acids and phenolic glycosides to be characterised by HPLC-PDA/MS or other chromatographic/spectrophotometric techniques. This technique was used by Velasco *et al.* [113] and Francisco *et al.* [114], to identify the acyl flavonol derivatives in kale and cabbage varieties, and turnip extracts respectively. In another study, Rochfort *et al.* identified 9 individual flavonol compounds in a cultivar of pak choi by alkaline hydrolysis [60]. Alkaline hydrolysis cleaves acid moieties such as *p*-coumaroyl (*m/z* 146) and caffeoyl (*m/z* 162), which have the same molecular ion as rhamnosyl and hexosyl residues respectively and may, therefore, provide misidentification in MS analysis [17].

2.6.2.3 **Hydroalcoholic Extraction**

The purpose of this extraction method is to obtain all the phenolic conjugates present in the plant material, including all phenolic glycosides and any acylated phenolic glycosides. Phenolic compounds are extracted from plants and food products using a mixture of water and either methanol or ethanol. Methanol is more frequently used than ethanol due to its higher extraction efficiency, even though both have a similar polarity [24]. In general, the solvent:plant ratios are between 5:1 and 10:1, and the phenolic compound recovery during the first extraction can reach yields of up to 90-95% of the total phenolic content of the sample [17]. The procedure usually involves maceration using a high-speed blender, followed by ultrasonication of a mixture of

freeze-dried, powdered plant material in aqueous methanol/acidic aqueous methanol at room temperature for 30 minutes. Extracts are then centrifuged and filtered, or simply filtered under vacuum, prior to analysis. Rochfort *et al.* [60] extracted the phenolic conjugates in freeze-dried, powdered immature leaves of pak choi with 20% aqueous methanol by stirring and sonication, followed by filtration under vacuum. Whereas, Harbaum *et al.* extracted the naturally occurring phenolic compounds in pak choi using a slightly different hydroalcoholic extraction method. The freeze-dried and ground plant material was extracted four times with acidic aqueous methanol (containing 1% metaphosphoric acid and 0.5% oxalic acid dihydrate) facilitated by ultrasonication and centrifuged [115].

Olsen *et al.* optimised the hydroalcoholic extraction of phenolic compounds in kale leaf samples. Ultrasound sonication and mechanical homogenisation were studied, as well as extraction solvent (acetone and methanol), sample size, and extraction duration. Mechanical homogenisation was found to be the most efficient extraction procedure. Methanol and acetone were equally efficient as the extraction solvent; however, methanol was chosen for all subsequent experiments. The optimal sample size was 5 g of frozen, ground leaf material, and after homogenisation, the extract was left for 15 minutes on ice before centrifugation [116].

2.6.2.4 **Enzyme Hydrolysis**

Enzymatic hydrolysis is predominately used to identify specific sugars and their linkage position on the aglycone, and when the phenolic compounds are sensitive to acid hydrolysis. For example, if a phenolic compound is hydrolysed with *β*-D-glucosidase to produce a glucoside as a product, then it confirms that the original phenolic compound was conjugated to a *β*-D-*O*-glucoside.

Enzymes such as glucuronidase and sulfatase are also utilised to synthesise conjugated metabolites like glucuronides and sulfates respectively, to aid in the quantification of these compounds. These conjugated metabolites are produced after metabolism in the human body and are usually analysed in biological fluids such as urine and plasma. Wittig *et al.* [117] synthesised quercetin glucuronides enzymatically using glucuronyl transferase from rabbit liver to confirm their presence in human plasma after consumption of fried onions and Day *et al.* [118] used chemical and enzymic synthesis to show that quercetin glucosides are not present in plasma of human subjects after consumption of onions. Another study by Oliveira and Watson [119] investigated the *in vitro* glucuronidation of quercetin and kaempferol by human UDP-glucuronosyltransferase microsomes using HPLC-MS. Both flavonols were extensively metabolised into monoglucuronides, and the characterisation of these metabolites was successfully achieved. The presence of quercetin glucuronides in human urine after consumption of *Gingko biloba* tablets was also demonstrated.

2.6.3 Purification

Phenolic compounds tend to be a part of a complex mixture in plant extracts which often makes purification of the extract necessary for adequate analysis. This is implemented for several reasons; to purify and enrich a certain phenolic compound or phenolic fraction, to eliminate any plant matrix components that may interfere with the analysis, or to remove highly lipophilic compounds that can adsorb onto reversed-phase HPLC columns [27].

The most widely used method is solid phase extraction (SPE) which enables a simple, rapid, and reproducible purification step prior to analysis. Small sample volumes can be used, and pre-concentration of phenolic compounds can quickly be achieved [120, 11, 121]. Other chromatographic techniques that serve not only to purify extracts, but also to enable fractions or single components to be collected for further analysis, include preparative or semi-preparative liquid chromatography, column chromatography (CC), countercurrent chromatography (CCC) and centrifugal partition chromatography (CPC). These techniques are often used in tandem for both the clean-up of plant extracts and the purification of compounds.

2.6.3.1 Solid Phase Extraction

SPE involves passing a liquid sample over a solid sorbent material and the components of interest within the sample adsorb onto the surface of the solid material. The target compounds are eluted using an appropriate solvent while any interfering compounds are retained on the solid material, or vice versa. Once eluted the extracts can usually be analysed directly or are sometimes concentrated or evaporated to dryness to resuspend in an alternate solvent. Incomplete recoveries can occur, so care should be taken when using SPE for quantification purposes. SPE is, however, widely accepted as the ideal method for sample clean up, purification, and pre-concentration of phenolic compounds because of the selectivity and saturation of the adsorbents [122]. It is also possible to combine SPE with HPLC as an online extraction method, where the crude plant extract can be injected directly into the SPE-HPLC system [121].

2.6.3.2 Preparative HPLC

Preparative HPLC is a precise purification method which utilises the separation power of the HPLC to obtain fractions with a high degree of purity. Analytical HPLC can achieve much higher separation due to its smaller sample size, however, preparative HPLC can separate larger amounts of material. Preparative columns are much larger, with bigger particle sizes, and utilise much higher mobile phase flow rates (10-50 mL/min). The instrument conditions are often optimised by firstly developing the analytical technique before being scaled up to the preparative procedure. A fraction collector usually forms part of the instrument set up, as well as an online UV detector,

to enable the detection and collection of the different phenolic compounds/fractions as they elute off the column. This technique is particularly useful for the generation of purified compounds to be used as analytical standards in chemical and biological research. In previous studies in our laboratories, Caridi *et al.* successfully isolated and purified glucoraphanin from broccoli [123-125] and the major quercetin glucosides present in onions [126-127] using preparative HPLC. The compounds were fully characterised by HPLC-PDA-MS^n, ^1H, and ^{13}C NMR spectroscopy and were used as reference standards to quantify the levels of these analytes in a range of broccoli and onion samples. Other studies have used preparative HPLC to isolate and purify flavonoids from cauliflower [128], black chokeberries [129], and onions [130] in order to use these compounds as standards, both qualitatively and quantitatively.

2.6.3.3 Column Chromatography

Column chromatography is a classical separation technique that is also useful for the purification and fractionation of phenolic compounds in plant extracts. There are many different stationary phases that can be used; e.g. silica gel, reversed-phase C-18 or C8, polyamide and cellulose, and elution is carried out using a wide range of appropriate solvent mixtures. Compounds are eluted sequentially using gradual changes in solvent concentration or polarity. Different column systems can be used, with diameters ranging from 5 to 50 mm, and the technique has successfully been used to prepare quantities of purified phenolic compounds in the microgram to kilogram range [17].

2.6.3.4 Countercurrent Chromatography

Countercurrent chromatography is a liquid-liquid chromatographic technique in which the stationary phase is also a liquid that is held in place under a centrifugal force field. Separation occurs as solutes partition between two immiscible solvents; the mobile and stationary phases [131, 121]. There are a number of advantages associated with having a liquid stationary phase in chromatography, such as (i) an increased loading capability, (ii) a very simple solute retention mechanism (i.e. liquid-liquid partitioning), (iii) either phase can be used as a mobile phase within the biphasic system, (iv) no problematic, irreversible, solute adsorption, (v) no problems with pH, and (vi) a decrease in biological solute denaturation.

Examples of some chromatographic techniques and conditions that are commonly used in the purification of phenolic compounds in plant and food products are shown in Table 2.4.

Table 2.4 Chromatographic techniques and conditions used in the purification of phenolic compounds in plant and food products

Chromatographic Technique	Conditions	Reference
Column chromatography	1. (2.6 × 40 cm) Polygosil C-18 60-4063 (Macherey Nagel): rinse with H_2O, collect analytes with 20-40% CH_3OH/H_2O 2. (2.6 × 40 cm) Polyclar AT (Serva): 20% CH_3OH/H_2O followed by 50% CH_3OH/H_2O 3. Preparatory paper chromatography (Whatman No.3) with *n*-BuOH-HOAc-H_2O (12:3:5), dried and eluted with H_2O 4. All compounds finally purified on (40 × 2.6 cm) Sephadex LH-20, elute with H_2O	Nielsen *et al.*[132]
Column chromatography	1. Silica gel chromatography, eluting with $CHCl_3$-EtOAc and EtOAc-CH_3OH 2. Sephadex LH-20, eluting with 80% CH_3OH-2 flavonoids isolated 3. Diaion HP-20 resin eluting with 25%, 50%, 75% and 100% CH_3OH 4. 25% CH_3OH fraction purified with sephadex LH-20 eluting with 30% CH_3OH-1 flavonoid isolated	Farag *et al.*[133]
Semi preparative HPLC	(250 × 10 mm) 5 μm C18, 100A Luna with (10 ×10 mm) C18 security guard (Phenomenex). Flow rate of 2.7 mL/min (A) H_2O + 0.5% HOAc, (B) CH_3CN: 0-5% B (0-5 min), 5% B isocratic (5-30 min), 5-10% B (30-35 min), 10 % B isocratic (35-65 min), 10-15 % B (65-75 min), 15% B isocratic (75-100 min), 15-20 % B (100-110 min), 20% isocratic (110-125 min), 20-50% B (125-130 min), 50% B isocratic (130-135 min), 50-0% B (135-140 min) and 0% B (140-147 min). No further purification required.	Fiol *et al.*[134]
Preparative, semi preparative HPLC	1. Preparative HPLC (250 × 40 mm) 10 μm Nucleosil 100-10 VarioPrep C18 (Macherey Nagel): Constant gradient from 65% A (H_2O + 0.6 % HOAc) to 20 % A in B (CH_3OH) within 200 min, flow rate 10 mL/min (UV at 350 and 460 nm) 2. Semi preparative HPLC (250 × 4 mm) 5 μm Nucleosil C18 (Macherey Nagel): System 1; 87% (A) H_2O with 1% HOAc, 13 % (B) CH_3CN for 60 min; System 2; 82% (A) H_2O with 1% HOAc, 18 % (B) CH_3CN for 60 min; System 3; 83% (A) H_2O with 1.5% H_3PO_4, 17 % (B) CH_3CN for 40 min; System 4; (A) H_2O with 1.5% H_3PO_4, (B) CH_3CN constant gradient from 0% (B) to 60% (B) in (A+B) within 40 min; flow rate of 1 mL/min (UV at 350 and 460 nm)	Schliemann *et al.*[135]

Chromatographic Technique	Conditions	Reference
Preparative HPLC	(250 × 21 mm) 5 µm C-18 Nucleodur flow rate of 20 mL/min (A) H_2O, (B) CH_3OH: 20% B at 0 min, 30% B at 10 min, 60 % B at 25 min, 100 % B at 30 min. Injection volume 1800 µl, (UV at 230 and 330 nm). 13 fractions collected, 2 fractions further purified, lyophylized and further purified by prep HPLC	Harbaum *et al.*[136]
Preparative HPLC	(250 × 21.2 mm) Polaris 5 µm C18 (Varian), flow rate 10 mL/min (A) H_2O + 0.1% HOOH, (B) CH_3OH + 0.1% HOOH. 5% B (0-5 min), 5-45% B (5-45 min), 45-80% B (45-55 min), 80% B (55-56 min) 80-5% B (56-58 min)	Caridi *et al.*[137]
Preparative HPLC	(250 × 21 mm) 5 µm C18 (2) 100 A Luna flow rate 21 mL/min (A) 90:10 H_2O:CH_3OH pH 3.5 HOOH, (B) CH_3CN:H_2O:CH_3OH pH 3.5 HOOH. 100% A (0-5min), 20% B (5-10 min), 20-60% B (10-20 min), 60-80% B (20-30 min), 80-100% B (30-35 min), 100% B (35-40 min), 100% A (40-42 min), 100% A (42-50 min)	Singh *et al.*[138]
High speed counter current chromatography	High speed CCC-1000 (HSCCC) Pharma-Tech Research Corp equipped with 3 prep coils in series, total volume 850 mL. Revolution speed 1000 rpm and flow rates of 2-5-3 mL/min	Degenhardt *et al.*[139]
Centrifugal partition chromatography	Semi-preparatory FCPC Kromaton with 200 mL hydrostatic column. Separation performed at 1300 rpm and a flow rate of 3 mL/min	Michel *et al.*[140]
Solid-phase microextraction (SPME)	Supelco with four fibres: 100 µm polydimethylsiloxane (PDMS), 65 µm polydimethylsiloxane-divinylbenzene (PDMS-DVB), 85 µm polyacrylate (PA), and 50 µm Carbowax-templated poly(divinylbenzene) resin (CW-TPR) Dynamic desorption with flow rate 0.02 mL/min of CH_3OH:H_2O (55:45) for 6 min	Satterfield *et al.*[141]

2.6.4 Instrumental Analysis

A key requirement in this research is the ability to accurately identify and quantify specific phenolic compounds found in the plant and food products. The separation, identification, and quantification of phenolic compounds from plant materials can be performed within a matter of minutes using "state of the art" analytical equipment [101]. High performance liquid chromatography (HPLC), and more recently ultra high performance liquid chromatography (UHPLC), are by far the most widely used methods to separate and analyse phenolic compounds in plant and food products [24]. Capillary electrophoresis (CE) and gas chromatography (GC) have also been used to a lesser extent. The main spectroscopic techniques that are used for the structural characterisation of phenolic compounds are ultraviolet spectrophotometry (UV), mass spectrometry (MS), and nuclear magnetic resonance spectroscopy (NMR).

2.6.4.1 Capillary Electrophoresis

In CE, compounds in a solution separate when they are exposed to an electric field within small capillaries due to the ions having differences in their electrophoretic motilities [24]. Capillary zone electrophoresis (CZE) separates the phenolic compounds according to their charge/mass ratio; the higher the ratio, the faster the compounds will migrate through the capillary column. Furthermore, the separation of different phenolic hexosides, which is rarely seen in HPLC separations [17] can be achieved with the use of a borate buffer through complex formation between the borate and the sugar hydroxyls corresponding to their stereochemical structure.

Another mode of CE is Micellar electrokinetic capillary chromatography (MEKCC), is a hybrid chromatographic and electrophoretic technique based on the addition of micelles and organic solvents to the buffer and can be used for the separation of both ionic and neutral substances. Separation of phenolic compounds occur because of interactions with the micelles in addition to electrophoretic migration [17].

The application of CE to phytochemical research was reviewed in 1995 by Tomas-Barberan [142], as well as more recently by de Rijke *et al.* [143] and Huck *et al.* [144], including the applicability of CE-MS [145-146]. The first application was reported in the 1990's on the analysis of flavones in complex extracts [142]. CE/ESI-MS was used to separate, identify, and quantify phenolic compounds present in olive mill wastewater. Levels of 1 pg to 386 pg were achieved for the compounds of interest in selective ion monitoring (SIM) mode [147].

Advantages of this technique compared to HPLC include inexpensive columns (fused silica capillary columns), the use of buffer solutions which decreases consumption of organic solvents,

and rapid analysis times. However, despite its advantages and high expectations, CE has found limited use in the analysis of phenolic compounds.

2.6.4.2 Gas Chromatography

GC has been used for the study of phenolic compounds for the past 30 years. Although the technique has been around for many years and has many applications, it is not the method of choice when it comes to separating, identifying, and quantifying phenolic compounds in plant and food products as many phenolic compounds are not volatile. Phenolic compounds are converted to more volatile derivatives via methylation, or converted into trimethylsilyl derivatives, or transformed with the use of N-(*tert*-butyldimethylsilyl)-N-methyltrifluoroacetamide [121]. This conversion step makes analysis of phenolic compounds using GC very time consuming, and moreover, when coupled to MS, can make the fragmentation patterns of the derivatives more difficult to interpret. The use of GC and GC-MS in the analysis of phenolic acids and flavonols was reviewed by Stalikas [121].

2.6.4.3 High Performance Liquid Chromatography

HPLC is the most widely used chromatographic technique to separate, detect, and quantify phenolic compounds in plant extracts. There are various combinations of columns and mobile phases that have been developed for the analysis of all the different subclasses of phenolics (refer to Figure 2.2 for classification), including phenolic acids, stilbenes, coumarins, tannins, and flavonoids such as flavonols and anthocyanins. The most widely used HPLC technique for the separation of phenolic compounds is reverse phase HPLC. Reverse phase HPLC consists of a non-polar stationary phase with a relatively polar mobile phase, and compounds are separated and eluted by either isocratic or gradient elution in order of decreasing polarity. The most commonly employed columns range in length from 100 to 250 mm, with internal diameters between 3.9 to 4.6 mm, and the stationary phase is typically a C_{18} bonded silica [121]. Mobile phases are generally made up of aqueous mixtures of methanol or acetonitrile with the addition of low concentrations of an organic acid to prevent ionisation of hydroxyl groups as well as minimise peak tailing. It is recommended to maintain the pH of the mobile phase between 2 and 4, as the ionisation of phenolic hydroxyls will occur above pH 4. By preventing the ionisation of the phenolic hydroxyls, the acidic conditions promote better interaction with the stationary phase which ultimately results in less ‘tailing’ and better peak resolution. The most frequently used acids to adjust the pH are acetic, formic and phosphoric [121]. The analysis of phenolic compounds is performed with the column at ambient temperature, however, in some cases a column oven may be used to maintain a constant temperature at or above ambient temperature to improve reproducibility and separation of analytes.

Normal phase HPLC consists of a polar stationary phase with a relatively non-polar mobile phase and compounds are separated and eluted from the column in order of increasing polarity. Normal phase HPLC has been used for the analysis of procyanidins (oligomers of flavanols) because it provides better separation of higher oligomers [148]. Additionally, other phytochemical compounds not part of the phenolic class, such as carotenoids, tocopherols, and tocotrienols, are better separated using normal phase HPLC methods due to their non-polar characteristics [149-150].

Post-column detection methods for phenolic compounds include ultraviolet-visible (UV-Vis), photodiode array (PDA), fluorescence, electrochemical (ECD) and mass spectrometry (MS). Many phytochemicals, in particular those belonging to the phenolics group, absorb in the UV range, therefore, detection is primarily achieved using UV-Vis or PDA, and more recently, hyphenated with MS.

There have been countless studies on a vast variety of fruits, vegetables, and other plant foods/plant products that have employed HPLC coupled to various detectors. Included are studies on broccoli [57], pak choi [60, 136], cauliflower [128, 151], kale [67, 116, 152, 134], onions [130, 153, 126, 154], strawberries [155], cranberries [156], other assorted berries [157-161], apples [162], citrus fruits [163-164], dragon fruit [165], rocket [166], buckwheat [167], and fennel [168].

2.6.4.4 Ultra High Performance Liquid Chromatography

UHPLC is a relatively new analytical separation technique. It is the same in principle as HPLC, however, it uses smaller columns with smaller particle sizes (<2.5 µm) and higher flow rates to produce greater efficiency with increased speed of separation and peak capacity, i.e. the number of peaks resolved per unit time in gradient separations [169]. Improvements in instrumentation to withstand higher operating pressures and to the development of stationary phase particles, has led to shorter columns in line with particle size reduction without loss of resolution. The technique is fast, provides high resolution and highly sensitivity (i.e. obtains sharper peaks). The sensitivity of UHPLC is typically 2-3 times greater than with HPLC, depending on the detection technique, and analysis times can be reduced from several minutes to less than a minute using UHPLC [169]. Furthermore, it uses only a fraction of the mobile phase that is normally required for conventional HPLC (i.e. less than 0.2 mL/min for smaller particle columns, as compared to 1 mL/min for conventional, larger particle columns), therefore solvent consumption is significantly reduced making the method more environmentally friendly and "greener" [170].

The technique has been increasingly employed for the separation of phenolic compounds. Researchers developed a UHPLC-PDA-MS/MS method to separate glucosinolates and phenolic compounds in *Brassica oleracea* L. var. *botrytis* (white and green cauliflower) [171]. The phenolic content of various cocoa sources was also investigated using UHPLC and it was determined that

the nib contained the richest phenolic profile compared to the other cocoa sources [172]. Another study used UHPLC-QTOF MS for detailed compound characterisation of flavonols in blueberries. It was the first time that the identification and quantification of the phenolics laricitrin and syringetin was reported for V. *corymbosum* [173]. More recently, UHPLC was employed in combination with QTOF MS and PDA/ESI-TOF MS by Farag *et al.* [133] and Gozales *et al.* [151] respectively. In both studies the technique was used to separate complex flavonoid glycosides which were then detected, and structures elucidated using the respective mass analysers. The first study analysed rapeseed [133], whereas the latter analysed a cauliflower waste extract [151].

2.6.4.5 Ultraviolet-Visible and Photo Diode Array Detection

Most phytochemicals can be detected using UV-Vis spectrophotometry, however, there is no single wavelength that can detect all the different classes of compounds. Anthocyanins are typically detected between 515-535 nm, flavonols are in the range 360-370 nm, phytosterols absorb at 200-210 nm, some phenolic acids are detected at 280 nm whereas hydroxycinnamic acids have a maximum absorbance at 320-330 nm, stilbenes have a maximum absorbance at 306 nm (trans) and 285 nm (cis), and hydrolysable tannins have a characteristic absorbance at 300 nm [17, 24]. To overcome this, many studies use PDA detection as it allows for simultaneous scanning of a range of wavelengths, as opposed to just a single wavelength. PDA detectors give full UV-Vis spectra of individual compounds, provide peak purity data and have the capability to use spectral library matching with standard compounds to give further proof of the compounds identity [121, 101].

Flavonoids are easily identified by their characteristic UV-Vis spectra as many exhibit two distinct bands; a maximum in the long UV range (band I) between 325 and 400 nm, and a second maximum at shorter wavelengths (band II) between 240 and 295 nm. Both band I and band II can be split into two maxima, or alternatively, one maximum and a shoulder/inflection. In some cases, a third maximum between 295 and 325 nm (band III) can also be distinguished [17]. Band I arises from the absorption of the flavonoid B-ring (refer to Section 2.3.2), which is associated with the cinnamoyl moiety of the compound, while band II arises from the A-ring, which corresponds to the benzoyl moiety (Figure 2.10). For example, quercetin, which is di-substituted on the B-ring, displays two maxima in band II at 255 and 266 nm. Whereas, kaempferol, which is a mono-substituted on the B-ring, has only a single maximum for band II at 266 nm. Band I for the flavones group shows a distinct absorption maximum between 325 and 255 nm, however, the maximum of band I for flavonols is shifted to 360-380 nm due to the free hydroxyl group found at position 3.

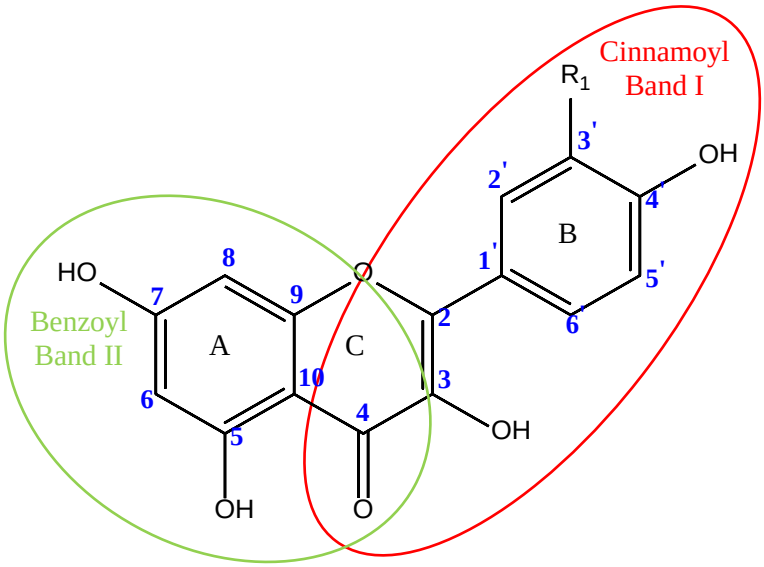


Figure 2.10 Generic structure of a flavonol compound illustrating the origin of the two characteristic absorbance bands observed in a typical UV spectrum for flavonoids. Band I occurs between 325 and 400 nm, and band II between 240 and 295 nm.

The λ_{max} of the compound changes as a flavonoid aglycone becomes substituted with sugars and organic acids. The maximum absorbance for band I shifts to a lower wavelength with the addition of glucose moieties, especially at the 3-*O* position for flavonols [116]. Furthermore, the λ_{max} will shift lower if the flavonol glycoside is further substituted with a hydroxycinnamic acid residue. Flavonoids that are acylated with hydroxycinnamic acids exhibit a UV-Vis absorption spectrum of the flavonoid overlapped with that of the acid. They typically show a maximum with a high absorption between 310-335 nm due to the hydroxycinnamic acid, and a small maximum that coincides with the flavonoid band II between 255-268 nm [57]. In poly-acylated flavonoid glycosides, in which two or even three hydroxycinnamate residues are conjugated to the flavonoid glycoside, the absorption of the maximum at 310-335 nm becomes predominant and the contribution from the band I and band II absorptions from the flavonoid are decreased. These shifts were observed by Rochfort *et al.* where the λ_{max} of quercetin was recorded as 256 and 374 nm, quercetin-3-sophoroside-7-glucoside had maximum absorbencies at wavelengths 196, 254 and 352 nm respectively, and the UV-Vis spectrum of quercetin-3-sophoroside-(caffeoyl)-7-glucoside displayed λ_{max} values of 194, 252 and 336 nm [60]. This is referred to as a hypsochromic shift, and was previously reported by Vallejo *et al.* [57] in the study of flavonols in broccoli and more recently by Olsen *et al.* [116] in the study of flavonols in curly kale.

The fact that flavonols have a characteristic UV-Vis spectrum with two distinct λ_{max} bands at 250-270 nm and 360-370 nm makes quantification of total aglycone levels, after hydrolysis, in plant and food products using HPLC-PDA relatively straight forward [67, 12-15].

2.6.4.6 Fluorescence Detection

Fluorescence detection is based on the ability of a molecule to emit light after its electrons have been excited by a beam of light or higher energy. It can be a very selective detection method as both the excitation and emission wavelengths are specific for a given molecule. Fluorescence detection has been found to be useful for compounds that possess specific functional groups or substitution patterns [121, 17] e.g. the analysis of procyanidins (oligomers of flavanols) where the detection limit using fluorescence is approximately 100 times lower than using UV detection. Combining fluorescence with UV detection presents the opportunity to differentiate between fluorescent and non-fluorescent compounds which may co-elute. It is important to establish the correct excitation and emission wavelengths with fluorescence detection, and therefore a rapid scanning fluorescence detector (the fluorescence equivalent of a PDA detector) is preferred, as it has the ability to apply multiple excitation and emission wavelengths [121, 17].

2.6.4.7 Electrochemical Detection

ECD is a very sensitive detection method for compounds that can be oxidised or reduced at low voltage potentials. Either an oxidation or reduction reaction occurs at an electrode, which results in a flow of electrons at its surface. This reaction will continue until the analyte in the sample is depleted and a concentration gradient is formed between the surface of the electrode and the rest of the solution. This is ultimately proportional to the concentration of the analyte in the sample. ECD can be used to give useful information about phenolic compounds in addition to spectra obtained by PDA detectors. It can be used to determine redox potentials of phenolics, as well as to identify the mechanism of oxidation. Furthermore, phenolic compounds can be identified based on comparisons made with an authentic standard, and redox potentials for unknown phenolics can be determined [24].

Although it is not the most widely used detection method for the analysis of phenolic compounds in plant and food products, it has been used quite frequently in the analysis of phenolics in plasma and urine samples due to its increased sensitivity and allowing for quantification of low concentration of analytes [17, 121174-175]. It has also been used more recently for the analysis of flavonoids in four varieties of Portuguese red grape skins [107] and for various phenolic compounds including anthocyanins, flavonols, and ellagitannins in strawberries [176].

2.6.4.8 **Mass Spectrometry**

The use of MS as a detector for the structural analysis of phenolic compounds in plant and food products has constituted a fundamental step forward within the area of phytochemical research [17]. MS analysis involves the ionisation of chemicals which results in charged species that can be separated according to their mass (*m*) to charge (*z*) ratio (*m/z*), thus enabling the detection, identification, quantification, and potentially structural characterisation of compounds using MS^n.

MS can easily be coupled to different separation techniques such as CE (2.5.4.1), GC (2.5.4.2), HPLC (2.5.4.3) and more recently UHPLC (2.5.4.4). This has allowed the screening (metabolite profiling and fingerprinting) and characterisation of phenolic compounds in complex matrices as well as structural identification of complex phenolic conjugates without the need for isolation and purification. This is critical where compounds may only be present in trace amounts.

HPLC-MS has become the most widely used technique for the analysis of phenolic compounds [101, 17, 24]. The success of the technique is strongly dependent upon the interaction of the analyte with the stationary phase, as well as the composition of the HPLC mobile phase and flow rate. Flow rates are typically 0.3 mL/min or lower with conventional 3-4.6 mm i.d. or 1-2.1 mm i.d. microbore columns [101]. If a conventional 3-4.6 mm i.d. column is used, it is frequently operated in combination with a flow splitter to make the flow rates compatible with the mass spectrometer [27]. The mobile phase typically contains easily ionised volatile compounds, e.g. ammonium acetate, ammonium formate, acetic acid, formic acid, or trifluoroacetic acid, from which a charge is transferred to the phenolic compound. This gives a positive or negatively charged species, depending on whether there was a loss $[M-H]^-$ or the gain of a proton $[M+H]^+$ [101]. Acidification provides better retention and separation on the C8 and C18 reversed phase columns that are predominately used in the analysis of phenolic compounds [27].

There have been a variety of ionisation techniques and mass analysers used for the analysis of phenolic compounds. Early ionisation techniques include electron impact, thermospray, fast atom bombardment (FAB), and particle bean ionisation. These techniques have, for the most part, been replaced by newer soft ionisation techniques such as atmospheric pressure ionisation (API) [177]. The fact that API sources do not require high vacuum enables easier coupling with HPLC and currently make them the most widely used ionisation technique [17]. There are two main sources of API; atmospheric pressure chemical ionisation (APCI), and electrospray ionisation (ESI), both of which can be used in the positive and negative ion mode. Matrix-assisted laser desorption ionisation (MALDI) has also been used for the analysis of phenolic compounds. These ionisation techniques will be discussed in greater detail in 2.5.4.8.1.

Once the compounds have been ionised, they enter a mass analyser which measures the mass-to-charge ratio (*m/z*) of the ions. The simplest mass analyser is the single quadrupole (MS). It consists of four parallel rods that allow only selected ions with a specific *m/z* to reach the detector, according to the voltage and current that has been applied to the rods. All other ions do not have a stable trajectory through the quadrupole mass analyser and cannot reach the detector. A single quadrupole MS has a mass range of up to *m/z* 4000 and primarily yields information about the molecular ion, however, some structural information can be gleaned from in-source collisions [17]. A triple quadrupole (3Q, QqQ) is a type of tandem mass analyser (MS/MS) and consists of three quadrupoles in a linear configuration which enables fragmentation of the molecular ion with the subsequent analysis of these fragment ions providing further structural information. Another type of tandem mass analyser is the ion trap mass spectrometer (IT), which is also a form of multiple MS (MS^n). IT mass analysers provide even further structural information to be obtained as fragment ions can be further fragmented, where the fragmentation process can be repeated up to ten times. Time of flight mass spectrometer (TOF) instruments provide primarily accurate mass data for the molecular ion and other fragment ions present in the spectrum. MS^n is often used for the elucidation of phenolic structures, as it provides information on the molecular weight of the compound, the nature of the aglycone core, as well as the number of sugar and acyl moieties and their overall position(s) within the molecule, all without the need to isolate and hydrolyse the compounds [57, 60, 116, 67].

In recent years, there have been an array of new hybrid MS/MS instruments developed that combine two different mass analysers, e.g. Q-TRAP MS and Q-TOF. Q-TRAP MS instruments merge a triple quadrupole with an ion trap mass spectrometer to combine high sensitivity with high selectivity [17] and Q-TOF is the combination of a triple quadrupole with a time of flight mass spectrometer. Arapitsas *et al.* [178] used a HPLC-PDA/Q-TRAP to characterise anthocyanins in red cabbage. Wolfender *et al.* [179] evaluated and compared the applicability of Q-TOF and IT MS for the on-line identification of natural products in crude plant extracts. More recent studies by Vallverdu-Queralt *et al.* [180] and Farag *et al.* [133] also utilised Q-TOF mass analysers for the screening of the polyphenol content of tomato-based products and to determine the phytochemical composition of *Brassica napus* L., respectively.

The application of MS to the characterisation of phenolic compounds, and their glycosylated derivatives, has been widely reviewed [181, 143, 145, 27, 182-183182] and some of the common mass spectroscopic methods are discussed in the following sections in greater detail.

2.6.4.8.1 Ionisation Techniques

The ionisation source is a key component of the mass spectrometer. As mentioned in the previous section, some of the earlier ionisation techniques included electron impact, thermospray, fast atom bombardment (FAB), and particle bean ionisation. More recent ionisation sources are based on what is referred to as "soft ionisation" [177] since there is very little fragmentation. Among these, the three most frequently used are atmospheric pressure chemical ionisation (APCI), electrospray ionisation (ESI), and more recently matrix-assisted laser desorption ionisation (MALDI). Another API source known as atmospheric pressure photo-ionisation (APPI) is not commonly used and will not be discussed here.

ESI is used more frequently than other ionisation sources for the analysis of phenolic compounds, particularly glycosylated phenolic compounds [145]. A review by Rauha *et al.* [184] compared the efficiency of different API sources (ESI, APCI, and APPI) on the analysis of flavonoids and reported that the highest sensitivity was obtained using the ESI source in the negative ion mode with an eluent system consisting of an acidic ammonium acetate buffer. However, both APCI [185, 179, 182] and ESI [186, 184, 187] are favoured by different authors for the analysis of flavonoids.

2.6.4.8.1.1 Electrospray Ionisation

ESI involves a liquid sample which is nebulized and dispersed into tiny electrically charged droplets at atmospheric pressure as it emerges from the column. A drying gas and heat causes the solvent to evaporate, and the droplets are forced apart into smaller droplets by coulombic repulsion until each droplet contains only one charged molecule [177]. This results in either a positively or negatively charged gaseous ion, depending on whether a proton was added or removed, which is then drawn into the mass analyser by a vacuum through a series of focussing lenses [188] (Figure 2.11). This technique is considered a soft ionisation technique because minimal fragmentation occurs during ionisation, except when the potential difference between the analyser and the transfer capillary is increased.

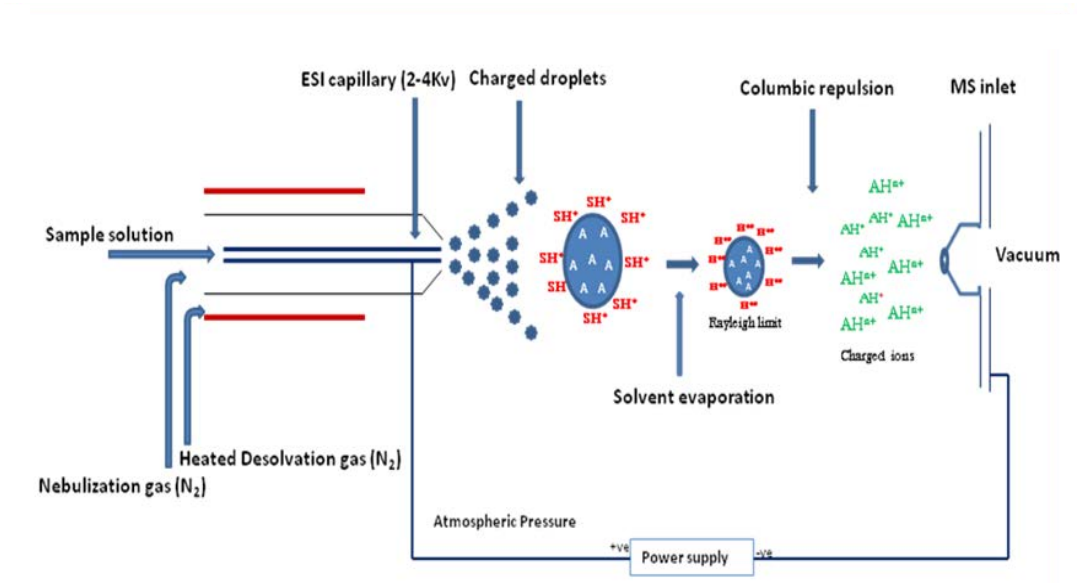


Figure 2.11 Schematic diagram of an ESI source. Diagram obtained from http://www.chem.pitt.edu/facilities/mass-spectrometry/mass-spectrometry-introduction [188].

2.6.4.8.1.2 Atmospheric Pressure Chemical Ionisation

APCI is similar to ESI except that the solvent is vapourised prior to ionisation so that ionisation occurs in the gas phase instead of the liquid phase (Figure 2.12). The analyte in solution is introduced into a heated capillary along with the nebuliser gas (N_2) to produce a mist of fine droplets. The emerged mist is then converted into a gas stream by the combined effects of heat and gas flow. Once the gas stream arrives in the ionisation region under atmospheric pressure, molecules are ionised at corona discharge (i.e. a pin that has a high potential applied to it and produces an electrical discharge). This process makes APCI less suitable for the analysis of polar phytochemicals that are thermally labile and not easily volatilised [177].

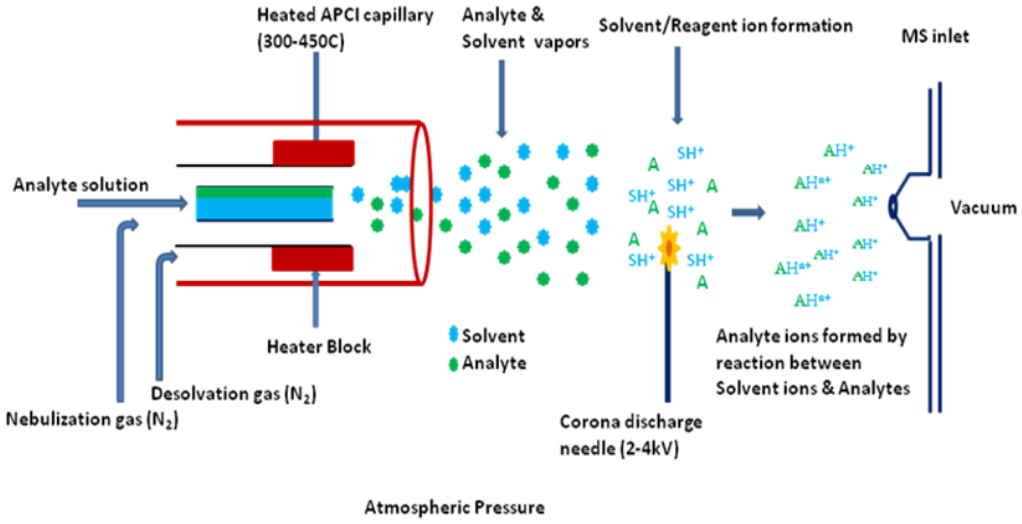


Figure 2.12 Schematic diagram of an APCI source. Diagram obtained from http://www.chem.pitt.edu/facilities/mass-spectrometry/mass-spectrometry-introduction [188].

2.6.4.8.1.3 Matrix-Assisted Laser Desorption Ionisation

MALDI is also a soft ionisation technique and involves mixing or coating the sample to be analysed with a solution consisting of an energy-absorbent, organic compound referred to as matrix (usually an aromatic organic acid). The matrix compound crystallises upon drying, causing the entrapped analyte to also co-crystallise. The analyte within the matrix is then ionised with a laser beam (nitrogen laser light, wavelength 337 nm). As the matrix absorbs the laser light it converts it to heat energy which results in a small part of the top outer surface of the matrix/analyte mixture being heated rapidly and vapourising. Singly protonated ions are formed because of this desorption and ionisation process, which are then accelerated at a fixed potential and separated based on their *m/z* ratio [181] (Figure 2.13). This technique is particularly useful for the transformation of high molecular weight compounds that are not easily ionised, such as organic macro molecules. It can desorb analyte molecular ions with relative masses up to 300 KDa into the gaseous phase as intact ions. Although different mass analysers can be used with the MALDI source, it is predominately used in conjunction with a TOF MS (Section 2.5.4.8.4) [189-191].

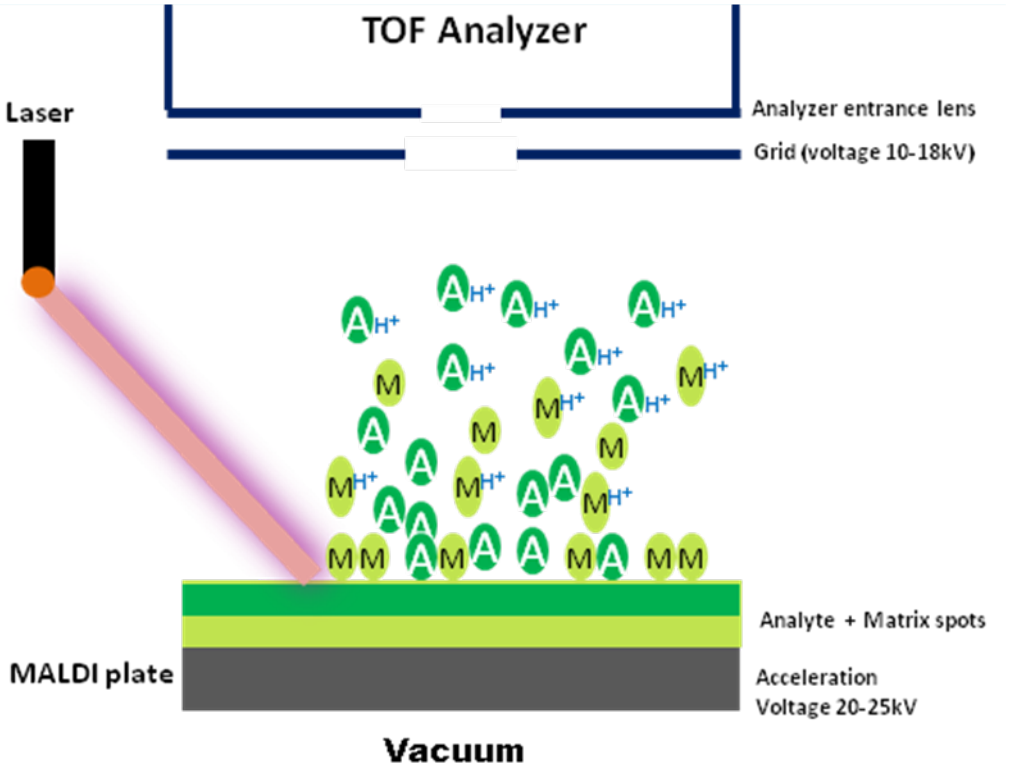


Figure 2.13 Schematic diagram of a MALDI source. Diagram obtained from http://www.chem.pitt.edu/facilities/mass-spectrometry/mass-spectrometry-introduction [188].

2.6.4.8.2 Triple Quadrupole Mass Spectrometry

A triple quadrupole mass spectrometer (3Q, QqQ, MS/MS) consists of a series of three quadrupoles (Q1, Q2, and Q3) where the first (Q1) and third (Q3) quadrupoles act as mass filters, and the second quadrupole (Q2) is a non-mass-resolving quadrupole with the primary function of causing fragmentation of the analyte through interaction with a collision gas. Q2 is a radiofrequency only quadrupole, whereas Q1 and Q3 are controlled by direct current in addition to radiofrequency. A triple quadrupole MS operates under the same principle as the single quadrupole mass analyser, whereby Q1 and Q3 (the two mass filters) commonly contain four parallel, cylindrical metal rods that allow only selected ions with a specific *m/z* to pass through. It can either be operated in a full scan mode or using a selected ion monitoring (SIM) mode. Some advantages of this technique compared to a single quadrupole mass analyser include increased selectivity, improved signal-to-noise (S/N), lower limits of quantification, wider dynamic range and improved accuracy and reproducibility especially at low concentrations. Gratacos-Cubarsi *et al.* [171] estimated the levels of 20 glucosinolates and phenolic compounds in cauliflower using a UHPLC-PDA/ESI-MS/MS in the negative ion mode.

2.6.4.8.3 Ion Trap Mass Spectrometry

An ion trap mass analyser is the three-dimensional analogue of the linear triple quadrupole mass spectrometer. The ions in an IT MS are also subjected to forces applied by a radio frequency field, but in this case the forces occur in all three dimensions, as opposed to just two. Ions are trapped within a system of three electrodes with hyperbolic surfaces; the central ring electrode, and two adjacent end-cap electrodes (the entrance and exit electrodes). The electrodes form a chamber in which it is possible to trap and analyse ions while they rotate and oscillate in an 8-shaped trajectory. The ring electrode is located halfway between the two end cap electrodes, both of which have a small hole in their centres through which the ions can travel. Ions that are produced from the ionisation source enter the trap through the entrance cap electrode and various voltages are applied to the electrodes to trap and eject ions according to their *m/z* ratios. Trapped ions can either be isolated or successively fragmented to provide MS^n spectra, therefore providing greater structural information than the MS/MS. Consequently, the IT MS is a valuable mass analyser when it comes to structural analysis of unknown compounds. Some of the main advantages of an IT MS include compactness and mechanical simplicity, ability to fragment ions multiple times, ion/molecule reactions can be studied for mass-selected ions; i.e. selected ion monitoring (SIM) and selected reaction monitoring (SRM), and ions of high *m/z* are accessible using resonance experiments. For these reasons, IT MS has been a popular choice for the analysis of phenolic compounds in a variety of fruits, vegetables, and other plants foods and there are a myriad of studies in literature that have utilised this type of instrumentation.

Velasco *et al.* [113] used HPLC-PDA coupled with an IT MS and an ESI interface to identify flavonoids, hydroxycinnamic acids, and glucosinolates in different *Brassica* species such as kale, cabbage and leaf rape. Another study by Olsen *et al.* [111] used HPLC-PDA/ESI-MS^n in both negative and positive ionisation modes to characterise and quantify polyphenols in a red variety of curly kale. A total of 47 phenolic compounds were identified using complementary information from the PDA and ESI-MS^n. Francisco *et al.* [114] simultaneously identified and characterised 12 glucosinolates and more than 30 phenolic compounds in turnip greens and turnip tops using an HPLC-PDA/ESI-MS^n. Furthermore, Schmidt *et al.* [67] also used HPLC-PDA/ESI-MS^n in the negative ionisation mode to identify complex naturally occurring flavonoid glycosides with different glycosylation and acylation patterns in kale. A total of 71 quercetin, kaempferol, and isorhamnetin derivatives were detected including 27 non-acylated, 30 monoacylated, and 14 diacylated glycosides. Harbaum *et al.* conducted a number of studies on the identification of flavonoids and hydroxycinnamic acids in pak choi [136], the analysis of free and bound phenolic compounds in leaves of pak choi and Chinese leaf mustard [115] and the impact of fermentation on these compounds [192], as well as the influence of pre-harvest UV-B irradiation and storage conditions on flavonoid and hydroxycinnamic acid content of pak choi [77] using HPLC-PDA/ESI-MS^n.

2.6.4.8.4 Time of Flight Mass Spectrometry

TOF analysers separate ions based on their flight times across a known distance, without the use of an electric or magnetic field, but instead separation of the ions occurs based on their kinetic energy and velocity. It is based on the principle that the time of flight of an ion in the flight tube is directly proportional to the root of the *m/z* ratio. TOF MS can either be performed in a linear or reflectron configuration. In the linear mode, ions simply travel in a straight line through the flight tube to the detector at the end, however, resolution can become difficult for ions of higher masses due to longer flight times. This can be rectified with the addition of a reflectron to the analyser, which increases resolution and mass accuracy [17]. A series of reflectors bend the flight path of the ions, which ultimately lengthens the travel path and corrects for any differences in initial kinetic energy among ions with the same *m/z*. In this way, both slow and fast ions of the same *m/z* value, reach the detector at the same time rather than at different times, narrowing the bandwidth for the output signal. This technique imparts some interesting characteristics and the speed at which the analysis takes place, as well as its sensitivity, have been viewed as important advantages when compared to other mass analysers [24].

TOF MS is most commonly used in tandem with the MALDI ion source (2.5.4.8.1) and has found applications for the analysis of various phenolic compounds from different sources. A study by Reed *et al.* [193] analysed oligomeric polyphenols in several fruits such as cranberries, grape seed extracts, and pomegranate using MALDI-TOF MS. It has also been applied to the analysis of isoflavones in soy products [190] and was shown to be a powerful tool in identifying these compounds, providing an isoflavone profile within two minutes. Similarly, it was applied to the analysis of flavonol glycosides [191] in both positive and negative ionisation modes. Frison-Norrie *et al.* [189] used MALDI-TOF MS to identify and quantify several flavonol glycosides in almond seedcoats, four of which were identified for the first time. A subsequent study by Monagas *et al.* [194] analysed the phenolic composition of almond skins, and applied the technique to characterise proanthocyanidins.

2.6.4.9 Nuclear Magnetic Resonance Spectroscopy

Modern NMR spectroscopy with all its modes has an important role in the structural elucidation of phenolic compounds, as well as for determining the content and purity of a sample. It is particularly useful for the differentiation of isomers, and determining sugar configurations and substitution patterns on aromatic ring systems [143]. NMR alone, however, will not provide sufficient spectroscopic information for complete identification of an unknown compound and is usually used alongside tandem MS, which can obtain initial information such as molecular mass and functional groups. Preparative HPLC is generally also required alongside NMR, where sufficient amounts of samples can be isolated and purified prior to analysis. NMR experiments that have been applied to the analysis of phenolic compounds include ^{1}H NMR, ^{13}C NMR, and 2D NMR such as correlation spectroscopy (COSY), heteronuclear chemical shift correlation NMR (C-H HECTOR), nuclear Overhauser effect spectroscopy (NOESY), rotating frame Overhauser effect spectroscopy (ROESY), total correlation spectroscopy (TOCSY), heteronuclear single-quantum correlation (HSQC), heteronuclear multiple-quantum coherence (HMQC), and heteronuclear multiple-bond correlation (HMBC) [24, 17]. A study by Schliemann *et al.* [135] used ^{1}H (1D and 2D COSY, 2D HMQC, HMBC, TOCSY, and ROESY), in addition to ^{13}C (1D) NMR to characterise several flavonol conjugates and an indole alkaloid containing identical acylated glycosidic substitutions. Another study analysed the flavonoids and hydroxycinnamic acid compounds present in pak choi using ^{1}H, ^{13}C, COSY, HSQC, and HMBC [136]. Fiol *et al.* [134] also analysed similar glycosylated flavonol-hydroxycinnamic acids in kale, using ^{1}H, ^{13}C, COSY, TOCSY, selTOCSY, NOESY, HSQC, and HMBC. Furthermore, similar compounds were characterised in rapeseed by Farag *et al.* [133] using ^{1}H, ^{13}C, COSY, HSQC, and HMBC.

In recent years, NMR has been coupled to HPLC for on-line identification of phenolic compounds in plant extracts [195-196]. HPLC-NMR as a stand-alone technique, however, does not provide sufficient spectroscopic information for the complete identification of phenolic compounds and complementary information is usually required from other hyphenated techniques such as HPLC-PDA and HPLC-MS^n. Furthermore, HPLC-NMR experiments are time consuming and often the peak first needs to be pre-screened with HPLC-PDA-MS. In one study by Lommen *et al.* [197], directly coupled HPLC-NMR-MS was applied to the analysis and identification of quercetin glycosides and phloretin glycosides in apple peel. The additional information obtained from the NMR data enabled the authors to determine the position of the glycosides on the quercetin and phloretin, as well as deduce whether they were α or β conformations.

2.6.5 Method Development and Validation/Verification

Analytical methods that are used to quantify specific analytes, such as phenolic compounds, must be shown to be fit for purpose, i.e. the data resulting from the method is suitable for the particular application. For scientific research, it is particularly important that the variation in analytical data is less than the biological variation seen in nature, i.e. variations in the levels of an analyte caused by growing conditions (e.g. soil type, amount of water, sunlight, location etc.), which can be quite large. NATA 'Technical Note 17 - Guidelines for the validation and verification of quantitative and qualitative test methods' [198] details the various steps that are necessary to validate/verify an analytical method. The extent of validation/verification required will depend on the status of the method under consideration. Method verification studies are typically less extensive than those required for method validation and can be used for minor variations of methods published by organisations such as Standards Australia, or the United States Environmental Protection Agency (USEPA), which have already been subjected to full validation. This also applies to methods published in peer reviewed literature. The method characteristics to be evaluated for validation/verification are summarised in Table 2.5. Not all characteristics need be evaluated for method verification, so long as the final method is "fit for purpose", in this case, the quantification (detector linearity, reproducibility, and recovery) of the major flavonols (as their aglycones) present in bok choy.

Table 2.5 Characteristics to be evaluated for validation or verification of an analytical method, as outlined by NATA Technical Note 17 [198]

Characteristics to be evaluated	Procedure to be followed
Linearity	Analysis of calibration standards.
Sensitivity	Analysis of spiked samples or standards prepared in sample extract solution.
Selectivity	Consideration of potential interferences; analysis of samples spiked with possible interferences. Method development may have overcome potential issues.
Trueness; bias	Analysis of: Certified Reference Materials (CRMs) Other Reference Materials (RMs) Sample spikes Comparison with Standard Methods Results from collaborative studies
Precision; Intra laboratory reproducibility	Replicate analysis of samples: if possible, selected to contain analytes at concentrations most relevant to users of test results.
Limits of Detection (LOD) Limits of Quantification (LOQ)	Analysis of samples containing low levels of analytes. The determination of LOD and LOQ is normally only required for methods intended to determine analytes at about these concentrations.
Working range	Evaluation of data from bias and possibly LOQ determinations.
Ruggedness	Consider those steps of the method, which if varied marginally, would possibly affect the results. Investigate if necessary (I) single variable test and (II) multi variable test.
Measurement uncertainty (MU); calculates a reasonable fit-for-purpose estimate of MU. Ensures estimates are aligned with the concentrations most relevant to the users of the results.	Utilise other validation data, combined with any other complementary data available (e.g. results from collaborative studies, proficiency tests, round robin tests, in house QC data).

2.6.6 Quantification

The quantification of individual phenolic compounds is dependent upon the availability of standard reference compounds. This is the case for many flavonol aglycones, which can easily be quantified after acid (2.5.2.1) or enzymatic hydrolysis (2.5.2.4) [24]. Commercially available standards for the more complex flavonol glycosides and acylated/further conjugated flavonol glycosides are not readily available and costly, thus direct quantification of these compounds is difficult. However, to get an estimate of the levels of these compounds in the plant material for profiling purposes, it is possible to quantify the relevant compounds present in the hydroalcoholic extract (2.5.2.3) against quercetin-3-rhamnosylglucoside (rutin) and express the amounts as rutin equivalents. Olsen *et al.* [111] quantified the naturally occurring flavonols, anthocyanins, and

phenolic acids in red curly kale using rutin, cyanidin-3-glucoside, and chlorogenic acid respectively. The results were then expressed as rutin, cyanidin, and chlorogenic equivalents.

There is a large volume of published analytical data on phytochemicals in food, including the quantification of flavonols. A database compiled by the United States Department of Agriculture (USDA) contains such data, including values for 506 food items and for 26 predominant dietary flavonoids, one of which is the flavonols group [29]. For further information, refer to tables in Appendix A (pages 296-297) where some data relevant to this study has been extracted from this database. Table A.1 (page 296) consists of a list of foods that contain the highest content of the flavonols of interest in this study; quercetin, kaempferol, and isorhamnetin, and Table A.2 (page 297) lists all the *Brassica* foods reported in the database and their relevant quercetin, kaempferol, and isorhamnetin content.

Two major studies have focussed on the characterisation and quantification of flavonols in *Brassica rapa* L. ssp. *chinensis*; referred to as bok choy in this study. Rochfort *et al.* [60] quantified the levels of quercetin, kaempferol and isorhamnetin in 11 different varieties of bok choy using HPLC-PDA/ESI-MS^n and found that the content varied significantly between the different varieties. In contrast to the data in the USDA database, the authors reported high levels of kaempferol and isorhamnetin and less amounts of quercetin. The kaempferol content ranged between 36 to 103 mg/100 g of dry weight (DW), equivalent to approximately 4.0-10 mg/100 g FW (moisture content is approximately 90% [199]). Isorhamnetin content ranged from 8.0 to 24.0 mg/100 g DW, equivalent to 0.8-2.4 mg/100 g FW. Quercetin levels ranged from 3.0 to 7.0 mg/100 g DW, equivalent to 0.3-0.7 mg/100 g FW.

Harbaum *et al.* [136] also analysed 11 different varieties of bok choy and quantified the flavonol content in the leaf blades, confirming that both kaempferol and isorhamnetin were the two main flavonol compounds present in the plant. However, unlike Rochfort *et al.* [60], this study did not detect any quercetin present in the bok choy, which the authors attributed to different growing and climate conditions. This study did not quantify the flavonol aglycones individually but reported the flavonol aglycone content based on the kaempferol aglycone; and quantified the flavonol derivatives, expressed as the main flavonol glycoside-hydroxycinnamic acid derivative equivalents (kaempferol-3-*O*-hydroxyferuloyldiglucoside-7-*O*-glucoside).

2.7 CHEMICAL ANALYSIS OF FLAVONOLS IN *BRASSICA* SPECIES

The following sections will review research on flavonols in *Brassica* species, and in particular the quantification and structure elucidation of quercetin, kaempferol, and isorhamnetin aglycones and their glycosylated and acylated conjugates. The *Brassica* species (2.4) has been widely investigated for both glucosinolate [200-204, 113, 205-207, 171, 114, 125] and phenolic composition [208-209, 128, 210-211, 114, 212, 57, 113, 74, 213, 136, 115, 60, 214, 205, 116, 111, 206, 215, 112, 216, 171, 134, 92, 217-218, 178].

Phenolic compounds and glucosinolates are beneficial for human health and may work in concert to provide synergistic effects in some *Brassica* species that contain both types of compounds. The phenolic and glucosinolate profiles of different *Brassica* species is quite well established, although in most cases the analysis is carried out separately as the two groups of phytochemicals usually require different methods. Bennett *et al.* [219, 166] developed a method for analysing both phenolic compounds and glucosinolates simultaneously in plants, which was later applied to the analysis of turnip green and turnip top samples (*Brassica rapa* var. *rapa*) by Francisco *et al.* [114]. Simultaneous identification of phenolic compounds and glucosinolates was also conducted by Gratacos-Cubarsi *et al.* [171] in white and green cauliflower, Velasco *et al.* [113] in kale, cabbage, and leaf rape, and Park *et al.* [205] in green and purple kohlrabies.

Instrumental analysis of flavonols in *Brassica* species is typically conducted using some form of HPLC-PDA coupled with a tandem mass spectrometer. Examples are given in the following table (Table 2.6), which lists the analytical instrumentation and conditions used by previous studies on the analysis of flavonol compounds in *Brassica* species. Negative ion ESI is the most widely used ionisation technique [26], offering higher sensitivity when coupled to HPLC. This is due to flavonols containing OH groups and are, therefore, more amenable to losing a proton. Mobile phases typically consist of a combination of water and acetonitrile or methanol, with low percentages of either formic, acetic, or trifluoro acetic acid.

Table 2.6 Analytical instrumentation and conditions used by other studies in the analysis of flavonols and their glycosylated and acylated derivatives in *Brassica* species. *NB: Do not belong to the *Brassica* genus, but to the larger family *Brassicaceae*.

***Brassica* Species**	**Analytical Instrumentation and Conditions**	**Reference**
Cabbage (*Brassica oleracea* var. *capitata*)	HPLC-PDA/ESI-MS^n (Agilent series 1200 Q-trap in negative and positive ion mode) Waters XTerra MS (150 x 3.9 mm, 5 µm) C18, 30°C H_2O + 0.1% HCOOH (A) and CH_3OH (B) gradient	Park *et al.*[214]
Kohlrabi (*Brassica oleracea* var. *gongylodes*)	HPLC-PDA/ESI-MS^n (Agilent series 1200 Q-trap in positive ion mode) RStech (250 x 4.6 mm, 5 µm) C18, 30°C CH_3CN (A) and H_2O + 0.15% AcOH (B) gradient	Park *et al.*[205]
Kale (*Brassica oleracea* var. *acephala*), Cabbage (*Brassica oleracea* var. *capitate*), and Leaf rape (*Brassica napus* var. *pabularia*)	HPLC-PDA/ESI-MS^n (Agilent series 1100 ion trap in negative ion mode) Phenomenex Luna (250 x 4.6 mm, 5 µm) C18 H_2O + 0.1% TFA (A) and CH_3CN + 0.1% TFA (B) gradient	Velasco *et al.*[113]
Red mustard greens (*Brassica juncea* var. *coss*)	UHPLC-PDA/ESI-MS^n (Thermo Orbitrap ion trap in positive and negative ion mode) Thermo Hypersil Gold (200 x 2.1 mm, 1.9 µm), C18 H_2O + 0.1% HCOOH (A) and CH_3CN + 0.1% HCOOH (B) gradient	Lin *et al.*[215]
Kale (*Brassica oleracea* var. *sabellica*)	HPLC-PDA/ESI-MS^n (Agilent series 1100 ion trap in negative ion mode) Phenomenex Prodigy (150 × 3.0 mm, ODS 3, 5 µm, 100 Å) C18, 30°C H_2O + 0.5% AcOH (A) and CH_3CN (B) gradient	Schmidt *et al.*[67]
Red Curly Kale (*Brassica oleracea* L. convar. *acephala* var. *sabellica* cv. 'Redbor')	HPLC-PDA/ESI-MS^n (Agilent series 1100 ion trap in negative and positive ion mode) Thermo Betasil (250 x 2.1 mm, 5 µm) C18, 30°C H_2O + 2% AcOH (A) and 50% CH_3CN + 48% H_2O + 2% AcOH (B) gradient	Olsen *et al.*[111]
17 different *Brassica* vegetables (other than *Brassica oleracea*)	HPLC-PDA/ESI-MS (Agilent single quadrupole in positive and negative ion mode) Waters Symmetry (250 x 4.6 mm, 5 µm) C18, 25°C H_2O + 0.1% HCOOH (A) and CH_3CN + 0.1% HCOOH (B) gradient	Lin and Harnly [112]
White and green cauliflower (*Brassica oleracea* L. var. *botrytis*)	UHPLC-PDA/ESI-MS/MS (Waters Acquity triple quadrupole in negative ion mode) Waters BEH Shield (150 x 1.0 mm, 1.7 µm), C18, 35°C 95% CH_3CN + 5% H_2O + 0.1% HCOOH (A) and 40% CH_3CN + 60% H_2O + 0.1% HCOOH (B) gradient	Gratacos-Cubarsi *et al.*[171]
Oilseed rape (*Brassica napus* L.)	HPLC-PDA/ESI-MS^n (Agilent series 1100 LCQ Deca ion trap in negative ion mode) Agilent Eclipse (150 x 2.1 mm, 3.5 µm) C18, 30°C H_2O + 0.1% HCOOH (A) and CH_3CN + 0.1% HCOOH (B) gradient	Auger *et al.*[218]

***Brassica* Species**	**Analytical Instrumentation and Conditions**	**Reference**
Turnip greens and turnip tops (*Brassica rapa* var. *rapa*)	HPLC-PDA/ESI-MS^n (Agilent series 1100 ion trap in negative ion mode) Phenomenex Luna (250 x 4.6 mm, 5 µm) C18 H_2O + 0.1% TFA (A) and CH_3CN + 0.1% TFA (B) gradient	Francisco *et al.*[114]
Curly Kale (*Brassica oleracea* L. convar. *acephala* var. *sabellica*)	HPLC-PDA/ESI-MS^n (Agilent series 1100 ion trap in negative and positive ion mode) Thermo Betasil (250 x 2.1 mm, 5 µm) C18, 30°C H_2O + 2% AcOH (A) and 50% CH_3CN + 48% H_2O + 2% AcOH (B) gradient	Olsen *et al.*[116]
Collard greens (*Brassica oleracea* L. var. *viridis*), Kale (*Brassica oleracea* L. var. *acephala*), and Chinese broccoli (*Brassica oleracea* var. *alboglabra*)	HPLC-PDA/ESI-MS (Agilent single quadrupole in positive and negative ion mode) Waters Symmetry (250 x 4.6 mm, 5 µm) C18, 25°C H_2O + 0.1% HCOOH (A) and CH_3CN + 0.1% HCOOH (B) gradient	Lin and Harnly[152]
Pak choi (*Brassica campestris* L. ssp. *chinensis* var. *communis*) and Chinese leaf mustard (*Brassica juncea* Coss)	HPLC-PDA/ESI-MS^n (Agilent series 1100 ion trap in negative ion mode) Macherey-Nagel Nucleodur (250 mm x 4 mm, 5 µm) C18, 20°C H_2O + 0.15% TFA (A) and CH_3CN (B) gradient (quantification of polyphenols) H_2O + 0.1% HCOOH (A) and CH_3CN (B) gradient (analysis of bound phenolic compounds)	Harbaum *et al.*[115]
Tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC), Kale (*Brassica oleracea* L. var. *acephala*), and Turnip (*Brassica rapa* var. *rapa* L.)	HPLC-PDA (Gilson) Waters Spherisorb ODS2 (250 x 4.6 mm, 5 µm) C18 H_2O + 0.1% HCOOH (A) and CH_3OH (B) gradient (analysis of *B. oleracea* varieties) H_2O + 10% HCOOH (A) and CH_3OH (B) gradient (analysis of *B. rapa*)	Sousa *et al.*[212]
Kale (*Brassica oleraceae* L. var. *acephala* DC.)	HPLC-PDA/ESI-MS (Waters single quadrupole in negative ion mode) Phenomenex Luna (250 x 2.0 mm, 5 µm) C18, 35°C 5 mM HCOOH in H_2O (A) and CH_3OH (B) gradient	Ayaz *et al.*[217, 220]
Pak choi (*Brassica campestris* L. ssp. *chinensis* var. *communis*)	HPLC-PDA/ESI-MS^n (Agilent series 1100 ion trap in negative ion mode) Macherey-Nagel Nucleodur (250 mm x 4 mm, 5 µm) C18, 20°C H_2O + 0.15% TFA (A) and CH_3CN (B) gradient (PDA analysis) H_2O + 0.1% HCOOH (A) and CH_3CN (B) gradient (MS analysis)	Harbaum *et al.*[136]
Turnip (*Brassica rapa* var. *rapa* L.)	HPLC-PDA (Gilson) Waters Spherisorb ODS2 (250 x 4.6 mm, 5 µm) C18 H_2O + 10% HCOOH (A) and CH_3OH (B) gradient	Fernandes *et al.*[221]

***Brassica* Species**	**Analytical Instrumentation and Conditions**	**Reference**
Turnip tops (*Brassica rapa* L. subsp. *sylvestris* L.)	HPLC-PDA/ESI-MS (Agilent quadrupole in negative and positive ion modes) Merck LiChroCART (250 x 4.0 mm, 5 µm) C18, 27°C, H_2O + H_3PO_4 (A) and CH_3OH (B) gradient. Phenomenex Luna (150 x 3.0 mm, 5 µm) C18, 27°C, H_2O (A) and CH_3CN (B) gradient	Romani *et al.*[213]
Salad Rocket (*Eruca sativa*), Wall Rocket (*Diplotaxis erucoides*) Wild Rocket (*Diplotaxis tenuifolia*), and Turkish Rocket (*Bunias orientalis*) *NB	HPLC-PDA/ESI-MS (Agilent quadrupole in negative and positive ion modes) and HPLC-PDA/ESI-MS/MS (Micromass Quattro II triple quadrupole in negative and positive ion modes) Phenomenex Luna (250 x 4.6 mm, 5 µm) C18, 25°C H_2O + 0.1% TFA (A) and CH_3CN + 0.1% TFA (B) gradient	Bennett *et al.*[166, 222]
Pak choi (*Brassica rapa* L. Ssp. *chinensis* L. (Hanelt.))	HPLC-PDA/ESI-MS^n (Agilent series 1100 HPLC with Thermo Fisher LTQ ion trap in negative ion mode) Alltech Alltima (250 x 4.6 mm, 5 µm) C18, 30°C, H_2O + 30% CH_3OH + 0.5% H_3PO_4 (A), CH_3OH + 0.5% H_3PO_4 (B) gradient. (quantification of flavonol aglycones) Thermo BDS Hypersil (150 x 2.1 mm, 3 µm) C18, H_2O (A), CH_3CN (B), and H_2O + 10% HCOOH (C) gradient. (qualitative analysis of flavonol derivatives)	Rochfort *et al.*[60]
Collards (*Brassica oleracea* L. cv. Top Bunch), and pak choi (*Brassica rapa* L. cv. Mei Qing)	HPLC-PDA (Beckman) Alltech Alltima (250 x 4.6 mm, 4 µm) C18 CH_3OH (A) and H_2O + 10% AcOH gradient	Young *et al.*[223]
Tronchuda Cabbage (*Brassica oleracea* L. var. *costata* DC)	HPLC-PDA/ESI-MS^n (Agilent series 1100 ion trap in negative ion mode) Merck LiChroCART (250 x 4 mm, 5 µm) C18 H_2O + 0.1% HCOOH (A) and CH_3OH (B) gradient	Ferreres *et al.*[208]
Broccoli (*Brassica oleracea* L. var. *italica*)	HPLC-PDA/ESI-MS^n (Agilent series 1100 ion trap in negative ion mode) Merck LiChroCART (250 mm x 4.0 mm, 5µm) C18 H_2O + 0.1% HCOOH (A) and CH_3OH (B) gradient	Vallejo *et al.*[57]
Cauliflower (*Brassica oleracea* L. var. *botrytis*)	HPLC-PDA/ESI-MS^n (Agilent ion trap in negative ion mode) Merck LiChroCART (250 x 4.0 mm, 5 µm) C18 H_2O + 0.5% HCOOH (A) and CH_3OH (B) gradient	Llorach *et al.*[128]
Black cabbage (*Brassica oleracea* var. *acephala* DC. subvar. v*iridis* cv. *serotina*)	HPLC-PDA/ESI-MS, (Agilent quadrupole in negative and positive ion modes) Phenomenex Luna (150 x 3.0 mm, 5 µm) C18, 26°C H_2O + HCOOH (A), CH_3CN (B), and CH_3OH (C) gradient	Romani *et al.*[74]
Cabbage (*Brassica oleracea* L. convar. *capitata* L. Alef. var. *alba* DC)	HPLC-UV/MS (FAB MS in negative ion mode) Superpac ODS2 (100 x 4.0 mm, 5 µm) C18 H_2O + 20% CH_3OH + 0.1% TFA isocratic elution	Nielsen *et al.*[132]

2.7.1 Structure Elucidation Using HPLC-MS^n

Recent advances in analytical techniques, particularly MS techniques, has allowed for detailed structure elucidation of naturally occurring plant phenolics. These techniques can provide information on structure characterisation including the following; the aglycone moiety; the type of sugar moiety (i.e. mono-, di-, tri- or tetra-saccharides and hexoses, deoxyhexoses or pentoses); any other conjugated substituent (i.e. hydroxycinnamic acids); the stereochemical assignment of terminal monosaccharide units (i.e. α or β); the sequence of the glycan part (i.e. *O*- or *C*-glycosylation); the interglycosidic linkages (i.e. (1→2) or (1→6)); and the point of attachment of the substituents to the aglycone core.

The glycosylation patterns of flavonoids in plant foods have been comprehensively studied [183, 27, 224, 26, 182]. Furthermore, a study by Ferreres *et al.* [225] concluded that it is possible to differentiate between the (1→2) and (1→6) interglucosidic linkages of *O*-glycosylated flavonoids, as well as determine the isomers of the flavonoids with two, three, and four glucoses attached. For example, if a flavonoid compound had two glucose moieties, it was possible to distinguish whether it was a sophoroside, gentiobioside or a diglucoside with the glucose moieties attached at positions *X* and *Y* on the aglycone core.

Some examples of complex flavonol derivatives from plant sources that have been identified using MS are shown in Table 2.7. The way in which structural identification is ascertained via the analysis of fragmentation patterns obtained from MS^n will be further discussed in the following sections.

Table 2.7 Characterisation of some complex flavonol derivatives in plant foods via MS^n structure elucidation.

Flavonols/Main Compounds	Plant	Reference
Kaempferol and quercetin derivatives: Kaempferol-3-*O*-sophoroside-7-*O*-glucoside Kaempferol-3-*O*-(2-caffeoyl-sophoroside)-7-*O*-glucoside	Black cabbage	Romani *et al.*[74]
Kaempferol derivatives: Kaempferol-3- [2-sinapoylsophoriside]-7-diglucoside Kaempferol-3-[2-feruloylsophoriside]-7-diglucoside	Italian kale	Heimler *et al.*[226]
Kaempferol and quercetin derivatives: Kaempferol-3-sinapoyl-diglucoside-7-diglucoside Quercetin-3-sinapoyl-diglucoside-7-diglucoside	Curly kale	Olsen *et al.*[116]
Kaempferol, quercetin and isorhamnetin derivatives: Kaempferol-3-*O*-sophoroside-7-*O*-glucoside Kaempferol-3-*O*-caffeoyl-sophoroside-7-*O*-glucoside Quercetin-3-*O*-sinapoyl-sophoroside-7-*O*-glucoside Kaempferol-3-*O*-feruloyl-sophoroside-7-*O*-glucoside	Kale	Ferreres *et al.*[227]
Kaempferol, quercetin and isorhamnetin derivatives: Major compounds were kaempferol glycosides and acylgentiobiosides	Collard greens, kale and Chinese broccoli	Lin and Harnly [152]
Kaempferol, quercetin and isorhamnetin derivatives: Major components not specified, however, did identify 29 compounds	Pak choi	Rochfort *et al.*[60]
Kaempferol and quercetin derivatives: Kaempferol-3-*O*-sophoroside-7-*O*-glucoside Kaempferol-3-*O*-caffeoyl-sophoroside-7-*O*-glucoside Kaempferol-3-*O*-feruloyl-sophoroside-7-*O*-glucoside Kaempferol-3-*O*-sinapoyl-sophoroside-7-*O*-glucoside Kaempferol-3-*O*-coumaroyl-sophoroside-7-*O*-glucoside Quercetin-3-*O*-feruloyl-sophoroside-7-*O*-glucoside Quercetin-3-*O*-caffeoyl-sophoroside-7-*O*-glucoside	Cabbage	Nielsen *et al.*[132]
Kaempferol, quercetin and isorhamnetin derivatives: Kaempferol-3-*O*-sophoriside Isorhamnetin-3-*O*-glucoside Isorhamnetin-3,7-*O*-diglucose	Rapeseed/Canola	Farag *et al.*[133]

2.7.1.1 Flavonol Aglycone Fragmentation Patterns

The fragmentation patterns of flavonoid aglycones has been studied in both the positive [228] and negative [229] ionisation modes and the most useful fragmentations, in terms of flavonoid aglycone identification, are those that require cleavage of two C—C bonds of the C ring [27] (Figure 2.14). The fragments produced can be designated according to the nomenclature proposed by Ma *et al.* [228] and are the most diagnostic since they provide information on the number and type of substitutions on the A and B rings. For free aglycones the labels ^{i,j}A and ^{i,j}B are assigned to fragments containing intact A and B rings respectively, The superscript letters i and j refer to the bonds on the C ring that have been broken. An additional subscript number 0 is used to the right of the letter for conjugated aglycones, to avoid confusion with the A_i and B_i labels that are used in the nomenclature for the fragmentation of carbohydrates (refer to Figure 2.15). Figure 2.14 represents one of the possible C ring cleavages; the $^{1,3}A_0$ ion is observed for all flavonoid groups and is typically the most abundant fragment ion.

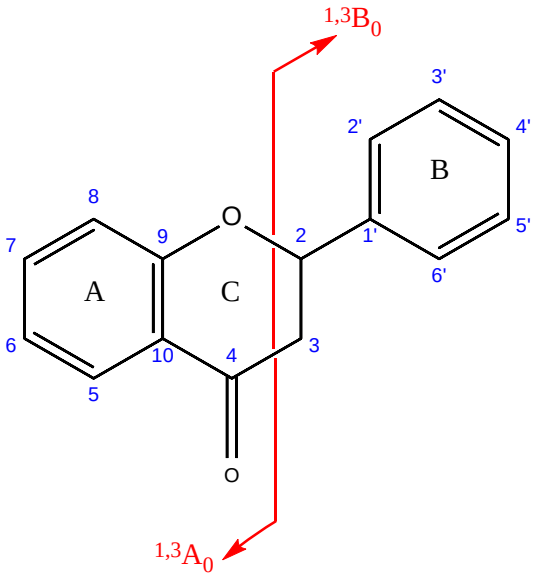


Figure 2.14 The most common cleavage observed in the fragmentation of flavonoids and the resulting A and B fragments assigned as per proposed nomenclature by Ma *et al.* [228] Numbers in blue represent the carbon numbers and the superscript numbers to the left of the fragment labels in red refer to the bond number.

The other major cleavages of C—C bonds that result in A and B ions (apart from 1/3) include 0/2, 0/3, 0/4, and 2/4. The fragmentation pathway depends heavily on the substitution pattern, in addition to the class of flavonoid. For example, flavonols contain an additional hydroxyl group in position 3 which results in more, and different, fragmentation possibilities as compared to flavones. Therefore, it is possible to differentiate between the flavonol kaempferol and the flavone luteolin, both of which have the same molecular mass, based on their fragmentation patterns [27].

In addition to the $^{i,j}A_0$ and $^{i,j}B_0$ ions, other fragments that may be observed include losses of small molecules and/or radicals; e.g. a loss of 18 amu (H_2O), 28 amu (CO), 42 amu (C_2H_2O), and the loss of 15 amu (CH_3) is particularly characteristic in the fragmentation of methoxylated flavonoids.

The MS fragmentation of aglycones is no longer routinely reported or necessary for the identification of these compounds due to the availability of commercial standards. As previously mentioned in this chapter, flavonols commonly occur as glycosides and furthermore, acylated glycosides, in which one or more of the sugar hydroxyls are esterified with an acid. The fragmentation of these compounds is the main focus of recent literature.

2.7.1.2 Flavonol Glycoside Fragmentation Patterns

In nature, flavonoid compounds commonly occur as flavonoid *O*-glycosides, whereby one or more hydroxyl groups of the aglycone core are conjugated to a sugar moiety. Hydroxyl groups at certain positions are usually favoured, for example the -OH at positions 3 and 7 in flavonols are the most common glycosylation sites [27]. The most widely encountered sugar is glucose, followed by galactose, rhamnose, xylose, and arabinose. Disaccharides are also regularly seen conjugated to flavonoids, with sophoroside (glucose (1→2) glucose) and gentiobioside (glucose (1→6) glucose) being common, as well as rutinose (rhamnosyl (1→6) glucose) and neohesperidose (rhamnosyl (1→2) glucose), and less frequently tri and tetrasaccharides. It is also possible for glycosylation to occur via a direct C—C bond between the sugar moiety and the flavonoid's basic nucleus, forming a flavonoid *C*-glycoside. This type of glycosylation is typically found at the C6 or C8 positions of the flavonoid nucleus [27].

Nomenclature devised by Domon and Costelo [230] is widely used to describe the fragmentation patterns of glycoconjugates. It was first used in the analysis of glycoconjugate ions produced from Fast Atom Bombardment (FAB) MS and Collision Induced Dissociation (CID) MS/MS spectra, however, it has since been applied to a variety of ionisation techniques and can also be applied to spectra obtained in both the positive and negative ionisation modes [26].

The simplest fragmentation of a carbohydrate molecule results from the cleavage of the glycosidic bond. As illustrated in Figure 2.15, fragment ions are designated as A_i, B_i and C_i where the subscript i represents the number of the glycosidic bond cleaved, counting from the terminal sugar unit. Fragment ions that contain the flavonol aglycone are labelled as X_j, Y_j and Z_j where the subscript j is the interglycosidic bond counted from the aglycone end, with the glycosidic bond that links the sugar moiety to the aglycone core being numbered as 0.

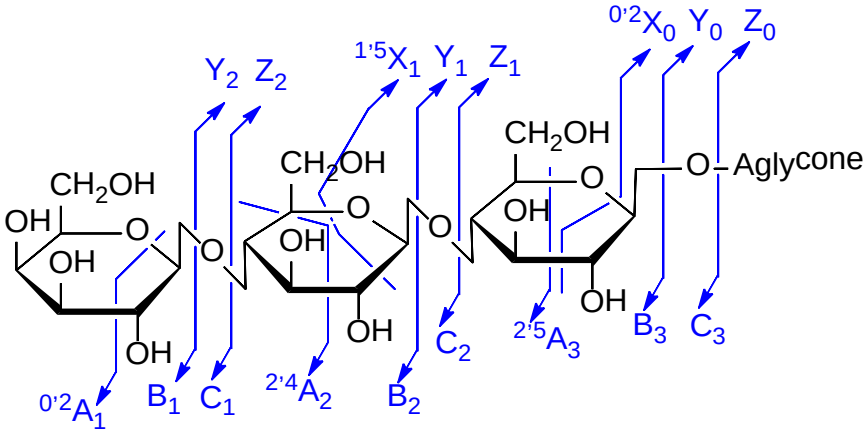


Figure 2.15 Common glycoconjugate cleavages and their respective fragment ions, as denoted using nomenclature devised by Domon and Costelo [230].

A more complex fragmentation process involves the cleavage of carbon-carbon bonds within the sugar ring itself. The resulting ions from this type of fragmentation are labelled as either $^{k,l}A_i$ or $^{k,l}X_j$, where the X ion contains the flavonol aglycone and the A ion the remaining sugar fragment. Since these types of fragmentations can occur in several ways, across several bonds, additional symbols are used to indicate the bonds that have been broken. Two superscripts, k and l, are added to the left of the letter to indicate the numbers of the bonds. Figure 2.16 depicts the numbering of the sugar ring bonds for glucose.

The Y and Z ions also include an additional superscript number (n) to the right of the letter; Y^n_j and Z^n_j (not shown in Figure 2.15). The n represents the carbon position of the phenolic hydroxyl where the sugar moiety that is being cleaved to produce that ion is attached.

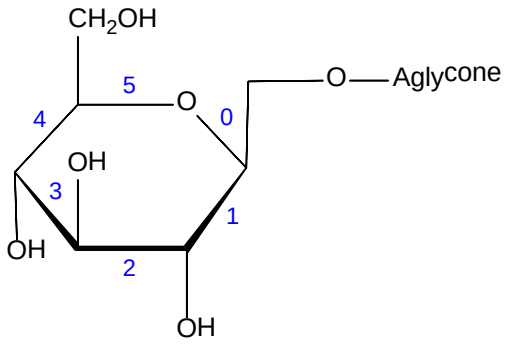


Figure 2.16 Structure of glucose showing the numbering of bonds.

The most common fragment observed in the MS spectra of flavonol glycosides arises from the cleavage of the glycosidic bond between the aglycone core and the sugar moiety, with the retention of the glycosidic oxygen atom by the aglycone core. This ion is represented by Y_0 (Figure 2.15). Another fragment that is frequently observed in the negative ion mode is fragmentation of the glycosidic bond with the loss of the glycosidic oxygen atom (i.e. the sugar moiety that is cleaved retains the glycosidic oxygen, it is not retained with the aglycone core). The remaining aglycone core ion is assigned as Z_0. In general, it is possible to determine the type of sugar conjugated to the flavonol by means of the MS losses, in addition to the relative abundance of the ions that are formed. In many cases, it is also possible to determine the glycosylation position and the interglycosidic linkage, as well as the stereochemical identity of the terminal sugar residue [17].

Ferreres *et al.* [225] reported that both flavonol sophorosides (1→2 interglucosidic linkage) and flavonol gentiobiosides (1→6 interglucosidic linkage) are characterised by the fragment ion $[M-H-324]^-$ as their base peak (i.e. the most abundant peak in the MS spectrum) in MS^2 experiments. The typical MS^2 fragmentation of 3-*O*-sophorosides consists of a base peak $[M-H-324]^-$, the fragment ion $[M-H-180]^-$ and the fragment ion $[M-H-162]^-$ [225, 67]. This fragmentation pattern suggests a sophoroside at the 3-*O* position. Sophorosides of quercetin and kaempferol have been observed in kale [227, 67] and several *Brassica oleracea* [208, 128, 57, 231] and *Brassica rapa* [128, 60, 136, 213] species. In contrast, the absence of the fragment ion $[M-H-180]^-$ in the MS^2 spectra revealed that the interglycosidic linkage at the 3-*O* position as a gentiobioside.

Flavonol di-glucosides with sugar moieties linked to different hydroxyl positions of the flavonol core have the fragment ion $[M-H-162]^-$ as their base peak, which corresponds to the loss of one glucose moiety at the 3-*O* position. Similarly, the same fragment ion is observed in the MS^2 spectra of mono-glucosides, in addition to the fragment ion $[M-H-120]^-$. This was observed in studies of flavonols in kale [67] and pak choi [60]. Mono-glucosides of quercetin, kaempferol and isorhamnetin have been reported in kale [152, 67] and other *Brassica oleracea* species, such as broccoli [57], tronchuda cabbage [208], cauliflower [128], and *Brassica rapa* species such as pak choi [60, 136] and turnip leaves [213]. In all cases, the glucose moiety was bound to the 3-*O* position of the aglycone.

Flavonol glycosides are typically *O*-glycosides, whereas flavones tend to occur as *C*-glycosides. The MS spectra of a flavonol-*O*-glycoside will always generate a deprotonated aglycone ion because of the fragmentation of the glycosidic linkage, which is the weakest bond. The aglycone ion is normally the base peak when the glycosylation occurs on only one phenolic hydroxyl. If the flavonol is glycosylated on more than one phenolic hydroxyl, then the base peak originates from the loss of one of the glycosidic units over one of the phenolic hydroxyls and the aglycone

ion is less abundant, or non-existent. A sugar unit that is conjugated at position 7 will cleave more readily than a sugar unit that is conjugated at position 3 [225]. In contrast to *O*-glycosides, a cleavage of the glycosidic bond between the sugar and the aglycone is not possible with *C*-glycosides. Therefore, an internal fragmentation of the sugar bonds occurs instead. For more detailed information regarding the characterisation and differentiation between *C*-glycosides conjugated at the C6 or C8 positions refer to a report by Waridel *et al.* [232].

2.7.1.3 Acylated Flavonol Glycoside Fragmentation Patterns

Flavonol glycosides frequently occur as acylated derivatives in nature, with either an aliphatic or aromatic acid. The most widely seen acyl moieties conjugated to flavonol glycosides are hydroxycinnamic acids; commonly *p*-coumaroyl, caffeoyl, feruloyl, or sinapoyl. These can be identified in MS fragmentations as losses of 146, 162, 176, or 206 amu respectively. *Brassica* species in particular are rich in flavonol glycosides acylated with hydroxycinnamic acids, with a vast number of them following the general structure of flavonol-3-*O*-(hydroxycinnamoyl)-glycoside-7-*O*-glycoside (refer to Table 2.7). For these compounds, the main fragmentation ions that are observed in the MS^2 $[M-H]^-$ spectrum are due to the breakdown of the *O*-glycosidic bond at the C7 position and the loss of that sugar. In addition to this, the loss of the hydroxycinnamoyl residue can be observed. If the acylated glycosidic residue at C3 is further fragmented via MS^3 $[(M-H) \rightarrow {Y^7}_0]^-$, the daughter ions observed from this fragmentation are typically due to the loss of the hydroxycinnamoyl residue [57, 67].

Schmidt *et al.* [67] studied the fragmentation patterns of flavonoid glycosides in kale (*Brassica oleracea* var. *sabellica*). The study used HPLC-PDA/ESI-MS^n to successfully identify 71 flavonoid glycosides (including 27 non-acylated, 30 mono-acylated and 14 di-acylated flavonol glycosides) of quercetin, kaempferol and isorhamnetin. Flavonol glycosides were present as mono-, di-, tri- and tetra-glycosides, as well as more complex acylated compounds with various hydroxycinnamic acids (*p*-coumaric, caffeic, ferulic, hydroxyferulic and sinapic acid). Mono-acylated compounds were found to exist as di-, tri- and tetra-glycosides, whereas di-acylated glycosides were found to occur as tetra- and even penta-glycosides [67]. The particular variety that was studied was called ‘Altmärker Braunkohl’ with green leaves. The main flavonol was kaempferol, followed by quercetin and isorhamnetin. These flavonol aglycones were glycosylated with glucose, the only sugar moiety found, which was also reported by Olsen *et al.* [116], Ferreres *et al.*[227], and Lin *et al.*[152].

2.7.2 Structure Elucidation Using NMR Spectroscopy

As previously discussed in 2.5.4.9, NMR is a powerful tool that has been used, in combination with HPLC-MS^n, for structural elucidation of phenolic compounds, including complex glycosylated and acylated flavonols. Some examples of these complex flavonol derivatives, the NMR instruments, and various 1D and 2D experiments utilised to characterise and confirm structure elucidation, are shown in Table 2.8. The two universally used solvents are deuterated dimethyl sulfoxide (DMSO-d_6) and deuterated methanol (MeOH-d_4).

Table 2.8 Characterisation of some complex flavonol derivatives in plant foods via NMR.

Flavonol Derivative	Instrument	Experiments	Solvent	Reference
kaempferol-3-*O*-sophoroside-7-glucoside	Bruker AVANCE DMX 600, DRX 500, AMX 400 or DPX 300 instrument recorded at 300 K.	^{1}H, ^{13}C, COSY, HSQC, HMBC, TOCSY, and ROESY	MeOH-d_4	Schliemann *et al.*[135]
kaempferol-3-*O*-sophoroside-7-glucoside	Jeol P-100 FT spectrometer at 95°C	^{13}C	DMSO-d_6	Markham *et al.*[233]
kaempferol-3-*O*-sophoroside-7-glucoside	Instrument not specified	^{1}H and ^{13}C	DMSO-d_6	Nielsen *et al.*[132]
kaempferol-3-*O*-hydroxyferuloylsophoroside-7-glucoside	Bruker BioSpin Avance 600 MHz with CryoProbe	^{1}H, ^{13}C, COSY, HSQC, and HMBC	MeOH-d_4	Harbaum *et al.*[136]
kaempferol-3-*O*-hydroxyferuloylsophoroside-7-glucoside	Bruker Avance 400 MHz (^{1}H) and 100.6 MHz (^{13}C)	^{1}H, ^{13}C, COSY, TOCSY, selTOCSY, NOESY, HSQC, and HMBC	DMSO-d_6	Fiol *et al.*[134]
kaempferol-3-*O*-sophoroside	Varian VNMRS 600 NMR with 5 mm inverse detection cryoprobe	^{1}H, ^{13}C, COSY, HSQC, and HMBC	Not specified	Farag *et al.*[133]
quercetin-3,4'-diglucoside, quercetin-4'-glucoside	Bruker 800 MHz with cryoprobe at 25°C	^{1}H and 2D (not specified)	DMSO-d_6	Caridi *et al.*[137]

2.7.3 Characterisation of Complex Flavonol Derivatives: A Summary

The key criteria used in the characterisation of flavonols and their glycosylated and acylated derivatives in various fruits, vegetables and plants using UV-Vis absorbance spectra and typical MS^n fragmentation patterns are summarised below.

- λ_{max} of quercetin, kaempferol, and isorhamnetin aglycones are ≈260 and ≈370 nm
- A shift in λ_{max} from ≈370 to ≈350 nm suggests that the 3 hydroxyl is blocked and therefore indicates that the position is glycosylated
- A further shift in λ_{max} from ≈350 to ≈330 nm is indicative of another substitution such as a hydroxycinnamic acid
- A loss of glucose with glycosidic oxygen is observed as a loss of 180 amu
- A loss of glucose without the glycosidic oxygen is observed as a loss of 162 amu
- The most common fragmentation through the glucose ring is observed as a loss of 120 amu
- Sugar residues as *O*-glycosides typically substitute on the flavonol core at positions 3 and 7
- If the first fragment for 7-*O*-glycosides in the MS^2 spectra is a loss of 162 amu, it indicates that one sugar is present, and a loss of 324 amu indicates two sugars
- If multiple ions are seen in the MS^2 spectra, it indicates that it is either a 3,7-diglucoside or it is un-substituted at position 7
- For normal di-glucosides; if it is a 3,7-diglucoside a loss of 162 amu is observed in the MS^2 spectra, or if it is a 3-diglucoside (i.e. gentiobioside with a 1→6 glycosidic link) a loss of 324 amu is observed in the MS^2
- For sophorosides (1→2 glycosidic link), a loss of 162 amu and 324 amu will be observed in the MS^3 spectra, in addition to losses of 120 amu (fragmentation through the sugar ring) and 180 amu (fragmentation of the glycosidic bond to the sugar including the glycosidic oxygen). These latter two losses are not detected for normal di-glucosides [60]
- Sophorosides will produce a high relative abundance of the 162 amu and 180 amu ions, whereas these ions are in very low abundance or absent for di-glucosides (1→6 linkages) [225]
- The Z ion is formed when the aglycone does not retain the glycosidic oxygen (i.e. the oxygen is lost with the sugar)
- The Y ion is formed when the aglycone does retain the glycosidic oxygen (i.e. the oxygen is not lost with the sugar)

- The X ion is formed when fragmentation occurs through the sugar ring. X ions have superscript numbers (k,l) to the left where cleavage occurs (i.e. the number of the bond in the sugar ring) and are denoted as $^{k,l}X$
- The subscript number (j) on a Z, Y or X ion is the number of the glycosidic bond that is cleaved, counting 0 from the aglycone, and is denoted as Y_j
- The superscript number (n) on a Z, Y, or X ion to the right of the letter indicates the carbon number that the sugar is attached to on the aglycone and is denoted as Y^n.

2.8 BIOACTIVITY AND ANTI-CANCER PROPERTIES

Phytochemicals have played important roles in human medicine many centuries before the active compounds were identified. Traditional herbal remedies, for example, have been used for a vast array of pharmaceutical and dietary applications for several millennia in East Asia, China, Japan, India, and Thailand. [90, 234]. Cancer therapy using natural substances and its prevention through dietary intervention has become an important issue. Considerable attempts have been made to identify natural compounds found in plants that have the ability to inhibit, retard, or reverse multi-stage carcinogenesis. Flavonols such as quercetin, kaempferol, and isorhamnetin, including their 3-sophoroside-7-glucosides and hydroxycinnamate derivatives, have been reported in *Brassicaceae* and have been attributed to many beneficial health effects, including displaying remarkable anti-cancer properties [94, 93, 212].

There are a number of articles that have reviewed dietary flavonoids and their application to cancer therapy and prevention, including their possible mechanisms of action [7, 23, 235-238, 37, 239]. The two main ways in which the anticarcinogenic activity of flavonoids are investigated include *in vitro* via cell culture models, and *in vivo* via animal models. Studies conducted *in vitro* have concentrated on both the direct and indirect actions of flavonols on cancer cells and have reported a variety of anti-cancer effects, in addition to *in vivo* experiments which have displayed anti-tumour activities. Flavonols can affect the initiation and promotion stages of the carcinogenic process, i.e. they have the ability to aid in cancer chemoprevention as well as chemotherapy [23, 235, 238].

2.8.1 Cell Culture Models: HT-29

Biological activities of phytochemicals are often assessed by using cultured cells as tissue models. Human colorectal carcinomas have been used in many studies to assess such effects, and are of particular interest due to the direct contact the digestive tract has with these compounds when consumed in the diet through fruits and vegetables [240]. A human colorectal adenocarcinoma cell

line known as HT-29 is one cell model that has been used in a plethora of published work and is the model chosen for this current study. The HT-29 cell line was originally isolated from a primary tumour of a 44-year-old Caucasian female in 1964 [241]. It was initially used to study different biological aspects of human cancers but has also been the focus of food digestion and bioavailability studies due to its ability to express characteristics of mature intestinal cells and simulate the *in vivo* environment of the small intestine. HT-29 cells have been used to investigate the transport of drugs and food compounds, the intestinal immune response to bacterial infection and microorganism survival, adhesion and invasion [241].

Numerous studies have investigated the effects of different compounds within the flavonoid group on HT-29 colon cancer cells including flavanones [242-243, 240, 244], flavones [245, 244] and anthocyanidins [246]. A number of studies have also screened different subclasses of flavonoids using this cell culture model to determine if there were any structure-activity relationships [247-250]. Other research that has utilised the HT-29 cell culture model have included studies on quercetin and its derivatives [251-252, 63], kaempferol [253-254], and isorhamnetin glycosides [255]. Furthermore, there have been several studies on a variety of fruit and vegetable extracts such as cranberries [256], cherries [257], strawberries [258-260] and other berries [261], apples [262-263], grapes [264], acai [265], kale [266], pomegranate juice [267], and other fruits [268]. Other plant extracts, including a number that are used in traditional herbal medicine, have also been examined for their effects on HT-29 cells [255, 269-272, 244, 273].

2.8.2 Antiproliferative Effects: MTT Assay and IC_{50}

One of the most widely used methodologies to determine cell viability and the antiproliferative effects of plant compounds on cell culture models is the MTT cell proliferation assay. It is based on the reduction of the yellow tetrazolium (MTT) salt (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) to a water-insoluble purple formazan salt. The MTT salt is reduced by metabolically active cells, thought to be by mitochondrial succinate dehydrogenase, after which the water-insoluble formazan salt is solubilised with an organic solvent and measured spectrophotometrically. Since the reduction of MTT can only occur in metabolically active cells, the level of activity and hence the intensity of the purple colour produced, is a measure of the viability of the cells [274].

A measure of antiproliferative effects that is commonly used in studies is the IC_{50}. The IC_{50} (IC = Inhibitory Concentration) indicates the concentration of an inhibitor where the response of the target, in this case the proliferation of the cancer cells, is reduced by half. It can be used as a way to evaluate the effectiveness of a potential anti-cancer agent and to make a comparison between different compounds. The lower the IC_{50} value, the more potent the compound is as an anti-cancer agent (i.e. the less that is required to achieve the desired effect, and the less likely the agent will

have some undesirable off-target effect). An example of some IC_{50} values obtained for quercetin, kaempferol, and isorhamnetin, and their ability to inhibit cell proliferation in a variety of cancer cell lines are displayed in Table 2.9.

Table 2.9 Examples of IC_{50} values published for quercetin, kaempferol, and isorhamnetin for a variety of cancer cell lines *in vitro*.

Flavonol	Cancer Type/ Cell Culture Model	Exposure Time (h)	IC_{50} (μM)	Reference
Quercetin	Colon (LoVo)	48	40.2	Zhang *et al.*[62]
	Breast (MCF-7)	48	30.8	Zhang *et al.* [62]
	Colon (HT-29 and HCT116) Breast (MCF-7) Liver (HepG2) Lung (A549)	72	>80	You *et al.*[252]
	Cervical (HeLa)	24	80	Priyadarsini *et al.*[65]
	Bladder (253J)	72	45.5	Kim *et al.*[275]
	Oesophagus (OE33)	24	78	Zhang *et al.*[276]
	Liver (HepG2)	72	80.0	Guo *et al.*[277]
	Colon (HT-29 and HCT116) Breast (MCF-7 and MDA-MB231)	72	>100	Li *et al.*[278]
Kaempferol	Liver – Rat (H4IIE)	24	41	Niering *et al.*[279]
	Lung (H460)	24	50	Leung *et al.*[280]
	Lung (A549)	48	35	Nguyen *et al.*[281]
	Oesophagus (OE33)	24	111	Zhang *et al.*[276]
	Bone (U-2 OS)	24	148.4	Huang *et al.*[282]
	Liver (HepG2)	72	57.3	Guo *et al.*[277]
Isorhamnetin	Embryonic – Mouse (NIH3T3)	Not specified	13	Lee *et al.*[283]
	Pancreas (CRL-4038)	Not specified	25	Lee *et al.*[283]
	Colon (SW48)	Not specified	64	Lee *et al.*[283]
	Colon (SW620)	Not specified	71	Lee *et al.*[283]
	Liver (BEL-7402)	72	235.3	Teng *et al.*[284]
	Oesophagus (ECA-109)	48	126.5	Ma *et al.*[285]
	Colon (HCT-116)	24	224	Jaramillo *et al.*[286]
	Colon (HCT-116)	48	72	Jaramillo *et al.*[286]
	Lung (A549)	48	140.6	Li *et al.*[287]
	Liver (HepG2)	72	29.0	Guo *et al.*[277]
	Colon (HT-29)	72	>100	Li *et al.*[278]
	Colon (HCT116)	72	72	Li *et al.*[278]
	Breast (MCF-7)	72	97	Li *et al.*[278]
	Breast (MDA-MB231)	72	71	Li *et al.*[278]
	Colon (HT-29)	48	75.3	Antunes-Ricardo *et al.*[255]
	Colon (Caco-2)	48	81.3	Antunes-Ricardo *et al.*[255]

2.8.2.1 Mechanisms of Antiproliferative Activity: Cell Cycle Arrest and Apoptosis

There are two types of cell death, apoptosis and necrosis, which are differentiated via distinct morphological and biochemical processes. Necrosis is less orderly than apoptosis and does not involve cell signals being sent to nearby phagocytes of the immune system to engulf the dying cell. A cell undergoing necrosis will release its intracellular content after damage to its cellular membrane, resulting in the release of harmful chemicals that may damage other nearby cells and cause inflammation [240].

Apoptosis is the process of programmed cell death. It is a vital process that is required to maintain a balance between cell death and cell proliferation, and the disruption of this cellular balance, or of the mechanisms that control it, can lead to human disease such as cancer. Complex signal transduction pathways govern the process and bring about gene-mediated cell death. Being a process that is regulated by specific gene activity, it is therefore very sensitive to DNA mutations [288].

Cell cycle arrest is another protective mechanism involved in the regulation of cellular homeostasis. The natural cell cycle of duplication and division includes a number of checkpoints that enable the cell to determine whether to proceed with division or stop. Cell cycle arrest is a stopping point in the cell cycle which might occur if the cell identifies that it is no longer functional due to DNA damage that might cause functional problems or lead to the development of a tumour.

Apoptosis and cell cycle arrest are often inhibited in cancerous cells which leads to a higher cell proliferation rate. Therefore, an ideal method for cancer chemoprevention or chemotherapy is for the anti-cancer agent to overcome such proliferation by inducing apoptosis or cell cycle arrest [261]. It is also vitally important that the dose of the anti-cancer agent does not cause significant toxicity for necrosis to occur, but rather for it to induce apoptosis or alternatively cell cycle arrest.

2.8.3 Antiproliferative Effects of Flavonols on Cancer Cells *in vitro*

Cancer chemoprevention using natural substances and its prevention through dietary intervention has become an important issue. Over the last three decades there has been considerable interest in assessing the antiproliferative effects of flavonols on cancer cells. Flavonols are the most ubiquitous group of flavonoids found in foods, therefore dietary flavonols and their antiproliferative activities may play an important role in cancer chemoprevention, especially cancer of the gastrointestinal tract because of the direct contact with food. In a study conducted by Kuo *et al.* [248], the antiproliferative potency of several flavonoid compounds on colon cancer cells including HT-29 and Caco-2 cell lines showed that one of the most potent flavonoids out of all those tested was the flavonol quercetin. Studies by Agullo *et al.* [249] and Daskiewicz *et al.* [250] also demonstrated that the flavonol subclass was one of the most potent in terms of *in vitro* antiproliferative abilities. Quercetin, kaempferol, and isorhamnetin, which are widely found in fruits and vegetables, have demonstrated an ability to inhibit cancer cell proliferation *in vitro*, whether it be in colon cancer cells or other human or rat cell culture models.

2.8.3.1 Antiproliferative Effects of Quercetin, Kaempferol, and Isorhamnetin

Evidence supporting the health claims of flavonols for protection against cancer is reflected from the large number of studies reporting inhibitory effects of quercetin, kaempferol, and isorhamnetin on the proliferation of cancer cell lines *in vitro*. This overwhelming amount of data suggests that these compounds could potentially be used as anti-cancer agents against a variety of carcinomas. Some of these studies are outlined in Table 2.10. For detailed reviews on the health benefits, including anti-cancer activities, and their potential use in cancer chemoprevention refer to publications by Jan *et al.* [53] for quercetin and Chen and Chen [289] for kaempferol. Isorhamnetin has also been shown to display potent anti-cancer activities, including antiproliferative effects on numerous cell culture models *in vitro*. Interestingly, isorhamnetin is one of the metabolites of quercetin in the human body, therefore the anti-cancer effects that are observed from quercetin may in part be mediated through isorhamnetin [290].

Table 2.10 Methodology including antiproliferative assay, concentration, and exposure time used for *in vitro* studies on quercetin, kaempferol and isorhamnetin on a variety of cancer cell lines.

Flavonol	Cancer Type/ Cell Culture Model	Method	Exposure Concentration (μM)	Exposure Time (h)	Reference
Quercetin	Colon (HCT15 and CO115)	MTT and BrdU	5,10,20 (HCT15) 5,8,12,15 (CO115)	48	Xavier *et al.*[291]
	Colon (LoVo) Breast (MCF-7)	MTT	25,50,100	24, 48	Zhang *et al.*[62]
	Colon (HT-29 and HCT116) Breast (MCF-7) Liver (HepG2) Lung (A549)	MTT	10-200	72	You *et al.* [252]
	Colon (SW480) Mouse colorectal (clone 26 cells)	MTT	10,20,40,80,160	24,48,72	Shan *et al.* [66]
	Cervical (HeLa)	MTT	20,40,60,80,100	24	Priyadarsini *et al.* [65]
	Colon (HT-29)	MTT	50,100	48	Kim *et al.* [63]
	Mouse embryonic (F9) Thyroid (TPC-1, FTC-133, NPA, FRO, and ARO)	MTT	1,10,50,100	24,48,72,96,120	Kang *et al.*[292]
	Bladder (253J)	XTT	50	72	Kim *et al.*[275]
	Oral cavity (SCC-1483, SCC-QLL1 and SCC-25)	Cell Titer 96 Aqueous One	20,40,80	24	Kang *et al.*[293]
	Colon (HCT-116 and HT-29) Breast (MCF-7)	LDH-leakage and BrdU	0-100	24	van der Woude *et al.* [251]
	Bladder (T24)	Cell counting (hemocytometer)	10,30,50,75,100,200	24,48,72	Rockenbach *et al.* [294]
	Pancreatic (EPP85-181P and EPP85-181RDB)	Colorimetric – sulphorhodamine B	3,6,12	24	Borska *et al.* [61]
	Lung (SPC111 and SPC212)	Cell Titer 96 Aqueous One	5,10,50,100	24,48,72,96	Demiroglu-Zergeroglu *et al.*[295]
	Leukaemia (CCRF-CEM, HL-60 and K-562)	XTT and trypan blue	12.5,25,50,75,100	48,72	Avci *et al.* [296]
	Oesophageal (OE33)	MTT	10,20,40,80	24,48,72	Zhang *et al.*[276].

Flavonol	Cancer Type/ Cell Culture Model	Method	Exposure Concentration (μM)	Exposure Time (h)	Reference
Kaempferol	Colon (HT-29)	MTT	20,40,60	24,48,72	Cho *et al.* [253]
	Rat liver (H4IIE)	MTT and neutral red uptake	50,100,250	24	Niering *et al.*[279]
	Breast (MCF-7) Stomach (SGC-7901) Cervical (Hela) Lung (A549)	MTT	25,50,75,100	48	Liao *et al.* [297]
	Breast (MCF-7)	MTT	50,100	96	Kim *et al.*[298].
	Oral cavity (SCC-1483, SCC-QLL1 and SCC-25)	Cell Titer 96 Aqueous One	20,40,80	24	Kang *et al.*[293]
	Lung (H460)	Trypan blue	30,50,80	24	Leung *et al.*[280].
	Lung (A549)	MTT and cell proliferation ELISA kit	17.5,35,52.5,70	24,48	Nguyen *et al.*[281]
	Breast (MCF-7, T47D, HC11 and MDA-MB-231)	MTT	0-100	48	Kim *et al.*[299]
	Gastric (MKN28, SGC7901 and GSE-1)	Cell counting kit-8	25,50,75,100,150,200	24,48,71	Song *et al.* [300].
	Oesophageal (OE33)	MTT	10,20,40,80	24,48,72	Zhang *et al.* [276]
	Bone (U-2 OS, HOB and 143B)	MTT	25,50,100,150,200	24	Huang *et al.* [282]
Isorhamnetin	Mouse embryonic fibroblast (NIH3T3, K-RAS, H-RAS) Colon (SW620)	WST-1	0.1-100	48	Lee *et al.*[283]
	Liver (BEL-7402)	MTT	25-300	12,24,48,72	Teng *et al.*[284]
	Oesophageal (ECA-109)	MTT	10,20,40,80	48	Ma *et al.*[285]
	Colon (HCT-116)	MTT	0-100	24,48	Jaramillo *et al.*[286]
	Lung (A549)	MTT	10,20,40,80,160,320	48	Li *et al.*[287]
	Liver (HepG2)	Methylene blue	0-200	72	Guo *et al.*[277]
	Gastric (AGS, SNU5 and MKN45)	MTT	10,25,50	24,48,72	Ramachandran *et al.* [301]
	Colon (HCT116 and HT-29) Breast (MCF-7 and MDA-MB-231)	MTS	Not specified	72	Li *et al.*[278]

2.8.3.2 Antiproliferative Effects of Aglycones vs Glycosides

It is important to assess the antiproliferative effects of flavonoid glycosides in addition to their aglycones, as almost all flavonoids in plants and foods occur in the form of glycosylated derivatives. Moreover, the addition of the sugar moiety/moieties affects the bioavailability of the compound and the mechanisms of its biological activities. Studies conducted *in vitro* have reported varying antiproliferative effects when comparing flavonoid aglycones to their conjugated glycosides. Veeriah *et al.* reported that individual apple flavonoid aglycones possess strong cell growth inhibitory activities and are biologically more active than their glycoside derivatives [262]. In a subsequent study, the effects of anthocyanins on HT-29 colon cancer cells found that cyanidin, but not its glycosides cyanin and idaein, was able to inhibit the cellular growth of the cultured colon carcinoma cells *in vitro* [246]. Similarly, another study by Manthey *et al.* [242], reported that glycosylation of citrus flavonoids removed their antiproliferative activity against six human cancer cell lines, including breast, prostate, colon, lung, and melanoma cells. More recently, a study by Guo *et al.* [277] reported that flavonoid di-glycosides displayed very weak antiproliferative activities in human liver cancer cells (HepG2) compared to their aglycones and mono-glycosides.

In contrast, one study that assessed the antiproliferative effects of anthocyanidins found in grapes on HT-29 cells found that the glycosides are at least as, or even more, effective than the aglycones [302]. In addition, You *et al.* [252] compared the antiproliferative activities of quercetin and two of its glycosylated derivatives, quercetin-3-*β*-D-glucoside and quercetin-3-*O*-rutinoside (commonly known as rutin) on six different human cancer cell lines including colon, breast, liver, and lung. Quercetin-3-*β*-D-glucoside was the most potent anti-cancer agent, with IC_{50} values ranging between 15 and 25 μM between the different cell lines, and rutin showed the least potency. Yang and Liu [303] also reported that quercetin-3-*β*-D-glucoside exhibits significant antiproliferative activity against human breast cancer cells (MCF-7) *in vitro*. Furthermore, a study by Guon and Chung [271] investigated the effects of two quercetin glycosides, quercetin-3-*O*-rutinoside (rutin) and quercetin-3-*O*-galactoside (hyperoside) on HT-29 colon cancer cells. Hyperoside and rutin significantly inhibited the proliferation of HT-29 human colon cancer cells in a dose dependent manner. Another study by Katsube *et al.* [304] showed that not only the aglycone anthocyanidins, but also their anthocyanin glycoside derivatives, inhibit the proliferation of cancer cells and induce apoptosis. Antunes-Ricardo *et al.* [255] evaluated the cytotoxic effects of the most abundant isorhamnetin tri and di-glycosides obtained from *Opuntia Ficus-indica* extracts against two different human colon cancer cells (HT-29 and Caco2). Isorhamnetin di-glycosides had stronger antiproliferative activities on HT-29 cells as compared to isorhamnetin aglycone and the tri-glycosides. This research demonstrates that the glycosylation pattern of flavonoids can affect their

bioactivity and antiproliferative effects against cancer cells *in vitro* and should therefore be taken into account.

2.8.4 Synergistic Effects of Flavonols on Cancer Cells *in vitro*

Studies have shown that flavonoids can act synergistically to enhance their antiproliferative effects. A synergistic therapeutic effect is defined as an increased effect due to the combination of two or more compounds, as compared to the individual compounds at equal concentrations [303].

The antiproliferative effects of cranberry extracts compared to individual phytochemicals and various fractions of the extracts were investigated using cell lines including human oral (KB, CAL27), prostate (RWPE-1, RWPE-2, 22Rv1), and colon cancer (HT-29, HCT116, SW480, SW620). The total polyphenol fraction was the most active inhibitory fraction against all cell lines. The enhanced antiproliferative activity of the total polyphenol extract compared to its individual phytochemicals suggests synergistic or additive antiproliferative interactions of the flavonoids within the cranberry extract [256]. Ackland *et al.* [68] utilised human gut and breast cancer cells to show the synergistic effect of quercetin and kaempferol in reducing cell proliferation. Combined treatments with quercetin and kaempferol were more effective than the additive effects of each individual flavonol. Another study by Yang and Liu [303] examined whether apple extracts in combination with quercetin-3-*β*-D-glucoside have additive and/or synergistic effects on human breast cancer (MCF-7) cell proliferation. The inhibitory activity toward the proliferation of the MCF-7 cells *in vitro* was increased significantly after the addition of the apple extracts and quercetin-3-*β*-D-glucoside in combination, when compared to the treatment with apple extracts and quercetin-3-*β*-D-glucoside alone. A further study by Demiroglu-Zergeroglu *et al.* [295] investigated the antiproliferative effects of quercetin in combination with cisplatin, a common chemotherapy drug, on two lung cancer cell lines. The combination of quercetin with cisplatin resulted in an enhanced antiproliferative effect on the growth of the lung cancer cells *in vitro*.

2.8.5 Antiproliferative Effects of Plant Extracts on Cancer Cells *in vitro*

Research has shown that fruit and vegetable phytochemical extracts exhibit strong antiproliferative activities *in vitro*, therefore, the additive and synergistic effects of phytochemicals in plant foods may actually be responsible for their potent anti-cancer activities. Table 2.11 lists a number of *in vitro* studies that investigated the antiproliferative effects of a variety of different plant extracts on different types of cancers, including the antiproliferative/cell viability assay used, the extract dose that the cells were exposed to, and the exposure time. The results from this research suggests that the varying magnitude of antiproliferative activities exhibited by different cultivars of the same fruit or vegetable on cancer cell proliferation might potentially be used to select cultivars for health-promoting properties.

Table 2.11 Studies that assessed the antiproliferative effects of fruit, vegetable, or plant extracts on a variety of cancer cells *in vitro*, including antiproliferative assay used, extract dose, and exposure time. DW = Dry weight.

Fruit, Vegetable, or Plant Extract	Cancer Type/ Cell Culture Model	Method	Extract Dose	Exposure Time (h)	Reference
Strawberries	Colon (HT-29) Breast (MCF-7)	WST-1	0.025, 0.05, 0.25, 0.5% DW	24	Olsson *et al.*[258]
	Liver (HepG2)	MTS	5,10,20,30,40,50,75 mg/mL	96	Meyers *et al.*[305]
	Oral cavity (KB, CAL-27) Breast (MCF-7) Prostate (LNCaP, DU145) Colon (HT-29, HCT116)	CellTiter-Glo Luminescent Cell Viability Assay	250 μg/mL	48	Zhang *et al.*[260]
	Colon (HT-29)	Perkin-Elmer ATP1step lite luminescence kit	3 mg/mL	24,48,72,86	Wang *et al.*[259]
Sea buckthorn berries	Liver (HepG2)	Methylene blue	0-10 mg/mL	72	Guo *et al.*[277]
Various Berries	Oral cavity (KB, CAL-27) Breast (MCF-7) Prostate (LNCaP) Colon (HT-29, HCT116)	CellTiter-Glo Luminescent Cell Viability Assay	25,50,100,200 μg/mL	48	Seeram *et al.*[261]
	Leukaemia (HL60) Colon (HCT116)	Cell counting (hemocytometer) and trypan blue	0.5,1,2,4,6 mg/mL	24,48	Katsube *et al.*[304]
Various fruits and berries	Colon (HT-29) Breast (MCF-7)	WST-1	0.025, 0.05, 0.25, 0.5% DW	24	Olsson *et al.*[268]
Apples	Colon (HT-29)	DAPI staining and fluorescence	Extracts containing 5-100 μM phloridzin	24,48,72	Veeriah *et al.*[262]
	Colon (LT97 and HT-29)	DAPI staining and fluorescence	0-900 μg/mL DW	24,48,72	Veeriah *et al.*[306]
	Colon (HT-29) Gastric (MKN45)	MTT	0-200 mg/mL	96	Serra *et al.*[307]
	Breast (MCF-7)	MTS and methylene blue	10,20,30,40,50,60,75,100,125 mg/mL	24, 96	Yang and Liu [303]
Cherries	Colon (HT-29) Gastric (MKN45)	MTT	0-20 mg/mL DW	96	Serra *et al.*[257]

Fruit, Vegetable, or Plant Extract	Cancer Type/ Cell Culture Model	Method	Extract Dose	Exposure Time (h)	Reference
Grapes	Liver (HepG2) Lung (A549)	MTT	5,15,50,75 μg/mL	24,48	Pacifico *et al.*[308]
	Colon (HT-29 and Caco-2)	Methylene blue	0.31,1.25,2.5,5 g/L	24,72,96	Parry *et al.*[264]
Dragon fruit	Gastric (AGS) Cervical (HeLa) Breast (MCF-7)	MTT	125-1000 μg/mL	72	Kim *et al.*[165]
Acai	Colon (HT-29)	Beckman Coulter Particle Counter	0.04-12 μg/mL gallic acid equivalents	48	Pacheco-Palencia *et al.*[265]
Shallots and various onions	Liver (HepG2) Colon (Caco-2)	MTS	10,25,50,75,100,125,150 mg/mL	96	Yang *et al.*[309]
Angelica sinensis (traditional medicinal plant)	Lung (A549) Colon (HT-29) Brain (DBTRG-05MG) Liver (J5)	MTT and trypan blue	1,10,40,100,400,1000 μg/mL	12,24,48,72	Cheng *et al.*[269]
Moringa oleifera (traditional medicinal plant)	Oral cavity (KB)	MTT	0-200 μg/mL	48	Sreelatha *et al.*[288]
Pistacia atlantica sub kurdica (traditional medicinal plant)	Colon (HT-29)	MTT	0.5,0.75,1,2 mg/mL	48,72,96	Rezaei *et al.*[270]

2.8.5.1 Antiproliferative Effects of *Brassica* Species

Several epidemiological studies have indicated that a high intake of *Brassica* vegetables is associated with a reduced risk of cancer [93]. *Brassica* vegetables are known to contain a complex mixture of health-related phytochemicals, including vitamin C, phenolic compounds and glucosinolates. The studies conducted on anti-cancer effects are usually confined to the glucosinolates or phenolic compounds, and generally do not take into consideration other potentially bioactive compounds found in *Brassica* vegetables. Therefore, there is limited research published on the antiproliferative activities of *Brassica* vegetable extracts on cancer cells *in vitro* and it is not well understood which constituents are responsible for these anti-cancer effects, or whether the effects may result as a consequence of synergistic action of several constituents. Some *in vitro* studies that have examined the antiproliferative effects of *Brassica* specie extracts on a variety of cancer cell lines are shown in Table 2.12, along with the antiproliferative/cell viability assay used, the extract dose that the cells were exposed to, and the exposure time.

Table 2.12 Studies that assessed the antiproliferative effects of *Brassica* species on a variety of cancer cells *in vitro*, including antiproliferative assay used, extract dose, and exposure time. FW = Fresh weight.

***Brassica* Species**	**Cancer Type/ Cell Culture Model**	**Method**	**Extract Dose**	**Exposure Time (h)**	**Reference**
Curly Kale (*Brassica oleracea* L. convar. *acephala* var. *sabellica*)	Colon (Caco-2, HT-29, HCT116)	MTT	10,20,40,60 g/L FW	24	Olsen *et al.*[266].
Rutabaga (*Brassica napus* L. var. *napobrassica)*	Liver (HepG2)	MTT	0.5,1,5,10 μg/mL	24	Pasko *et al.*[310]
Cabbage (*Brassica oleracea* var. *sabauda*)	Colon (Caco-2)	MTT and crystal violet	0.2,0.4 μg/μL	48	Ombra *et al.*[311]
Broccoli (*Brassica oleracea Italica*)	Brain (U251) Breast (MCF-7) Kidney (786-0) Lung (NCI-H460) Colon (HT-29)	Sulforhodamine B assay	0.25,2.5,25,250 μg/mL	48	Bachiega *et al.*[312]
Broccolini (*Brassica oleracea Italica* x *Alboglabra*)	Lung (A549) Ovarian (OVCAR-3)	MTT	10,30,60,90,120 μg/mL	24	Yang and Zhang [313].
Cabbage Broccoli	Liver (HepG2)	MTS	1,5,10,20,30,40,50 mg/mL	96	Chu *et al.*[314]
Cabbage (*Brassica oleracea* var. *capitata* f. *alba*)	Lung (Calu-6) Gastric (SNU-601)	MTT	10,30,100,300,1000 μg/mL	72	Gorinstein *et al.*[315].
Cauliflower juice (*Brassica oleracea*)	Breast (MCF-7, BT474, MDA-MB-231, BT20)	Cell counting (hemocytometer) and trypan blue	5,10,20 mL/L	72	Brandi *et al.*[316]

2.9 SUMMARY

Accumulating evidence demonstrates that fruit and vegetable consumption has health-promoting properties and research has been conducted in numerous scientific fields to identify the constituents that are responsible for these effects. Fruits and vegetables, as well as other plant products, are rich sources of diverse phytochemicals that vary dramatically with regards to structure and quantity in various plants. Flavonoids are a class of phytochemicals, and are further classified into flavones, flavanols (catechins), isoflavones, flavonols, flavanones, and anthocyanins. These compounds have been found in a variety of different plant foods and are prominent within the *Brassicaceae* plant family, including broccoli, Brussels sprouts, cabbage, cauliflower, kale and bok choy. Bok choy contains relatively high levels of the flavonols isorhamnetin and kaempferol, with smaller amounts of quercetin. The need to correctly identify and determine the levels of flavonols and their glycosylated and acylated derivatives in bok choy is crucial in understanding the health benefits of this plant, as different varieties can contain varying levels of flavonols, implying that certain varieties may be healthier, as they may contain higher amounts of bioactive compounds.

The chemical analysis of flavonols in plant products usually involves quantification of target compounds, as well as profiling and identifying complex conjugated derivatives. The analysis of these compounds generally consists of four main steps: sample preparation, extraction, purification and instrumental analysis via a combination of chromatographic and spectroscopic techniques. Extraction methods such as acid hydrolysis, alkaline hydrolysis, and hydroalcoholic extraction isolate the various forms/conjugates of flavonol compounds found within the plant material. Purification techniques such as SPE and preparative chromatography are often necessary to purify and enrich certain compounds or fractions, or to eliminate any plant matrix components that may interfere with analysis. The most frequently used analytical technique for flavonol analysis is reversed-phase high performance liquid chromatography (HPLC) with photodiode array detection (PDA), in conjunction with mass spectrometry (MS). There are several different types of mass spectrometers and ionisation sources available and HPLC-PDA/ESI-MS^n has been used extensively to determine the total aglycone levels of flavonols in plants after hydrolysis of the glycosides, as well as to identify the complex sugar and acyl moieties attached to the aglycone without the need to isolate the compounds. NMR is also used in conjunction with HPLC-PDA/ESI-MS^n for structure elucidation.

The study of phytochemicals as anti-cancer agents and their potential roles in the prevention and/or treatment of cancer has become of particular interest to scientists. Anti-cancer activities, such as antiproliferative effects of flavonols and their mechanisms of action, are often assessed by using cultured cells as tissue models. Human colorectal carcinomas have been used in many studies to assess such effects, including the cell line HT-29, due to the direct exposure of the intestinal tract to dietary components. Colorectal cancer is the fourth most common malignant tumour worldwide and the second most commonly diagnosed cancer in both men and women in Australia, hence any advancements towards the prevention and/or treatment of this type of cancer would be invaluable. The anti-cancer properties of flavonol compounds are mostly studied in their aglycone form and quercetin, kaempferol, and isorhamnetin have all demonstrated potent anti-cancer activities against a variety of different carcinomas, both *in vitro* and *in vivo*. Flavonols can affect the initiation and promotion stages of the carcinogenic process, i.e. they have the ability to aid in cancer chemoprevention as well as chemotherapy.

Some important things to consider when assessing the antiproliferative abilities of these compounds is their form, i.e. whether they are glycosylated or acylated as this affects their bioactivities, as well as whether they act synergistically in plants. There have been limited studies conducted on glycosylated derivatives of flavonols and the reported data between studies can be contradictory. Some research has also shown that flavonols can act synergistically to enhance their antiproliferative effects *in vitro*, which supports the theory that the anti-cancer properties of fruit/vegetable/plant extracts may be attributed to the complex mixture of phytochemicals present in the whole plant, not just attributed to individual constituents within the plant. Many *in vitro* studies on fruit and vegetable phytochemical extracts have also shown that the crude plant extracts exhibit stronger antiproliferative activities then individual phytochemical compounds, therefore, the additive and synergistic effects of phytochemicals in plant foods may actually be responsible for their potent anti-cancer activities.

CHAPTER III
MATERIALS AND METHODS

3.1 INTRODUCTION

This chapter outlines all the materials used and methods applied for the experiments conducted as part of this study. All methods utilised have been previously validated and published in literature [60, 137], with minor adjustments made in some cases (as discussed in Chapter IV).

3.2 BOK CHOY SAMPLES

Three locally grown cultivars of bok choy (*Brassica rapa* L. ssp. *chinensis*) seedlings, Sumo, Karate, and Miyako were purchased from Boomeroo Nurseries in Lara, Victoria (Figure 3.1).

Figure 3.1 Bok choy seedlings at approximately 6 weeks old, prior to transplanting. Cultivars include Sumo, Karate, and Miyako, and were purchased from Boomeroo Nurseries in Lara, Victoria.

In all trials, bok choy seedlings were transplanted into 150 mm pots containing a soil-free potting mix approximately 6 weeks after germination. The seedlings were grown in a replicated block design (n = 5) in a glasshouse at the Department of Primary Industries (DPI) Knoxfield with a temperature range of 13-25°C and a mean maximum light level of 53.8 klx (approximately 900 µmol/m^2s PAR). The potting mix contained approximately 88% pine bark and 12% sand. The nutrient content is summarised in Table 3.1. At harvest (28 days after re-potting), the roots were removed, and the aerial parts were placed in calico bags and immediately frozen under liquid nitrogen, freeze dried, and powdered by grinding in a domestic coffee grinder (approximately 500 g per sample). The finely powdered samples were placed in air tight containers and stored in a freezer at -20°C until required.

Table 3.1 Nutrient content of potting mix used for growing bok choy samples.

Potting Mix	
pH	6.2
Total soluble salts (% w/w)	-
Organic matter (% w/w)	-
C (% w/w)	2.1
N (% w/w)	<10 mg/L
P (mg/kg)	1.1
K (mg/kg)	81
S (mg/kg)	85
Ca (meq/100 g)	130 mg/L
Mg (meq/100 g)	31 mg/L
Na (meq/100 g)	22 mg/L

3.3 CHEMICALS, REAGENTS, AND OTHER MATERIALS

Chemicals, reagents, and all other materials used in this study, including their commercial sources, are listed in Table 3.2.

Table 3.2 List of all chemicals, reagents, and other materials used in this study.

Chemical/Reagent/Material	Source
Water with 0.1% Formic Acid – HPLC-MS Chromasolv (Fluka Analytical)	Sigma-Aldrich #34673
Methanol with 0.1% Formic Acid – HPLC-MS Chromasolv (Fluka Analytical)	Sigma-Aldrich #34671
Acetonitrile with 0.1% Formic Acid – HPLC-MS Chromasolv (Fluka Analytical)	Sigma-Aldrich #34668
Methanol – HPLC-MS Chromasolv (Fluka Analytical)	Sigma-Aldrich #34966
Water – HPLC-MS Chromasolv (Fluka Analytical)	Sigma-Aldrich #39253
Acetonitrile – HPLC (LiChrosolv)	Merck #100030
Methanol – HPLC (LiChrosolv)	Merck #106007
Formic Acid – GR Grade	Merck #100264
Methanol – AR Grade	Merck #1230
Ethanol Absolute – AR Grade	Merck #1170
Sodium Hydroxide Pellets – AR grade	Merck #106498
tert-Butylhydroquinone – purum HPLC (Fluka)	Sigma-Aldrich #19986
Rutin trihydrate – HPLC ≥90% (Fluka Biochemika)	Sigma-Aldrich #84082
Quercetin dihydrate – HPLC ≥98% (Sigma)	Sigma-Aldrich #Q0125 – 10g
Kaempferol-3-*O*-glucoside – HPLC ≥99%	Extrasynthese #1243 S
Isorhamnetin-3-*O*-glucoside – HPLC ≥95%	Extrasynthese #1228
Quercetin-3-*β*-D-glucoside – HPLC ≥90% (Fluka Biochemika)	Sigma-Aldrich #17793
Isorhamnetin – HPLC ≥95.0% (Fluka Analytical)	Sigma-Aldrich #17794
Kaempferol – HPLC ≥97.0% (Fluka Analytical)	Sigma-Aldrich #60010
Hydrochloric Acid	Ajax Finechem #174 – 2.5L
Dimethyl Sulfoxide-d_6	Sigma-Aldrich #151947
Methanol-d_4	Sigma-Aldrich #156914
Tetramethylsilane	Sigma-Aldrich #551333
MilliQ water	Millipore Milli-Q Plus
Strata X SPE tube 100 mg/6 mL	Phenomenex #8B-S100-ECH
Oasis HLB (SPE) extraction cartridges 20 cc/1g	Waters #100 186000117
13 mm PTFE HPLC syringe filter 0.45 µm	Alltech #2165
25 mm Nylon HPLC syringe filter 0.45 µm	Alltech #2047
13 mm Cellulose Acetate HPLC syringe filter 0.45 µm	Alltech #2643
HT-29 Human colorectal adenocarcinoma cells	American Type Culture Collection (ATCC) #HTB-38

Chemical/Reagent/Material	Source
McCoy's 5A medium (GIBCO)	Invitrogen #16600
Foetal Bovine Serum	Bovogen #SFBS
0.25% Trypsin-EDTA (GIBCO)	Invitrogen #25200
Phosphate Buffered Saline pH 7.2 1x (GIBCO)	Invitrogen #20012
Trypan Blue Solution 0.4% (Sigma)	Sigma-Aldrich #T8154
Dimethyl Sulfoxide (Sigma)	Sigma-Aldrich #D2438
5-Fluorouracil (Sigma)	Sigma-Aldrich #F6627 – 1g
Crystal Violet	BDH Lab Supplies #34024
Triton-X-100 Solution	Sigma-Aldrich #93443
5 mL Serological Sterile Pipettes	Greiner Bio One (GBO) #606180
10 mL Serological Sterile Pipettes	Greiner Bio One (GBO) #607180
25 mL Serological Sterile Pipettes	Greiner Bio One (GBO) #760180
Standard Cell Culture Flasks 50mL, 25cm^2 (Cellstar)	Greiner Bio One (GBO) #690160
Standard Cell Culture Flasks 250mL, 75cm^2 (Cellstar)	Greiner Bio One (GBO) #658170
96 Well Polystyrene Cell Culture Microplates	Greiner Bio One (GBO) #655180
6 Well Cell Culture Multiwell Plates (Cellstar)	Greiner Bio One (GBO) #657160
1000 µL Pipette Tips, Blue	Greiner Bio One (GBO) #686271
200 µL Pipette Tips, Yellow	Axygen Scientific #T-200-Y
0.5-10 µL Pipette Tips, Clear	Axygen Scientific #T-300
Cryo Sterile Freezing Tubes, 2 mL	Greiner Bio One (GBO) #122263
PP Tube, 50 mL, Conical Bottom, Sterile	Greiner Bio One (GBO) #210270
PP Tube, 15 mL, Conical Bottom, Sterile	Greiner Bio One (GBO) #188261
1.5 mL Microtubes	Axygen Scientific #MCT-150
0.6 mL Microtubes	Axygen Scientific #MCT-060
CellTiter 96 Non-Radioactive Cell Proliferation Assay	Promega #G4100
Cellular DNA Fragmentation ELISA	Roche #11 585 045001

3.4 EXTRACTION PROCEDURES

Three different extraction procedures were used to analyse flavonol compounds present in the freeze-dried, powdered bok choy samples:

(1) acid hydrolysis was used to extract the flavonol aglycones,

(2) alkaline hydrolysis was used to extract the flavonol glycosides, and

(3) a hydroalcoholic solvent extraction using a mixture of methanol/water was used to extract all acylated flavonol glycoside compounds (i.e. flavonol glycoside-hydroxycinnamic acid conjugates) that occur naturally within the plant.

Optimisation studies were carried out on a freeze-dried, powdered onion sample ('Tango' variety) that was available in the laboratory from a previous study while waiting for the bok choy seedlings to grow. All freeze-dried, powdered samples were weighed on an analytical balance and recorded to four decimal places.

3.4.1 Hydroalcoholic Extraction of Freeze-Dried Onion Sample

Approximately 2 g of the freeze-dried, powdered onion sample was extracted with 50 mL of a 70/30 v/v methanol/water mixture using a hand-held blender (Braun – Control Plus Vario) for 5 minutes. The mixture was filtered under vacuum and the remaining residue and filter paper were re-extracted with another 50 mL of 70/30 v/v methanol/water for 5 minutes and filtered under vacuum. The two filtrates were combined and diluted to 100 mL with methanol. The extract was filtered through a 0.45 μm nylon syringe filter prior to HPLC analysis. This extraction method was adapted from Caridi *et al.* [137].

3.4.2 Acid Hydrolysis of Freeze-Dried Bok Choy Samples: Flavonol Aglycones

A mixture of 0.5 g freeze-dried, powdered bok choy, 40 mL of 62.5% aqueous methanol containing 2 g/L *tert*-butylhydroquinone (TBHQ) and 10 mL of 8 M HCl was heated under reflux with stirring for 4 hours. The solution was cooled to room temperature, diluted to 100 mL with methanol, and sonicated for 10 minutes. The extract was filtered through a 0.45 μm polytetrafluoroethylene (PTFE) syringe filter prior to HPLC analysis. This extraction method was adapted from Rochfort *et al.* [60].

3.4.3 Alkaline Hydrolysis of Freeze-Dried Bok Choy Samples: Flavonol Glycosides

A mixture of 1 g freeze-dried, powdered bok choy and 60 mL of 53% aqueous methanol containing 2.6 M sodium hydroxide was stirred for 20 hours at room temperature. The mixture was filtered under vacuum, after which the filtrate was neutralised with concentrated hydrochloric acid and filtered through a 0.45 μm nylon syringe filter prior to HPLC analysis. This extraction method was adapted from Rochfort *et al.* [60].

3.4.4 Hydroalcoholic Extraction of Freeze-Dried Bok Choy Samples: Flavonol Glycoside-Hydroxycinnamic Acid Conjugates

Approximately 1 g of freeze-dried, powdered bok choy was extracted with 100 mL of an 80/20 v/v methanol/water mixture using a hand-held blender (Braun – Control Plus Vario) for 5 minutes. This mixture was sonicated for 20 minutes and filtered under vacuum. 5 mL of the filtered solution was dried under a stream of nitrogen, re-suspended in 1 mL of 70/30 v/v methanol/water and filtered through a 0.45 μm nylon syringe filter prior to HPLC analysis. This extraction method was adapted from Rochfort *et al.* [60].

3.5 PREPARATION OF STANDARDS

The following flavonol aglycone standard compounds were purchased for use in this study: quercetin dihydrate (Sigma-Aldrich #Q0125 ≥98.0%), isorhamnetin (Sigma-Aldrich #17794 ≥95.0%), and kaempferol (Sigma-Aldrich #60010 ≥97.0%). These compounds were used to prepare standard solutions to confirm the identity and to quantify the total flavonol aglycone content in the bok choy samples after acid hydrolysis, and for recovery experiments.

3.5.1 Preparation of Standards for Identification

Standard solutions for each flavonol aglycone were prepared to identify the flavonols present in the bok choy (after acid hydrolysis) using HPLC-PDA/ESI-MS^n. After obtaining an estimate of the amount of each flavonol present in the bok choy samples, standard solutions were prepared at a concentration that was within the range present in the bok choy, e.g. 1 mg/L for quercetin, 3 mg/L for isorhamnetin, and 6 mg/L for kaempferol. Standard solutions were prepared as outlined in the following sections.

3.5.1.1 Stock Standards

A 500 mg/L stock solution was made for each individual flavonol. For isorhamnetin and kaempferol, this involved weighing accurately 5.0 mg of each compound into a separate 10 mL volumetric flask and making each flask up to the mark with HPLC-MS grade 90/10 v/v methanol/water + 0.1% v/v formic acid. For quercetin the stock solution was prepared by accurately weighing 5.6 mg of quercetin dihydrate (equivalent to 5.0 mg of quercetin). The stock solutions were sonicated for 5-10 minutes to ensure complete dissolution of the compound.

3.5.1.2 Intermediate Standards

100 mg/L and 10 mg/L solutions of each of the individual flavonols were prepared from the stock solutions by dilution with HPLC-MS grade 90/10 v/v methanol/water + 0.1% v/v formic acid to a final volume of 5 mL.

3.5.1.3 Working Standards

Individual working standards were prepared from the 10 mg/L intermediate standard solutions by pipetting 0.1, 0.3, and 0.6 mL for quercetin, isorhamnetin and kaempferol respectively using a micropipette. Each standard was made up to a final volume of 1 mL with HPLC-MS grade 90/10 v/v methanol/water + 0.1% v/v formic acid.

3.5.2 Preparation of Standards for Quantification

The initial 500 mg/L stock solution and 100 mg/L intermediate stock solution for each individual flavonol was prepared as outlined in 3.5.1.1 and 3.5.1.2. After which, a 10 mg/L mixed standard solution was prepared by adding together 0.5 mL of each flavonol standard (100 mg/L) into a 5 mL standard flask and making up to the mark with HPLC-MS grade 90/10 v/v methanol/water + 0.1% v/v formic acid. Ten mixed standard solutions (each containing quercetin, isorhamnetin, and kaempferol) ranging from 0.1 to 10 mg/L were prepared for use in the quantification studies. Figure 3.2 shows a flow diagram of the preparation of standards.

The final concentrations of the working standards prepared for quantification included 0.1, 0.2, 0.5, 1, 2, 3, 4, 6, 8 and 10 mg/L, where the linear range used for quercetin was 01, 0.2, 0.5, 1, 2 and 3 mg/L, isorhamnetin was 0.2, 0.5, 1, 2, 3 and 4 mg/L, and kaempferol was 2, 3, 4, 6, 8 and 10 mg/L. Calibration curves and linearity data is presented in Section 5.2.2.2 and quantification results are discussed in Section 5.2.3.

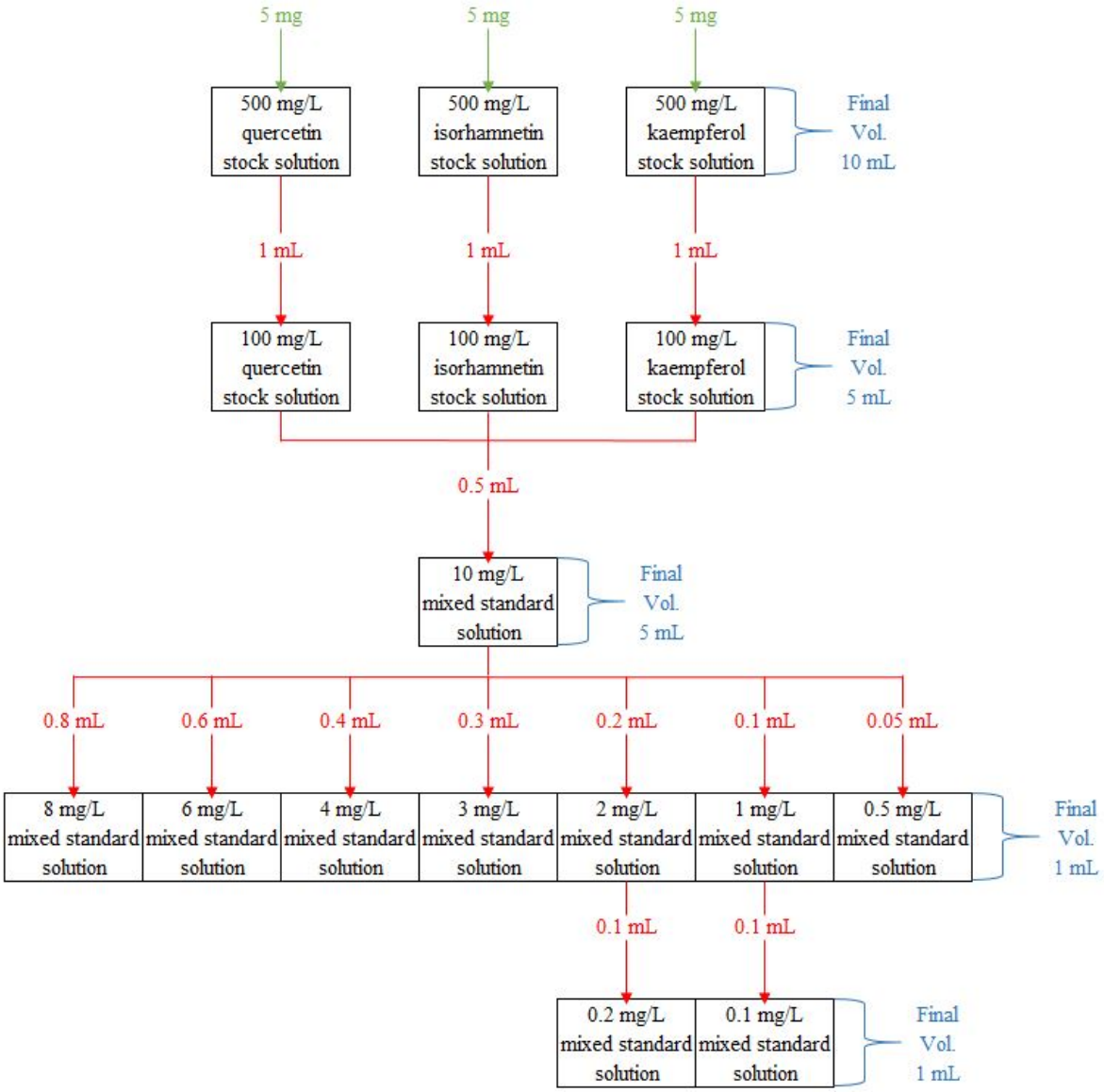


Figure 3.2 Flow diagram showing preparation of flavonol aglycone standard solutions used for quantification. NB: 5.6 mg of quercetin dihydrate was accurately weighed to obtain the equivalent of 5 mg of quercetin.

3.5.3 Preparation of Standards for Recovery Studies

Separate standards for each flavonol aglycone (50 mg/L quercetin, 100 mg/L isorhamnetin, and 100 mg/L kaempferol) were prepared for recovery studies (results are presented in Section 5.2.2.3). These standards were prepared similar to those in 3.5.1. Freeze-dried, powdered bok choy samples were spiked with one of the respective standards prior to extraction as follows.

3.5.3.1 Quercetin

A sample of the Miyako bok choy cultivar (0.5 g) was spiked with 1 mL of a 50 mg/L quercetin solution prior to acid hydrolysis. 0.05 mg/0.5 g is equivalent to 10 mg/100 g of bok choy.

3.5.3.2 Isorhamnetin

A sample of the Sumo bok choy cultivar (0.5 g) was spiked with 1 mL of a 100 mg/L isorhamnetin solution prior to acid hydrolysis. 0.1 mg/0.5 g is equivalent to 20 mg/100 g of bok choy.

3.5.3.3 Kaempferol

A sample of the Karate bok choy cultivar (0.5 g) was spiked with 2 mL of a 100 mg/L kaempferol solution prior to acid hydrolysis. 0.2 mg/0.5 g is equivalent to 40 mg/100 g of bok choy.

3.5.3.4 Calculations for Percent Recovery

An example of how the percent recovery values presented in 5.2.2.3 (Table 5.8) were calculated is shown below:

- Kaempferol concentration obtained from calibration graph (Figure 5.15) = 6.7 mg/L
- Therefore, amount of kaempferol (mg) in 100 mL digest = 0.67 mg
- Therefore, amount of kaempferol (mg) in 0.5 g of freeze-dried, powdered bok choy = 0.67 mg
- Therefore, amount of kaempferol (mg) in 100g of dry weight (DW) bok choy = 133 mg (expressed as 133 mg/100 g DW bok choy)

Percent recovery was calculated as follows [198]:

$$\%\, recovery = \frac{spiked\ bok\ choy\ (mg/100\ g)\ -\ bok\ choy\ (mg/100\ g)}{added\ flavonol\ standard\ (mg/100\ g)} \times 100$$

All results are expressed as mg/100 g dry weight (DW) bok choy.

3.6 ANALYTICAL HPLC-PDA/ESI-MS

All analytical HPLC-PDA/ESI-MS^n experiments were conducted with a Thermo Fisher Surveyor Plus HPLC equipped with a degasser, quaternary gradient pump, auto sampler with sample cooler (maintained at 4^{o}C), column oven (maintained at 25^{o}C), and Surveyor PDA plus detector coupled with a Thermo Fisher LCQ Deca XP Max ion trap mass spectrometer. 5 µL of extract was injected onto a 150 x 2 mm ID, 3 µm Varian Polaris C18-A column with a matching Varian Polaris 3 µm C18-A 2 mm ID guard column. As part of the standard operating procedure, quality control checks to monitor instrument (chromatographic and detector) reproducibility involved analysing a calibration standard (including quercetin, isorhamnetin, and kaempferol) every 12 injections and any carry over was monitored by analysing solvent blanks in between different sample types.

3.6.1 Separation of Flavonol Aglycones after Acid Hydrolysis

The flavonol aglycones were separated using a binary gradient of 0.1% v/v formic acid in water (mobile phase A) and 0.1% v/v formic acid in acetonitrile (mobile phase B). The following gradient was used: 90/10 – 10/90 A/B (0-30 minutes), 10/90 A/B (30-35 minutes), 10/90 – 90/10 A/B (35-36 minutes), 90/10 A/B (36-46 minutes). The flow rate was 0.2 mL/min and the column was maintained at 25°C.

3.6.2 Separation of Flavonol Glycosides after Alkaline Hydrolysis

The flavonol glycosides were separated using a binary gradient consisting of 0.1% v/v formic acid in water (mobile phase A) and 0.1% v/v formic acid in methanol (mobile phase B) with the following gradient: 95/5 – 5/95 A/B (0-40 minutes), 5/95 A/B (40-45 minutes), 5/95 – 95/5 A/B (45-46 minutes), 95/5 A/B (46-60 minutes). The flow rate was 0.2 mL/min and the column was maintained at 25°C.

3.6.3 Separation of Flavonol Glycoside-Hydroxycinnamic Acid Conjugates after Hydroalcoholic Solvent Extraction

The flavonol glycoside-hydroxycinnamic acid conjugates were separated using the same conditions as that for the flavonol glycosides (3.6.2).

3.7 PREPARATIVE HPLC

All preparative HPLC experiments were conducted on a Varian Pro Star preparative HPLC equipped with a binary gradient pump and a UV-Visible detector set to either 350 nm or 330 nm for the analysis of alkaline or hydroalcoholic methanol/water bok choy extracts respectively. 1 mL of extract was manually injected onto a 250 x 21.2 mm ID, 5 µm Varian Polaris C18-A column with a matching Varian Polaris 5 µm C18-A 21.2 mm ID guard column. The same binary gradient was used for both alkaline and hydroalcoholic extracts and consisted of 0.1% v/v formic acid in water (mobile phase A) and 0.1% v/v formic acid in methanol (mobile phase B). The following gradient elution program was used: 95/5 – 5/95 A/B (0-60 minutes), 5/95 A/B (60-65 minutes), 5/95 – 95/5 A/B (65-66 minutes), 95/5 A/B (66-76 minutes). The flow rate was 10 mL/min and the column was at room temperature.

3.7.1 Fraction Collection

Two fractions were collected from an alkaline hydrolysate bok choy extract and six were collected from a hydroalcoholic bok choy extract using the preparative HPLC. Some of these were used for further structure elucidation via NMR (refer to Sections 5.3.4 and 5.4.4), as well as in the cancer cell studies to assess their antiproliferative activity on human colon cancer cells (refer to Section 6.3.3). Fractions were collected manually by watching the real-time view of the chromatogram and placing the column flow directly into a round bottom flask as the peaks eluted. At least 20 injections were performed for each collection. The vast majority of solvent was then evaporated using a rotary evaporator set at 40°C, after which the remaining solution was passed through a SPE cartridge (refer to 3.8). The purified compound or fraction was eluted off the SPE cartridge with methanol directly into a pre-weighed glass vial. The methanol was then evaporated using a stream of N_2. The glass vial containing the purified compound or fraction residue was left in a vacuum desiccator overnight, or until required, after which it was weighed and dissolved in the required solvent for NMR (3.10) or cancer cell studies (3.11.4.2).

3.8 SOLID PHASE EXTRACTION

The fractions collected from the preparative HPLC were further purified for NMR (3.10) and cancer cell studies (3.11.4.3) by solid phase extraction (SPE) using Waters Oasis HLB cartridges. These cartridges consist of a polymeric reversed-phase sorbent which was developed for the extraction of a wide range of acidic, basic, and neutral compounds from various matrices [317]. The cartridges had a barrel size of 20 mL, particle size of 60 µm, pore size of 80 Å, and a sorbent weight of 1 g. The cartridge was conditioned with methanol (20 mL), equilibrated with water (20 mL) prior to loading the sample (between 0.08-0.1 g of dried matter) onto the cartridge. The

cartridge was washed with water (20 mL), allowed to dry (1 minute) and the analyte/s (i.e. flavonol compound/s) eluted with methanol (20 mL).

3.9 MASS SPECTROMETRIC ACQUISITION PARAMETERS

Mass spectra were acquired on a Thermo Fisher LCQ Deca XP Max ion trap mass spectrometer equipped with an electrospray ionisation (ESI) source in the negative ion mode. Prior to data acquisition the system was tuned using a 250 mg/L standard solution of rutin made up in 80/20 v/v methanol/water containing 0.1% v/v formic acid (HPLC-MS grade). The rutin tune solution was introduced into the MS via direct infusion from a syringe pump through a T-piece at a rate of 5 µL/min with a HPLC flow rate of 0.2 mL/min and a mobile phase composition of 80/20 v/v methanol/water containing 0.1% v/v formic acid. Tune files were created using an auto tune function.

3.9.1 MS^1 Parameters Used for Identification and Quantification Studies of Flavonol Aglycones in Bok Choy

For all HPLC-ESI-MS experiments, the spray needle voltage was set at 4.5 kV in the negative ion mode. The ESI capillary voltage was -48.50 V and the capillary temperature was held at 350°C. The sheath gas was set at 20, the auxiliary at 2 and the sweep gas at 14 units. Scan event 1 was always a full MS scan between the mass ranges of 120-2000 amu. Selected ion monitoring (SIM) was used for quantification studies with the following additional scan events; Scan Event 2 for quercetin: MS SIM ranges 300.50-301.50 amu; Scan Event 3 for isorhamnetin: MS SIM ranges 314.50-315.50 amu; Scan Event 4 for kaempferol: MS SIM ranges 284.50-285.50 amu.

3.9.2 MS^n Parameters Used for Identification of Flavonol Compounds Present in Alkaline and Hydroalcoholic Extracts of Bok Choy

For the HPLC-ESI-MS^n experiments on the alkaline and hydroalcoholic extracts of bok choy cultivars, a data dependant “top five” protocol was used in ESI negative ion mode with a mass range of 120 to 2000 amu. Dynamic exclusion was engaged with a 25 second exclusion time. Data were acquired using automated MS^n settings with a target of 30,000, a normalised collision energy of 35% and an ion max time of 200 ms. The heated capillary was maintained at 350^{o}C and the sheath, auxiliary and sweep gases were at 20, 2 and 14 units respectively. Source voltage was set to 4.5 kV with a capillary voltage of –48.50 V.

3.10 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

The isolated compounds and fractions from the preparative HPLC (3.7.1) were reconstituted in deuterated dimethyl sulfoxide (DMSO-d_6) for initial experiments, followed by deuterated methanol (MeOH-d_4) for subsequent experiments. Proton (^{1}H), COSY, HSQC and HMBC NMR spectra were obtained on a Bruker AvanceIII spectrometer operating at 800.13 MHz and equipped with a CryoProbe (Bruker, Rheinstetten, Germany). The reference compound tetramethylsilane (TMS) was used as an internal standard for the determination of chemical shifts. A line broadening of 0.3 Hz was applied to all free induction decays (FIDs) prior to Fourier transformation. Spectra were manually phased, and baseline corrected in Topspin 3.0 (Bruker).

3.10.1 ^{1}H NMR Parameters

Proton data were acquired using 1D pulse sequence zg30 with 256 transients following 4 dummy scans over a spectral width of 13.95 ppm (11160.72 Hz) and a total acquisition time of 2.94 seconds. Size of FID was 65536.

3.10.2 COSY Parameters

COSY spectra were acquired using pulse sequence cosygpqf with 4 transients following 16 dummy scans over a spectral width of 13.95 ppm for ^{1}H (11160.72 Hz) and 13.95 ppm for ^{13}C (11160.68 Hz) and a total acquisition time of 0.09 seconds. Size of FID was 512.

3.10.3 HSQC Parameters

HSQC spectra were acquired using pulse sequence hsqcedetgpsisp2.2 with 58 transients following 64 dummy scans over a spectral width of 9.68 ppm for ^{1}H (7747.93 Hz) and 200 ppm for ^{13}C (40242.19 Hz) and a total acquisition time of 0.26 seconds. Size of FID was 256.

3.10.4 HMBC Parameters

HMBC spectra were acquired using pulse sequence hmbcgplphdqf with 22 transients following 64 dummy scans over a spectral width of 12.02 ppm for ^{1}H (9615.39 Hz) and 221.87 ppm for ^{13}C (44642.86 Hz) and a total acquisition time of 0.11 seconds. Size of FID was 300.

3.11 CELL CULTURE

A human colorectal adenocarcinoma cell line (HT-29) obtained from American Type Culture Collection (ATCC) was chosen as the cell model for this study. Cells were cultured in complete media comprised of McCoy's 5A modified medium (containing L-glutamine) supplemented with 10% fetal bovine serum (FBS). Cells were incubated in a standard cell culture incubator at 37ºC in a humidified atmosphere of 5/95 CO_2/air.

3.11.1 Routine Maintenance of HT-29 Cell Line

HT-29 cells were routinely cultured in 25 cm^2 (T-25) and 75 cm^2 (T-75) culture flasks in complete media as a monolayer. A total volume of 10 mL was used for T-25 flasks and 20 mL for T-75 flasks. When 70% confluency was reached, cells were passaged using 0.25% v/v trypsin in 0.5 mM ethylenediaminetetraacetic acid (EDTA) and plated into new flasks at a dilution factor of 1:10 (passage dilution optimisation discussed in 4.6.1). A T-25 setup at a dilution of 1:10, using McCoy's 5A medium with 10% v/v FBS, would reach approximately 70% confluency in three days. Cell passages used in the course of this work varied from passage 6-19, in order to maintain the genomic integrity of the cells.

3.11.1.1 Trypsinisation of HT-29 Cells

Once cells reached confluency, the culture medium was removed using sterile disposable pipettes and cells were rinsed with 1-2 mL pre-warmed phosphate buffered saline (PBS). Pre-warmed trypsin (to 37ºC in the incubator) was then added at a volume of 1 mL for T-25 flasks, or 2 mL for T-75 flasks, and cells were incubated and checked for release at 2 minute intervals. Once released, complete media was added to the flask in excess of the trypsin in order to neutralise it. Cells were gently pipetted at this time to facilitate the dissociation of any remaining clumps. The number of viable cells in the suspension were counted using 0.4% trypan blue solution at a ratio of 1:1, and a hemocytometer. The cell count was extrapolated to a quantity per millilitre and used to determine seeding number for the MTT assays (3.11.5).

3.11.2 MTT Cell Proliferation Assay

The cytotoxicity and inhibitory activity of selected flavonol compounds, fractions, and bok choy extracts against the HT-29 cell line was measured using the MTT Cell Proliferation Assay (Roche Applied Science). This micro plate assay is based on the reduction of the yellow tetrazolium salt (MTT) by metabolic activity of viable cells. The reaction produces a water-insoluble purple formazan salt, which is solubilised and quantified using an ELISA plate reader between 550 – 600 nm. The intensity of the purple colour produced is indicative of the number of viable cells

that are present. Photographs of one of the assays are displayed in Appendix B, showing the colour produced by the reaction.

The growth area per well of the 96-well plates was 0.34 cm^2 and the working volume per well was 25-340 µL. The total volume used in the experiments was 100 µL and each well was seeded with $1x10^5$ cells/mL, which is equivalent to $1x10^4$ cells/well. Initially the assay was conducted over four days (96 hours) with a measurement taken every 24 hours, including a time zero reading. Therefore, five plates were prepared for each experiment. Subsequent experiments were conducted at the 72 hour time point only. Triplicate wells were set up on the 96 well plate for each concentration of test compound/fraction/extract, as well as both controls and the blank. Figure 3.3 provides an example of a typical design for the cell proliferation assay experiments.

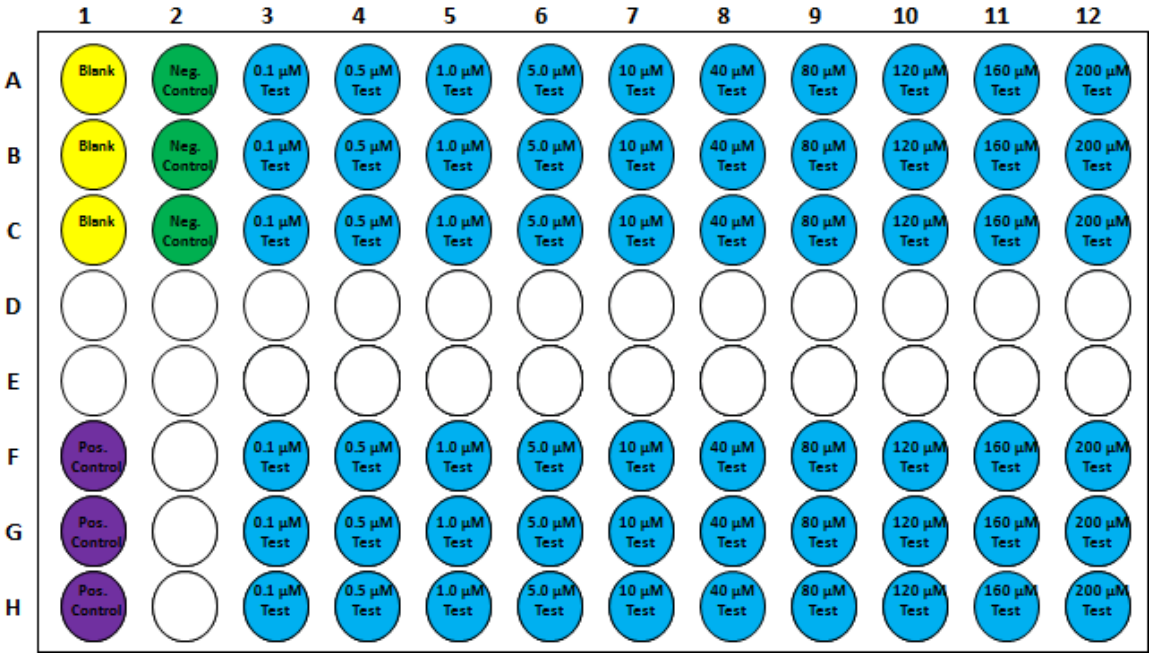


Figure 3.3 Illustration of a typical 96-well plate design for a MTT cell proliferation assay.

The cell proliferation assay was performed by adding 15 µL of dye solution to each well of the 96-well plate. The plate was then incubated at 37ºC for 1 hour in a humidified CO_2 incubator. After the incubation period the plate was removed from the incubator and 100 µL of solubilisation/stop solution was added to each well. Absorbance was recorded after 1 hour of incubation (the coloured formazan product is stable at 4ºC and absorbance can be recorded in one hour or up to several days later) at 570 nm using a BioRad iMark Microplate Absorbance reader (S/N 10094). A reference wavelength of 655 nm was used, and the reading mode was set to fast (6 sec/single wavelength reading, 10 sec/dual wavelength reading) with shaking turned on at a medium speed for 120 seconds. Three absorbance readings were obtained for each well.

3.11.3 HT-29 Growth Curve

A growth curve of HT-29 cells was conducted in order to determine optimum cell density and time parameters for the MTT proliferation assays on bok choy extracts, fractions, and flavonol compounds. Cells were trypsinised from a T2-5 flask and a cell count performed using trypan blue, as described in section 3.11.1.1. A range of cell concentrations (cells/mL) were then made to volume with complete media to make concentrations of $1x10^3$, $5x10^3$, $1x10^4$, $5x10^4$, $1x10^5$, $5x10^5$, and $1x10^6$ cells/mL. Concentrations were plated in triplicate on a 96-well plate with a final volume of 100 µL per well, resulting in $1x10^2$, $5x10^2$, $1x10^3$, $5x10^3$, $1x10^4$, $5x10^4$, and $1x10^5$ cells/well (0.34 cm^2 surface area). Cell viability/proliferation was assessed using the MTT assay over 8 days (192 hours) and a growth curve was plotted. The growth curve and results are discussed in 4.6.2.

3.11.4 Screening Procedure of HT-29 Cell Line

Test and reference compounds, fractions, and dried hydroalcoholic bok choy extracts were first solubilised in dimethyl sulfoxide (DMSO) and then further diluted in McCoy's 5A medium containing 10% v/v FBS to achieve the final test concentration. In order to minimise adverse effects on the cells from the DMSO solvent, the amount of DMSO was kept between 0.1-0.3% during treatments, which had no effect on cell growth. The positive and negative controls also contained the same percentage of DMSO in the medium as the percentage present in the treatment wells in that particular experiment.

Following trypsinisation (3.11.1.1) the confluent cells were seeded into 96-well plates at a density of $1x10^4$ cells/well (as chosen from the HT-29 growth curve assay), containing a total of 100 µL culture medium. The cells were then incubated for 24 hours to allow for the cells to adhere to the plate. After this initial 24 hour attachment period, the medium was aspirated using a multi-channel pipette and replaced with fresh medium containing the flavonol compound/s, fraction, or bok choy extract, to test for antiproliferative activity. Cells were treated with flavonol compounds at concentrations ranging from 0.1-200 µM, or in the case of the crude bok choy extracts from 0.2-10 mg/L. The plates were incubated for a total of 96 hours and the proliferation of the cancer cells was determined at 24 hour time intervals for the initial experiments (at 24, 48, 72, and 96 hours), after which the 72 hour time point was used for subsequent assays. The negative control wells contained cells with the same solvent used in the test wells (e.g. 0.1% DMSO in complete media) and the positive control used was 5-Flurouracil. The blank consisted of the test solvent (e.g. 0.1% DMSO in complete media) without any cells.

3.11.5 Preparation of Test Concentration Ranges

The concentration range of flavonol compounds, including aglycones and mono-glucosides which were obtained commercially, as well as purified compounds/fractions that were isolated from bok choy extracts was between 0.1-200 μM (0.1, 0.5, 1, 5, 10, 40, 80, 120, 160 and 200 μM). The crude hydroalcoholic bok choy extracts were between 0.2 -10 mg/L (0.2, 0.4, 0.6, 0.8, 1, 2, 4, 6, 8, and 10 mg/L). This equates to approximately $1x10^{-3}$ – $7x10^{-2}$ μM (depending on the cultivar of bok choy) of total flavonol (i.e. quercetin, isorhamnetin, and kaempferol aglycone) equivalents within the bok choy according to results obtained from quantification studies.

3.11.5.1 Preparation of Flavonol Aglycone and Mono-Glucoside Standards

Quercetin, isorhamnetin, and kaempferol aglycones, in addition to quercetin, isorhamnetin, and kaempferol 3-*O*-glucosides, were purchased commercially. 25.0 mg of isorhamnetin and kaempferol, and 28.0 mg of quercetin dihydrate (equivalent to 25.0 mg quercetin), was accurately weighed out using an analytical balance to make a stock solution of 200,000 μM in DMSO. A serial dilution was then performed to make standards with the following concentrations: 160,000, 120,000, 80,000, 40,000, 10,000, 5,000, 1,000, 500, and 100 μM using DMSO to a final volume of 400 μL. A 1:1000 dilution of each DMSO standard using complete media (McCoy's 5A containing 10% v/v FBS) was then made, making the final test concentrations of 200, 160, 120, 80, 40, 10, 5, 1, 0.5, and 0.1 μM containing 0.1% DMSO. The standards were made up in Eppendorf tubes and pre-warmed in the incubator prior to adding to the MTT cell proliferation test plates, of which 100 μL of solution was added to each well.

3.11.5.2 Preparation of Flavonol Compounds and Fractions Collected from Bok Choy

The two purified flavonol compounds, and the fraction containing an additional two compounds, were collected from bok choy extracts using preparative HPLC (3.7.1). The purified compounds that were tested on the HT-29 cells included kaempferol-3-sophoroside-7-glucoside which was isolated from an alkaline hydrolysate bok choy extract, and kaempferol-3-sophoroside(caffeoyl)-7-glucoside which was isolated from a hydroalcoholic extract. The additional fraction that was tested was collected from the alkaline hydrolysate bok choy extract and contained two flavonol glycosides, kaempferol-3,7-diglucoside and isorhamnetin-3,7-diglucoside, with isorhamnetin-3,7-diglucoside being the major component. The two purified compounds and the fraction were made up to a 200,000 μM stock solution, whereby the molecular weight of isorhamnetin-3,7-diglucoside was used to calculate the concentration for the fraction. The stock solution was prepared using DMSO and the volume added to the purified compound/fraction was calculated according to the weight that was obtained from the preparative HPLC fraction collection: 6.3 mg

of kaempferol-3-sophoroside-7-glucoside, 4.6 mg of the isorhamnetin-3,7-diglucoside /kaempferol-3,7-diglucoside fraction, and 1.3 mg of kaempferol-3-sophoroside(caffeoyl)-7-glucoside. The same process was then followed as described for the commercially purchased aglycones and mono-glucosides, whereby the stock solution was diluted successively using DMSO and then further diluted by a factor of 1000 using complete media to make test concentrations 200, 160, 120, 80, 40, 10, 5, 1, 0.5, and 0.1 μM containing 0.1% DMSO.

3.11.5.3 Preparation of Hydroalcoholic Bok Choy Extracts

For each bok choy cultivar, 20 mL of a hydroalcoholic methanol/water extract was evaporated to dryness using a rotary evaporator, freeze-dried, and stored in a vacuum desiccator until required (generally, between 0.08-0.1 g of dried matter was obtained). 0.01 g of the dried, extracted, plant material was dissolved in 1 mL of DMSO in order to create a 10,000 mg/L stock solution. This was then successively diluted to make concentrations of 8,000, 6,000, 4,000, 2,000, 1,000, 800, 600, 400, and 200 mg/L in pure DMSO with a final volume of 100 μL. Each standard in DMSO was then diluted 1:1000 using complete media (McCoy's 5A containing 10% v/v FBS) to give final concentrations of 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 4.0, 6.0, 8.0, and 10.0 mg/L with each solution containing 0.1% DMSO. The range of test bok choy extracts were made up in Eppendorf tubes and pre-warmed in the incubator prior to adding 100 μL of solution to each well of the MTT cell proliferation test plates, as described for the standards and purified compounds/fraction.

3.11.5.4 Data Analysis

The absorbance values obtained from the ELISA plate reader for the test compounds/fractions/extracts vs the negative control (containing cells and solvent only) were used to plot line graphs to demonstrate antiproliferative activity. The ratio of treated to untreated cells (i.e. % control values) were plotted on the y-axis, with test concentration on the x-axis. The % control values indicated cell proliferation, or cell viability, and were calculated using the following formula:

$$\frac{Absorbance\ test\ compound\ or\ extract}{Absorbance\ negative\ control} \times 100$$

The absorbance values were also used to calculate % cell inhibition of HT-29 cells using the following formula:

$$\frac{Absorbance\ negative\ control - Absorbance\ test\ compound\ or\ extract}{Absorbance\ negative\ control} \times 100$$

The % cell inhibition values were then used to calculate IC_{50} values for each flavonol compound/fraction/bok choy extract. IC_{50} values were calculated by nonlinear regression (curve fit), i.e. a dose-response inhibition curve consisting of log(inhibitor) vs. response – variable slope (four parameters), utilising GraphPad Prism 7.03 (GraphPad Software, Inc., San Diego, CA).

CHAPTER IV
METHOD OPTIMISATION AND OTHER PRELIMINARY STUDIES

4.1 INTRODUCTION

This chapter contains the results of the various preliminary studies and method optimisation that was required for this work. These include:

- Optimisation of HPLC-PDA/ESI-MS^n conditions for the hydroalcoholic, alkaline hydrolysate and acid hydrolysate bok choy extracts. Parameters optimised included:
    - HPLC Column
    - Mobile phase composition
    - Mobile phase gradient
    - Optimum PDA detector wavelength
    - ESI-MS^n conditions
- Optimisation of preparative HPLC conditions to collect fractions for further analysis using HPLC-PDA/ESI-MS^n, NMR, and cancer cell studies. Parameters optimised included:
    - Flow rate and injection volume (scaled up from the HPLC-PDA/ESI-MS^n method)
    - Mobile phase gradient
- Optimisation of acid hydrolysis conditions. Parameters optimised included:
    - Hydrolysis time
- Optimisation of syringe filter type for filtration of bok choy samples prior to chromatography, including the hydroalcoholic, alkaline hydrolysate, and acid hydrolysate extracts. Filter types investigated included:
    - Polytetrafluoroethylene (PTFE)
    - Cellulose acetate
    - Nylon
- Optimisation of cell culture conditions, including the following parameters:
    - Passage dilution for routine maintenance
    - Seeding density of cells for MTT proliferation assays
    - Time parameters for MTT proliferation assays

4.2 OPTIMISATION OF THE HPLC-PDA/ESI-MS SEPARATION OF FLAVONOLS IN BOK CHOY

Previous analytical studies on flavonols in various fruits and vegetables have utilised reverse-phase HPLC with PDA detection. Separation conditions usually employ C18 columns with a combination of acetonitrile/water or methanol/water mobile phases and PDA detection at 370 nm for analysis of aglycones [11-12, 318, 15, 161, 159-160, 223]. More recently, HPLC-PDA techniques have been combined with the powerful structure elucidation ability of mass spectrometry. The three types of mass analysers that are typically used are single quadrupole (MS)[319-322], triple quadrupole/tandem MS (MS/MS) [213] and ion trap (MS^n) [159, 158, 136, 60, 166, 208, 57, 74, 128, 67] where the latter is preferred when structure elucidation is a requirement. These studies also employed C18 columns with acetonitrile/water or methanol/water mobile phases, with the inclusion of various volatile acids as mobile phase modifiers: formic, trifluoroacetic, acetic or orthophosphoric acid being the most commonly used. Ionisation for MS studies has been conducted in either the negative or positive ion mode using either electrospray (ESI) or atmospheric pressure chemical ionisation (APCI). Negative ion mode has been the preferred method of ionisation in most studies as flavonols are more amenable to losing a proton, hence offering increased sensitivity [26]. This study utilised an ion trap mass analyser with an ESI interface in negative ion mode. Although a previously validated method for the separation of flavonol compounds in bok choy was implemented [60], a number of parameters were required to be optimised using the instrumentation available for this study.

4.2.1 Flavonol Glycosides in Onion: Hydroalcoholic Extract

Freeze dried bok choy samples were not available at the beginning of the study, and whilst waiting for the bok choy to be harvested, preliminary method development was carried out on freeze dried onion powder which was available in our laboratories from previous studies. This included column selection and mobile phase optimisation, which was carried out on a hydroalcoholic methanol/water extract of the onion sample (preparation of the extract is described in 3.4.1). Even though onion samples contain less complex flavonol compounds (mainly quercetin mono to diglycosides) [137, 15, 130, 126-127, 154, 323] as compared to bok choy (mainly acylated kaempferol di to triglycosides), given that there was a supply of onion powder in the laboratory, it provided a good basis to begin some optimisation work. The optimised conditions using the onion sample would then be tested for their suitability on the three bok choy extracts: hydroalcoholic, alkaline hydrolysate and acid hydrolysate.

Previous work by Caridi *et al*. [137] and Laugher [126] demonstrated that flavonol glycosides present in the hydroalcoholic extract of freeze dried onion could be successfully separated by HPLC-PDA/ESI-MS^n using either a 150 x 2.1 mm 3 µm Alltech Prevail Select C18 column or a 150 x 2.1 mm 3 µm BDS C18 Hypersil HPLC column with mobile phases consisting of methanol/water + 0.1% v/v formic acid and acetonitrile/water + 0.1% v/v formic acid respectively. HPLC columns with a 2 mm internal diameter were favoured as these allowed ESI-MS detection with no need for flow splitting from the HPLC, which would normally be required at the higher flow rates of larger ID columns. Three columns were available for this study: 150 x 2.1 mm 3 µm Varian Polaris C18-A, 150 x 2.0 mm 3 µm Alltech Prevail Select C18, and 150 x 2.0 mm 3 µm Phenomenex Gemini C18 110 Å column. Application notes for the separation of flavonols using these columns were available from the suppliers [324-326]. Initial work using the Varian Polaris column gave a suitable separation of the flavonol glycosides with minor changes to the published gradient profile. The separations are displayed in Figure 4.1 using both an acetonitrile/water mobile phase and a methanol/water mobile phase. The chromatograms were monitored at 370 nm (optimisation of the display wavelength is discussed in 4.2.5). The insert in Figure 4.1 shows the gradient profile using the following gradient program: 90/10 – 10/90 A/B (0-30 mins), 10/90 A/B (30-35 mins), 10/90 – 90/10 A/B (35-36 mins), 90/10 A/B (36-46 mins) where mobile phase A consisted of 0.1% v/v formic acid in water and mobile phase B was composed of either 0.1% v/v formic acid in acetonitrile, or 0.1% v/v formic acid in methanol.

Both mobile phases gave excellent separation and sharp peaks for the three major flavonol glycosides present in the onion extract. These were previously identified as quercetin 3,4'-diglucoside (peak 1), quercetin 4'-monoglucoside (peak 2) and isorhamnetin 4'-monoglucoside (peak 3) [137, 126]. The acetonitrile/water mobile phase showed similar selectivity, however, at a much shorter analysis time. Overall the results suggested that either mobile phases consisting of acetonitrile/water/formic acid or methanol/water/formic acid are suitable for this study.

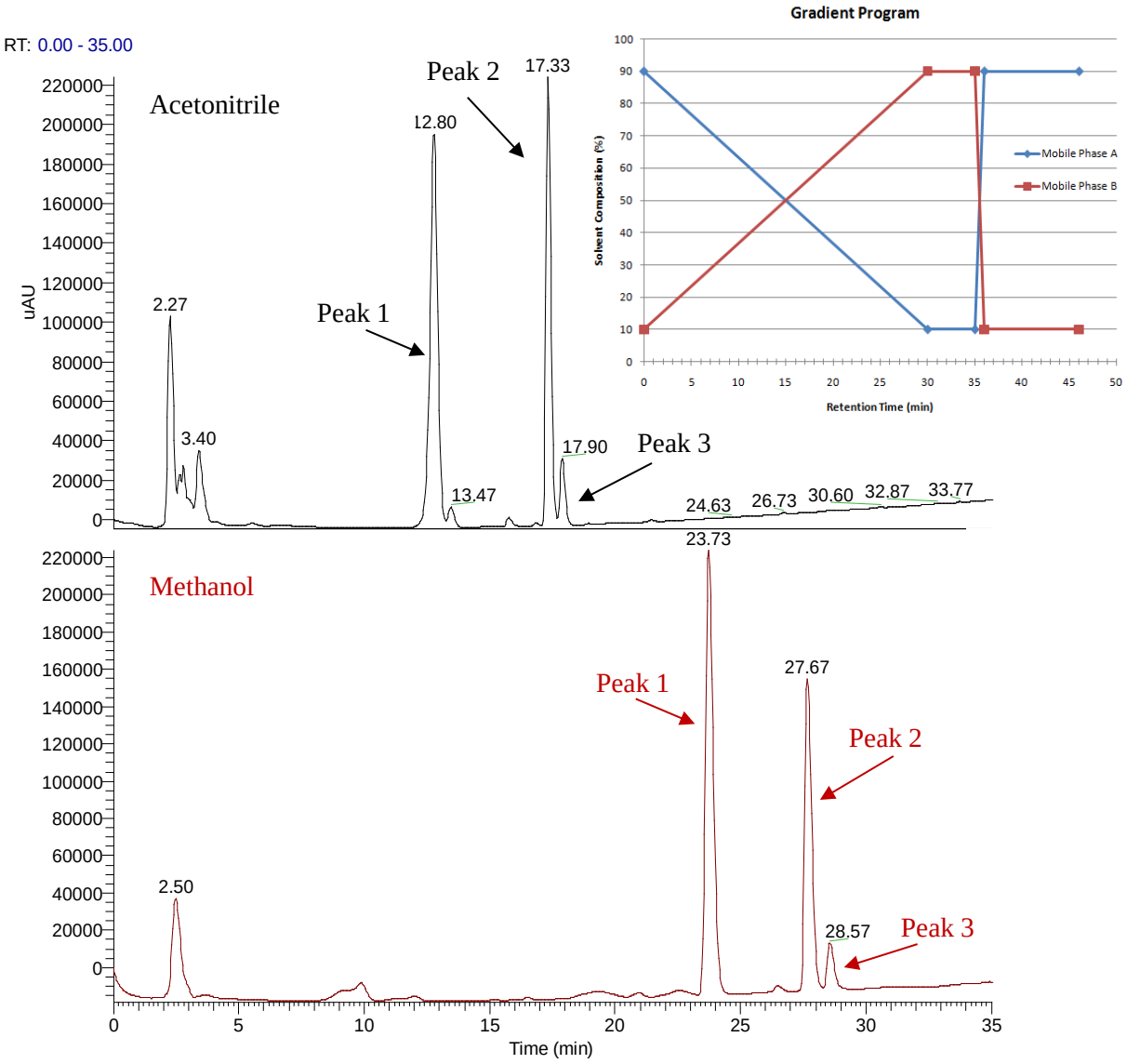


Figure 4.1 Separation of the flavonol glycosides present in a freeze dried onion hydroalcoholic extract. Mobile phase A consisted of 0.1% v/v formic acid in water, and mobile phase B was either 0.1% v/v formic acid in acetonitrile or 0.1% v/v formic acid in methanol. The gradient was as follows: 90/10 – 10/90 A/B (0-30 mins), 10/90 A/B (30-35 mins), 10/90 – 90/10 A/B (35-36 mins), 90/10 A/B (36-46 mins). The chromatogram was monitored at 370 nm.

The HPLC-PDA chromatograms of the freeze-dried onion extracts using the acetonitrile/water/formic acid mobile phase gradient with the Varian Polaris, Alltech Prevail and Phenomenex Gemini HPLC columns are shown in Figure 4.2. All three columns showed good separation of the flavonol glycosides, however, the Alltech Prevail column gave a slightly longer analysis time and, furthermore, the two minor peaks eluting after each major flavonol peak (as seen with the Varian Polaris and Phenomenex Gemini columns) were not observed. The separation achieved with the Polaris and Gemini columns were comparable, however, poor peak shape for quercetin 3,4'-diglucoside (peak 1) was seen with the Gemini column. These results suggested that the Varian Polaris column would be the most appropriate for this work.

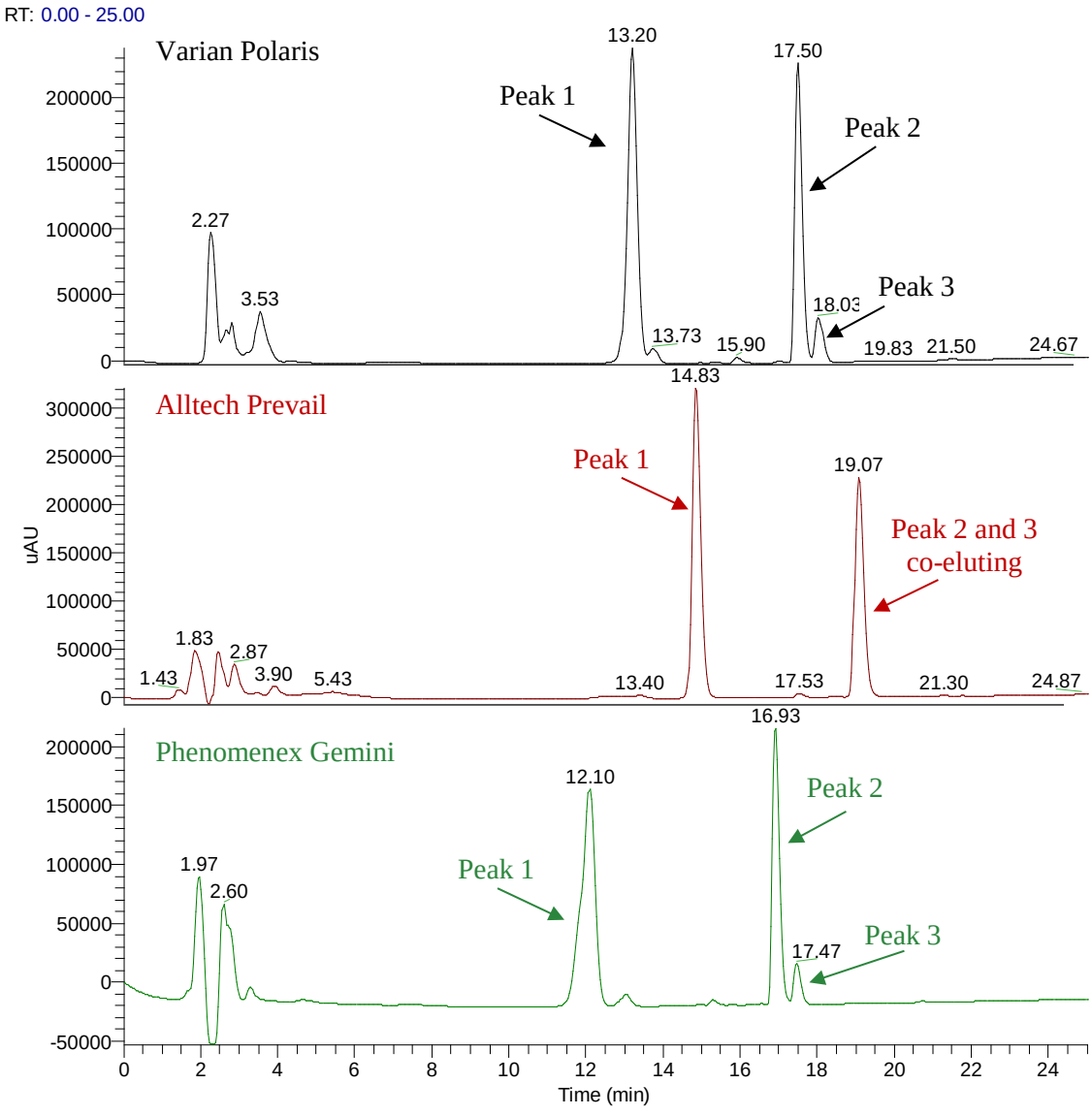


Figure 4.2 Separation of the flavonol glycosides present in a freeze dried onion hydroalcoholic extract using three different C18 columns: Varian Polaris, Alltech Prevail, and Phenomenex Gemini. Mobile phase A consisted of 0.1% v/v formic acid in water and mobile phase B was made up of 0.1% v/v formic acid in acetonitrile. The gradient program is shown in Figure 4.1. The chromatograms were monitored at 370 nm.

4.2.2 Flavonol Glycoside-Hydroxycinnamic Acid Derivatives in Bok Choy: Hydroalcoholic Extract

The Sumo variety of bok choy was used for method optimisation. A sample (1 g) was extracted using the hydroalcoholic methanol/water extraction method outlined in 3.4.4. Figure 4.3 shows the separation of the hydroalcoholic bok choy extract using the acetonitrile based mobile phase and gradient program optimised on the onion extract shown previously (see Figure 4.1 caption for conditions) and displayed at 330 nm (optimisation of display wavelength is discussed in 4.2.5). As can be seen in Figure 4.3, the resolution of peaks in the bok choy extract are not ideal using this mobile phase and gradient. Separation was further improved by making the gradient more shallow (i.e. extending to 40 min and then further to 50 min), which gave an expected increase in analysis time and resolution but there was no visible improvement in separation after extending the gradient slope beyond 40 minutes. Similar optimisation studies of varying the gradient slope and percent composition of mobile phases A and B were also conducted using the methanol/water/formic acid mobile phase.

The optimal separation was achieved with the following gradient: 95/5 – 5/95 A/B (0-40 min), 5/95 A/B (40-45 min), 5/95 – 95/5 A/B (45-46 min), 95/5 A/B (46-60 min) with mobile phase A consisting of 0.1% v/v formic acid in water and mobile phase B being made up of either 0.1% v/v formic acid in acetonitrile or 0.1% v/v formic acid in methanol. Figure 4.4 shows the separation of the flavonols in a hydroalcoholic methanol/water extract of freeze dried bok choy using this gradient program and both the acetonitrile and methanol mobile phases, with the chromatogram displayed at 330 nm.

Methanol gave different selectivity, particularly for the first group of peaks (19-23 min) and even though using acetonitrile significantly reduced analysis time it also significantly reduced resolution of the many compounds present in the extract. Therefore, since the methanol gradient displayed an increased ability to resolve more peaks it was chosen as the most appropriate solvent to be used in the analysis of the hydroalcoholic methanol/water extracts of bok choy.

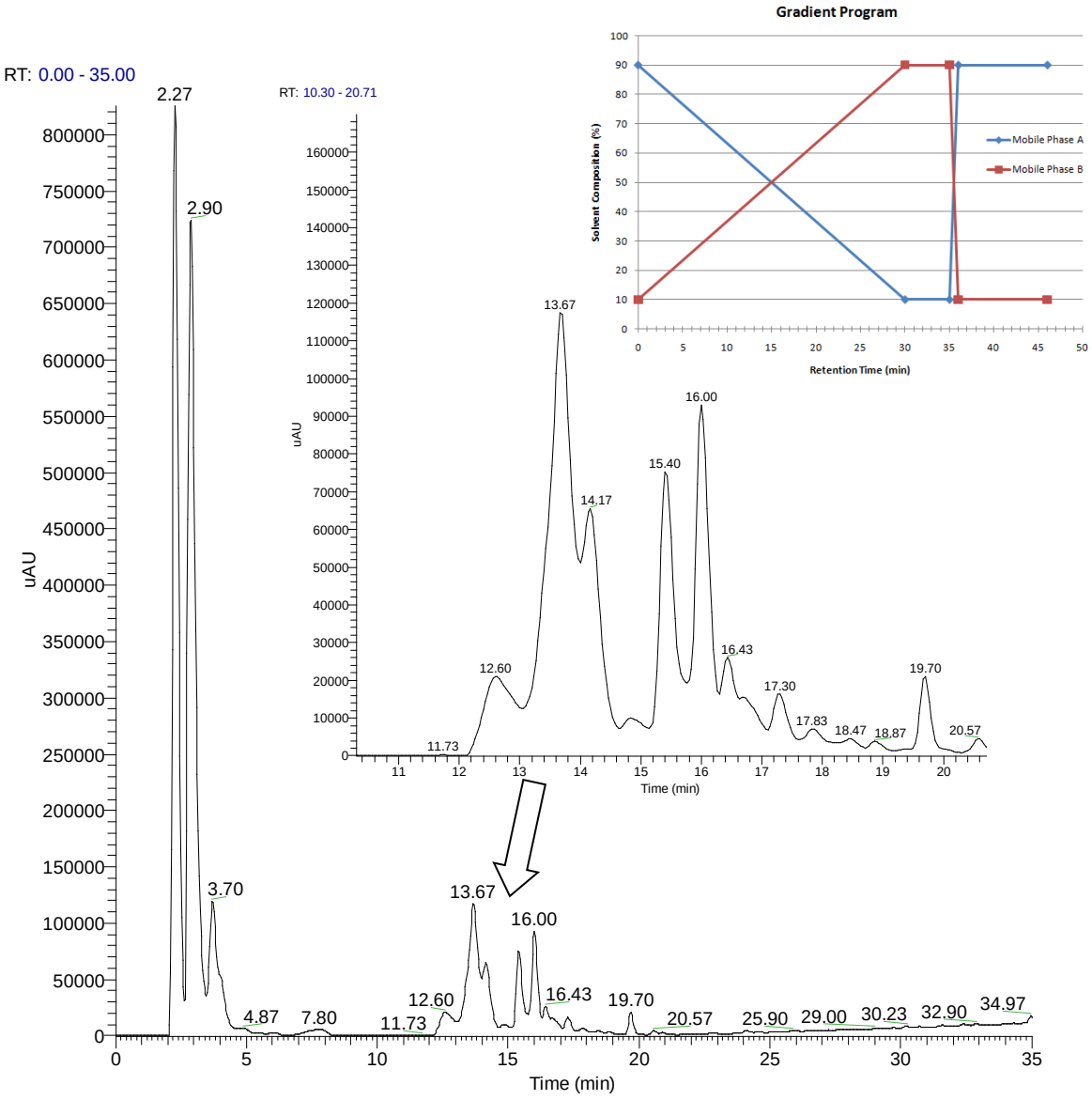


Figure 4.3 Separation of the flavonol derivatives present in a hydroalcoholic bok choy extract using the Varian Polaris column and previously optimised acetonitrile mobile phase and gradient program shown in Figure 4.1. The chromatogram was monitored at 330 nm. The insert shows a zoomed in section of the chromatogram between 12 and 20 minutes.

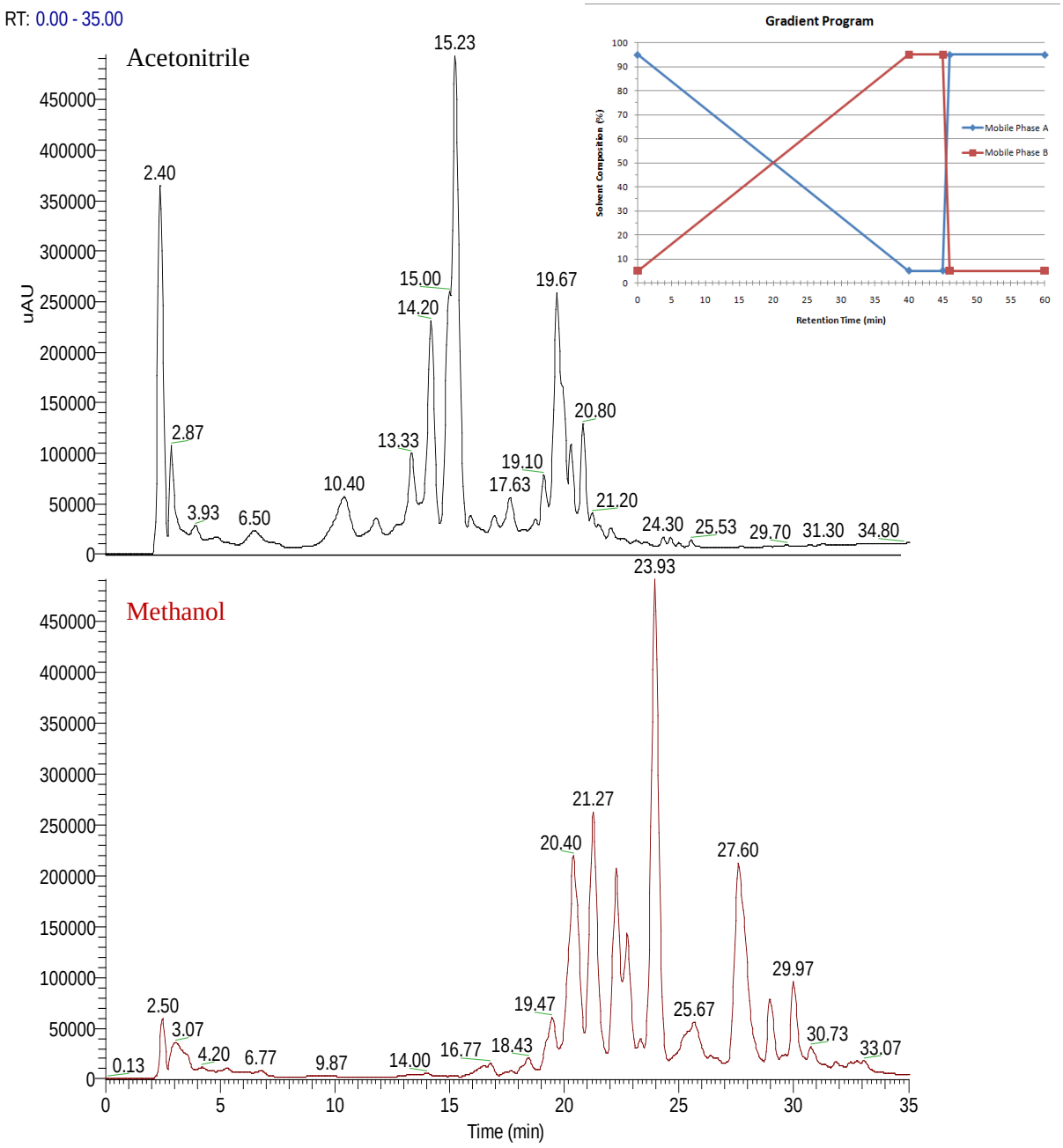


Figure 4.4 Separation of the flavonol derivatives present in a hydroalcoholic bok choy extract. Mobile phase A consisted of 0.1% v/v formic acid in water, and mobile phase B was either 0.1% v/v formic acid in acetonitrile or 0.1% v/v formic acid in methanol. The gradient was as follows: 95/5 – 5/95 A/B (0-40 min), 5/95 A/B (40-45 min), 5/95 – 95/5 A/B (45-46 min), 95/5 A/B (46-60 min). The chromatogram was monitored at 330 nm.

4.2.3 Flavonol Glycosides in Bok Choy: Alkaline Hydrolysate

The Sumo variety bok choy was again used for method optimisation, this time after extraction using the alkaline hydrolysis method outlined in 3.4.3. Figure 4.5 shows the separation of the alkaline hydrolysate extract displayed at 350 nm (optimisation of display wavelength is discussed in 4.2.5), using the acetonitrile and methanol mobile phases previously optimised for the hydroalcoholic methanol/water bok choy extract (shown in Figure 4.4).

In agreement with observations noted from Figure 4.4 on the separation of flavonol derivatives in a hydroalcoholic extract of bok choy, it was also observed with the alkaline hydrolysate (Figure 4.5) that the analysis time was longer using the mobile phase containing methanol. Furthermore, the separation and resolution of the flavonols from other compounds present in the extract using a methanol mobile phase is superior to that achieved with the mobile phase containing acetonitrile. Therefore, the methanol mobile phase was used for all further studies on the alkaline hydrolysate extracts of bok choy.

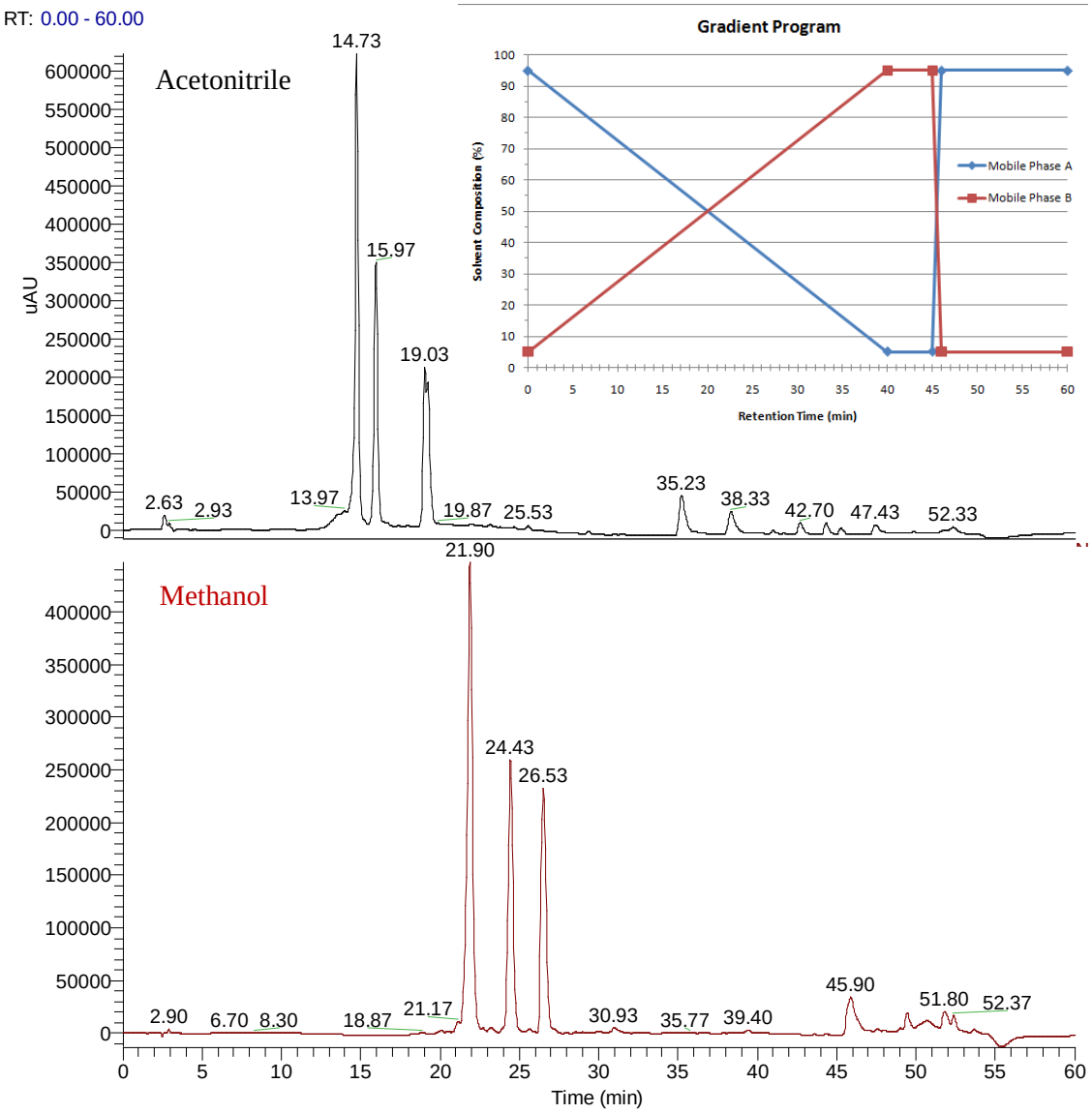


Figure 4.5 Separation of the flavonol glycosides present in an alkaline hydrolysate bok choy extract. Mobile phase A consisted of 0.1% v/v formic acid in water, and mobile phase B was either 0.1% v/v formic acid in acetonitrile or 0.1% v/v formic acid in methanol. The gradient program is shown in Figure 4.4. The chromatogram was monitored at 350 nm.

4.2.4 Flavonol Aglycones in Bok Choy: Acid Hydrolysate

The Sumo variety of bok choy was once again used for method optimisation. A sample (0.5 g) was extracted using the acid hydrolysis method outlined in 3.4.2. Initially, the same mobile phase composition and gradient program that was found to be suitable for the analysis of the hydroalcoholic methanol/water extract of bok choy, as well as the alkaline hydrolysate, was used for the separation of the acid hydrolysate. However, chromatographic resolution was unsatisfactory and in order to improve the separation of the flavonol aglycones the gradient elution program was altered a number of times. Complete separation of isorhamnetin and kaempferol proved to be very difficult due to similar chemical properties of these two flavonols, and ultimately could not be achieved. After completing these optimisation studies, the shorter gradient program that was optimised for the hydroalcoholic methanol/water onion extract (see Figure 4.1 caption for mobile phase conditions) was chosen for the analysis of the acid hydrolysate extracts. Extending the gradient run time by altering the slope did not show any improvement in the chromatographic resolution. Figure 4.6 shows the separation of the flavonol aglycones using both the acetonitrile and methanol mobile phases, with chromatograms displayed at 370 nm (optimisation of display wavelength is discussed in 4.2.5).

There is a significant difference in the separation of the compounds using the different mobile phases, and isorhamnetin and kaempferol co-elute with methanol in the mobile phase. Moreover, the peak shapes were broader overall, and the analysis time was much longer using the methanol mobile phase. A superior separation of isorhamnetin and kaempferol was achieved using acetonitrile in the mobile phase. Furthermore, the overall peak shapes were much sharper, and the separation was achieved in a shorter time. Therefore, the mobile phase containing acetonitrile was used for the analysis of the free flavonol aglycones in the acid hydrolysate extracts of the three cultivars of bok choy.

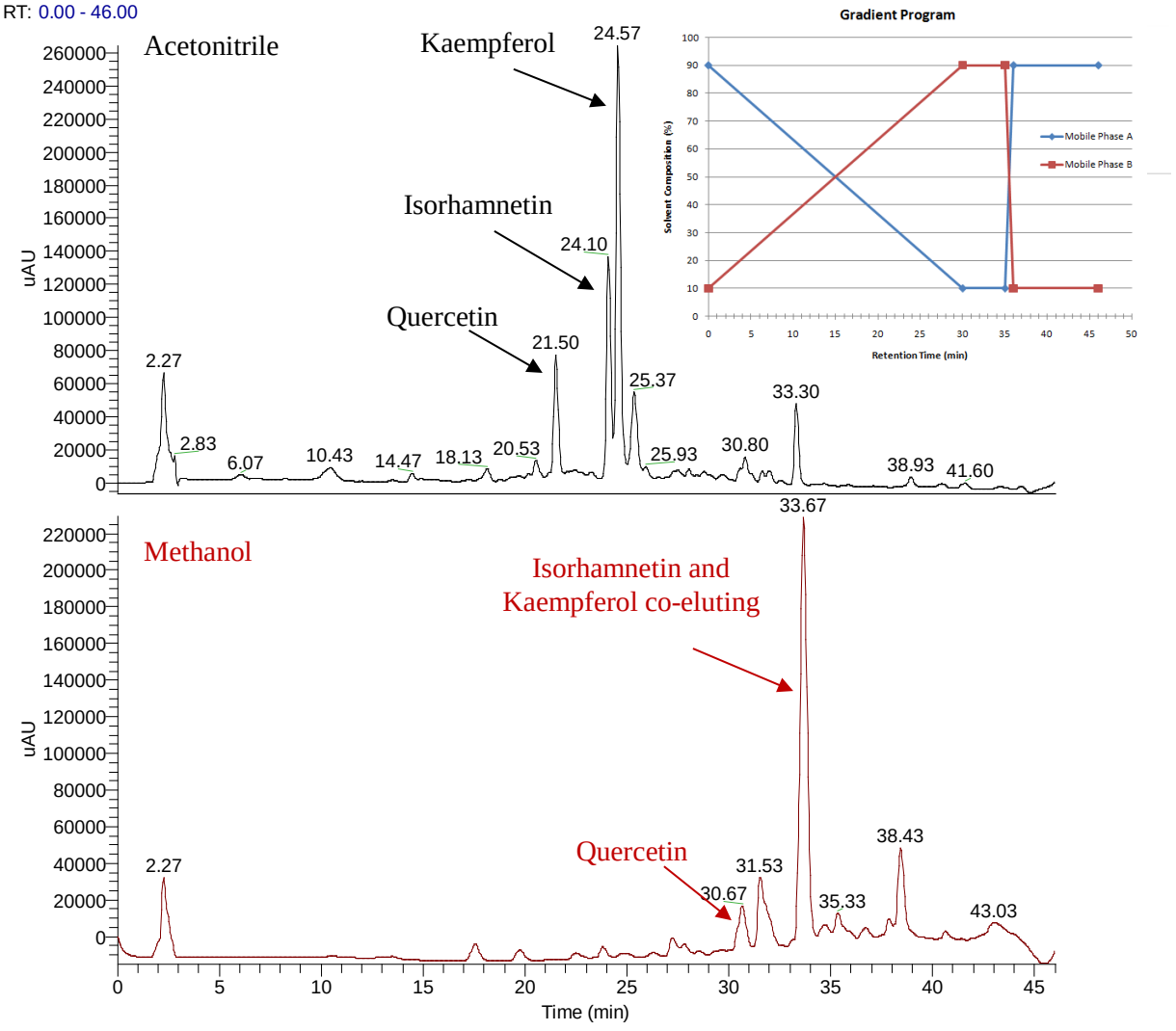


Figure 4.6 Separation of the flavonol aglycones present in an acid hydrolysate bok choy extract. Mobile phase A consisted of 0.1% v/v formic acid in water, and mobile phase B was either 0.1% v/v formic acid in acetonitrile or 0.1% v/v formic acid in methanol. The gradient program is shown in Figure 4.1. The chromatogram was monitored at 370 nm.

4.2.5 Optimisation of PDA Detector Wavelength for the Analysis of Flavonols in Bok Choy Samples

Quercetin, isorhamnetin, and kaempferol exhibit typical flavonol aglycone UV-Vis spectra with two distinct λ_{max} bands at 255 and 370 nm, 253 and 370 nm, and 265 and 367 nm respectively [227, 152]. The broad maximum at approximately 370 nm shifts lower to approximately 350 nm when the flavonol aglycone becomes substituted with a sugar unit, especially in the 7-*O* position [116]. The further addition of a hydroxycinnamic acid residue to the flavonol compound causes another shift to the broad maximum of around 330 to 340 nm. This phenomenon, referred to as a hypsochromic shift, was reported by Vallejo *et al.* [57] in the study of flavonols in broccoli. It was also observed by Rochfort *et al.* [60] in the study of immature leaves of pak choi, and more recently by Olsen *et al.* [116] in the study of flavonols in curly kale. These shifts in λ_{max} bands were also detected for all glycosylated and acylated flavonol compounds in this investigation, leading to the use of different detection wavelengths for the PDA in the analysis of the different extracts of bok choy: acid hydrolysate, alkaline hydrolysate, and hydroalcoholic.

4.2.5.1 Flavonol Aglycones in Bok Choy: Acid Hydrolysate

Quercetin, kaempferol and isorhamnetin were identified in the HPLC-PDA separation of freeze dried bok choy after acid hydrolysis by comparison with the retention times and UV-Vis spectra of pure standards (refer to Section 5.2.1). The extracted UV-Vis spectra of each flavonol are displayed in Figure 4.7 and the HPLC-PDA chromatograms, monitored at the two wavelengths of maximum absorbance, of an acid hydrolysate bok choy extract with the identified peaks is presented in Figure 4.8. As shown in Figure 4.7, all three flavonols have similar UV-Vis spectra consisting of a distinct broad absorbance band at approximately 370 nm and a stronger, sharper, absorbance band at approximately 230 nm.

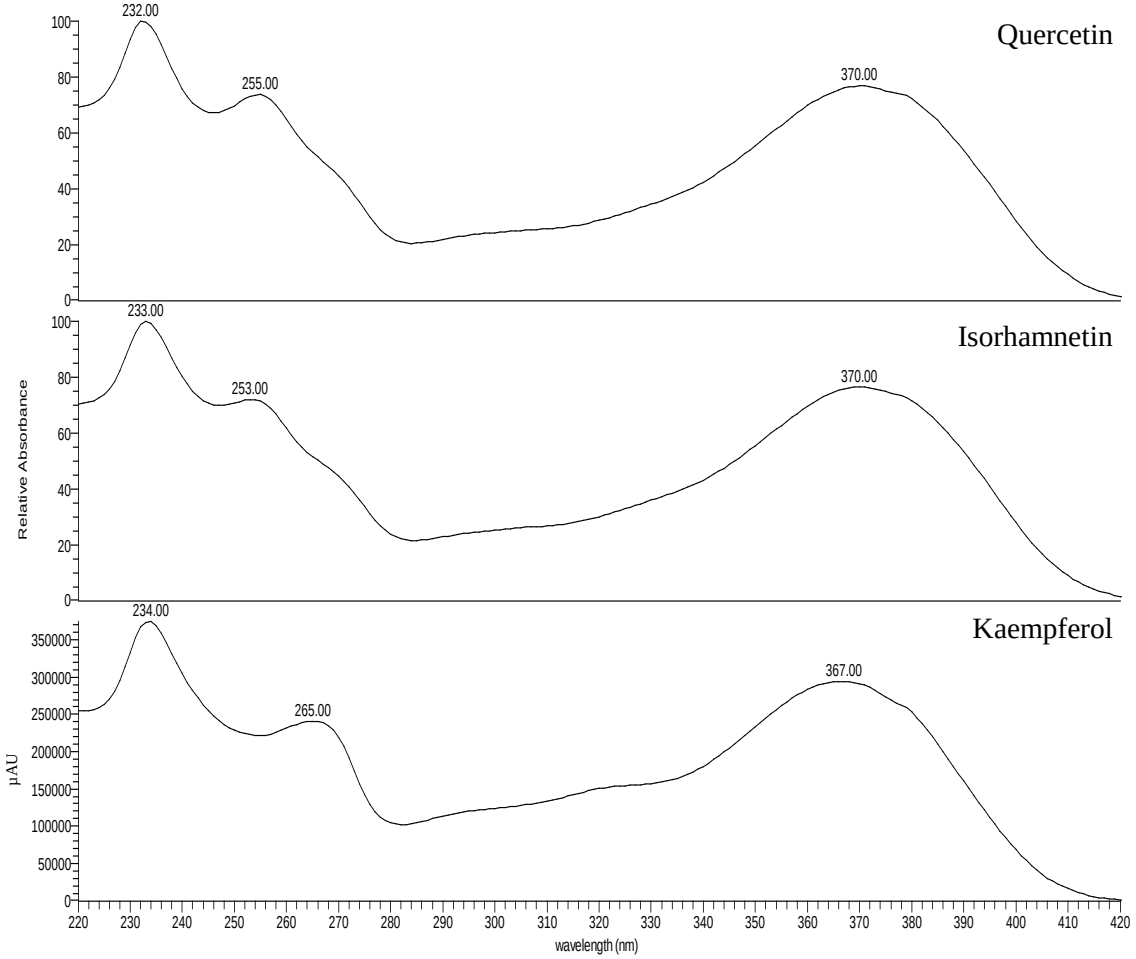


Figure 4.7 UV-Vis spectra of quercetin, isorhamnetin, and kaempferol recorded from the HPLC-PDA chromatogram of an acid hydrolysate Sumo bok choy extract scanned from 220-420 nm.

The separation of the three flavonols was monitored at 230 nm and 370 nm (Figure 4.8). Both wavelengths gave similar absorbance readings for the three flavonols, however, there were other strong UV-Vis absorbing species present at 230 nm with retention times of 6.80 min and 22.17 min which caused the flavonols to appear visually smaller. Therefore, 370 nm was chosen as the preferred display wavelength for the analysis of flavonol aglycones in acid hydrolysate bok choy extracts.

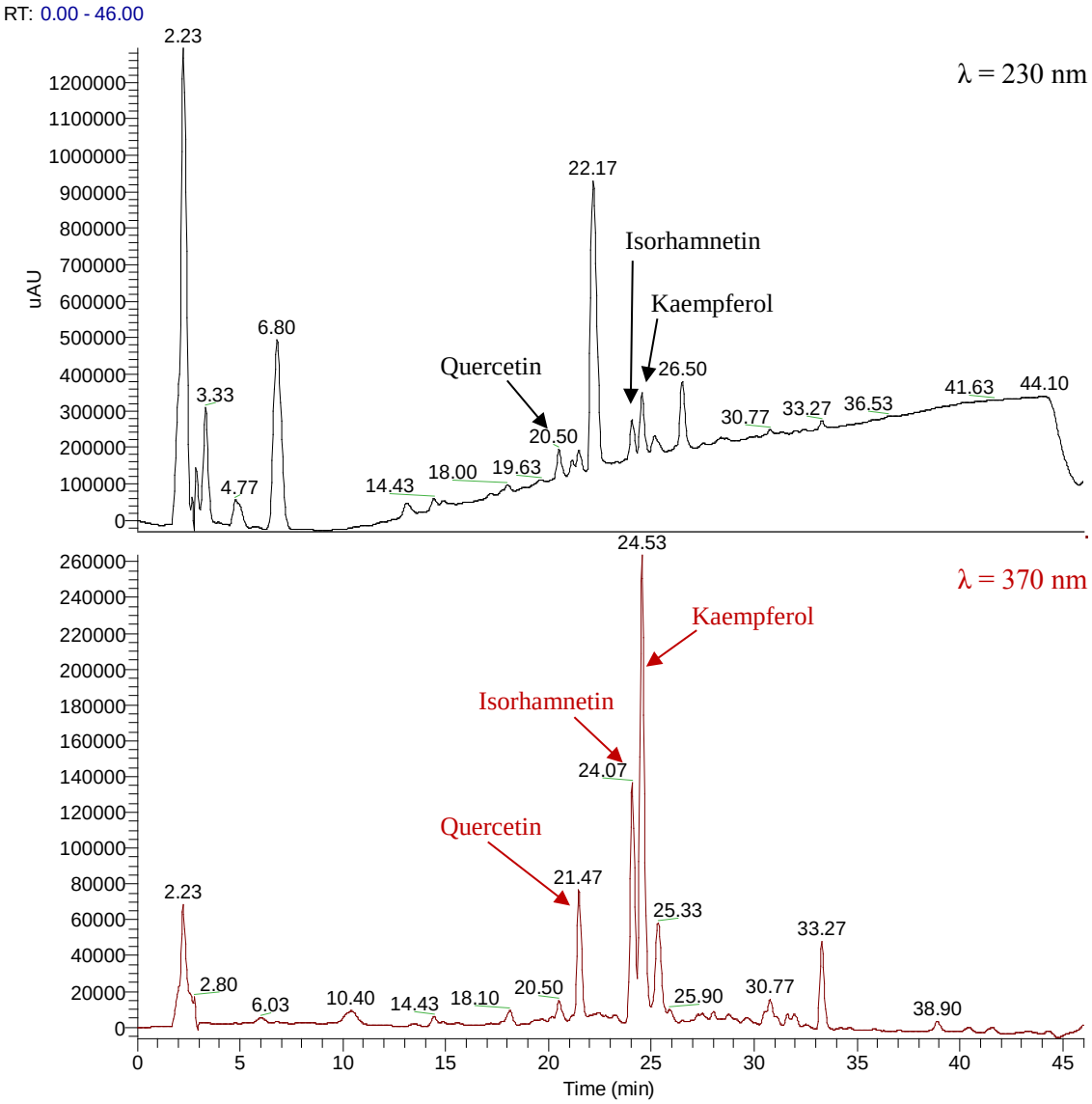


Figure 4.8 HPLC-PDA chromatograms of a Sumo bok choy acid hydrolysate extract monitored at 230 nm and 370 nm, using previously optimised acetonitrile mobile phase and gradient program shown in Figure 4.1.

4.2.5.2 **Flavonol Glycosides in Bok Choy: Alkaline Hydrolysate**

The two major flavonol glycosides present in the alkaline hydrolysate extract of a bok choy sample were later identified as kaempferol-3-sophoroside-7-glucoside and isorhamnetin-3,7-diglucoside (see Section 5.3 for the identification of these compounds using HPLC/ESI-MS^n). The UV-Vis spectra for these two compounds are shown below in Figure 4.9. Both flavonol glycosides have a distinct broad absorbance band at approximately 350 nm and a slightly stronger, sharper, absorbance band at approximately 260 nm.

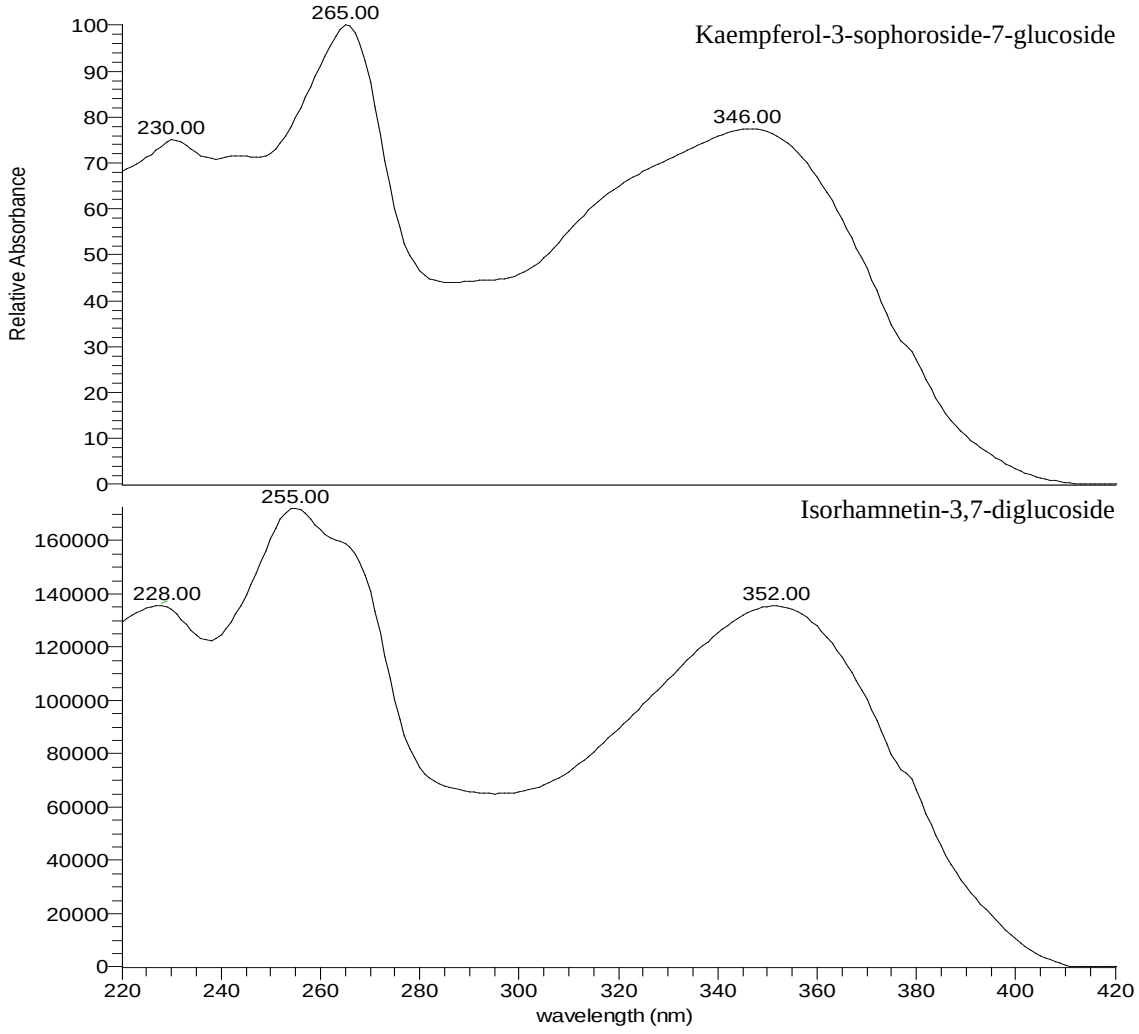


Figure 4.9 UV-Vis spectra of kaempferol-3-sophoroside-7-glucoside and isorhamnetin-3,7-diglucoside recorded from the HPLC-PDA chromatogram of an alkaline hydrolysate Sumo bok choy extract scanned from 220-420 nm.

The HPLC-PDA chromatograms of an alkaline hydrolysate bok choy extract recorded at both wavelengths of maximum absorbance (260 and 350 nm) are shown below in Figure 4.10. The chromatograms were similar and either one of these wavelengths could be used for this analysis. However, the chromatogram recorded at 350 nm displayed less absorbing species as compared to the chromatogram at 260 nm and was therefore chosen as the preferred display wavelength for alkaline hydrolysate bok choy extracts.

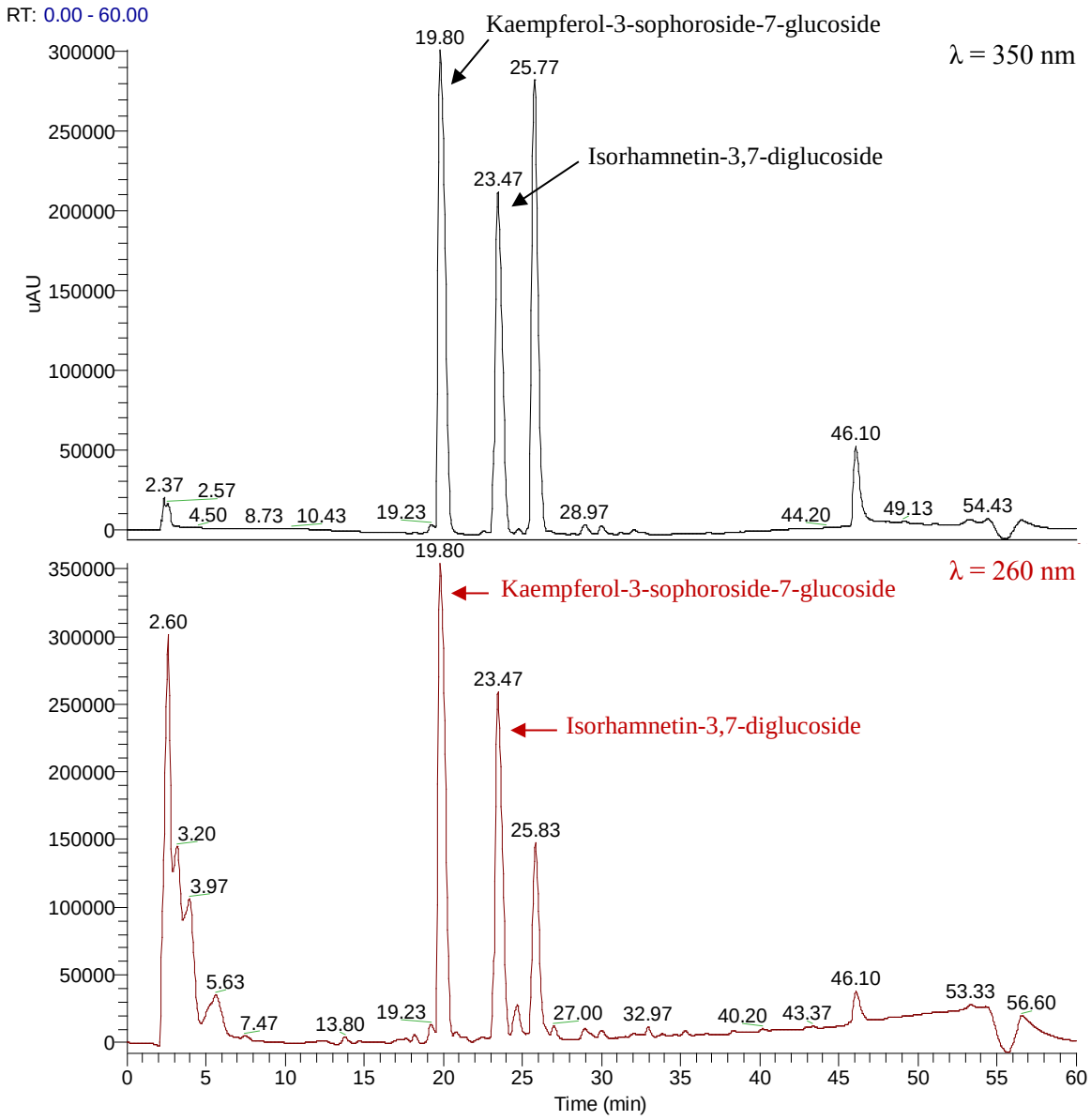


Figure 4.10 HPLC-PDA chromatograms of a Sumo bok choy alkaline hydrolysate extract monitored at 350 nm and 260 nm, using previously optimised methanol mobile phase and gradient program shown in Figure 4.4.

4.2.5.3 Flavonol Glycoside-Hydroxycinnamic Acid Derivatives in Bok Choy: Hydroalcoholic Extract

The two major flavonol glycoside-hydroxycinnamic acid derivatives present in the hydroalcoholic methanol/water extract of a bok choy sample were later identified as kaempferol-3-sophoroside(caffeoyl)-7-glucoside and kaempferol-3-sophoroside(feruloyl)-7-glucoside (see Section 5.4 for the identification of these compounds using HPLC/ESI-MS^n). The UV-Vis spectra for these two flavonol derivatives are shown below in Figure 4.11. Both flavonol glycoside-hydroxycinnamic acid conjugates have a distinct broad absorbance band at approximately 330 nm, as well as two other bands at about 235 nm and 268 nm of similar intensity.

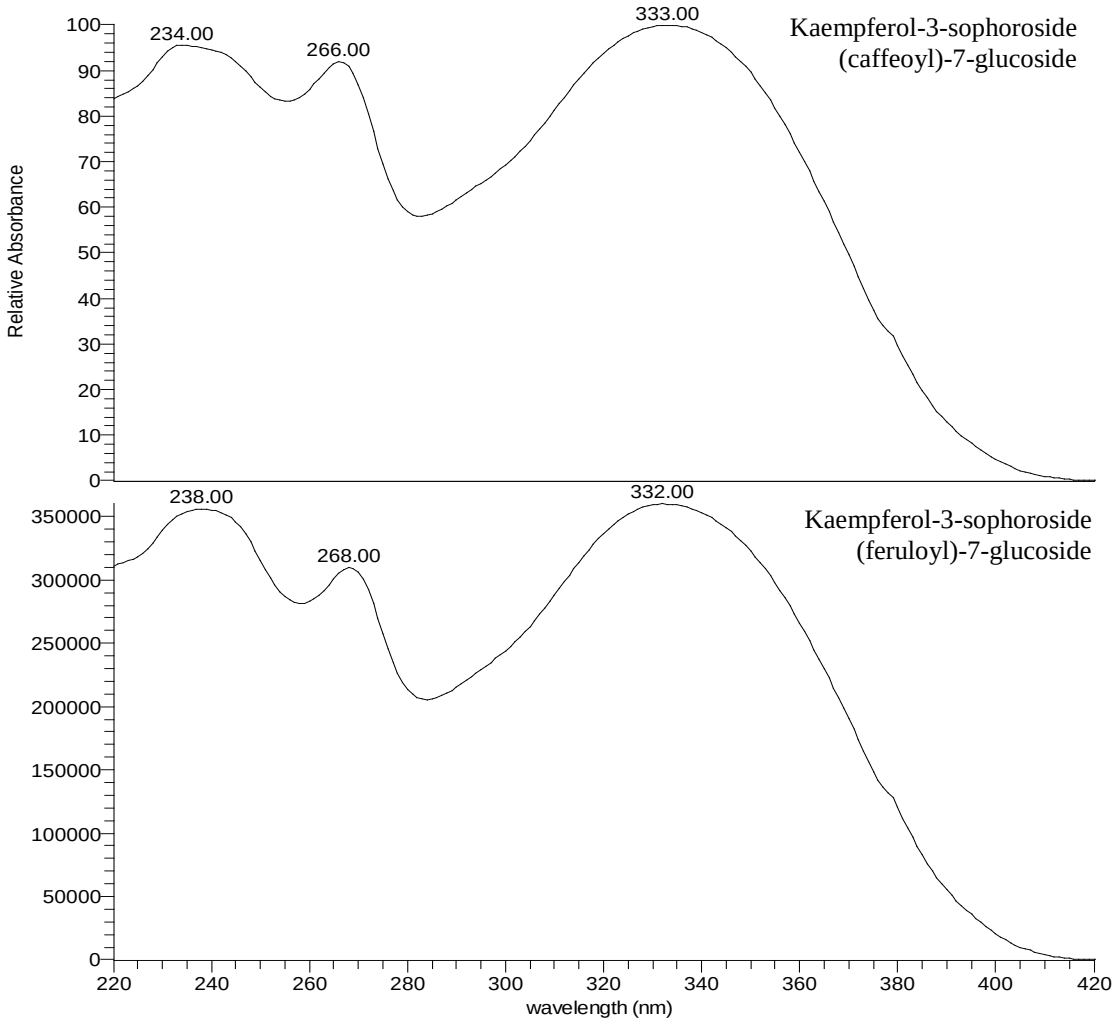


Figure 4.11 UV-Vis spectra of kaempferol-3-sophoroside(caffeoyl)-7-glucoside and kaempferol-3-sophoroside(feruloyl)-7-glucoside recorded from the HPLC-PDA chromatogram of a hydroalcoholic Sumo bok choy extract scanned from 220-420 nm.

The HPLC-PDA chromatograms of a hydroalcoholic methanol/water bok choy extract monitored at 330 nm and 260 nm are shown below in Figure 4.12. The chromatograms were similar; however, more compounds were visible at 330 nm, and it was therefore chosen as the preferred display wavelength for hydroalcoholic bok choy extracts.

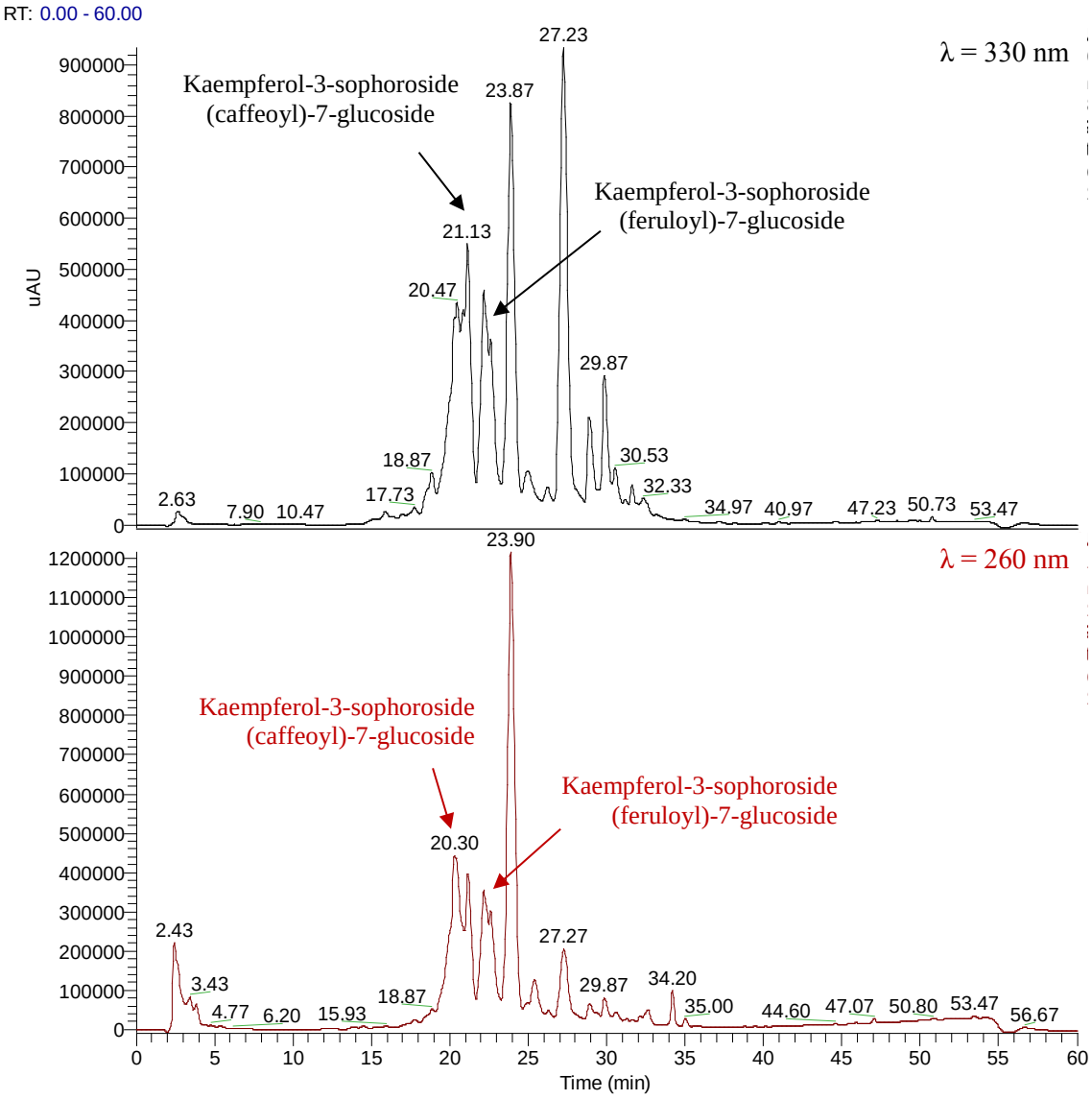


Figure 4.12 HPLC-PDA chromatograms of a Sumo bok choy hydroalcoholic extract monitored at 330 nm and 260 nm, using previously optimised methanol mobile phase and gradient program shown in Figure 4.4.

In conclusion, a hypsochromic shift of 20 nm was observed in the UV-Visible spectra of flavonol compounds when the aglycones were substituted with glucose and a further 20 nm with glucose-hydroxycinnamic acid substitution. This is displayed in Figure 4.13. The chromatograms for the flavonol aglycones in the acid hydrolysate, the flavonol glycosides in the alkaline hydrolysate, and the flavonol glycoside-hydroxycinnamic acid derivatives in the hydroalcoholic extract, were monitored/displayed at 370 nm, 350 nm and 330 nm respectively as these wavelengths were found to be more specific for these compounds.

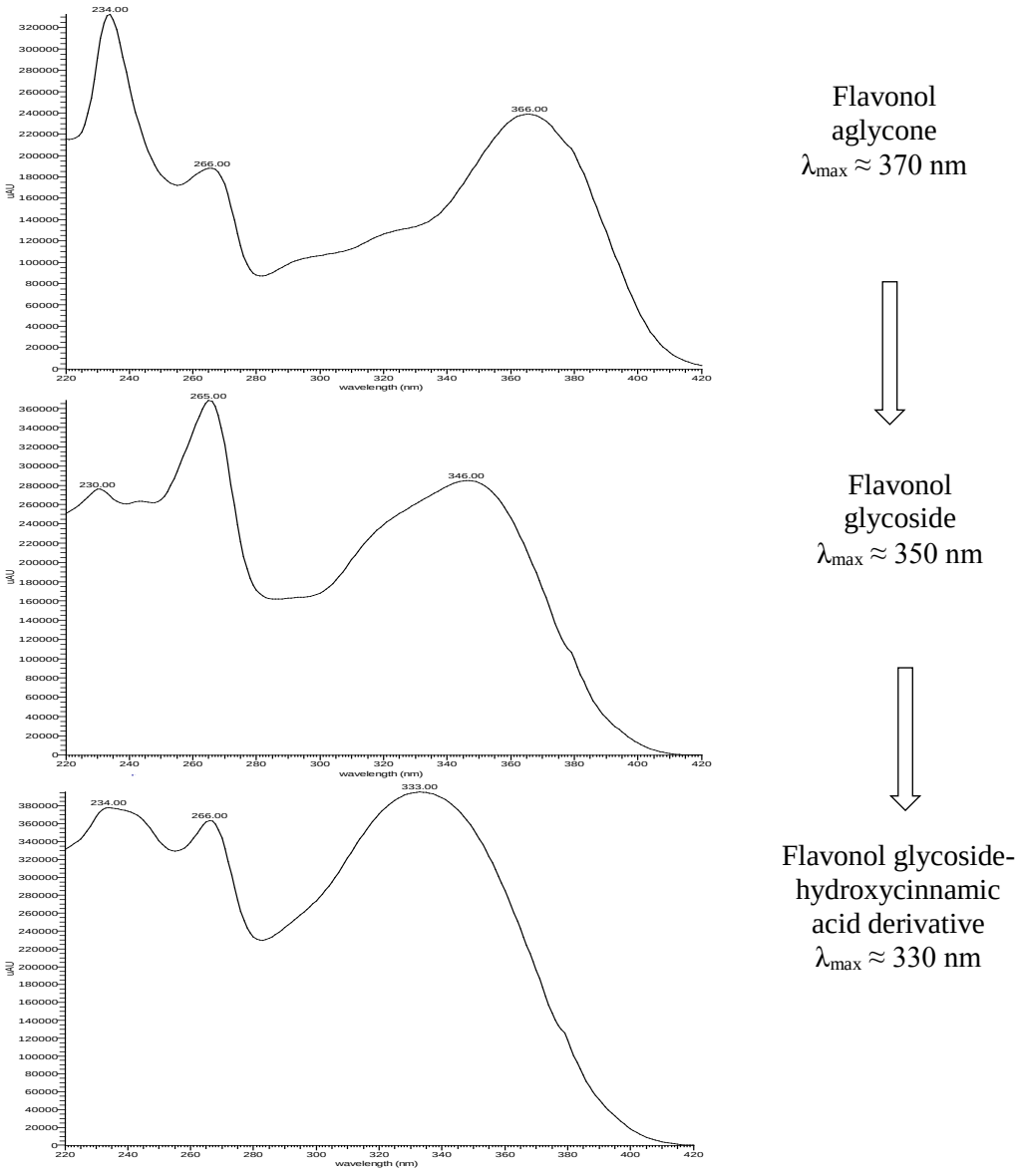


Figure 4.13 UV-Vis spectra showing hypsochromic shift of absorbance maxima from flavonol aglycone (370 nm) to flavonol glycoside (350 nm) to flavonol glycoside-hydroxycinnamic acid derivative (330 nm).

4.2.6 Optimisation of ESI-MS^n Conditions for the Analysis of Flavonols in Bok Choy Samples

Analytes can be ionised by a loss of protons (negative ionisation) or by a gain of protons (positive ionisation) using ESI-MS^n. Flavonols have successfully been analysed using both these ionisation modes, however, in general the negative ion mode is the method of choice. A review of both positive and negative ionisation modes, and the relative fragments observed, was presented by de Rijke *et al.* [143]. A number of studies have reported that the negative ion mode provides the highest instrument sensitivity [182, 187, 224, 319-320, 229] and is more suited for deducing the molecular mass of separated phenolic compounds due to limited fragmentation, especially when compounds are present in trace amounts [27]. Furthermore, a previous study on the flavonols present in pak choi [60] suggested that the negative ion mode is more robust in the analysis of these compounds (given that flavonols have multiple OH groups that can readily lose a H), and so negative ion ESI-MS^n was chosen for this study.

The volatility and solvent's ability to donate or accept a proton is also important in ESI. In the negative ion mode, the use of 0.1% formic acid [224] or ammonium acetate buffer at a concentration of 5 mM [184] as a mobile phase additive seems to be desirable, whilst the ESI source and HPLC separation are both negatively influenced by the addition of basic eluents. Numerous analyses of *Brassica* species via HPLC-PDA/ESI-MS^n utilised formic acid in the mobile phase, including studies on pak choi [136, 192, 60], kale [217], broccoli [57], cabbage [214, 208, 74], turnip [221], red mustard greens [215], cauliflower[171, 128], oilseed rape [218], and other varieties of *Brassica* vegetables [112, 152, 212]. Therefore, formic acid was also chosen as the mobile phase additive in this study.

4.2.6.1 **Tuning the ESI-MS^n**

The mass spectrometer was tuned using the molecular ion ([M-H]$^-$ = 609) of the glycoside rutin (i.e. quercetin-3-rutinoside, rutoside or sophorin). Rutin was chosen as it is structurally similar to the compounds of interest and it was readily available and inexpensive. The structure is shown below in Figure 4.14.

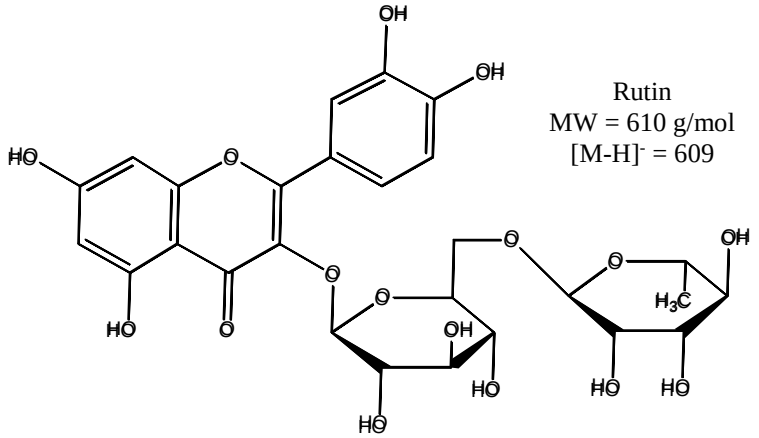


Figure 4.14 Chemical structure of rutin, the quercetin glycoside used to tune the ESI-MS^n prior to analysis of the flavonols in bok choy.

It was also important to tune the MS with similar HPLC solvent and flow conditions to that used for the HPLC-PDA/ESI-MS^n studies of bok choy, and therefore tuning was conducted using a methanol/water/formic acid mobile phase and a flow rate of 0.2 mL/min. A 250 mg/L rutin standard (≥90%) made up in 80/20 methanol/water containing 0.1% v/v formic acid (HPLC-MS grade) was introduced into the MS via direct infusion from a syringe pump through a T-piece at a rate of 5 µL/min. At the same time a mobile phase comprised of 20/80 water/methanol plus 0.1% v/v formic acid was delivered to the T-piece at a flow rate of 0.2 mL/min by the HPLC pump.

Firstly, the position of the probe was manually altered to maximise the normalised level (NL) value and minimise ion time. Tuning was conducted using the auto tune function. Capillary temperature and capillary voltage were optimised by observing ion time and relative response to the rutin ion (NL value). A series of temperatures (350ºC, 300ºC, 250ºC, 220ºC) and voltages (+54 V, +18.50 V, -18.50 V, -48.50 V) were investigated.

A capillary temperature of 350ºC and a capillary voltage of -48.50 V gave the optimum response; at this temperature and voltage settings rutin gave sufficient signal (Figure 4.15). There was also a small ion observed at 300.20 amu, which at first was thought to represent the quercetin aglycone coming from an impurity in the rutin, or perhaps some in source fragmentation. However, the quercetin aglycone would have a molecular ion of 301 amu in negative ion mode, and without further investigation the identification of this impurity could not be ascertained. Refer to 3.9 for the entire list of MS acquisition parameters.

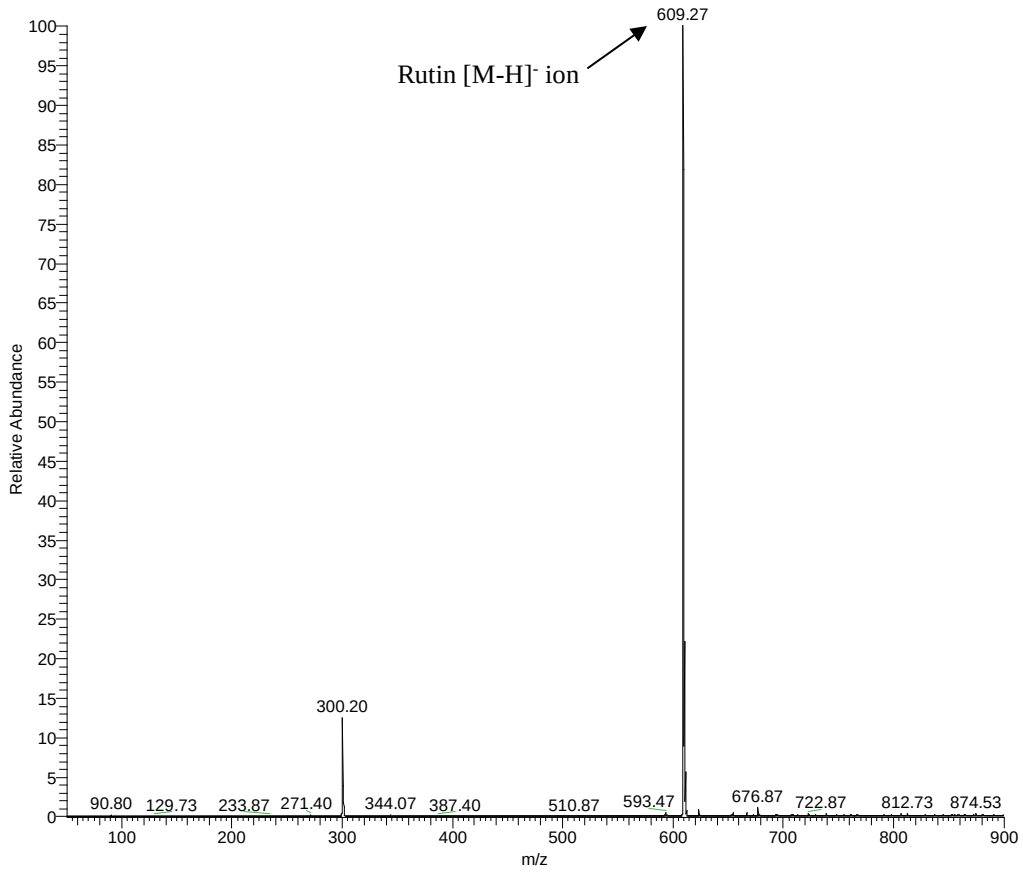


Figure 4.15 ESI-MS[1] spectra of the tune compound rutin in negative ion mode. Capillary temperature was set to 350ºC and capillary voltage -48.50 V.

4.3 OPTIMISATION OF PREPARATIVE HPLC CONDITIONS

Preparative HPLC was used to isolate compounds from a hydroalcoholic methanol/water extract and alkaline hydrolysate extract in sufficient quantities required for NMR characterisation and further cancer cell studies. A preparative HPLC column packed with a similar stationary phase as the analytical column was chosen as this allowed for a relatively straight forward optimisation of the injection volume and flow rate for the final separation/collection conditions. The analytical column that was chosen for the HPLC-PDA/ESI-MS^n analysis of flavonols in bok choy was a 150 x 2.0 mm 3 µm Varian Polaris C18-A. Therefore, a 250 x 21.2 mm 5 µm Varian Polaris C18-A preparative column was purchased for the preparative HPLC work. Flow rate and injection volume was determined by using the standard scale up equations shown below [327].

Injection Volume:

$$Vol_{PREP} = Vol_{ANALYTICAL} \times \frac{D^2_{PREP}}{D^2_{ANALYTICAL}} \times \frac{L_{PREP}}{L_{ANALYTICAL}}$$

Where Vol is the injection volume (µL), D is the inner diameter of the column (mm), and L is the column length (mm).

Flow Rate:

$$F_{PREP} = F_{ANALYTICAL} \times \frac{D^2_{PREP}}{D^2_{ANALYTICAL}}$$

Where F is flow rate (mL/min) and D is the inner diameter of the column (mm).

The theoretical flow rate and injection volume derived from these equations were 22.5 mL/min and 936.3 µL respectively. However, flow rates above 10 mL/min gave high back pressures which caused the HPLC tubing to burst from the fittings. Accordingly, 10 mL/min was chosen as the flow rate, and the injection volume was rounded up to 1000 µL.

The mobile phase composition and gradient program were also optimised for preparative HPLC. The methanol based mobile phase that was previously optimised for the separation of flavonol compounds in both the hydroalcoholic and alkaline hydrolysate extracts of bok choy was utilised, consisting of 0.1% v/v formic acid in water (A) and 0.1% v/v formic acid in methanol (B). The gradient program implemented for preparative HPLC was slightly longer than that used for the analytical HPLC as this produced a better separation for the hydroalcoholic methanol/water bok choy extract. Figures 4.16 and 4.17 show the separation of a hydroalcoholic bok choy extract using the gradient program previously optimised on the analytical HPLC, and the adjusted gradient program for preparative HPLC respectively. The same gradient program was also used

for the alkaline hydrolysate separation on the preparative HPLC. Table 4.1 shows the comparison of both analytical and preparative HPLC conditions.

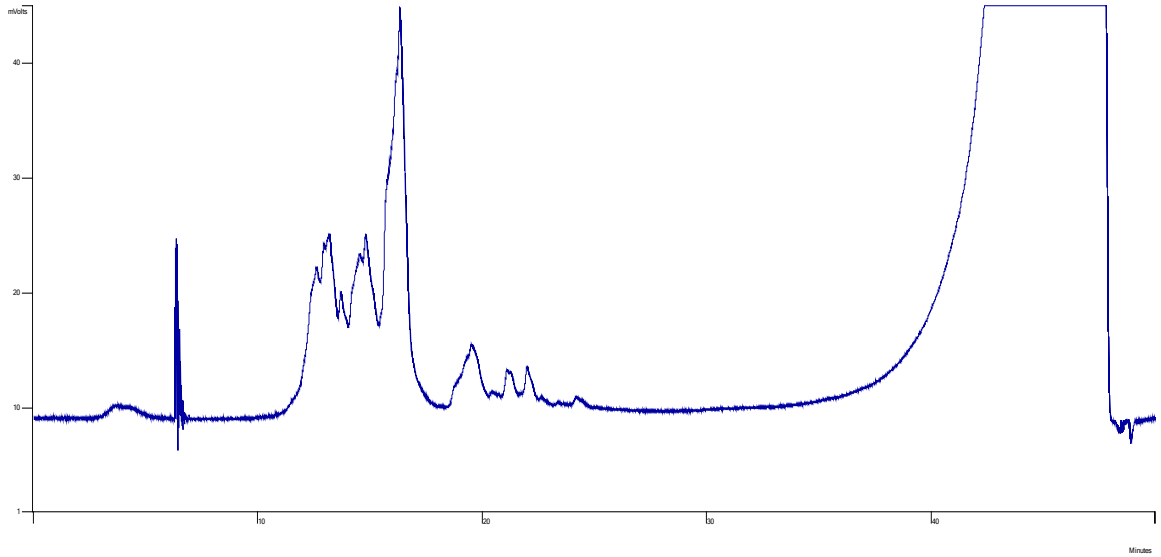


Figure 4.16 Separation of flavonol derivatives in a hydroalcoholic bok choy extract using preparative HPLC and previously optimised analytical HPLC gradient. Refer to Table 4.1 for analytical HPLC mobile phase composition and gradient program. The chromatogram was monitored at 330 nm.

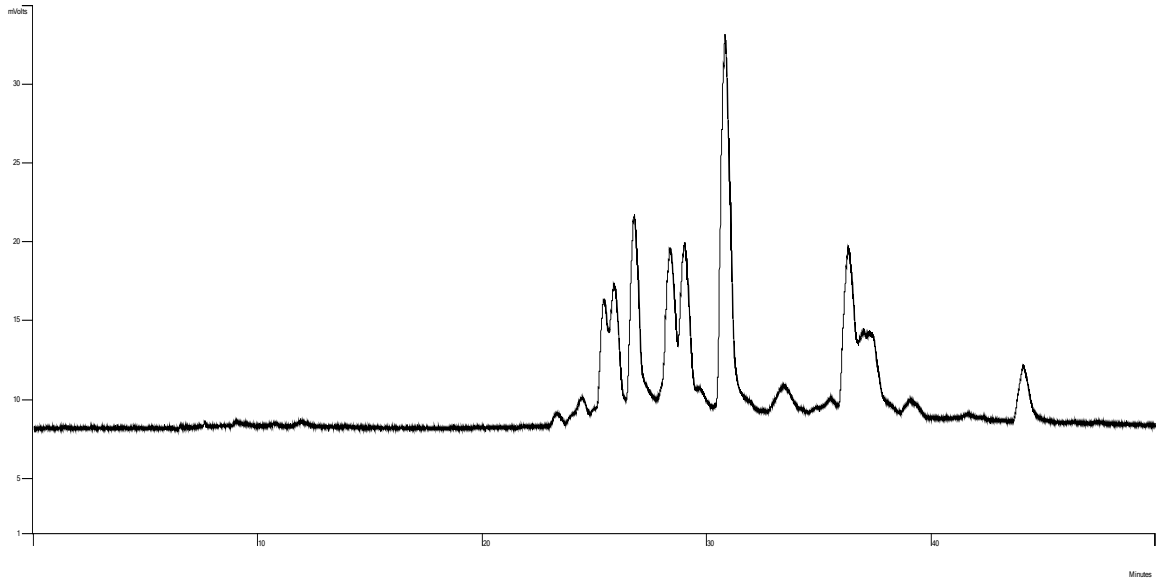


Figure 4.17 Separation of flavonol derivatives in a hydroalcoholic bok choy extract using preparative HPLC and newly optimised preparative HPLC gradient. Refer to Table 4.1 for preparative HPLC mobile phase composition and gradient program. The chromatogram was monitored at 330 nm.

Table 4.1 Comparison of analytical and preparative HPLC conditions with differences/changes in red.

Parameter	Analytical HPLC 150 x 2 mm ID, 3 µm Varian Polaris C18-A	Preparative HPLC 250 x 21.2 mm ID, 5 µm Varian Polaris C18-A
Flow Rate	0.2 mL/min	10 mL/min
Injection Volume	5 µL	1000 µL
Mobile Phase Composition	Mobile phase A: 0.1% v/v formic acid in water Mobile phase B: 0.1% v/v formic acid in methanol	Mobile phase A: 0.1% v/v formic acid in water Mobile phase B: 0.1% v/v formic acid in methanol
Gradient Program	95/5 A/B – 5/95 A/B (0 – 40 min), 5/95 A/B (40 – 45 min), 5/95 A/B – 95/5 A/B (45 – 46 min), 95/5 A/B (46 – 56 min)	95/5 A/B – 5/95 A/B (0 – 60 min), 5/95 A/B (60 – 65 min), 5/95 A/B – 95/5 A/B (65 – 66 min), 95/5 A/B (66 -76 min)
Column Temperature	25°C (maintained by column oven)	Room Temperature (≈25°C but not controlled)

4.4 OPTIMISATION OF ACID HYDROLYSIS CONDITIONS FOR THE ANALYSIS OF FLAVONOL AGLYCONES IN BOK CHOY SAMPLES

In order to determine the optimum hydrolysis time for the conversion of the flavonol conjugates to the base aglycones, the freeze dried bok choy was hydrolysed in boiling methanolic hydrochloric acid and the reaction monitored every two hours for a twelve-hour period (see 3.4.2 for acid hydrolysis methodology). At each two-hour time interval, the aglycone levels were analysed by HPLC-PDA/ESI-MS^n as described in 3.6.1 and quantified. The PDA chromatograms, monitored at 370 nm, for each hydrolysis time point are shown in Figure 4.18. The flavonol aglycones were absent in the acid hydrolysate at the commencement of the experiment (i.e. time zero), however, the flavonol aglycones are clearly present after refluxing for 2 hours. The aglycone levels (mg/100 g DW) quantified from both the PDA (at 370 nm) and MS SIM data for each hydrolysis time point (0, 2, 4, 6, 8, 10, and 12 hours) are plotted on graphs in Figures 4.19 and 4.20 respectively and displayed in Table 4.2. It was determined that the aglycone levels were relatively stable after 2 hours, however to ensure complete hydrolysis, 4 hours was chosen as the optimum hydrolysis time.

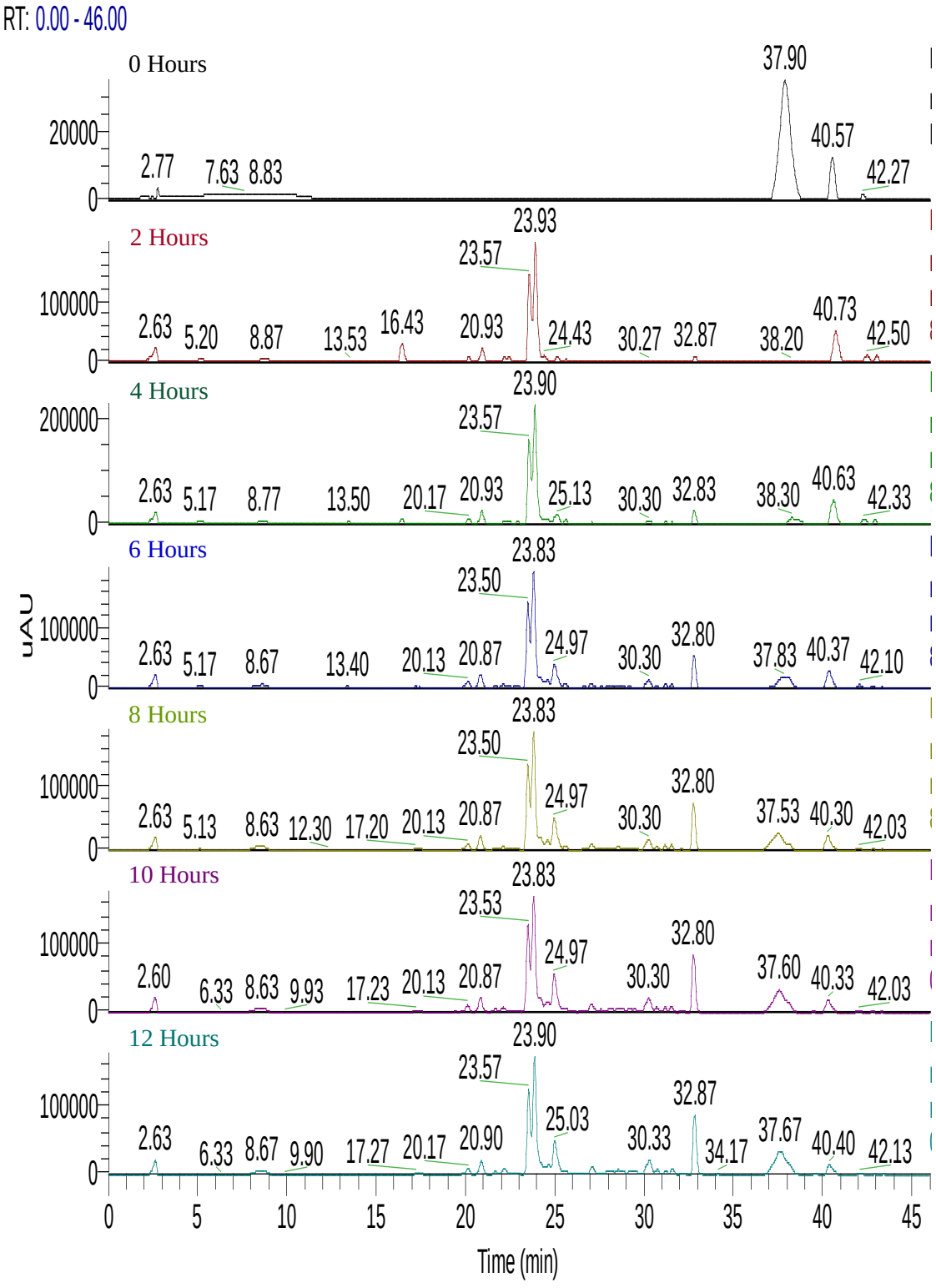


Figure 4.18 HPLC-PDA chromatograms of acid hydrolysate bok choy extracts hydrolysed for 0, 2, 4, 6, 8, 10, and 12 hours. Mobile phase composition and gradient program as optimised in 4.2.4. All chromatograms were monitored at 370 nm.

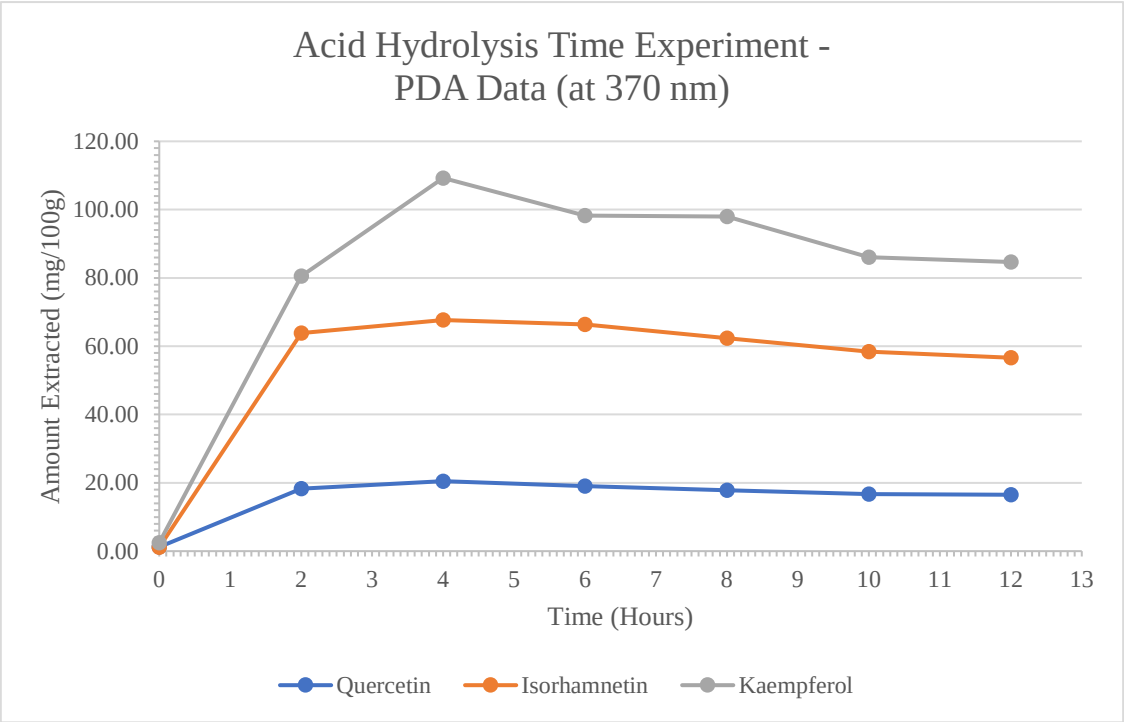


Figure 4.19 Quercetin, isorhamnetin, and kaempferol levels in an acid hydrolysate bok choy extract after 0, 2, 4, 6, 8, 10, and 12 hours. PDA data (at 370 nm).

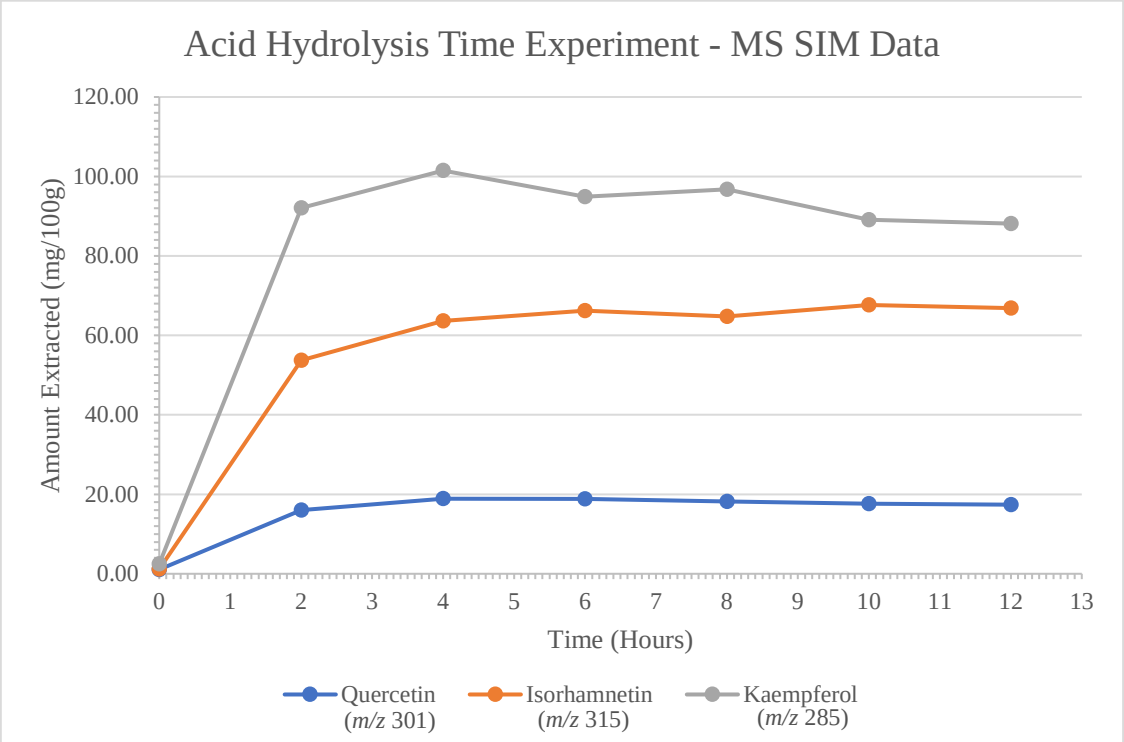


Figure 4.20 Quercetin, isorhamnetin, and kaempferol levels in an acid hydrolysate bok choy extract after 0, 2, 4, 6, 8, 10, and 12 hours. MS SIM data.

Table 4.2 Quercetin (Q), isorhamnetin (I), and kaempferol (K) levels in an acid hydrolysate bok choy extract after 0, 2, 4, 6, 8, 10, and 12 hours of hydrolysis. PDA and MS SIM data.

	PDA Data (at 370 nm) (mg/100 g)			MS SIM Data (mg/100 g)		
Hours	**Q**	**I**	**K**	**Q (*m/z* 301)**	**I (*m/z* 315)**	**K (*m/z* 285)**
0	0.00	0.00	0.00	0.00	0.00	0.00
2	18.3	63.88	80.6	16.0	53.7	92.1
4	20.5	67.7	109.3	18.9	63.8	101.5
6	19.1	66.4	98.3	18.9	66.2	94.9
8	17.9	62.4	97.9	18.2	64.8	96.7
10	16.7	58.4	8.0	17.6	67.6	89.1
12	16.5	56.7	84.7	17.4	66.9	88.1

4.5 SYRINGE FILTER STUDY FOR FILTRATION OF BOK CHOY SAMPLES PRIOR TO CHROMATOGRAPHIC ANALYSIS

The acid hydrolysate, alkaline hydrolysate and hydroalcoholic methanol/water extracts of bok choy were prepared as described in 3.4.2, 3.4.3, and 3.4.4 respectively, and a sample of each extract was filtered through a HPLC syringe filter prior to HPLC-PDA/ESI-MS^n analysis. There were three different syringe filter discs available in the laboratory: nylon, polytetrafluoroethylene (PTFE), and cellulose acetate, and the affinity of the flavonol compounds present in all three bok choy extracts for each of the filter discs was examined. Figure 4.21 shows the HPLC-PDA chromatograms for each syringe filter for the acid hydrolysate extract. As can be seen there is no difference in absorbance of the flavonol aglycone peaks using either PTFE or cellulose acetate, but there is a marked reduction in absorbance (approximately 60% µAU) when the nylon filter disc was used. These findings indicated that the flavonol aglycones adsorbed to the nylon disc and therefore nylon should not be used to filter acid hydrolysate extracts prior to chromatography. Accordingly, PTFE was used to filter the acid hydrolysate prior to HPLC-PDA/ESI-MS^n analysis of flavonol aglycones.

Figures 4.22 and 4.23 show the HPLC-PDA chromatograms for each syringe filter for the alkaline hydrolysate and hydroalcoholic methanol/water bok choy extracts respectively. As demonstrated in the chromatograms, there is no difference in absorbance for both the flavonol glycosides in the alkaline hydrolysate, and the flavonol glycoside-hydroxycinnamic acid derivatives in the hydroalcoholic extract, using either one of the three filters. In this work nylon was used to filter the alkaline hydrolysates and hydroalcoholic bok choy extracts prior to HPLC-PDA/ESI-MS^n analysis of flavonol glycosides and flavonol glycoside-hydroxycinnamic acid derivatives.

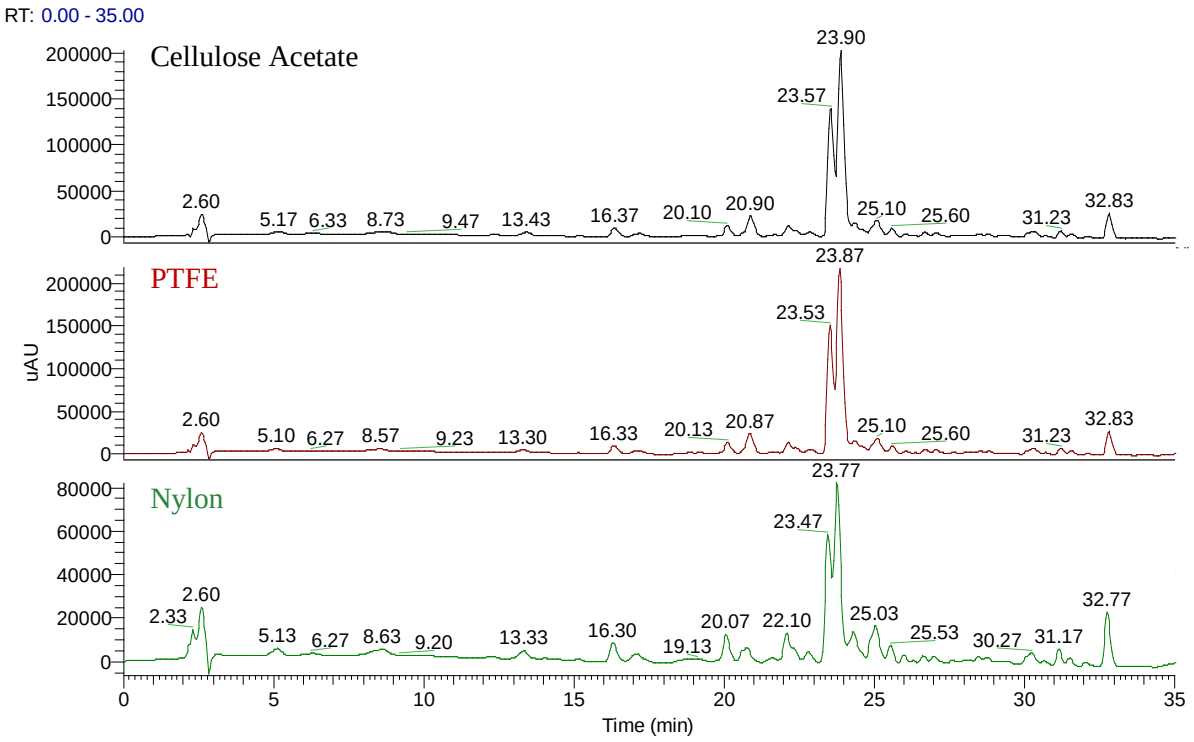


Figure 4.21 HPLC-PDA chromatograms of an acid hydrolysate bok choy extract filtered through a cellulose acetate syringe, a PTFE syringe, and a nylon syringe filter.

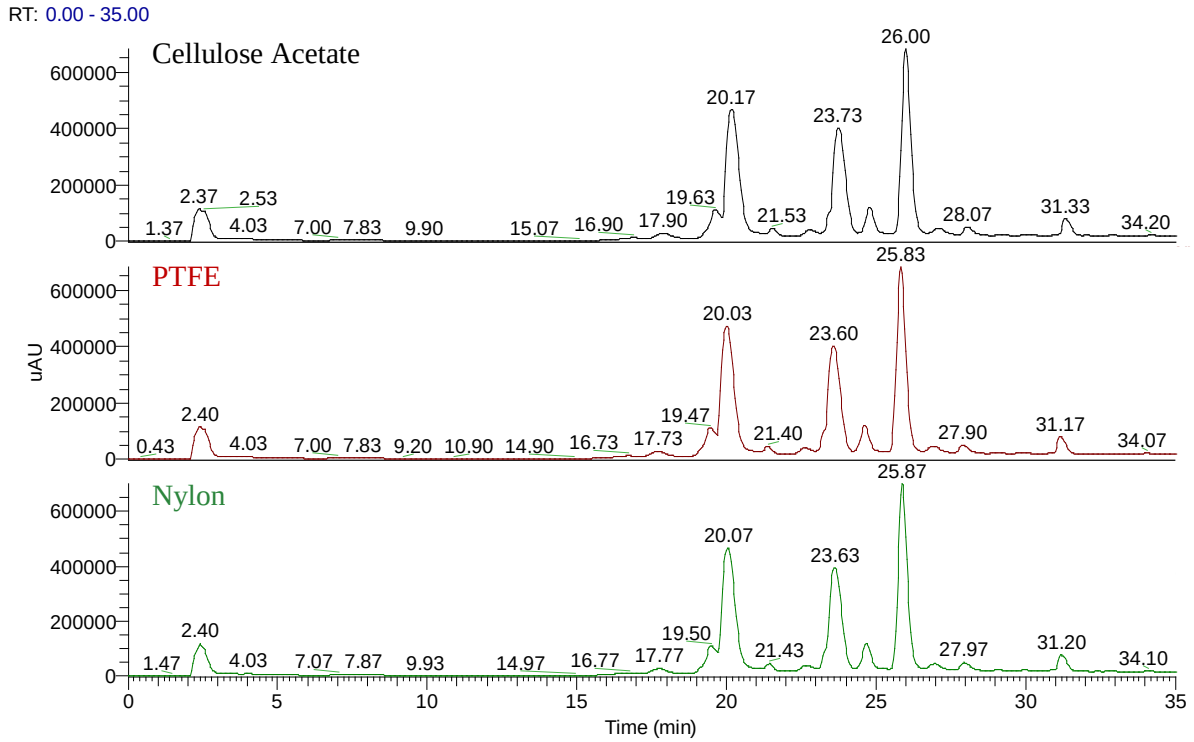


Figure 4.22 HPLC-PDA chromatograms of an alkaline hydrolysate bok choy extract filtered through a cellulose acetate, PTFE, and nylon syringe filter prior to analysis.

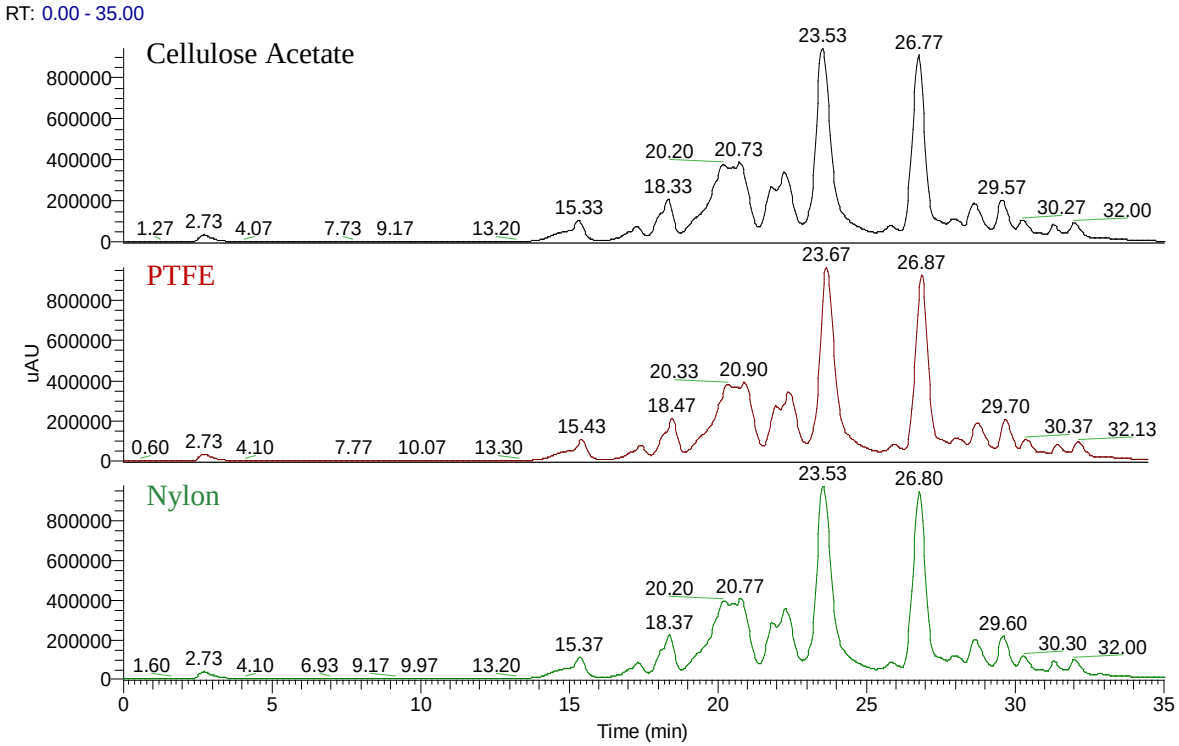


Figure 4.23 HPLC-PDA chromatograms of a hydroalcoholic bok choy extract filtered through a cellulose acetate, PTFE, and nylon syringe filter prior to analysis.

4.6 OPTIMISATION OF CELL CULTURE CONDITIONS

A number of parameters were required to be optimised for the HT-29 cell culture model. This included the passage dilution factor to be utilised throughout the study for routine maintenance of the cells, in addition to the cell density and time range to be used for the MTT proliferation assays on flavonol compounds, selected fractions, and crude bok choy extracts.

4.6.1 Passage Dilution Optimisation

In order to determine the optimum dilution factor required for the maintenance and passage of HT-29 cell cultures, a dilution series was performed. Cells were trypsinised from a T-25 flask and several dilutions of the cell suspension with complete media were made, including a 1:5, 1:10, 1:20, 1:50, and 1:100 dilution. Each dilution was made up in a T-25 flask and monitored regularly over a period of seven days. A passage of approximately 70% confluence within the seven days was preferable and at the conclusion of the experiment the 1 in 10 dilution was chosen for the purposes of this study. Figure 4.24 shows the photographs from the inverted microscope for the 1 in 10 dilution, with the progression of confluence demonstrated at key points over the seven days. As can be seen, approximately 70% confluency was reached on day three for a 1 in 10 dilution of cells, and this was the dilution factor chosen to passage cells in between conducting the MTT proliferation assays.

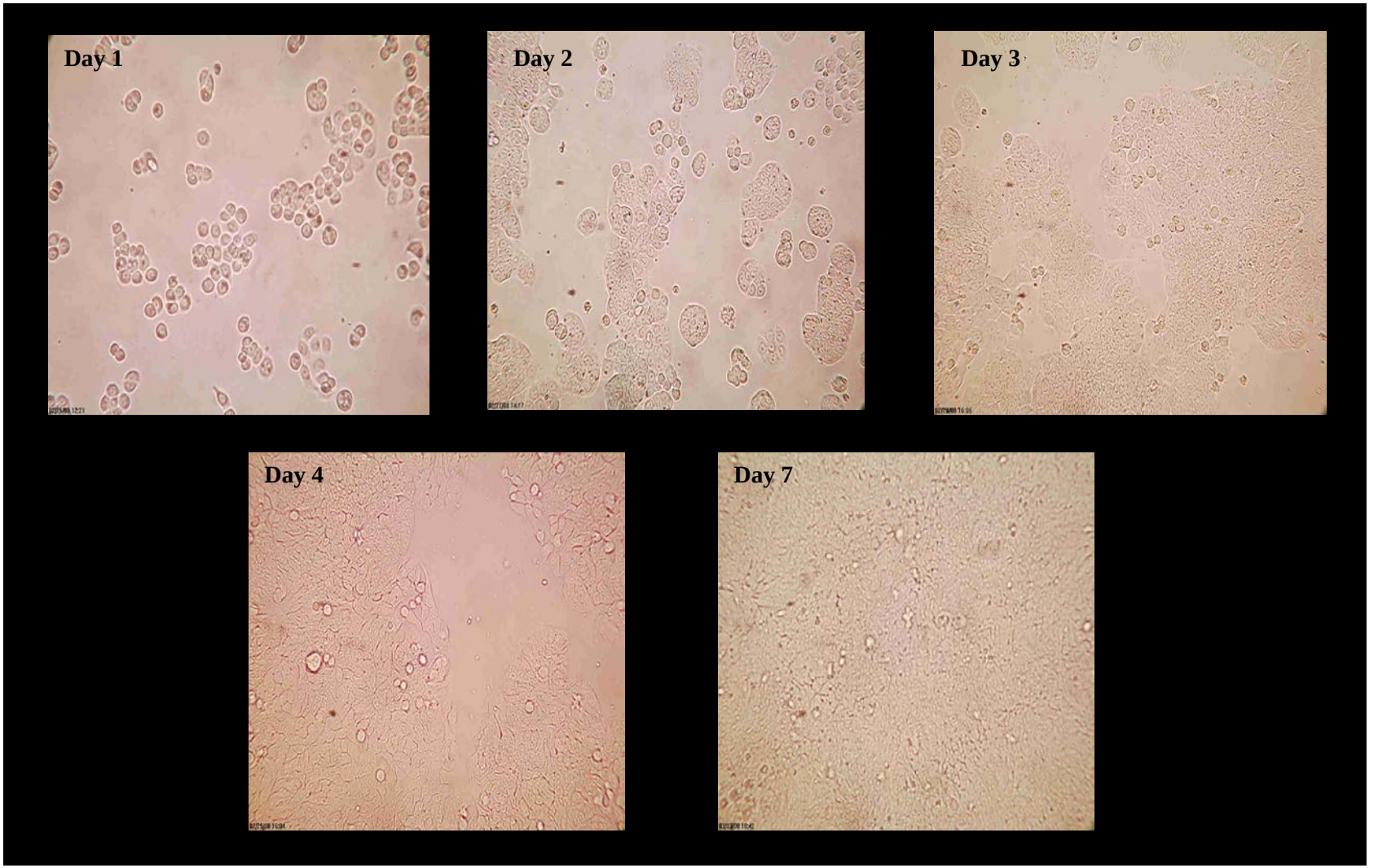


Figure 4.24 Inverted microscope photographs showing progression of confluence of a 1 in 10 dilution of HT-29 cells over 7 days.

4.6.2 Optimisation of Cell Density and Time Parameters

As part of the optimisation of cell culture conditions, it was also important to determine the optimum cell density to be seeded in the 96-well plates used for the MTT proliferation assays, as well as the time range that the assay would be conducted over. In order to determine these two parameters, a growth curve of the HT-29 cells was established (Figure 4.25). A range of concentrations of cells were made up and transposed to the number of cells per well, referred to as cell density, and cell viability was monitored over 192 hours using the MTT assay. The preparation of the range of cell densities is outlined in 3.11.2 and the MTT assay methodology is detailed in 3.11.5. The primary aim of this preliminary experiment/method optimisation was to ensure that the cells would have passed their initial lag phase and be in their exponential growth phase during the subsequent MTT proliferation assays on bok choy extracts, selected fractions, and flavonol compounds. It was observed that a cell density of 1,000 cells/well or lower had a long lag phase and required far too long to reach their growth phase, with the lowest density of 100 cells/well not even reaching a growth phase over the 192 hour time period. It was also observed that the two highest densities of 50,000 and 100,000 cells/well reached their exponential growth phase quite quickly and begun to die after 72 hours. This left the densities of 5,000 and 10,000 cells/well to be considered. The cell density of 10,000 cells/well was chosen for this study as the exponential growth phase of the cells at this density was between 24 and 96 hours, making it ideal for the MTT assays. Therefore, the growth curve provided a basis for the assays performed on the flavonol compounds, fractions, and bok choy extracts, in which the HT-29 cells were seeded at a density of 10,000 cells/well. Initially, for each group of flavonol compounds tested, as well as the collected fraction and bok choy extracts, an experiment was performed for one of the compounds/extracts over a 96 hour time period. The MTT proliferation assay was conducted at the 24, 48, 72, and 96 hour time points to observe if any antiproliferative effects were time dependent. After this initial experiment, a MTT proliferation assay for the remainder of the flavonol compounds within each group, and the remainder of the bok choy extracts, was performed at the 72 hour time point only. Chapter VI presents and discusses the results of these experiments.

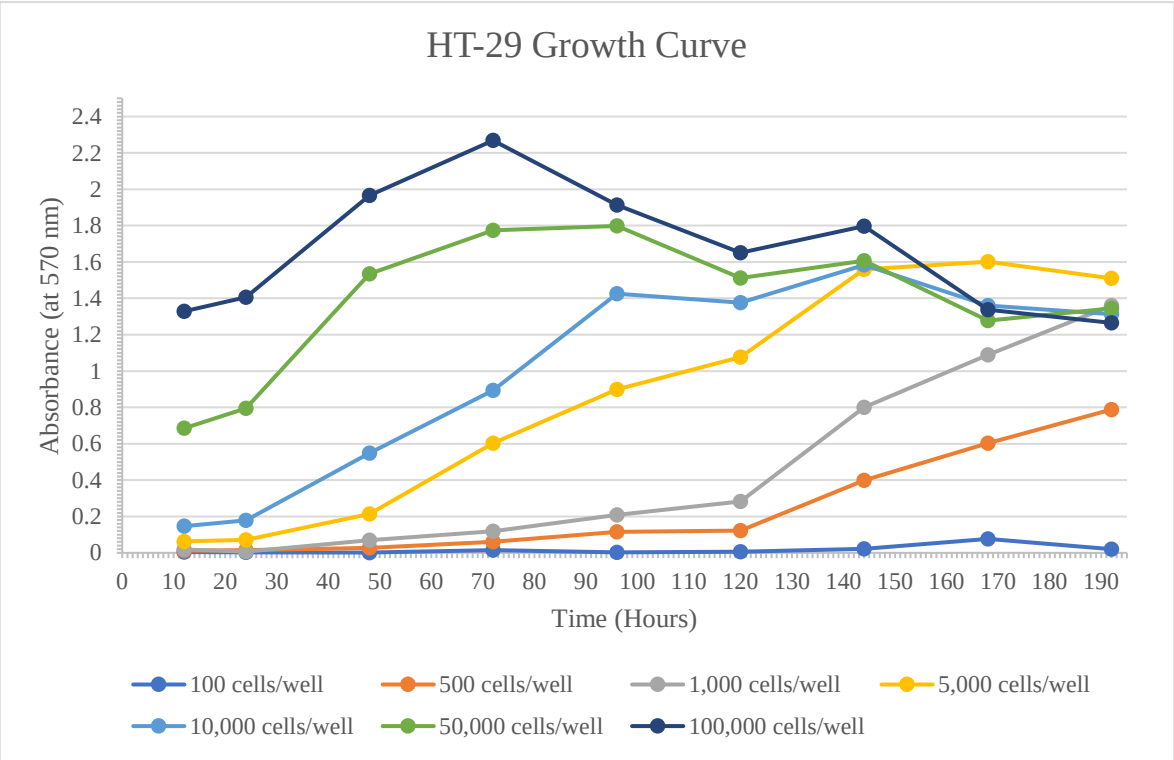


Figure 4.25 HT-29 growth curve over 8 days (192 hours) using MTT assay to assess cell viability.

4.7 CONCLUSION

This chapter presented and discussed the findings from several preliminary experiments and method optimisation studies that were required in the lead up to the primary experimental work conducted as part of this study. This included the optimisation of several parameters for HPLC-PDA/ESI-MS^n analysis, such as the HPLC column to be used, the composition of the mobile phase and the gradient program for each of the three bok choy extracts (i.e. hydroalcoholic, alkaline hydrolysate, and acid hydrolysate), as well as the optimum PDA detector wavelength for each extract, and the ESI-MS^n ionisation conditions.

The HPLC column chosen for this study was the Varian Polaris C18-A; 150 x 2.0 mm 3 µm as it gave the best resolution and peak shape of the major flavonol glycosides present in a hydroalcoholic onion extract. The best separation of the flavonol aglycones present in the acid hydrolysate bok choy extracts was achieved using a acetonitrile/water + 0.1% v/v formic acid mobile phase with the following gradient: 90/10 A/B – 10/90 A/B (0-30 minutes), 10/90 A/B (30-35 minutes), 10/90 A/B – 90/10 A/B (35-36 minutes), 90/10 A/B (36-46 minutes). A superior separation of the flavonol glycosides and the flavonol glycoside-hydroxycinnamic acid derivatives found within the alkaline hydrolysate and hydroalcoholic extracts respectively, was

achieved using a methanol/water + 0.1% v/v formic acid mobile phase with the following gradient: 95/5 A/B – 5/95 A/B (0-40 minutes), 5/95 A/B (40-45 minutes), 5/95 A/B – 95/5 A/B (45-46 minutes), 95/5 A/B (46-60 minutes). Therefore, the same conditions were used for both the alkaline hydrolysate and hydroalcoholic extracts, whereas the acid hydrolysate was analysed using a different mobile phase composition and gradient program.

The optimum PDA detector wavelength was determined to be 370 nm for flavonol aglycones in the acid hydrolysate extracts, 350 nm for flavonol glycosides in the alkaline hydrolysate extracts and 330 nm for the flavonol glycoside-hydroxycinnamic acid derivatives found in the hydroalcoholic extracts. It was observed that the broad absorbance maximum that arises from the absorption of the flavonol B-ring, referred to as band I, shifts approximately 20 nm lower with the addition of a glucose moiety to the flavonol aglycone, and a further 20 nm with the addition of a acyl moiety. This is known as a hypsochromic shift.

Analysis of the flavonol compounds present in the different bok choy extracts via ESI-MS^n was conducted in the negative ionisation mode using formic acid as the mobile phase additive. The MS was tuned using rutin, a similar flavonol compound to those analysed in the bok choy. The capillary temperature and capillary voltage were set to 350 ºC and -48.50 V respectively, which gave the optimal response for the tune compound.

The preparative HPLC conditions also required optimisation. The flow rate and injection volume required to be scaled up from the HPLC-PDA/ESI-MS^n method, and the mobile phase gradient program needed to be adjusted. The flow rate was scaled up from 0.2 mL/min to 10 mL/min for the preparative HPLC and the injection volume was scaled up from 5 µL to 1000 µL. The mobile phase composition was kept the same as the analytical HPLC method, however, the gradient was extended as this produced a better separation. The optimised gradient was as follows: 95/5 A/B – 5/95 A/B (0-60 minutes), 5/95 A/B (60-65 minutes), 5/95 A/B – 95/5 A/B (65-66 minutes), 95/5 A/B (66-76 minutes).

Other sample preparation/extraction parameters that were optimised included the hydrolysis time for the acid hydrolysis extraction method, as well as the syringe filter type used to filter each extract prior to chromatographic analysis. The optimum hydrolysis time was determined to be 4 hours for the acid hydrolysis method. Nylon filter discs were used for both the alkaline hydrolysate and hydroalcoholic bok choy extracts prior to analysis, however, PTFE filter discs were used for the flavonol aglycones in the acid hydrolysate extracts as it was observed that the flavonol aglycones adsorbed onto the nylon discs.

The passage dilution factor for routine maintenance of the HT-29 cell culture model, and the cell density and time range to be used for the MTT proliferation assays were also optimised. A 1:10 dilution of HT-29 cells was the ideal dilution factor to reach 70% confluency of cells in three days and was therefore used to passage cells in between the MTT proliferation assays. A cell density of 10,000 cells/well was chosen for the assays as the exponential growth phase of the cells was within an ideal timeframe at this density. Initial MTT proliferation assays were conducted between 24-96 hours, after which all subsequent assays were conducted at the 72 hour time point.

After completion of all preliminary and optimisation studies, the methods were employed for the primary experimental work, and all results from this work are presented and discussed in the following two chapters.

CHAPTER V
IDENTIFICATION AND QUANTIFICATION OF FLAVONOLS IN BOK CHOY CULTIVARS

5.1 INTRODUCTION

This chapter reports and discusses the results obtained from the HPLC-PDA/ESI-MS^n study of flavonol compounds present in three hardy varieties of bok choy ('Sumo,' 'Karate,' and 'Miyako') produced for the local market in Victoria, Australia. Information on these varieties, including growing conditions and preparation for analysis is given in 3.2 and 3.4.

The key flavonols of interest in this study include quercetin, isorhamnetin and kaempferol, their glycosylated derivatives, and other more complex conjugates that occur naturally within the bok choy plant, such as flavonol glycoside-hydroxycinnamic acid derivatives. This chapter is divided into the following sections:

1. Identification and quantification of the flavonol aglycones obtained after acid hydrolysis of freeze-dried, powdered bok choy (3.4.2), including verification of the method used.
2. Identification and structure elucidation of flavonol glycosides obtained after alkaline hydrolysis of freeze-dried, powdered bok choy (3.4.3).
3. Identification and structure elucidation of flavonol glycoside-hydroxycinnamic acid conjugates and other naturally occurring flavonol compounds obtained by hydroalcoholic methanol/water extraction of freeze dried, powdered bok choy (3.4.4).

5.2 QUANTIFICATION OF FLAVONOL AGLYCONES

This section discusses the identification and quantification of the aglycones quercetin, isorhamnetin, and kaempferol in three different cultivars of bok choy by HPLC-PDA/ESI-MS^n after acid hydrolysis, as well as the verification of the method employed. Cultivar information, growing conditions, sample preparation, and acid hydrolysis conditions are detailed in 3.2 and 3.4.2. Optimisation of HPLC-PDA/ESI-MS^n conditions are described in 4.2.

5.2.1 Identification of Flavonol Aglycones

Quercetin, isorhamnetin, and kaempferol standards were commercially available and were used to confirm the identity of the compounds in the bok choy acid hydrolysate extracts through retention time, UV-Vis spectra, and ESI-MS negative ion matching. The structures of the three compounds are shown in Figure 5.1. The molecular weights of the aglycones and the *m/z* for the MS^1 negative ions are shown in Table 5.1.

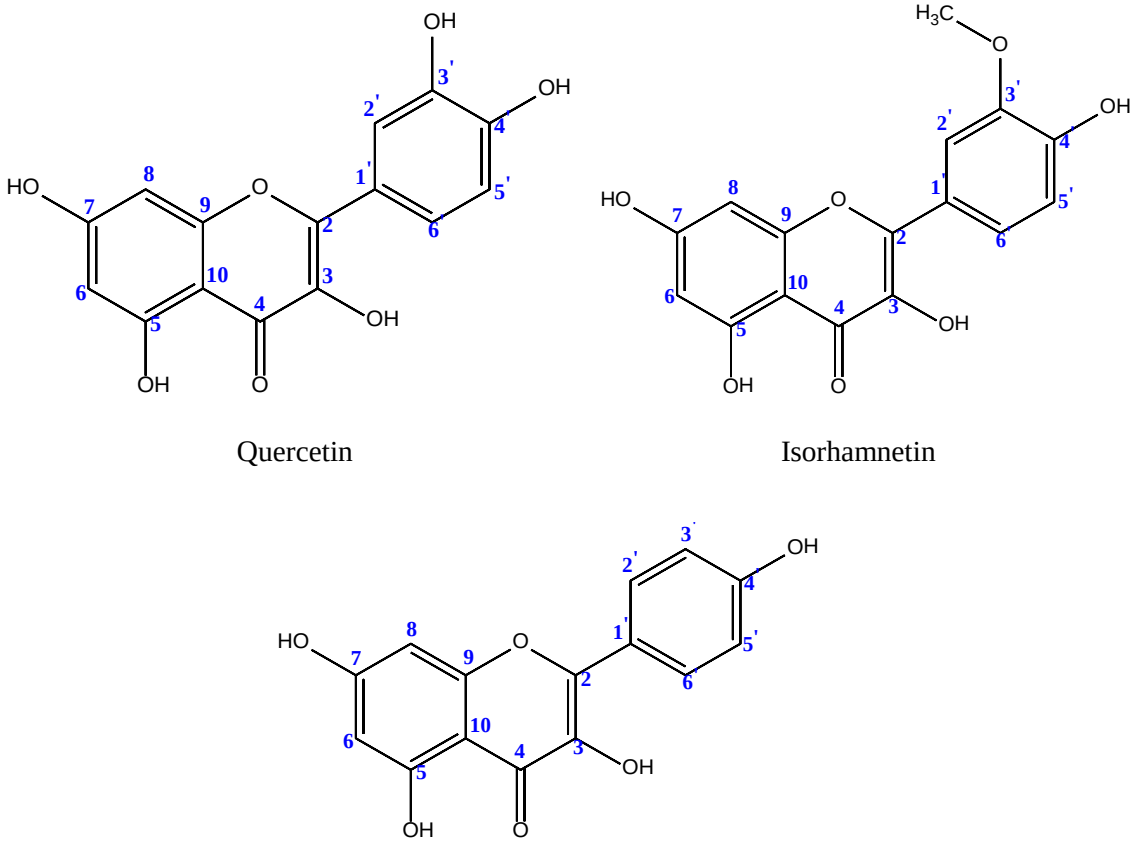


Figure 5.1 Chemical structures of quercetin, isorhamnetin and kaempferol.

Table 5.1 Molecular weights and MS^1 negative ions ($[M-H]^-$) of quercetin, isorhamnetin, and kaempferol.

Flavonol	Molecular Weight (g/mol)	$[M-H]^-$ (*m/z*)
Quercetin	302.24	301
Isorhamnetin	316.26	315
Kaempferol	286.24	285

The HPLC-PDA chromatograms, recorded at 370 nm, of the Sumo, Karate, and Miyako bok choy acid hydrolysate extracts are shown in Figure 5.2. The five peaks of interest are labelled 1-5 and the retention times for these peaks are shown in Table 5.2. Similar chromatograms were obtained for each cultivar.

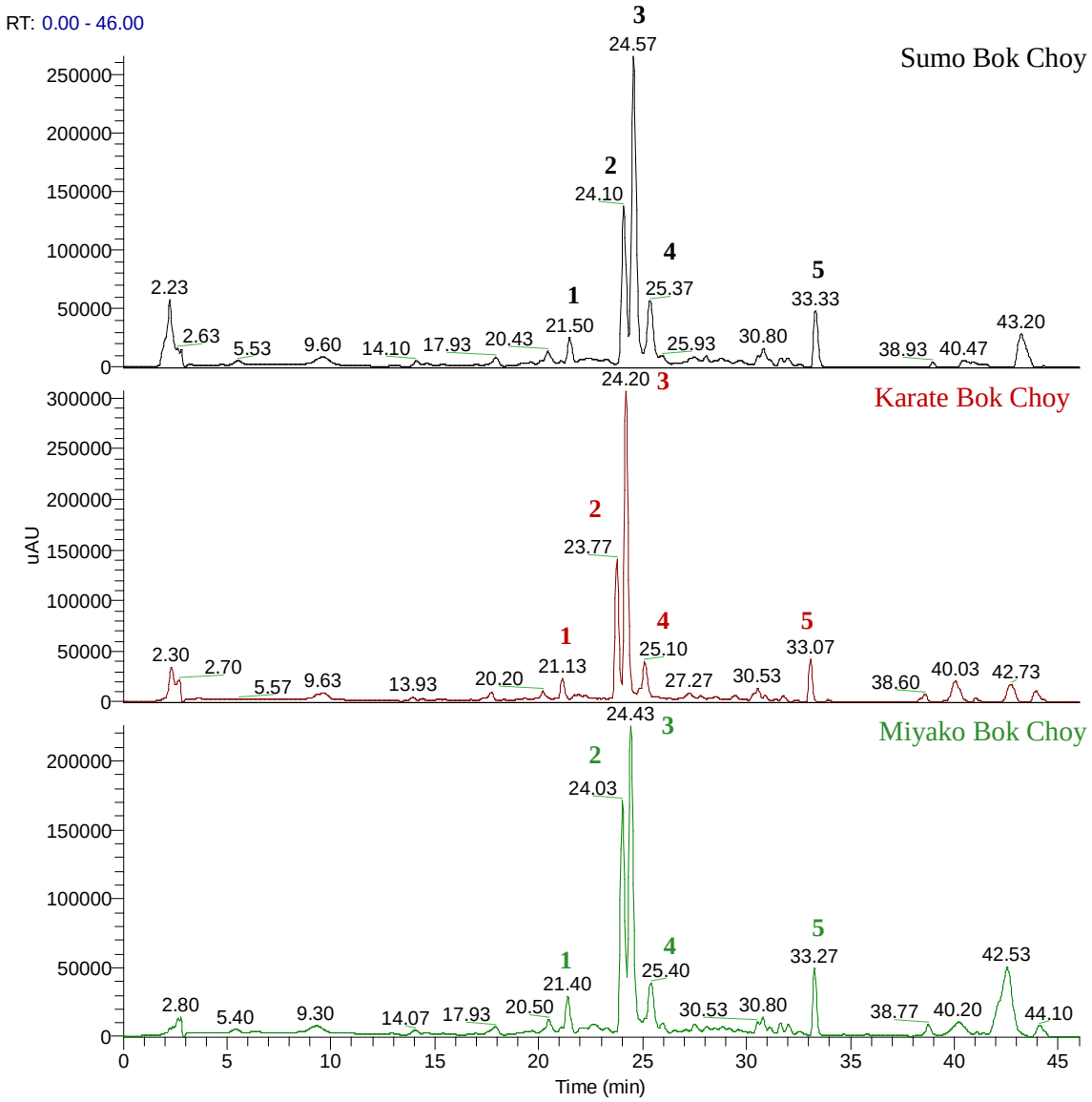


Figure 5.2 HPLC-PDA chromatograms, recorded at 370 nm, of the acid hydrolysate extracts of all three bok choy cultivars: Sumo, Karate, and Miyako. The five peaks of interest are labelled 1-5.

Table 5.2 HPLC-PDA retention times (t_R) of the five peaks of interest in the acid hydrolysate extracts of all three cultivars of bok choy.

Bok Choy Cultivar	Peak 1 t_R (min)	Peak 2 t_R (min)	Peak 3 t_R (min)	Peak 4 t_R (min)	Peak 5 t_R (min)
Sumo	21.50	24.10	24.57	25.37	33.33
Karate	21.13	23.77	24.20	25.10	33.07
Miyako	21.40	24.03	24.43	25.40	33.27

The HPLC-PDA chromatograms of the quercetin, isorhamnetin, and kaempferol standards are shown in Appendix C and based on retention times, peaks 1, 2, and 3 in the bok choy acid hydrolysate extracts were identified as quercetin, isorhamnetin, and kaempferol respectively.

The UV-Vis spectra for the three standards were compared with the UV-Vis spectra of peaks 1, 2, and 3 in each of the bok choy acid hydrolysate extracts for further confirmation of the identity of the peaks (Figure 5.3). The red spectra represent the standards and the blue, green, and orange spectra represent the matching peak in the Sumo, Karate, and Miyako bok choy cultivars respectively (the same solvent was used to make up all sample and standard solutions). All three display a good match, except for the extra absorbance bands observed at 310 nm and 292 nm for peaks 1 and 2 respectively. These may be due to other co-eluting species in the acid hydrolysate bok choy extracts (assuming there were no impurities in the solvent), which could potentially affect the quantification data obtained from the PDA detector.

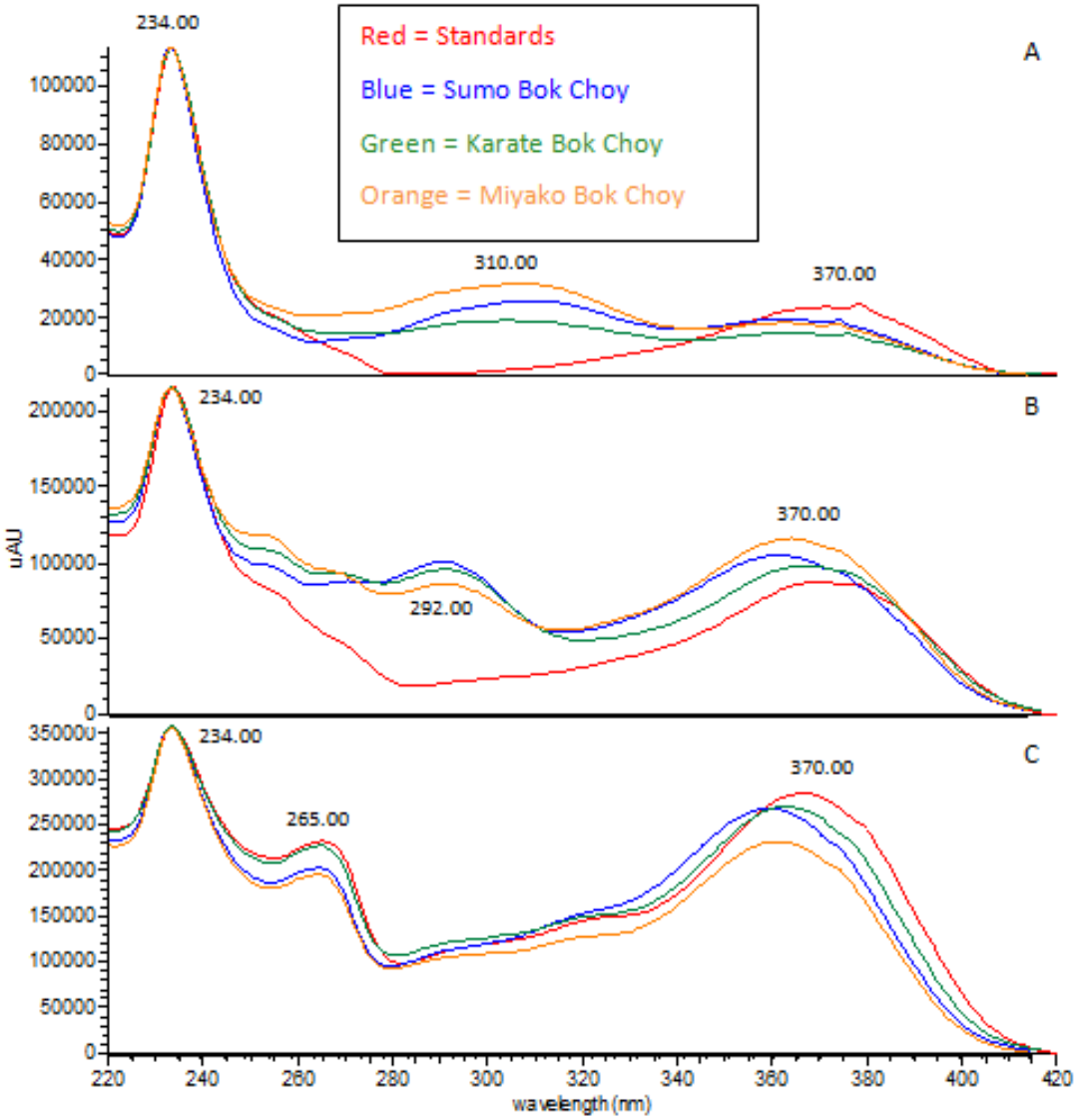


Figure 5.3 UV-Vis spectra (220 to 420 nm) of quercetin standard and peak 1 from each bok choy acid hydrolysate extract (A), isorhamnetin standard and peak 2 from each bok choy acid hydrolysate extract (B), and kaempferol standard and peak 3 from each bok choy acid hydrolysate extract (C).

The HPLC-ESI-MS total ion chromatograms (TIC) for the Sumo, Karate, and Miyako bok choy acid hydrolysate extracts are shown in Figure 5.4. The five peaks of interest are labelled 1-5 on the chromatograms and their corresponding retention times are given in Table 5.3. The three cultivars gave similar chromatograms displaying the same five peaks, with slight variation in peak abundances.

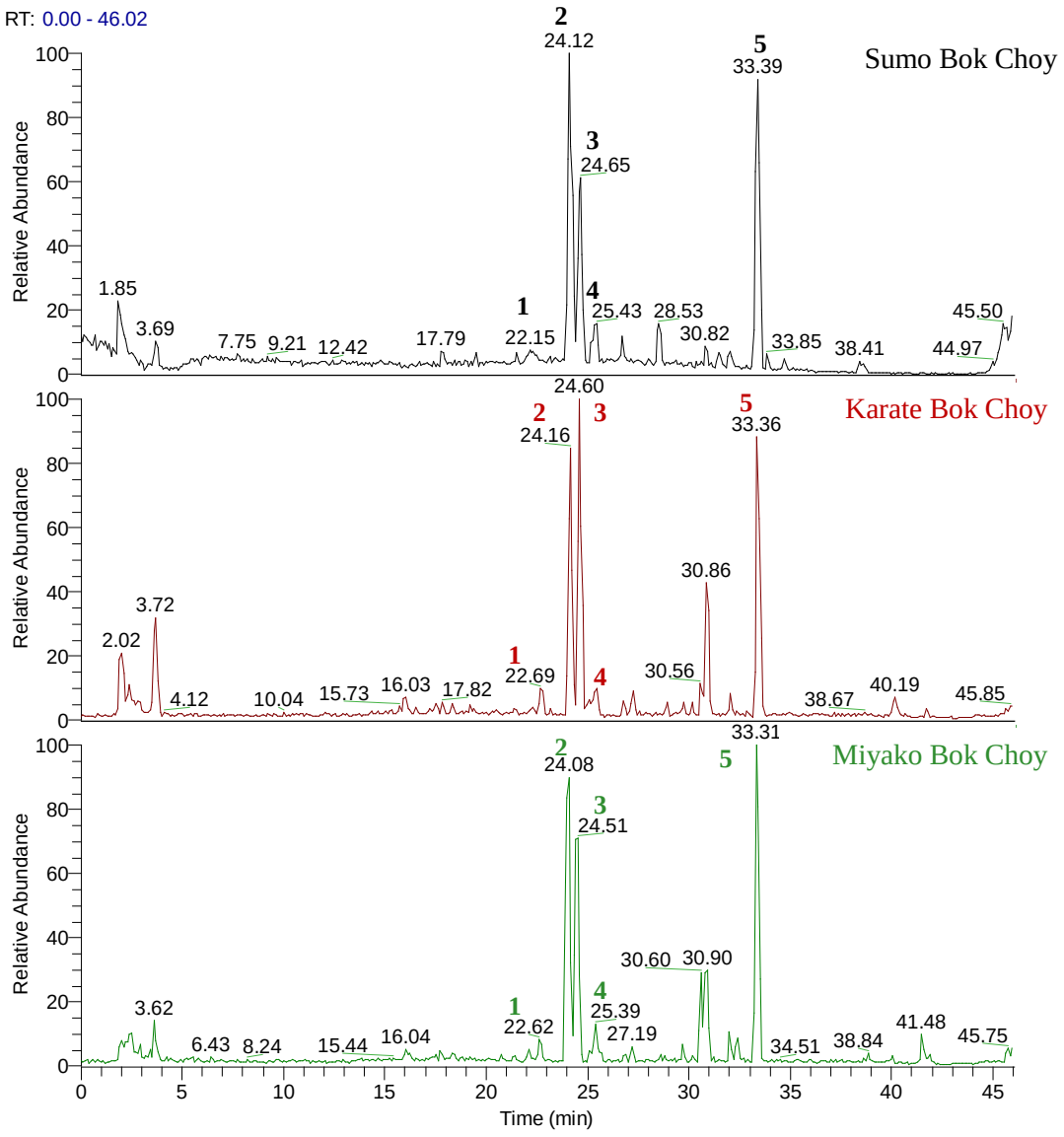


Figure 5.4 HPLC-ESI-MS TIC (scanning mass range 120-2000 amu) of the acid hydrolysate extracts of the three bok choy cultivars: Sumo, Karate, and Miyako. The five peaks of interest are labelled 1-5.

Table 5.3 HPLC-MS TIC retention times (t_R) of the five peaks of interest in the acid hydrolysate extracts of all three cultivars of bok choy.

Bok choy cultivar	Peak 1 t_R (min)	Peak 2 t_R (min)	Peak 3 t_R (min)	Peak 4 t_R (min)	Peak 5 t_R (min)
Sumo	22.15	24.12	24.65	25.43	33.39
Karate	22.69	24.16	24.60	25.27	33.36
Miyako	22.62	24.51	24.51	25.39	33.31

The negative ion MS^1 spectra for peaks 1-3 in the acid hydrolysate extracts for the Sumo, Karate, and Miyako bok choy cultivars are presented in Figures 5.5-5.7.

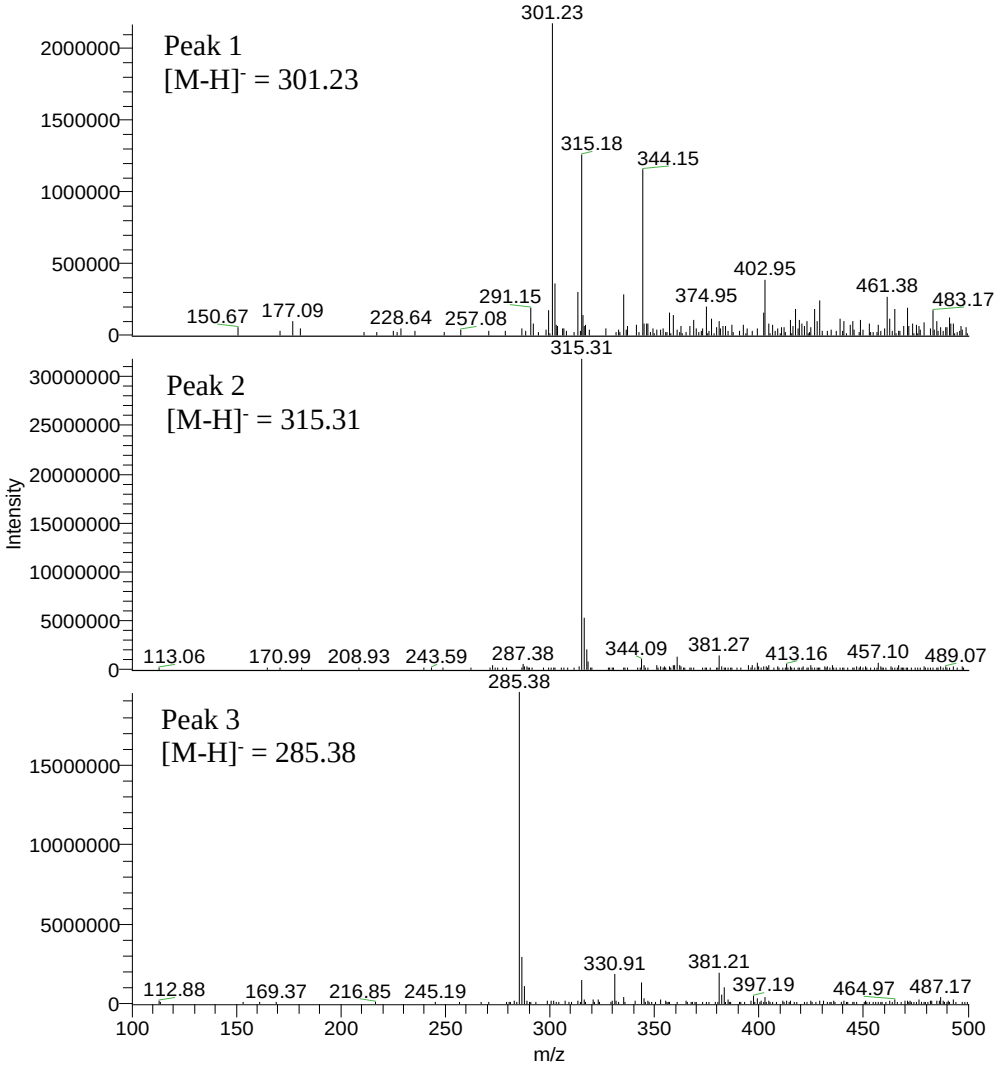


Figure 5.5 ESI-MS^1 spectra in negative ion mode of peaks 1, 2, and 3 in the acid hydrolysate extract of the Sumo bok choy cultivar.

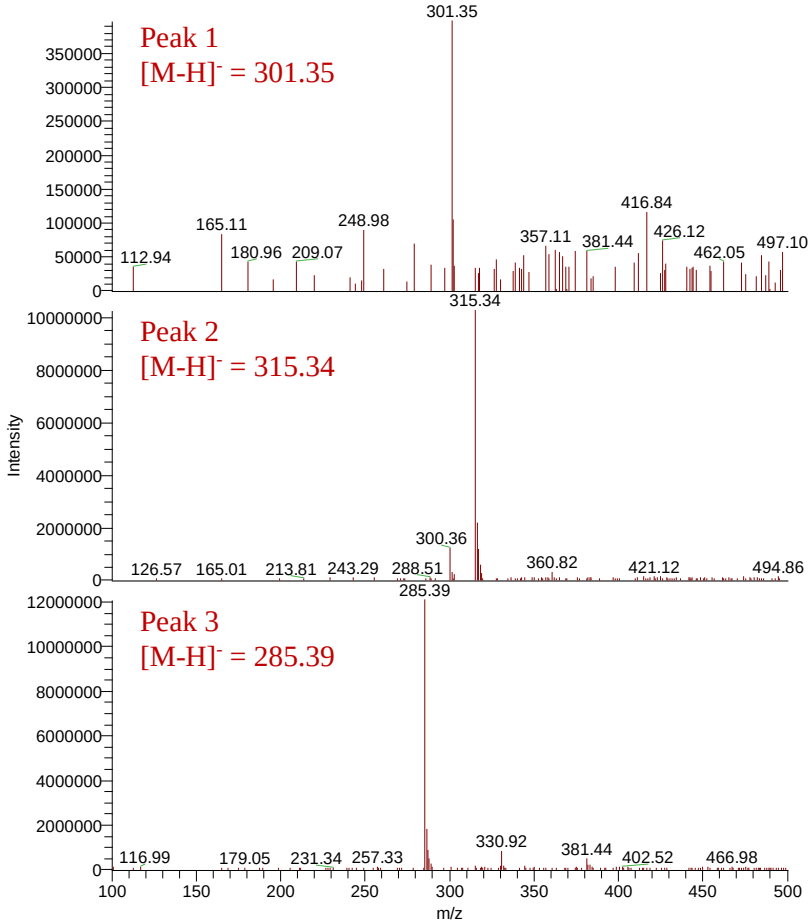


Figure 5.7 ESI-MS[1] spectra in negative ion mode of peaks 1, 2, and 3 in the acid hydrolysate extract of the Karate bok choy cultivar.

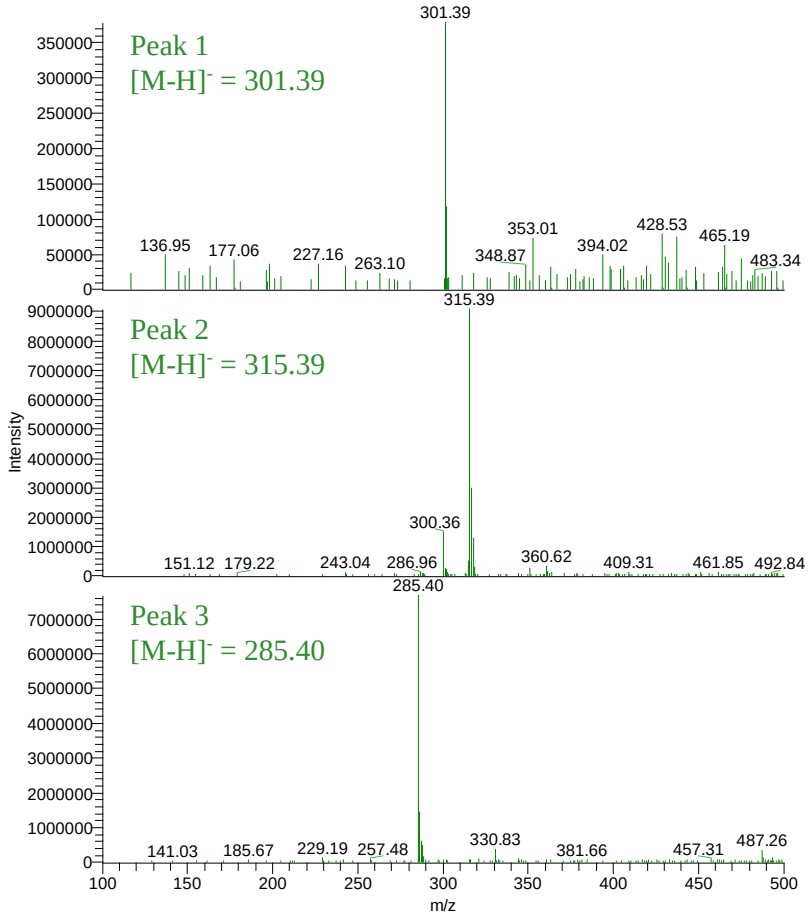


Figure 5.6 ESI-MS[1] spectra in negative ion mode of peaks 1, 2, and 3 in the acid hydrolysate extract of the Miyako bok choy cultivar.

Additional confirmation of the identity of peaks 1, 2, and 3 in the acid hydrolysate extracts of the bok choy samples was provided by the ESI-MS via retention time matching in the TIC's and negative ion MS^1 spectra matching with aglycone standards. The HPLC-MS TIC's of the quercetin, isorhamnetin, and kaempferol standards, along with their corresponding negative ion MS^1 spectra, are shown in Appendix C.

The following two tables provide a summary of the positive confirmation of the first 3 peaks in the acid hydrolysate extracts. The HPLC-PDA retention time and UV-Visible spectral data are presented in Table 5.4 and the ESI-MS TIC retention time and $[M-H]^-$ data are summarised in Table 5.5.

Table 5.4 HPLC-PDA retention time (t_R) and UV-Vis spectral data of flavonol aglycone standard compounds and bok choy acid hydrolysate extracts.

Sample	Quercetin (Peak 1)		Isorhamnetin (Peak 2)		Kaempferol (Peak 3)	
	t_R (min)	UV-Vis (nm)	t_R (min)	UV-Vis (nm)	t_R (min)	UV-Vis (nm)
Standards	21.23	233,369	23.90	234,370	24.23	234,265,366
Sumo	21.50	233,310,369	24.10	234,292,366	24.57	234,265,363
Karate	21.13	234,306,367	23.77	234,292,369	24.20	234,265,365
Miyako	21.40	234,310,367	24.03	234,293,369	24.43	234,265,366

Table 5.5 HPLC-ESI-MS TIC retention time (t_R) and $[M-H]^-$ data of flavonol aglycone standard compounds and bok choy acid hydrolysate extracts.

Sample	Quercetin (Peak 1)		Isorhamnetin (Peak 2)		Kaempferol (Peak 3)	
	t_R (min)	$[M-H]^-$ (*m/z*)	t_R (min)	$[M-H]^-$ (*m/z*)	t_R (min)	$[M-H]^-$ (*m/z*)
Standards	21.34	300.92	23.99	314.92	24.19	284.99
Sumo	22.15	301.23	24.12	315.31	24.65	285.38
Karate	22.69	301.35	24.16	315.34	24.60	285.39
Miyako	22.62	301.39	24.08	315.39	24.51	285.40

The other two peaks present in the acid hydrolysate extracts of all three varieties of bok choy (peaks 4 and 5 in Figures 5.2 and 5.4) were not characterised. The UV-Vis spectra were similar to those recorded for the three identified flavonols, however, the negative ion MS^1 spectra were not typical of flavonols. The UV-Vis spectra and negative ion MS^1 spectra for peaks 4 and 5, using the Sumo bok choy cultivar as an example, are shown in Appendix D.

5.2.2 Method Verification

The HPLC-PDA/ESI-MS^n method developed for the separation and quantification of aglycones in the acid hydrolysate bok choy extracts was based on the method described by Rochfort *et al.* [60]. The method was verified for quantification through peak area and retention time reproducibility, detector linearity, and recovery data. Both PDA and ESI-MS data were used for quantification, therefore, the linearity of both detectors was assessed. PDA data were collected at 370 nm and ESI-MS data were acquired in selected ion monitoring (SIM) mode. ESI-MS acquisition parameters were established to collect four chromatograms simultaneously: a total ion chromatogram (TIC), and three separate SIM chromatograms, one for each flavonol (*m/z* 301, *m/z* 315, and *m/z* 285 for quercetin, isorhamnetin, and kaempferol respectively), however, only the SIM chromatograms were used for quantification purposes. Figure 5.8 shows the four chromatograms used for quantification, i.e. the PDA chromatogram at 370 nm and the three SIM chromatograms (*m/z* 301, *m/z* 315, and *m/z* 285), using the Sumo bok choy cultivar as an example. The HPLC-PDA chromatograms and HPLC-ESI-MS SIM chromatograms for the other two bok choy cultivars, Karate and Miyako, are displayed in Appendix E. An example of the chromatograms collected for the mixed standard solutions (containing quercetin, isorhamnetin, and kaempferol aglycones) used for quantification is shown in Figure 5.9 (for the 10 mg/L mixed standard). Refer to 3.5.2 for the preparation of standard solutions.

These chromatograms convey the power of the MS as a detector when analysing and quantifying known compounds, especially when complete separation of compounds cannot be achieved using HPLC-PDA alone. As can be seen in the HPLC-PDA chromatograms (Figures 5.8 and 5.9), and as previously mentioned in 4.2.4, the isorhamnetin and kaempferol peaks are not completely resolved. Thus, the integration of the peak area data, and hence the quantification of these compounds, may not be 100% accurate using the PDA. The MS SIM mode, however, allows for highly selective and specific separation, and hence provides a more accurate quantification of these compounds.

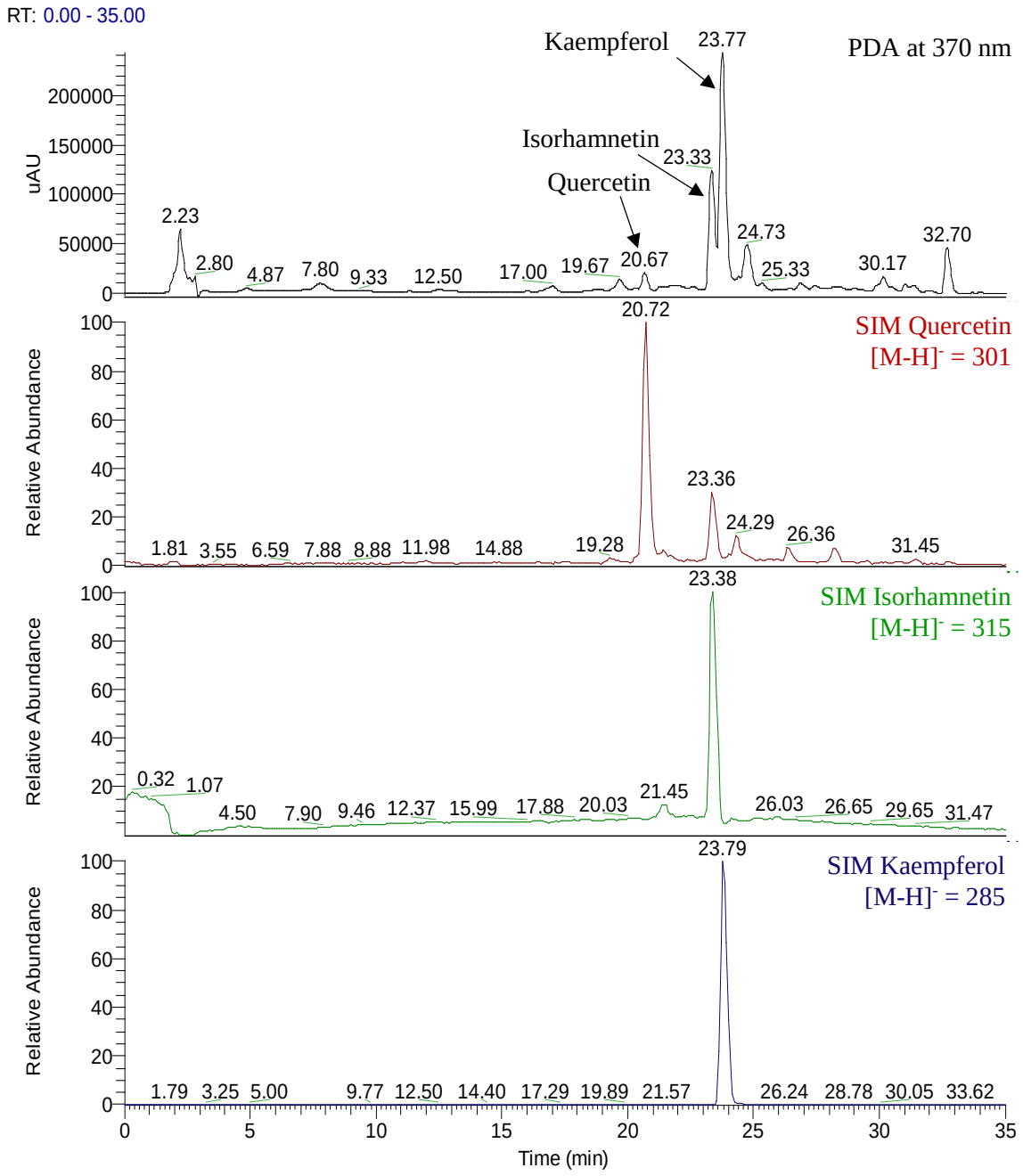


Figure 5.8 HPLC-PDA and HPLC-ESI-MS SIM chromatograms of a Sumo bok choy acid hydrolysate extract used for quantification.

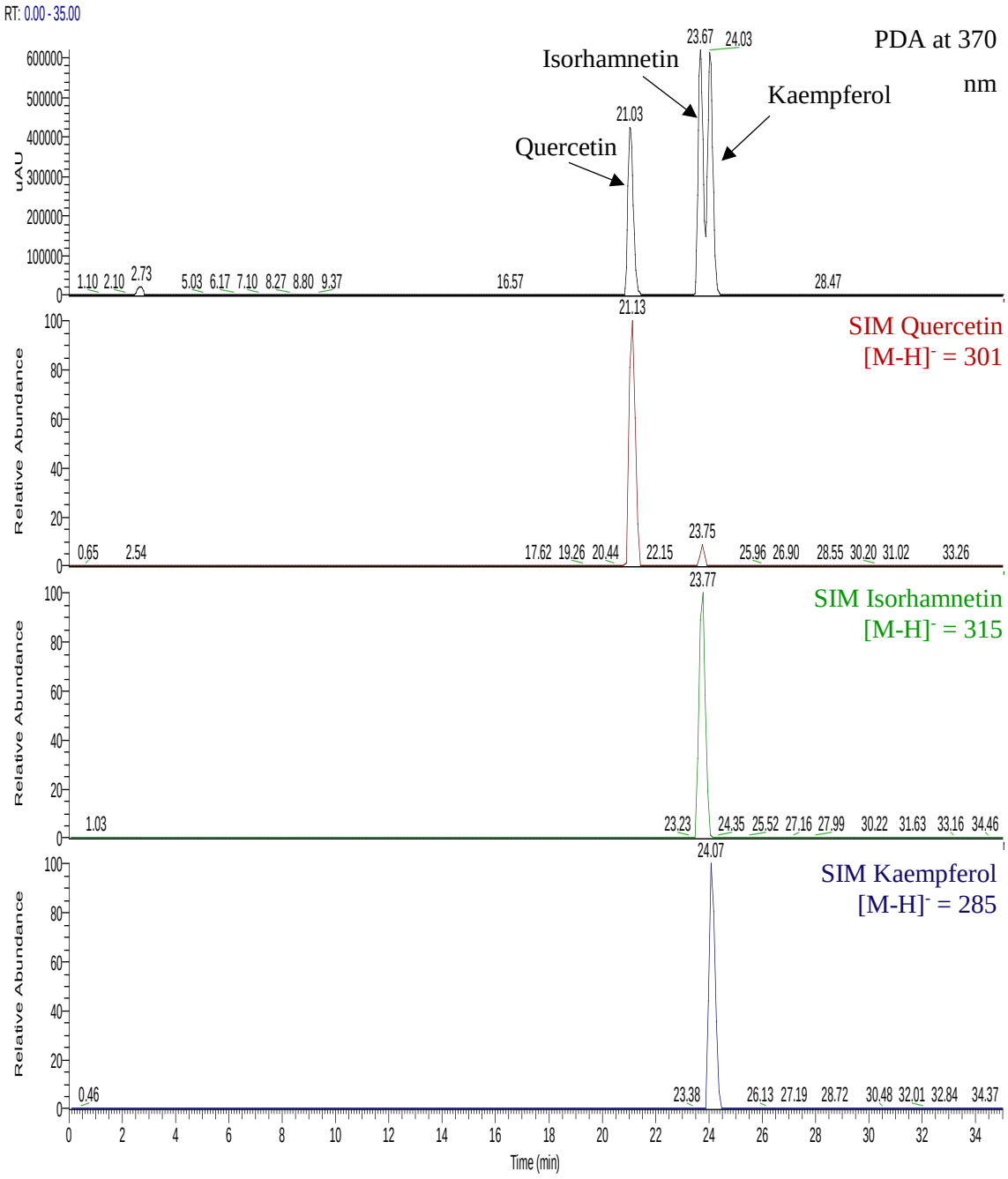


Figure 5.9 HPLC-PDA and HPLC-ESI-MS SIM chromatograms of the 10 mg/L mixed standard used for quantification.

5.2.2.1 Reproducibility Studies

Peak area and retention time reproducibility was assessed using both PDA and ESI-MS in the SIM mode using a standard solution containing 6 mg/L each of quercetin, isorhamnetin, and kaempferol, and a Sumo bok choy acid hydrolysate extract. The standard solution was prepared in 80/20 v/v methanol/water containing 0.1% v/v formic acid and gave detector responses similar to the flavonol aglycones in the Sumo bok choy extract. Peak area and retention time reproducibility data were recorded for 20 successive injections. The full set of data are tabulated in Appendix F. The isorhamnetin and kaempferol peaks were not fully resolved in the PDA chromatograms, and so the peak areas were obtained by manually splitting the peaks before integration. In contrast to the PDA chromatograms, the two compounds were completely resolved in the MS SIM chromatograms. The retention time and peak area % CV for each aglycone in the bok choy sample and standard solution are shown in Table 5.6. The HPLC-PDA/ESI-MS^n method shows very good retention time and peak area reproducibility for both PDA and MS SIM data collection. Peak area % CVs were more variable than the retention time % CVs, however, overall, the data was suitable for quantification of the flavonol aglycones.

Table 5.6 Summary of % CV for HPLC-PDA and HPLC-ESI-MS SIM retention time and peak area reproducibility data after 20 successive injections of a Sumo bok choy acid hydrolysate extract (Sample) and a 6 mg/L mixed standard containing quercetin, isorhamnetin and kaempferol (Std).

Flavonol	% CV Retention Time				% CV Peak Area			
	PDA		MS SIM		PDA		MS SIM	
	Std	Sample	Std	Sample	Std	Sample	Std	Sample
Quercetin	0.4	0.7	0.3	0.7	1.6	3.3	1.5	3.2
Isorhamnetin	0.4	0.4	0.4	0.4	1.8	2.1	2.1	4.4
Kaempferol	0.4	0.4	0.4	0.4	0.5	1.0	4.3	5.0

5.2.2.2 Detector Linearity

Detector linearity studies were conducted using mixed standard solutions of varying concentrations where the concentration range covered that expected for the aglycones in the bok choy extracts. The calibration plots for both the PDA (370 nm) and MS SIM data for quercetin, isorhamnetin and kaempferol are shown in Figures 5.10 – 5.15 (each data point is the average of 3 independent injections of 3 replicates) and calibration data is summarised in Table 5.7.

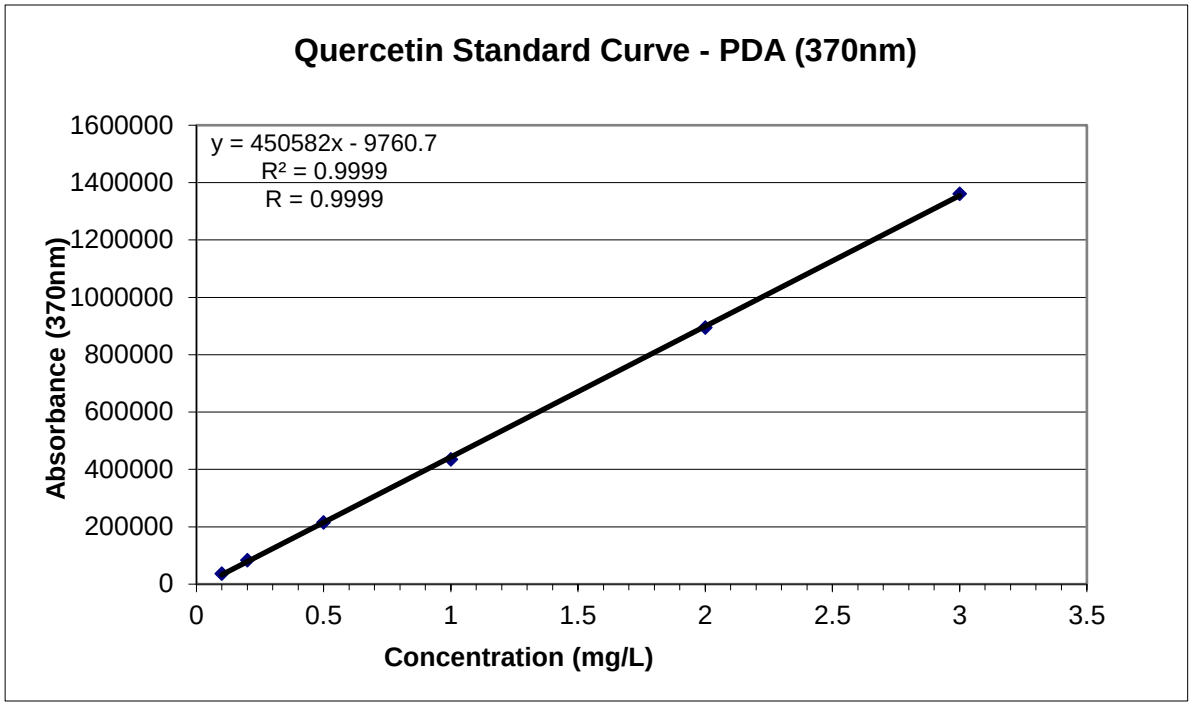


Figure 5.10 Calibration plot for quercetin using HPLC-PDA peak area recorded at 370 nm.

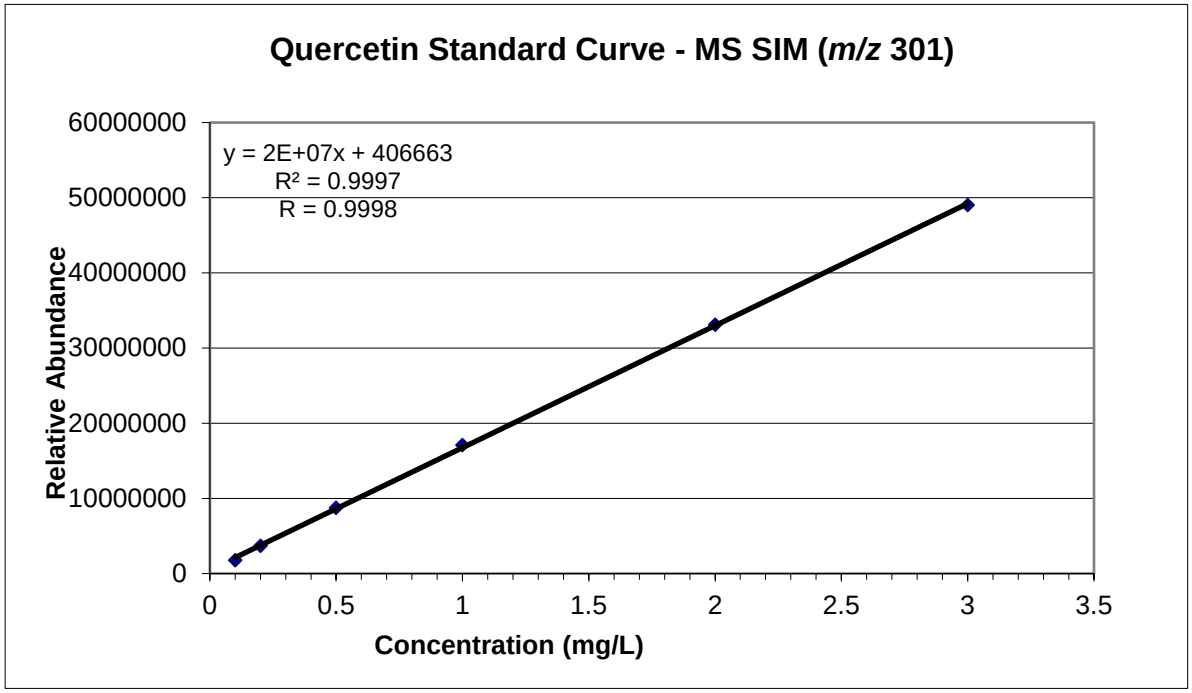


Figure 5.11 Calibration plot for quercetin using HPLC-ESI-MS SIM peak area of the *m/z* 301 ion.

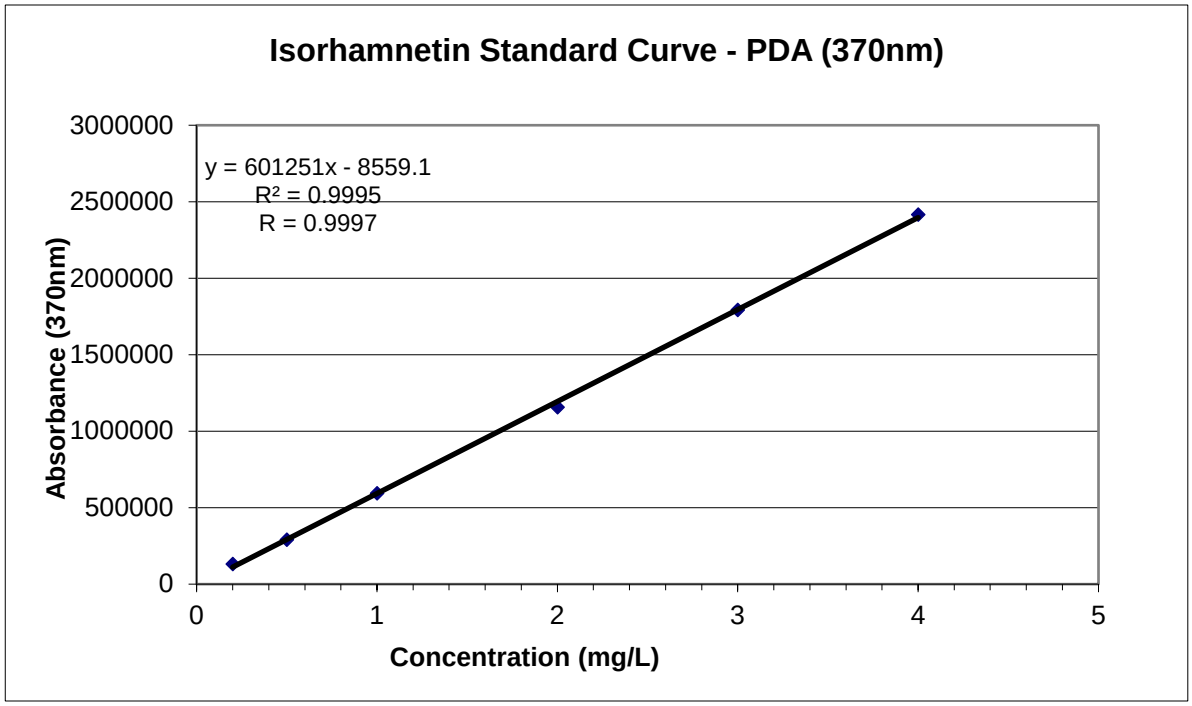


Figure 5.12 Calibration plot for isorhamnetin using HPLC-PDA peak area recorded at 370 nm.

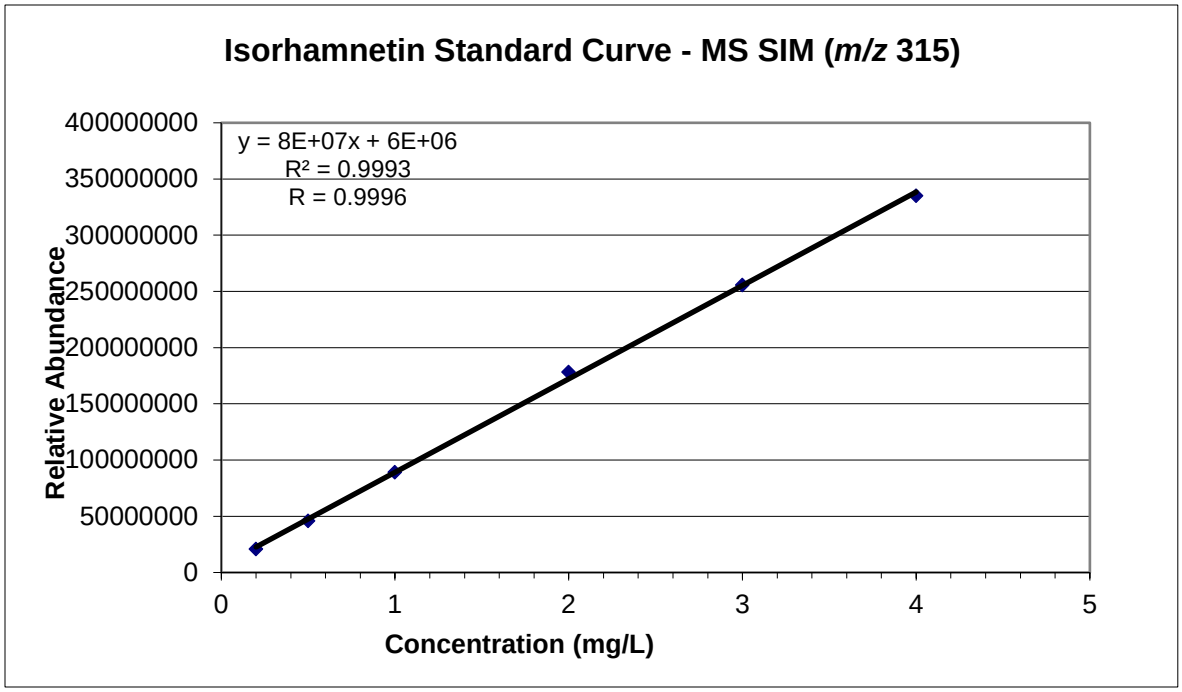


Figure 5.13 Calibration plot for isorhamnetin using HPLC-ESI-MS SIM peak area of the *m/z* 315 ion.

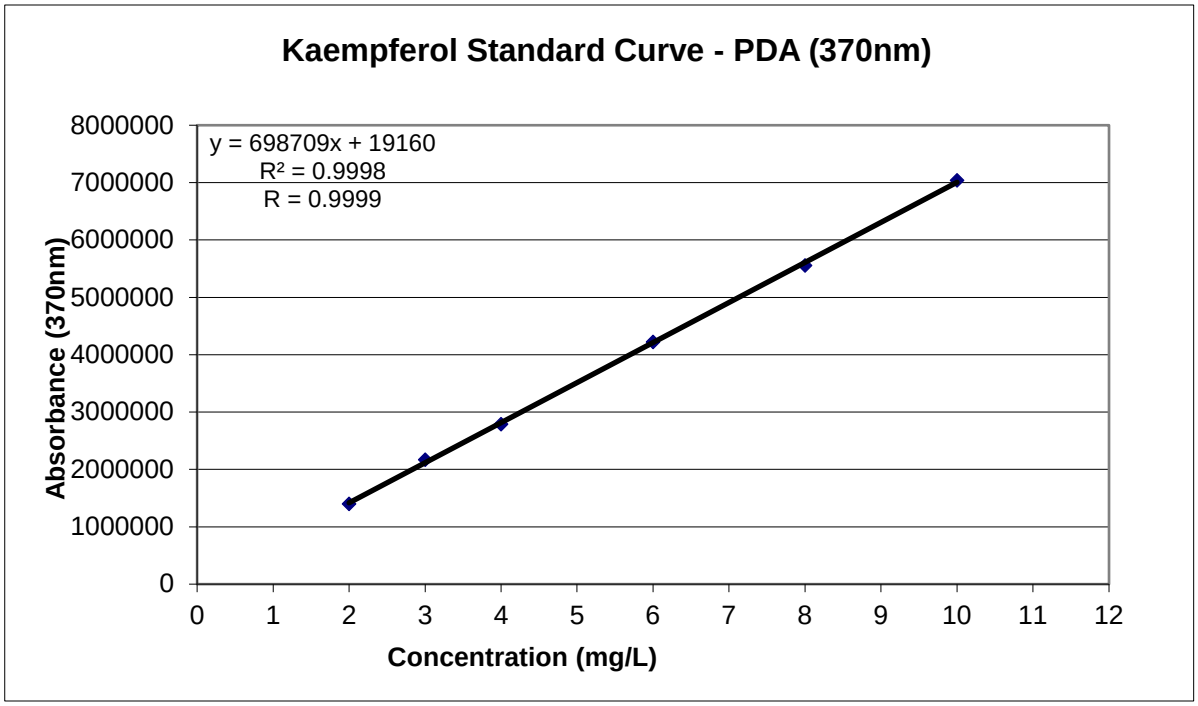


Figure 5.14 Calibration plot for kaempferol using HPLC-PDA peak area recorded at 370 nm.

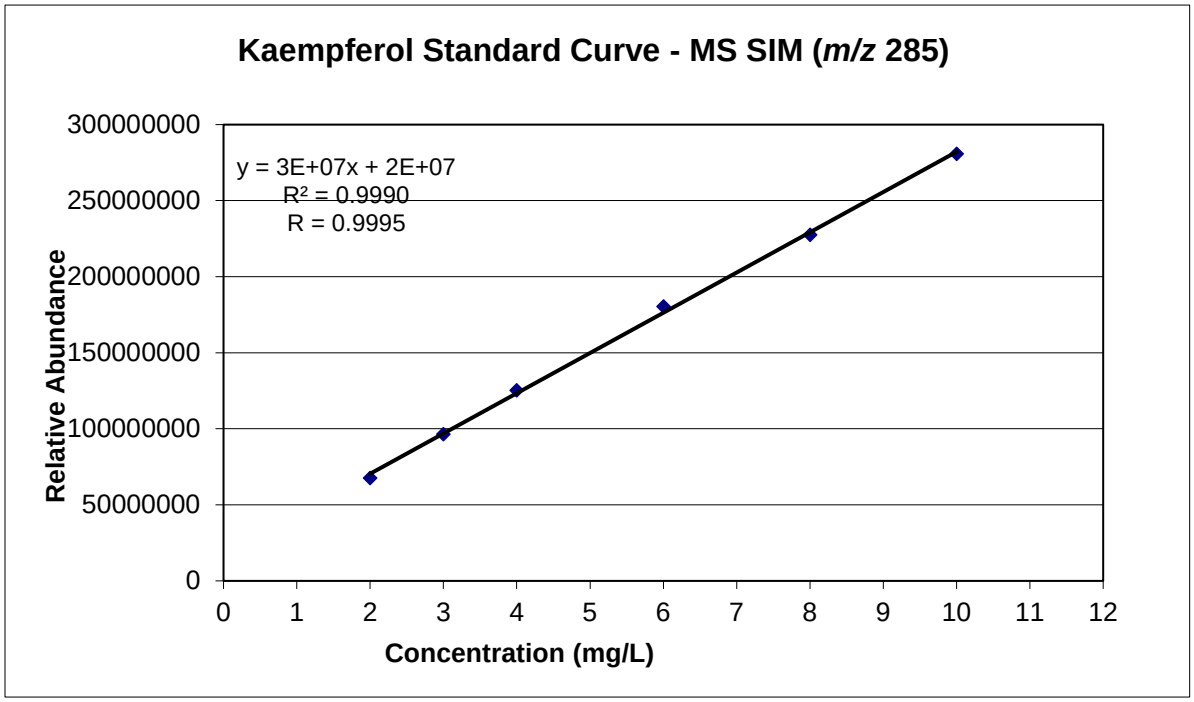


Figure 5.15 Calibration plot for kaempferol using HPLC-ESI-MS SIM peak area of the *m/z* 285 ion.

Table 5.7 Summary of R^2 and R values obtained from calibration plots of all three standards, quercetin, isorhamnetin and kaempferol using peak area data from HPLC-PDA and HPLC-ESI-MS SIM chromatograms.

Flavonol Aglycone	Concentration Range (mg/L)	Correlation Coefficient (R)		Coefficient of Determination (R^2)	
		PDA	MS SIM	PDA	MS SIM
Quercetin	0.1, 0.2, 0.5, 1.0, 2.0, 3.0	0.9999	0.9998	0.9999	0.9997
Isorhamnetin	0.2, 0.5, 1.0, 2.0, 3.0, 4.0	0.9997	0.9996	0.9995	0.9993
Kaempferol	2.0, 3.0, 4.0, 6.0, 8.0, 10	0.9999	0.9995	0.9998	0.9990

As can be seen in Table 5.7, the coefficient of determination (R^2) values for all calibration plots ranged from 0.9990 to 0.9999 and the correlation coefficient (R) values ranged from 0.9995 and 0.9999. The correlation coefficient values obtained indicate that each calibration plot had a strong positive linear relationship between detector response and concentration and the coefficient of determination values signify that between 99.90 and 99.99% of the variation in detector response can be explained by the linear relationship between detector response and concentration (i.e. the equation of the line). In other words, the calibration errors were extremely small.

5.2.2.3 Recovery Studies

Recovery studies were conducted for the various bok choy varieties whereby each bok choy sample was spiked with a known concentration of flavonol aglycone standard prior to acid hydrolysis, and then analysed via HPLC-PDA/ESI-MS^n. The Miyako bok choy cultivar was spiked with quercetin, and the Sumo and Karate varieties were fortified with isorhamnetin and kaempferol respectively. Samples of each bok choy variety were analysed in triplicate and quantified using both data from the PDA (370 nm) and MS detector in the SIM mode. The preparation of recovery standard solutions and an example of the percent recovery calculation are presented in 3.5.3. The acid hydrolysis extraction method is outlined in 3.4.2 and the HPLC-PDA/ESI-MS^n conditions are summarised in 3.6.1 and 3.9.1. The average percent recoveries for each bok choy cultivar are presented in Table 5.8. The percent recoveries ranged from 90-115% indicating that the accuracy, and efficiency of recovery, of the sample extraction method and processing steps of the analytical method was high.

Table 5.8 Average percent recoveries for bok choy acid hydrolysate extracts spiked with quercetin, isorhamnetin, and kaempferol.

Spiked Flavonol and Bok Choy Cultivar	% Recoveries	
	PDA (370nm)	MS SIM
Quercetin in Miyako	90%	90%
Isorhamnetin in Sumo	115%	100%
Kaempferol in Karate	95%	100%

5.2.3 Quantification of Quercetin, Isorhamnetin and Kaempferol in Three Cultivars of Bok Choy Using HPLC-PDA/ESI-MS^n

The levels of quercetin, isorhamnetin, and kaempferol aglycones in the bok choy acid hydrolysate extracts were determined by HPLC-PDA/ESI-MS^n using both the PDA (370 nm) and ESI-MS (SIM) detectors. HPLC-PDA/ESI-MS^n has been used extensively in literature to determine the aglycone levels of flavonols in plants after acid hydrolysis (refer to Chapter II). Methodology for acid hydrolysis is described in 3.4.2 and HPLC-PDA/ESI-MS^n conditions are outlined in sections 3.6.1 and 3.9.1. The PDA and MS detectors were linear over the following ranges: quercetin 0.1 to 3 mg/L, isorhamnetin 0.2 to 4 mg/L, and kaempferol 2 to 10 mg/L (5.2.2.2), and preparation of standards is described in 3.5.2. All determinations were carried out within the linear ranges. Three subsamples of each freeze dried, powdered bok choy cultivar (3.2) were extracted and quantified and each subsample was analysed in triplicate, i.e. injected onto the HPLC-PDA/ESI-MS^n three times. The results of the quantification of the three flavonol aglycones quercetin, isorhamnetin, and kaempferol, in the three bok choy cultivars are summarised in Table 5.9.

Table 5.9 Quercetin, isorhamnetin, and kaempferol aglycone content of the three cultivars of bok choy using both PDA (370 nm) and MS SIM data. Results are expressed as mg/100 g dry weight (DW) bok choy. The value for each of the three sub-samples of bok choy represents the mean of triplicate injections on the HPLC-PDA/ESI-MS^n.

Bok Choy Cultivar	Quercetin (mg/100 g DW)		Isorhamnetin (mg/100 g DW)		Kaempferol (mg/100 g DW)	
	PDA (370 nm)	MS SIM (*m/z* 301)	PDA (370 nm)	MS SIM (*m/z* 315)	PDA (370 nm)	MS SIM (*m/z* 285)
Sumo 1	15.4	13.7	53.0	65.7	133	120
Sumo 2	12.1	10.0	41.3	49.0	114	95.6
Sumo 3	14.1	12.1	47.9	57.8	120	114
Average	**13.9**	**11.9**	**47.4**	**57.5**	**122**	**110**
Range	**12.1 – 15.4**	**10.0 – 13.7**	**41.3 – 53.0**	**49.0 – 65.7**	**114 - 133**	**96 - 120**
Karate 1	11.0	10.0	36.0	41.5	104	88.7
Karate 2	12.8	11.3	39.0	49.2	114	100
Karate 3	11.0	11.3	40.0	44.1	110	104
Average	**11.6**	**10.9**	**38.3**	**44.9**	**110**	**98**
Range	**11.0 – 12.8**	**10.0 – 11.3**	**36.0 – 40.0**	**41.5 – 49.2**	**104 - 114**	**89 - 104**
Miyako 1	20.5	17.7	68.7	64.7	111	83.3
Miyako 2	20.3	17.4	64.4	64.4	113	81.1
Miyako 3	21.0	20.0	67.0	67.0	107	91.6
Average	**20.6**	**18.4**	**66.7**	**65.4**	**110**	**85.5**
Range	**20.3 – 21.0**	**17.7 – 20.0**	**64.4 – 68.7**	**64.4 – 67.0**	**103 - 111**	**81.1 – 91.6**

Kaempferol was present in the highest amount at 85.5 to 122 mg/100 g DW (ranging across the 3 different bok choy cultivars). The levels of isorhamnetin varied from 38.3 to 66.7 mg/100 g DW, and quercetin was present in the least amount at 10.9 to 20.6 mg/100 g DW. The water content in bok choy leaves is approximately 90% [199, 60], therefore, the kaempferol content is equivalent to approximately 9 to 12 mg/100 g fresh weight (FW), the isorhamnetin content is approximately 4 to 7 mg/100 g FW, and the quercetin content is approximately 1 to 2 mg/100 g FW bok choy.

Miyako contained a significantly greater amount of quercetin, as compared to Sumo and Karate, which were similar in quercetin content. Miyako also contained the highest levels of isorhamnetin, followed by Sumo, and then Karate. Kaempferol, on the other hand, is comparable in all three cultivars of bok choy. Although Miyako contained higher levels of quercetin and isorhamnetin, all three bok choy cultivars contained similar total aglycone levels (Sumo 183.3 mg/100 g DW, Karate 159.9 mg/100 g DW, and Miyako 197.3 mg/100 g DW bok choy). Therefore, no firm conclusions can be made as to whether one bok choy cultivar contained more health-promoting flavonols than

another. This will be further explored in Chapter VI by assessing the antiproliferative activity of the three cultivars on human colon cancer cells *in vitro*.

The levels of flavonols determined in this study concur with those previously reported for pak choi (i.e. bok choy) by Rochfort *et al.* [60], whereby kaempferol was also found to be the major flavonol present, followed by isorhamnetin, and quercetin was present only in trace amounts. Rochfort *et al.* investigated the flavonol aglycone content of 11 varieties of bok choy grown under glasshouse conditions in Australia and found that the kaempferol content ranged from 36 to 103 mg/100 g DW (equivalent to approximately 4 to 10 mg/100 g FW), isorhamnetin content ranged from 8 to 24 mg/100 g DW (equivalent to 0.8 to 2.4 mg/100 g FW), and quercetin content ranged from 3.2 to 6.1 mg/100 g DW (0.3 to 0.6 mg/100 g FW). The Sumo variety was common to both studies, however, the levels of the three flavonol aglycones in this study were higher than those reported by Rochfort *et al* [60]: kaempferol was 110 to 122 mg/100 g DW as compared to 81.1 mg/100 g DW, isorhamnetin was 47 to 58 mg/100 g DW as compared to 35.1 mg/100 g DW, and quercetin was 12 to 14 mg/100 g as compared to 7.1 mg/100 g DW. These differences could possibly be due to the growth age of the bok choy samples.

The other major study on the characterisation and quantification of flavonols in bok choy was conducted by Harbaum *et al.* [136] on 11 different varieties of bok choy grown under field conditions in China and glasshouse conditions in Germany. The authors also reported that the major flavonol aglycone present in all the varieties of bok choy was kaempferol, followed by isorhamnetin. Harbaum *et al.* did not, however, detect quercetin in the bok choy cultivars they studied. This difference could be attributed to many factors such as environmental, seasonal, and geographic variation. It was difficult to make a direct comparison with quantification results from this study as the authors reported total aglycone levels (in the leaf blade of the bok choy) based on kaempferol. The range of total aglycone content in the 11 bok choy cultivars was between 1.39 to 4.95 mg/g DW (i.e. 139 to 495 mg/100 g DW). The total aglycone levels obtained for this study for the three bok choy cultivars ranged from 153.8 to 197.3 mg/100 g DW.

The results in this study indicated that the vegetable bok choy may be a good additional source of both kaempferol and isorhamnetin in the Western diet.

5.2.3.1 Comparison of Detectors and Variation in Quantification Data

There were some differences in the calculated levels of quercetin, isorhamnetin, and kaempferol between the two detectors. In order to make a comparison between the PDA and MS results and determine whether there was a general trend, a ratio between the values obtained for each detector, using the levels of each flavonol (in mg/100 g DW bok choy), was calculated. Table 5.10 shows a clear comparison between the results obtained for the PDA and MS for each flavonol aglycone, as well as the ratio between the two (PDA/MS).

Table 5.10 Comparison of PDA (370 nm) and MS SIM quantification data showing bias between the two detectors. Results are expressed as mg/100 g dry weight (DW) bok choy. The value for each of the samples of bok choy represents the mean of triplicate injections on the HPLC-PDA/ESI-MS^n.

Bok Choy Sample	Quercetin (mg/100 g DW)			Isorhamnetin (mg/100 g DW)			Kaempferol (mg/100 g DW)		
	PDA	MS SIM	Ratio (PDA/ MS)	PDA	MS SIM	Ratio (PDA/ MS)	PDA	MS SIM	Ratio (PDA/ MS)
Sumo 1	15.4	13.7	1.12	53.0	65.7	0.81	133	120	1.11
Sumo 2	12.1	10.0	1.21	41.3	49.0	0.84	114	95.6	1.19
Sumo 3	14.1	12.1	1.17	47.9	57.8	0.83	120	114	1.05
Karate 1	11.0	10.0	1.10	36.0	41.5	0.87	104	88.7	1.17
Karate 2	12.8	11.3	1.13	39.0	49.2	0.79	114	100	1.14
Karate 3	11.0	11.3	0.97	40.0	44.1	0.91	110	104	1.06
Miyako 1	20.5	17.7	1.16	68.7	64.7	1.06	111	83.3	1.33
Miyako 2	20.3	17.4	1.17	64.4	64.4	1.0	113	81.1	1.39
Miyako 3	21.0	20.0	1.05	67.0	67.0	1.0	107	91.6	1.17
Average			**1.12**			**0.90**			**1.18**
Range			**0.97-1.21**			**0.81-1.06**			**1.05-1.39**

For quercetin and kaempferol, there is a clear bias towards the levels determined by the PDA being greater than the levels determined by the MS. In almost all cases, excluding the Miyako bok choy samples, the opposite is observed with isorhamnetin, whereby the levels determined by the MS appear to be greater than the levels determined by the PDA. The variation observed between the levels determined by the two detectors could be due to the error associated with the integration of the peak areas in the PDA, especially for isorhamnetin and kaempferol as these two peaks were not fully resolved in the PDA chromatograms. This was not the case with the MS SIM chromatograms as the peaks were fully resolved from each other.

The differences observed between the ratios of the sub-samples of bok choy (e.g. 1.12, 1.21, and 1.17 for quercetin in the Sumo cultivar) are most probably due to the homogeneity of the sample, as well as variations in operator extraction techniques and other "lower level errors;" e.g. injector reproducibility, ionisation, and the integration of peak areas.

5.3 IDENTIFICATION OF FLAVONOL GLYCOSIDES IN ALKALINE EXTRACTS OF THREE VARIETIES OF BOK CHOY

This section discusses the results of the HPLC-PDA/ESI-MS^n separation of flavonol glycosides present in alkaline hydrolysate extracts of freeze-dried, powdered bok choy samples in three different cultivars of bok choy. Bok choy cultivar information and growing conditions are described in 3.2, with the alkaline hydrolysis experimental procedure and sample preparation described in 3.4.3. HPLC-PDA/ESI-MS^n separation conditions are outlined in 3.6.2 and structure elucidation of these compounds was achieved using a data dependent protocol on the ion trap MS^n (3.9.2). Preparative liquid chromatography was used to collect two flavonol glycoside compounds from an alkaline hydrolysate bok choy extract for further characterisation using NMR. Preparative HPLC conditions and fraction collection are described in 3.7, and NMR conditions are outlined in 3.10.

5.3.1 HPLC-PDA/ESI-MS^n Analysis of Flavonol Glycosides in Three Bok Choy Cultivars

Flavonol glycoside standards were not available for this work, therefore PDA, MS^n and NMR data were used to identify and characterise the flavonol glycosides present in the alkaline hydrolysate extracts of the bok choy cultivars. The HPLC-PDA/ESI-MS^n conditions used for the separation of these glycoside conjugates were optimised in 4.2.3 and the HPLC-PDA chromatograms, recorded at 350 nm, for the Sumo, Karate, and Miyako bok choy alkaline hydrolysate extracts are shown in Figure 5.16. The four peaks of interest are labelled 1-4 and their respective retention times are shown in Table 5.11 for each cultivar. The three varieties have similar chromatographic profiles displaying the same four major peaks.

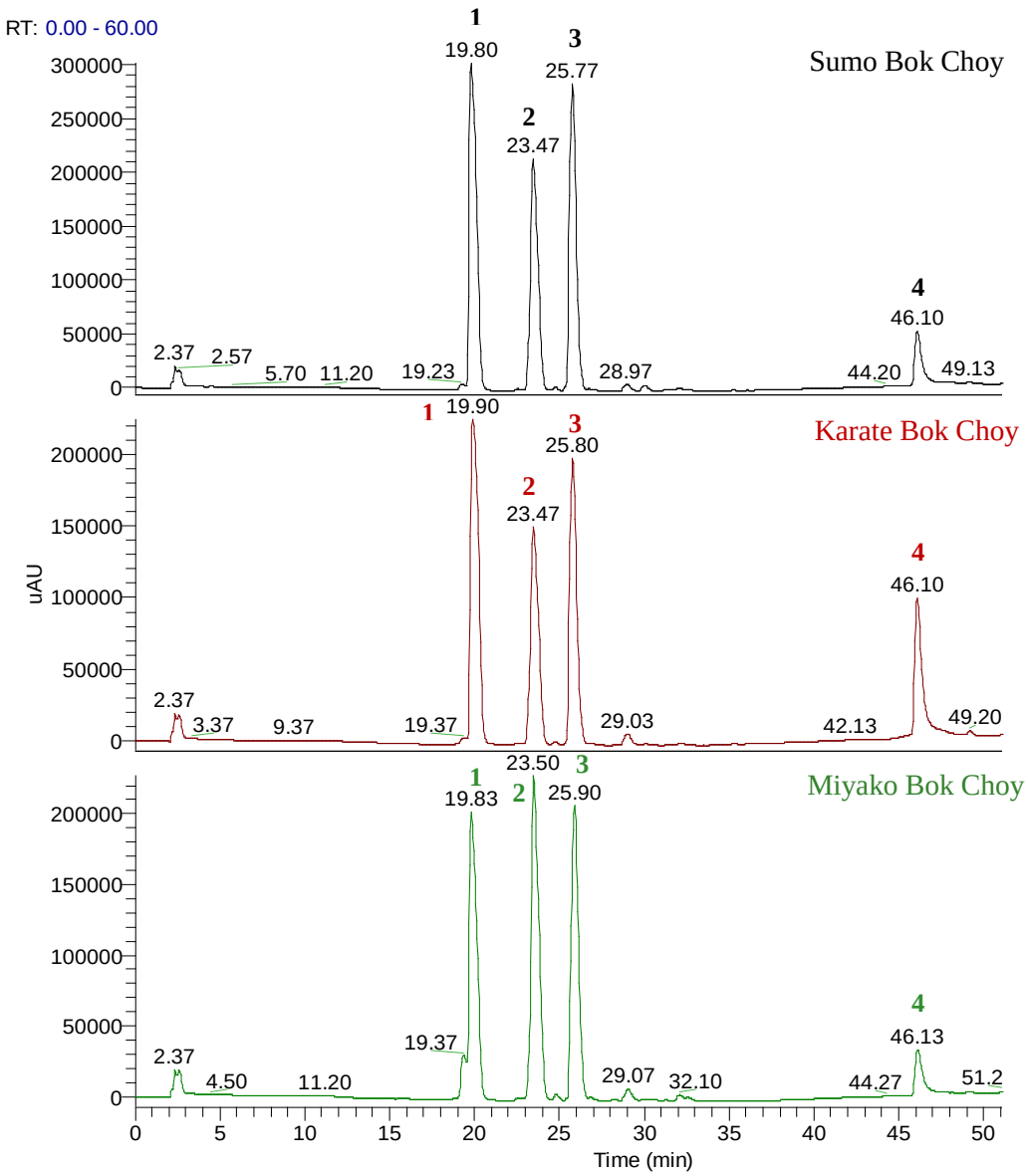


Figure 5.16 HPLC-PDA chromatograms, recorded at 350 nm, of the alkaline hydrolysate extracts of all three bok choy cultivars: Sumo, Karate, and Miyako. The four peaks of interest are labelled 1-4.

Table 5.11 HPLC-PDA retention times (t_R) of the four peaks of interest present in the alkaline hydrolysate extracts of all three cultivars of bok choy.

Bok Choy Cultivar	Peak 1 t_R (min)	Peak 2 t_R (min)	Peak 3 t_R (min)	Peak 4 t_R (min)
Sumo	19.80	23.47	25.77	46.10
Karate	19.90	23.47	25.80	46.10
Miyako	19.83	23.50	25.90	46.13

The UV Visible spectra for peaks 1-4 in each variety of bok choy are overlayed (blue, green and orange spectra represent the Sumo, Karate, and Miyako bok choy alkaline hydrolysate extracts respectively) and are shown in Figure 5.17.

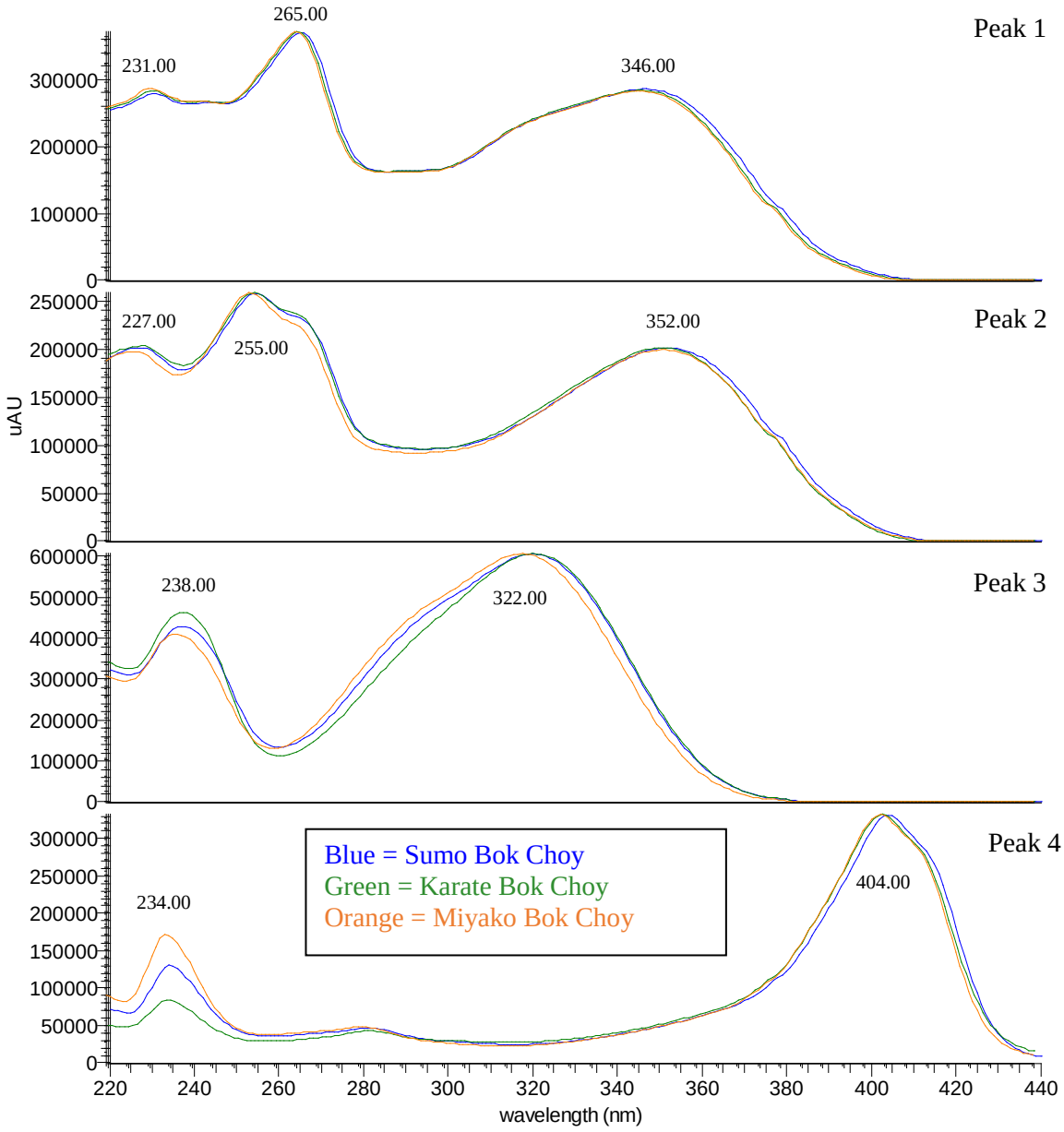


Figure 5.17 UV-Vis spectra (220 to 420 nm) of the four peaks of interest present in the alkaline hydrolysate extracts of each bok choy cultivar: peak 1, peak 2, peak 3, and peak 4.

The UV-Visible spectra are identical, suggesting that the same four compounds are present in the alkaline hydrolysate extracts of each bok choy variety. Peaks 1 and 2 showed typical flavonol glycoside profiles (refer to Section 4.2.5.2), with two broad bands at approximately 350 and 260 nm. Peak 3 has a UV-Visible profile similar to that of a flavonol glycoside-hydroxycinnamic acid conjugate. However, these compounds are not expected to be present in the alkaline hydrolysate, as they are hydrolysed to flavonol glycosides under alkaline conditions. Peak 4 did not have a typical flavonol profile and, as will be shown later, peaks 3 and 4 could not be ionised under the ESI-MS^n conditions used for the analysis. Therefore, these two compounds were not further studied in this work. The retention time and UV-Visible data for peaks 1-4 in each of the three bok choy varieties are shown in Table 5.12.

Table 5.12 HPLC-PDA retention time (t_R) and UV-Vis spectral data for the four peaks of interest in the alkaline hydrolysate extracts of each bok choy cultivar.

Bok Choy Cultivar	Peak 1		Peak 2		Peak 3		Peak 4	
	t_R (min)	UV-Vis (nm)	t_R (min)	UV-Vis (nm)	t_R (min)	UV-Vis (nm)	t_R (min)	UV-Vis (nm)
Sumo	19.80	231,265,346	23.47	227,255,352	25.77	237,320	46.10	234,404
Karate	19.90	231,265,347	23.47	228,255,352	25.80	238,321	46.10	234,404
Miyako	19.83	231,265,346	23.50	227,254,352	25.90	237,319	46.13	234,404

The HPLC-ESI-MS total ion chromatograms (TIC) for the Sumo, Karate, and Miyako bok choy alkaline hydrolysate extracts are shown in Figure 5.18, with the two major peaks labelled. From the four major peaks observed in the PDA chromatograms of each bok choy cultivar (Figure 5.16), peaks 1 and 2 are also clearly present in the TIC for each variety. However, as mentioned above, peaks 3 and 4 are absent in the TIC's, suggesting that these compounds are not flavonols. Both Sumo and Karate display comparable chromatograms with similar abundances for the two peaks. Peak 1 is the most prominent compound in both these cultivars, with peak 2 being much smaller in size. Miyako, on the other hand, produced a slightly different profile, whereby peak 2 was present at approximately the same level as peak 1.

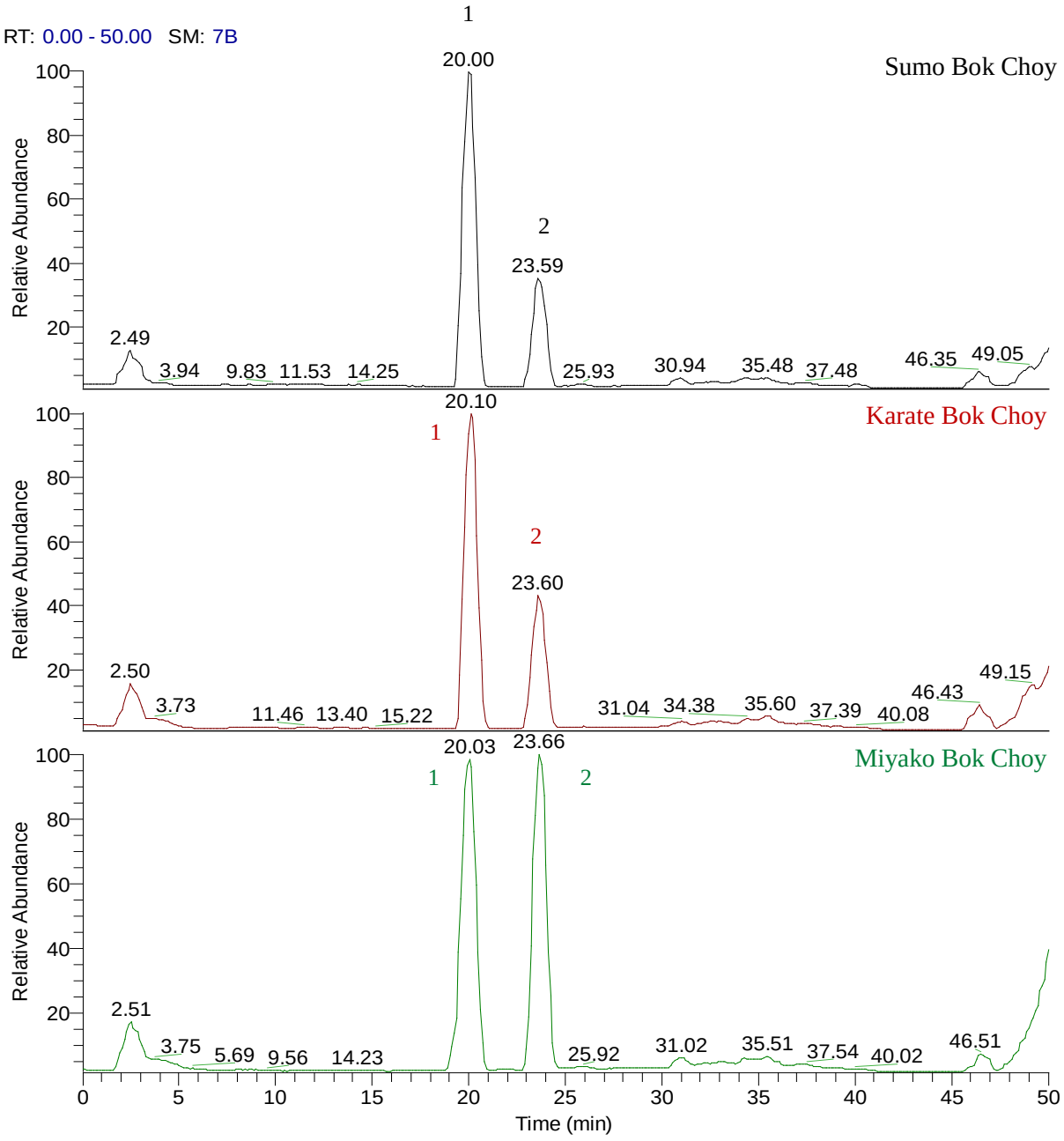


Figure 5.18 HPLC-ESI-MS TIC (scanning mass range 120-2000 amu) of the alkaline hydrolysate extracts of the three bok choy cultivars: Sumo, Karate, and Miyako. The two major peaks are labelled 1-2.

The negative ion ESI-MS^1 spectra for peak 1 in each bok choy cultivar is shown in Figure 5.19 and the negative ion ESI-MS^1 spectra for peak 2 in each bok choy cultivar is shown in Figure 5.20. The MS spectra confirm, along with the UV-Visible spectra discussed above, that all three varieties contain the same two compounds. As will be shown later in the data dependant studies, however, peak 2 actually contained two co-eluting compounds and there were also two other minor flavonol glycosides present in the total ion chromatograms.

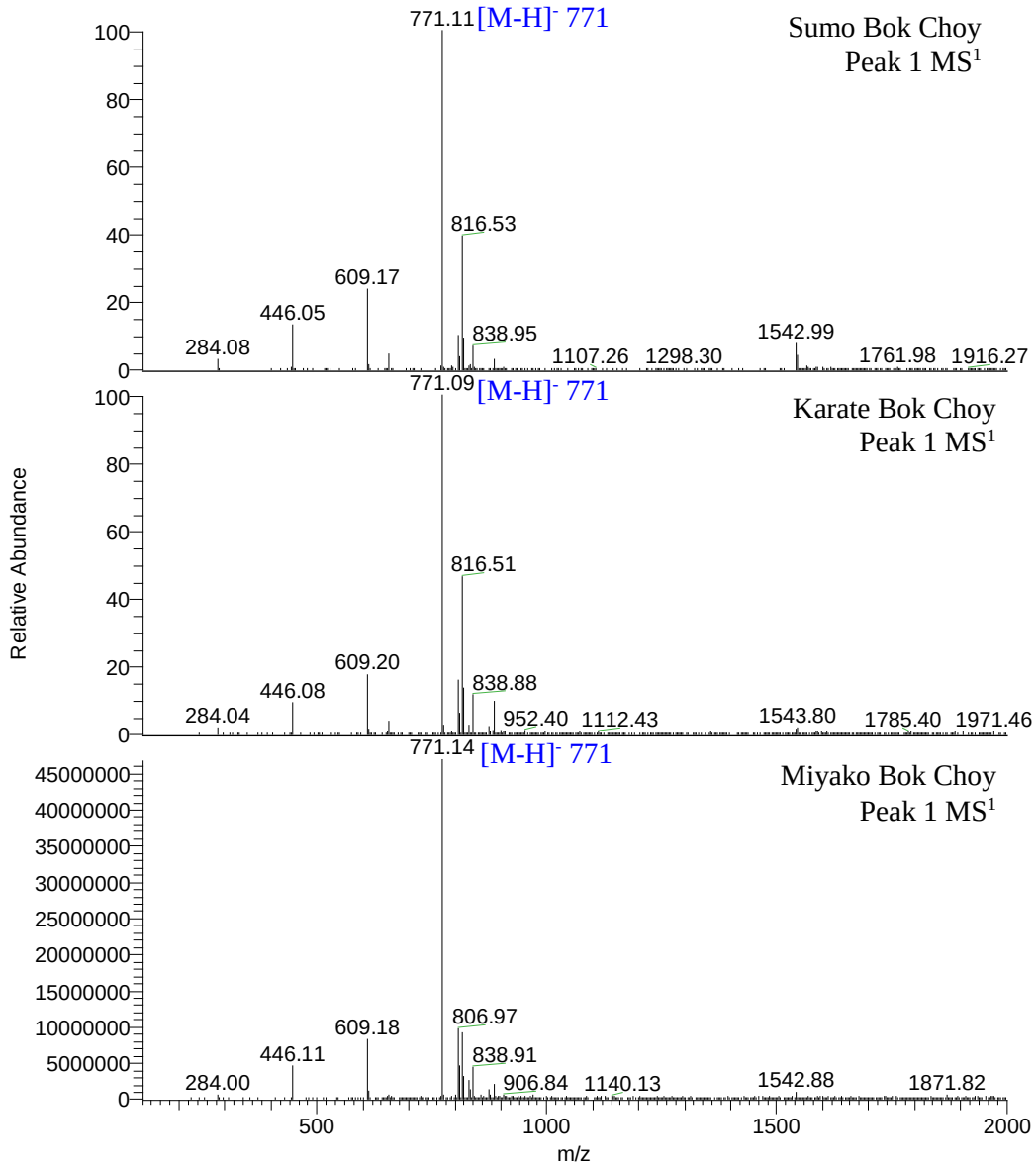


Figure 5.19 ESI-MS^1 spectra in negative ion mode of peak 1 in the alkaline hydrolysate extracts of the three bok choy cultivars: Sumo, Karate, and Miyako.

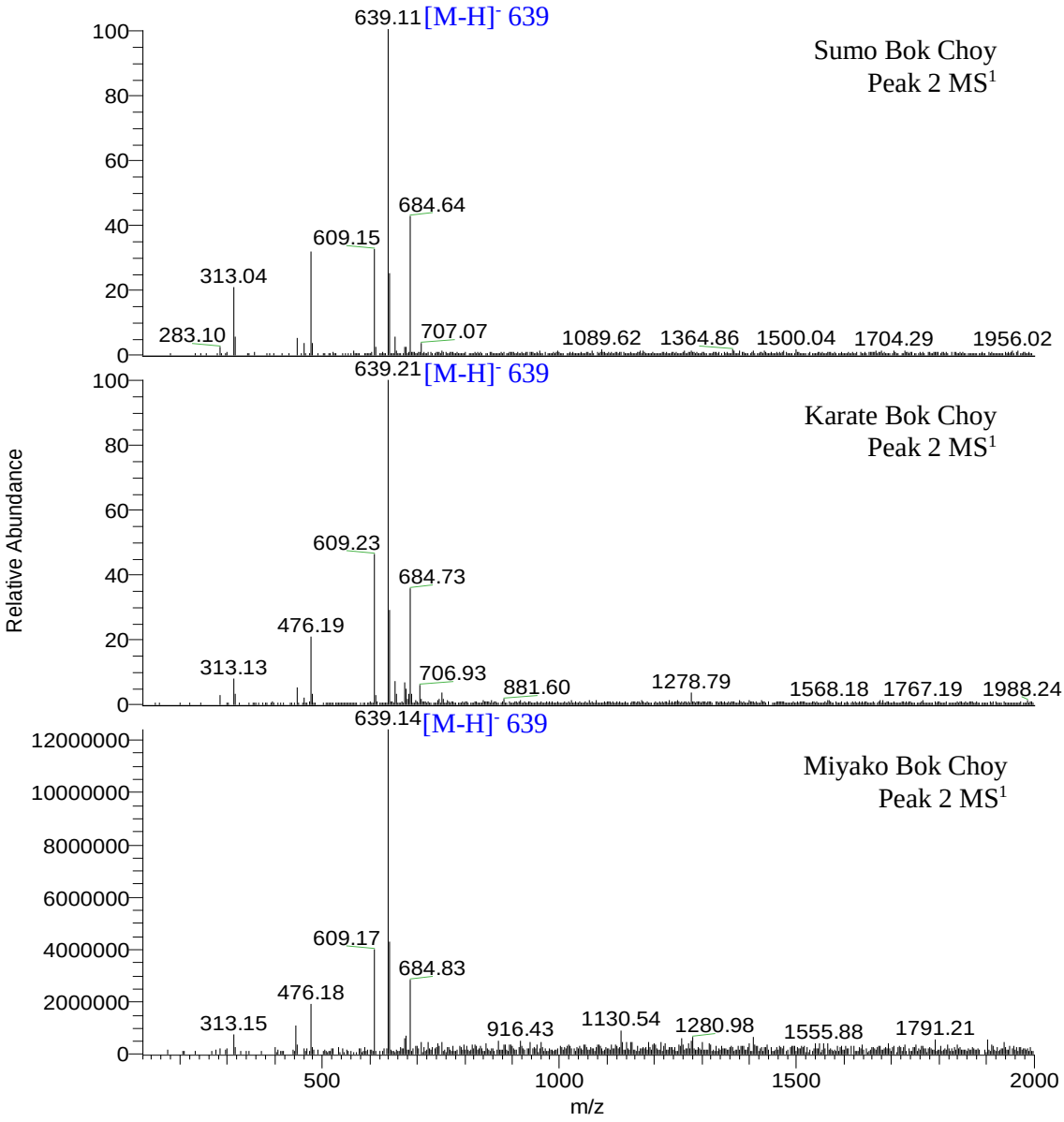


Figure 5.20 ESI-MS[1] spectra in negative ion mode of peak 2 in the alkaline hydrolysate extracts of the three bok choy cultivars: Sumo, Karate, and Miyako.

The retention times and parent/deprotonated molecular ions ([M-H]$^-$) observed for peaks 1 and 2 in each of the three bok choy varieties, and some of the other negative ions that were common to all three varieties are displayed in Tables 5.13 and 5.14 respectively. The various common ions appeared to be similar to those observed in the data dependent MS[2] and MS[3] studies, however, without further investigation their possible sources (such as in source fragmentation) could not be ascertained.

Table 5.13 HPLC-ESI-MS TIC retention time (t_R) and negative parent ion ($[M-H]^-$) data for peaks 1 and 2 present in the alkaline hydrolysate extract of each bok choy cultivar.

Bok Choy Cultivar	Peak 1 MS^1		Peak 2 MS^1	
	t_R (min)	$[M-H]^-$ (*m/z*) Parent Ion	t_R (min)	$[M-H]^-$ (*m/z*) Parent Ion
Sumo	20.00	771.11	23.59	639.11
Karate	20.10	771.09	23.60	639.21
Miyako	20.03	771.14	23.66	639.14

Table 5.14 Commonly observed negative ions in the MS^1 spectra for peaks 1 and 2 and their possible sources.

Negative Ion (*m/z*)	Possible Source
Peak 1	
1543	Dimer
817	Parent ion + formic acid adduct
771	Parent ion
609	In-source fragmentation (MS^2 ion)
446	In-source fragmentation (MS^3 ion)
284	In-source fragmentation - aglycone (MS^3 ion)
Peak 2	
1279	Dimer
685	Parent ion + formic acid adduct
639	Parent ion
609	Identified as a co-eluting compound based on 609 not being a product ion of 639 (as shown in data dependent studies)
476	In-source fragmentation (MS^2 ion)
313	In-source fragmentation - aglycone (MS^3 ion)

5.3.2 Characterisation and Structure Elucidation of Flavonol Glycosides in Alkaline Hydrolysate Bok Choy Extracts using Data Dependent MS^n

Structural information on the flavonol compounds present in the alkaline hydrolysate extracts of bok choy was obtained by multi-stage mass analysis using the Data Dependent MS^n technique on the LCQ Deca XP Max mass spectrometer. The data dependent acquisition parameters are described in 3.9.2. In the centroid scan mode, the LCQ Deca XP Max performs data dependent scans for up to 25 ions in a given spectrum.

The structure elucidation of the flavonol compounds present in the bok choy cultivars investigated in this study benefited from a number of earlier, detailed studies of the fragmentation of flavonols and carbohydrates by mass spectrometry. This included studies on broccoli [57], cauliflower [128], pak choi [60, 136], cabbage [74, 132], turnip [213], and kale [226, 116, 227, 152, 67]. The assignment of different sugar substitutions to the flavonol hydroxyl groups and their interglycosidic linkage was carried out with reference to these previous studies. The studies showed that the first fragmentation of the deprotonated molecular ion $[M-H]^-$ is due to the breakdown of the *O*-glycosidic bond at the 7-*O* position, with the remaining sugars on the flavonol molecule linked to the hydroxyl group at the 3-*O* position of the flavonol core [67, 57]. Ferreres *et al.* [225] reported that both flavonol sophorosides (1→2 interglucosidic linkage) and flavonol gentiobiosides (1→6 interglucosidic linkage) at the 3-*O* position are characterised by the fragment ion $[M-H-324]^-$ as the base peak in MS^2 experiments. The typical MS^2 fragmentation pattern of 3-*O*-sophorosides consists of a base peak $[M-H-324]^-$, the fragment ion $[M-H-180]^-$ and the fragment ion $[M-H-162]^-$ [225, 67]. In contrast, the absence of the fragment ion $[M-H-180]^-$ in the MS^2 spectra revealed that the interglycosidic linkage at the 3-*O* position is a gentiobioside. Sophorosides of quercetin and kaempferol have been observed in kale [227, 67] and several *Brassica oleracea* [208, 128, 57, 231] and *Brassica rapa* [128, 60, 136, 213] species.

Flavonol di-glucosides with sugar moieties linked to different hydroxyl positions of the flavonol core (X,Y-diglucosides), have the fragment ion $[M-H-162]^-$ as their base peak, which corresponds to the loss of one glucose moiety at the 3-*O* position. Similarly, the same fragment ion is observed in the MS^2 spectra of mono-glucosides, in addition to the fragment ion $[M-H-120]^-$. This was observed in studies of flavonols in kale [67] and pak choi [60]. Quercetin, kaempferol, and isorhamnetin mono-glucosides have been found in kale [67, 152] and other *Brassica oleracea* species, such as broccoli,[57] tronchuda cabbage, [208] cauliflower, [128], and *Brassica rapa* species, such as pak choi[60, 136], and turnip leaves [213]. In all cases, the glucose moiety was bound to the 3-*O* position of the aglycone.

Furthermore, the study published by Domon and Costello in 1998 [230] proposed a nomenclature for carbohydrate fragmentation, which has now become a widely accepted system in the characterisation of glycosylated compounds [60]. This nomenclature has also been adopted in this study to describe the MS^n fragmentation patterns of flavonol compounds in three bok choy cultivars and was described in detail in 2.6.1.2. A summary of the key criteria, collated from previous studies, that was used to characterise the flavonol compounds in this study was outlined in 2.6.3 and included both MS and UV-Vis characteristics of carbohydrates and flavonols, as well as the nomenclature used. Table 5.15 presents this information again in a summarised form. By using this information in conjunction with the data dependent MS^n data and UV-Vis spectra, five flavonol glycosides were identified in the alkaline hydrolysate bok choy extracts. A previous study by Rochfort *et al.* [60] was able to identify nine individual flavonol glycosides in the alkaline hydrolysate extract of a pak choi variety named 'Shanghai' using the more sensitive Thermo Fisher LTQ ion trap mass spectrometer.

Table 5.15 Summary of information collated from previous studies used to interpret data dependent MS^n and characterise flavonol compounds.

Information Used for Interpretation of Data and Structure Elucidation	
UV-Vis Spectra	λ_{max} of flavonol aglycones are ≈260 and ≈370 nm
	λ_{max} of broad peak for flavonol glycoside conjugates shifts to ≈350 nm
	λ_{max} of broad peak for flavonol glycoside-hydroxycinnamic acid conjugates shifts to ≈330 nm
MS^n Fragmentation	A loss of 180 amu indicates a loss of glucose with the glycosidic oxygen
	A loss of 162 amu indicates a loss of glucose without the glycosidic oxygen
	A loss of 120 amu is the most common fragmentation through the glucose ring
	O-glycosides typically conjugate at positions 3 and 7 on flavonols
	A loss of 162 amu in the MS^2 spectra for a 7-*O*-glycoside indicates that there is only one sugar moiety present
	A loss of 324 amu in the MS^2 spectra for a 7-*O*-glycoside indicates that there are two sugar moieties present
	If the MS^2 spectra consists of multiple ions it indicates that the compound is a 3,7-diglucoside or is un-substituted at position 7
	For di-glucosides; a loss of 162 amu as the base peak in the MS^2 spectra indicates it is a 3,7-diglucoside and a loss of 324 amu as the base peak in the MS^2 indicates it is a 3-diglucoside (i.e. gentiobioside with a 1→6 glycosidic link)
	For sophorosides (1→2 glycosidic link); a loss of 162 amu and 324 amu will be observed in the MS^3 spectra, in addition to losses of 120 amu and 180 amu
	The losses of 120 amu and 180 amu are not generally observed for di-glucosides
Nomenclature	The Z ion refers to the aglycone when it does not retain the glycosidic oxygen (i.e. the oxygen is lost with the sugar)
	The Y ion refers to the aglycone when it does retain the glycosidic oxygen (i.e. the oxygen is not lost with the sugar)
	The X ion is formed when fragmentation occurs through the sugar ring
	X ions have superscript numbers (k,l) to the left, which refers to the position where cleavage has occurred (i.e. the number of the bond in the sugar ring), and are denoted as $^{k,l}X$
	The Z, Y and X ions have a subscript number (j) to the right, which refers to the number of the glycosidic bond that is cleaved, counting 0 from the aglycone, and is denoted as Y_j
	The Z, Y, and X ions also have a superscript number (n) to the right, which refers to the carbon number that the sugar is attached to on the aglycone, and is denoted as Y^n
	Y_0 is the base peak in the MS^2 for a sophoroside (1→2 glycosidic link), whereas Y_1 is the base peak observed for a gentiobioside (1→6 glycosidic link) and Y_0 has a very low relative abundance.

The TIC generated from the data dependent MS^n study on the Sumo bok choy variety is displayed in Figure 5.21 below. The five flavonol glycoside compounds are labelled peaks 1-5, along with their corresponding retention times. The same five compounds were present in all three bok choy cultivars.

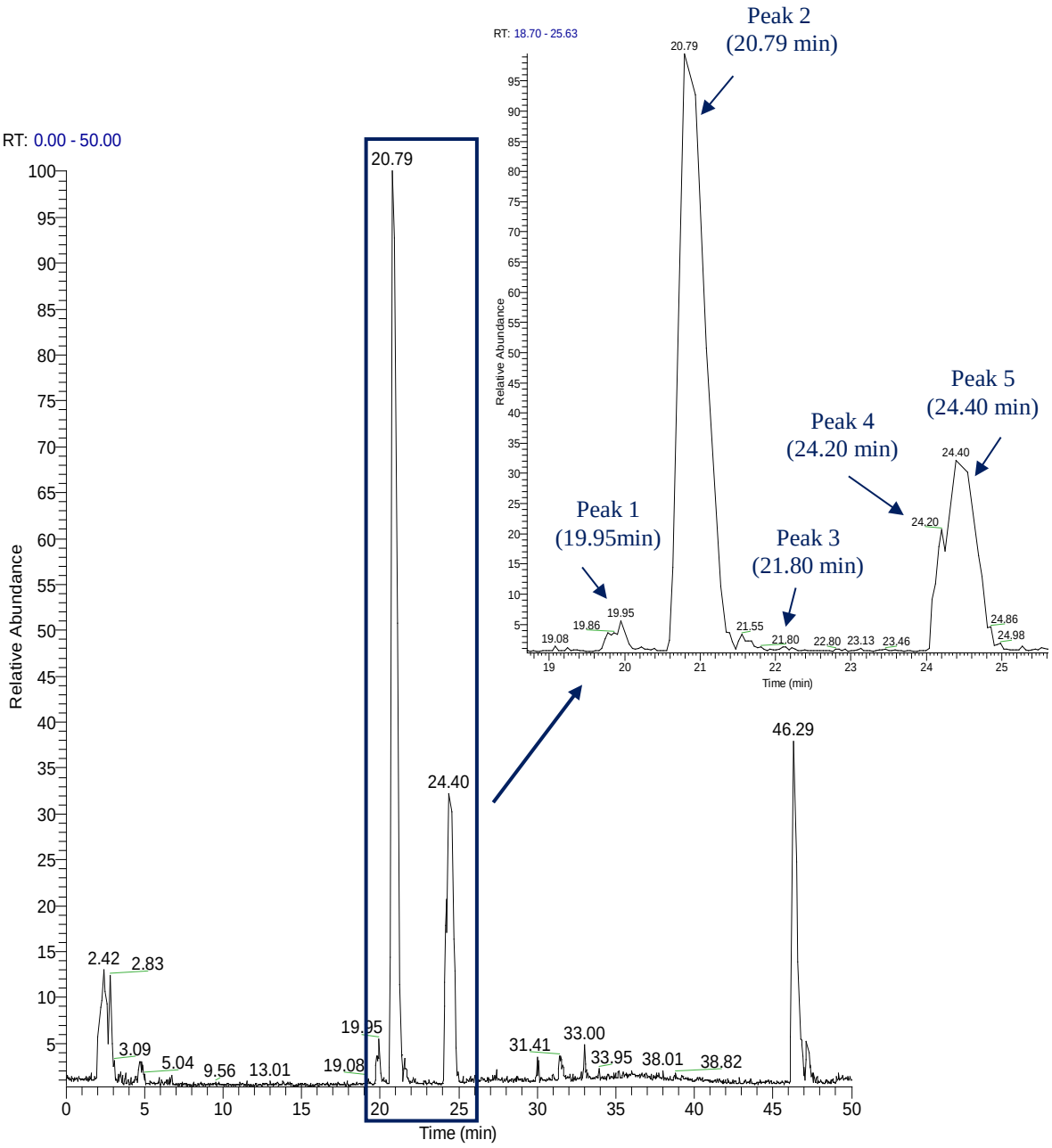


Figure 5.21 HPLC-ESI-MS TIC (scanning mass range 120-2000 amu) of the alkaline hydrolysate Sumo bok choy extract. The five flavonol glycoside conjugates that were identified in the data dependent studies are labelled peaks 1-5 in the insert.

Identification of the major flavonol aglycones present in the three cultivars of bok choy, after acid hydrolysis, revealed that quercetin (*m/z* 301), isorhamnetin (*m/z* 315) and kaempferol (*m/z* 285) where present in each of the extracts (5.2.1). The five flavonol glycoside conjugates (labelled 1-5 in Figure 5.21) that were identified in the alkaline hydrolysate extracts of the bok choy cultivars all contained one of the three flavonol aglycone ions in the MS^3 spectra. Structure elucidation was then based on the particular aglycone ion observed in the MS^3 spectra (i.e. *m/z* 301, 315, or 285). Only the data for the Sumo cultivar are presented in this section as the same five compounds were present in all three varieties.

Analysis of the UV-Vis spectra obtained from the PDA chromatograms of the alkaline hydrolysate bok choy extracts suggested that many of the compounds present were glycosylated, through the presence of a broad maximum at approximately 350 nm [57] (5.3.1). It has previously been reported that sugar substitution on flavonols usually occurs as *O*-glycosides, mainly at the 3-, 7-, and 4′-positions [225]. The UV-Vis spectra for the five compounds identified in the alkaline hydrolysate extracts all showed a broad maximum at ≈ 350 nm which suggested that the 3-hydroxyl on the flavonol core is blocked, and therefore there is glycosylation occurring at this position [60, 57]. Glycosylation was also assigned at position C7 based on similar studies in cauliflower, broccoli and pak choi [57, 128, 60]. Vallejo *et al.* [57] demonstrated that the first fragmentation from the deprotonated molecular ion $[M-H]^-$ is always due to the breakdown of the 7-*O*-glycosidic bond, which leads to a characteristic Y^{7-}_0 ion. This leads to a loss of 162 amu $[M-H-162]^-$ for mono-hexosides or a loss of 324 amu $[M-H-324]^-$ for di-hexosides [225]. Analysis of the flavonol compounds detected in the alkaline hydrolysate bok choy extracts suggested that peaks 1 and 2 were glycosylated at the 7-position with a clear Y^{7-}_0 $[M-H-162]^-$ ion obtained in the MS^2 spectrum, whereas peaks 3-5 showed multiple ions in their MS^2 spectra suggesting that they were either 3,7-diglycosides or un-substituted at position C7. As reported by Rochfort *et al.* [60], 3,7-diglycosides are characterised by a Y^{7-}_0 $[M-H-162]^-$ ion appearing in the MS^2, whereas 3-diglycosides are characterised by a Y^{3-}_0 $[M-H-324]^-$ ion. Furthermore, the 3-diglycosides are identified as sophorosides (Glucose 1→2 Glucose) by characteristic fragments corresponding to $^{0,2}X_0^-$ $[M-H-120]^-$ and Z_1^- $[M-H-180]^-$ ions that are typically not detected for 1→6 glycosides [225, 60]. The Z_1^- $[M-H-180]^-$ ion was not present at all for peaks 3-5, and although the $^{0,2}X_0^-$ $[M-H-120]^-$ and Y^{3-}_0 $[M-H-324]^-$ ions were, their relative abundances were much lower as compared to the Y^{7-}_0 $[M-H-162]^-$ ion, which was the base peak in all three of the MS^2 spectra for those peaks. Therefore, it was deduced that position C3 was not substituted with a sophoroside and peaks 3-5 were all identified as 3,7-diglycosides.

The characterisation and structure elucidation data for each of the five peaks are presented in the subsequent sections as follows:

- A flow diagram showing the fragmentation of ions selected by the data dependent MS^n protocol
- The ESI-MS^n spectra
- The UV-Visible spectrum
- The chemical structure elucidation and fragmentation pattern flow diagram

5.3.2.1 Identification and Structure Elucidation of Peak One in the Alkaline Hydrolysate Bok Choy Extracts

Peak 1 had a retention time of 19.95 min in the HPLC-ESI-MS TIC of the alkaline hydrolysate Sumo bok choy extract shown in Figure 5.21 and was identified as quercetin-3-sophoroside-7-glucoside. The flow diagram shown in Figure 5.22 below depicts the product ion spectra selected from the data dependent information obtained, whereby only the most intense ions were investigated. The MS^n spectra for these ions are shown in Figure 5.23.

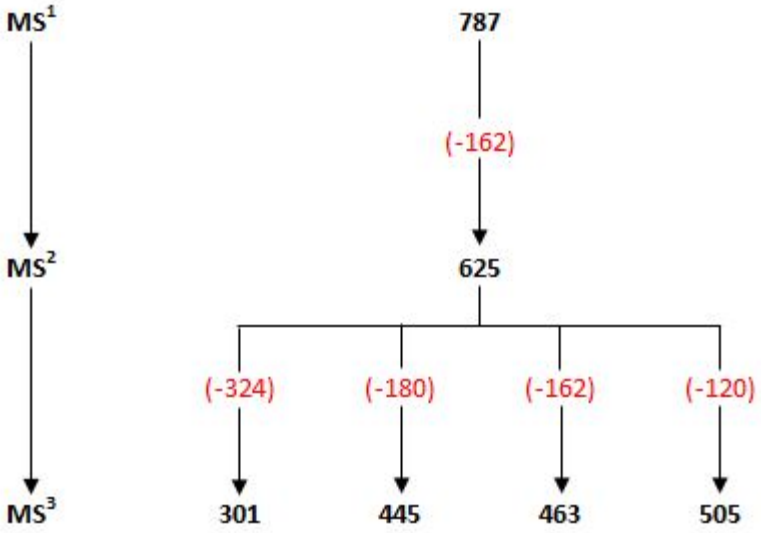


Figure 5.22 Breakdown of the MS^n fragmentation of ions in peak 1 of the alkaline hydrolysate bok choy extract, as selected by the data dependent protocol.

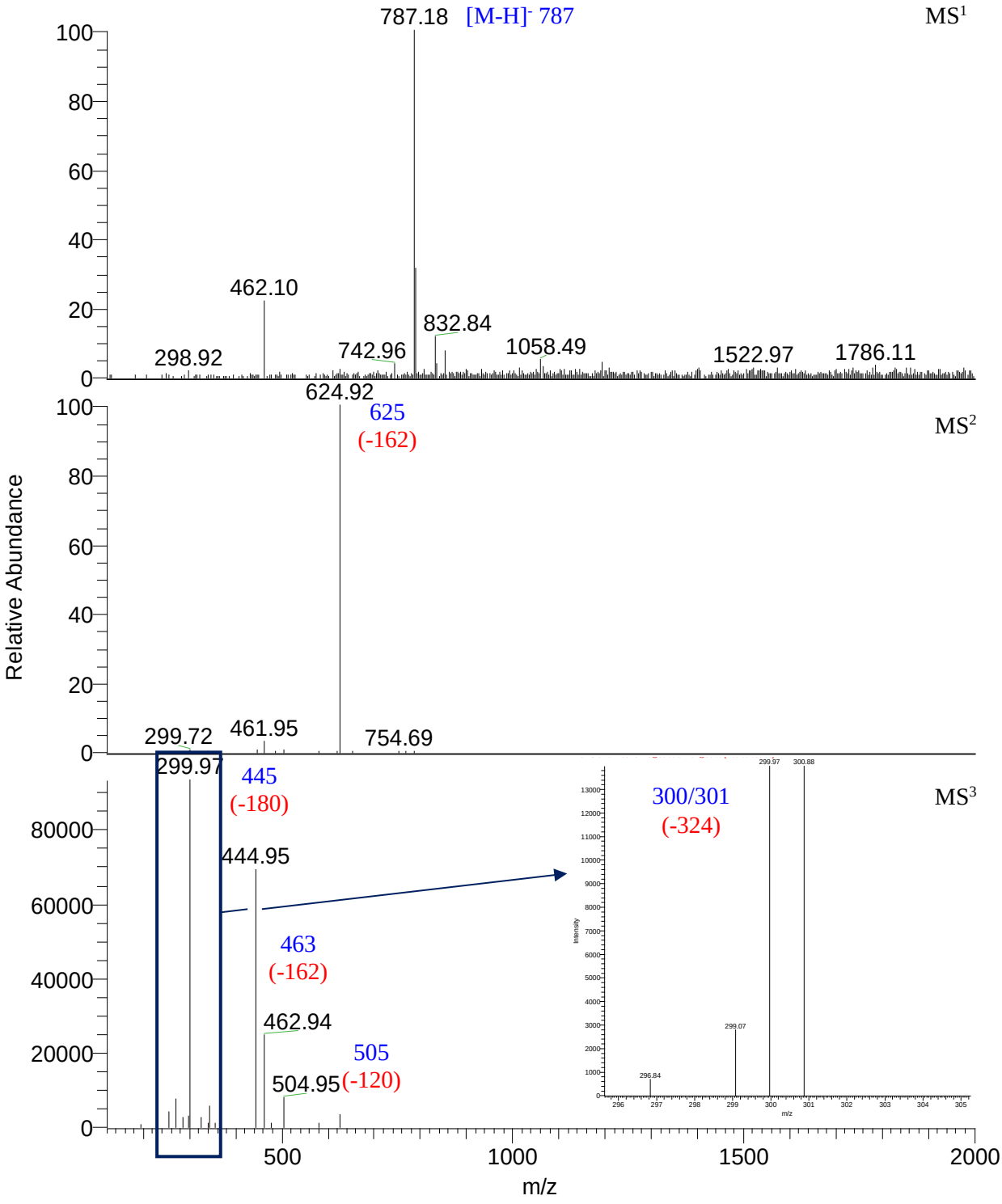


Figure 5.23 MS^n spectra collected during data dependent studies on peak 1 of the alkaline hydrolysate bok choy extract.

It was firstly confirmed that the core structure of the flavonol compound was quercetin due to the ion at *m/z* 301 (Y^{7}_{0} $Y^{3}_{0}$$^{-}$ = [M-H-162-324]$^{-}$) displayed in the MS^3 spectrum, as shown in the insert of Figure 5.23. The MS^2 spectrum showed a single product ion Y^{7-}_{0} (*m/z* 625) which resulted from the loss of a 162 amu fragment (glucose unit without the glycosidic oxygen); this signified the fragmentation of the aglycone from the C7 position. The MS^3 spectrum showed four product ions, including the aglycone Y^{7}_{0} $Y^{3}_{0}$$^{-}$(*m/z* 301) and three others: Y^{7}_{0} $^{0,2}X^{3}_{1}$$^{-}$ (*m/z* 505), Y^{7}_{0} $Y^{3}_{1}$$^{-}$ (*m/z* 463), and Y^{7}_{0} $Z^{3}_{1}$$^{-}$ (*m/z* 445), which resulted from losses of 324 amu, 120 amu, 162 amu, and 180 amu from the Y^{7-}_{0} (*m/z* 625) product ion respectively. This MS^3 fragmentation pattern is typical of a sophoroside (Glucose 1→2 Glucose) where the 324 amu fragment represents two sugars attached to the C3 position of the aglycone. This was further supported by the UV-Vis spectrum shown in Figure 5.24, where the UV-Vis absorbance maximum at approximately 350 nm indicated glycosylation at the C3 position.

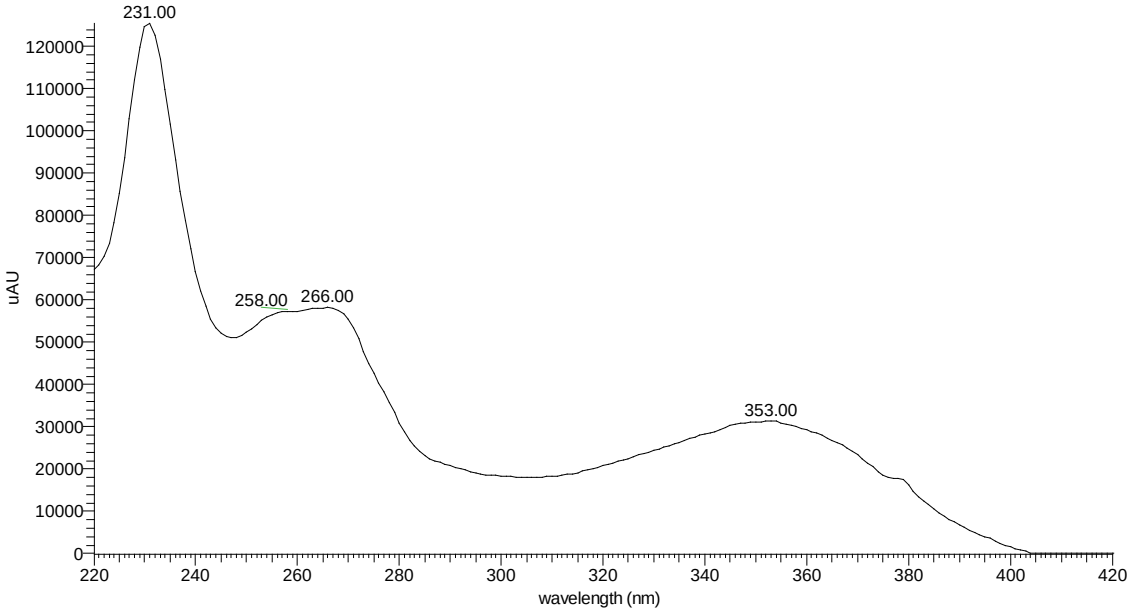


Figure 5.24 UV-Vis spectrum of peak 1 from the alkaline hydrolysate bok choy extract.

The high abundance of the [M-H-162-162]$^{-}$ (*m/z* 463) and [M-H-162-180]$^{-}$ (*m/z* 445) ions also supported the sophoroside identity. The -120 amu (fragmentation through the sugar ring), -162 amu (loss of glucose without the glycosidic oxygen), and -180 amu (loss of glucose with the glycosidic oxygen) represented losses from the sophoroside moiety. Peak 1 was therefore identified as quercetin-3-sophoroside-7-glucoside and the fragmentation of this compound as observed in the data dependent studies is shown in Figure 5.25. A summary of the above findings is displayed in Table 5.16 at the end of this section.

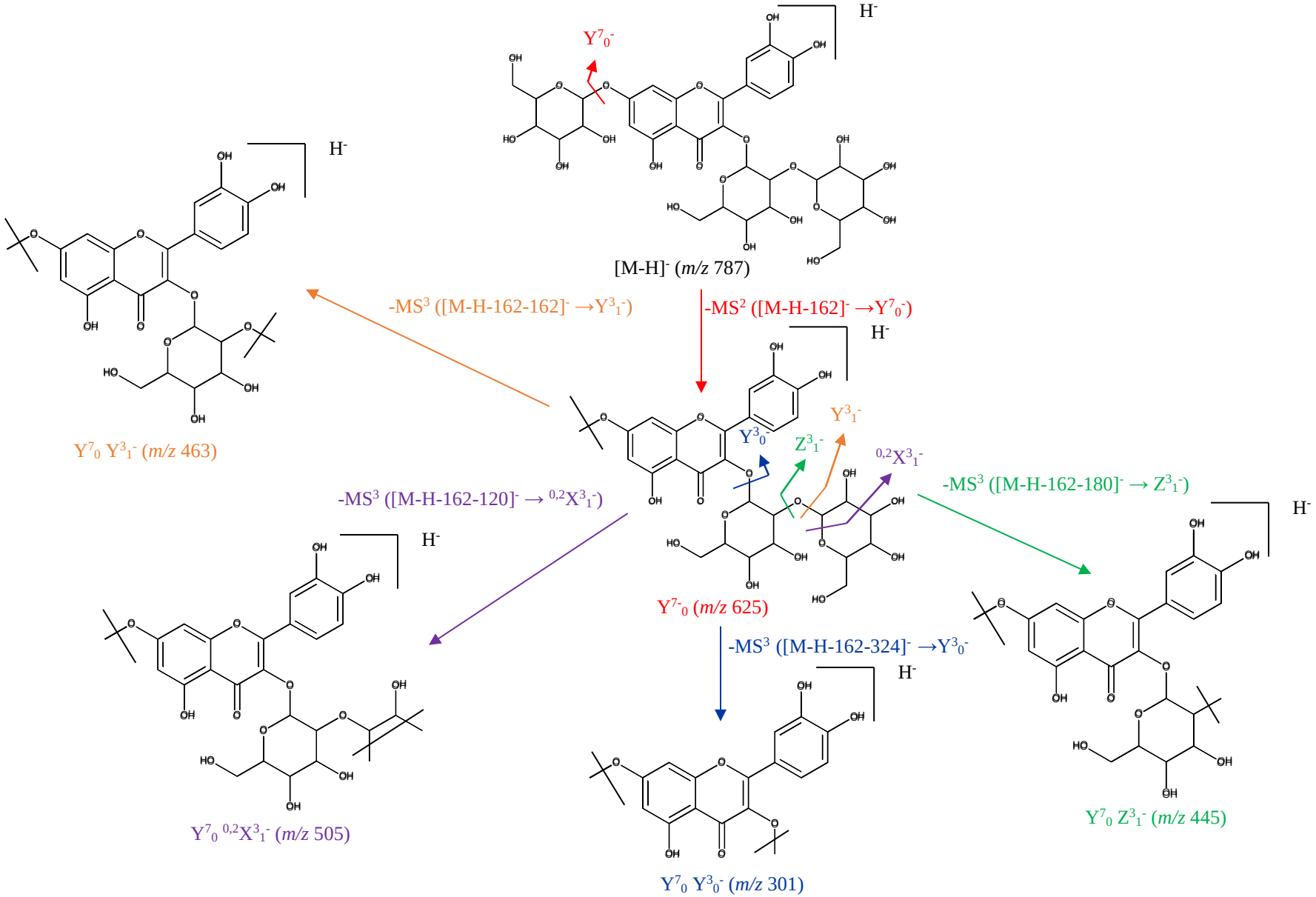


Figure 5.25 Flow diagram showing the chemical structure elucidation and fragmentation pattern of peak 1, identified as quercetin-3-sophoroside-7-glucoside.

5.3.2.2 Identification and Structure Elucidation of Peak Two in the Alkaline Hydrolysate Bok Choy Extracts

Peak 2 had a retention time of 20.79 min in the HPLC-ESI-MS TIC of the alkaline hydrolysate Sumo bok choy extract shown in Figure 5.21 and was identified as kaempferol-3-sophoroside-7-glucoside. The flow diagram shown in Figure 5.26 below depicts the product ion spectra selected from the data dependent information obtained, whereby only the most intense ions were investigated. The MS^n spectra for these ions are shown in Figure 5.27.

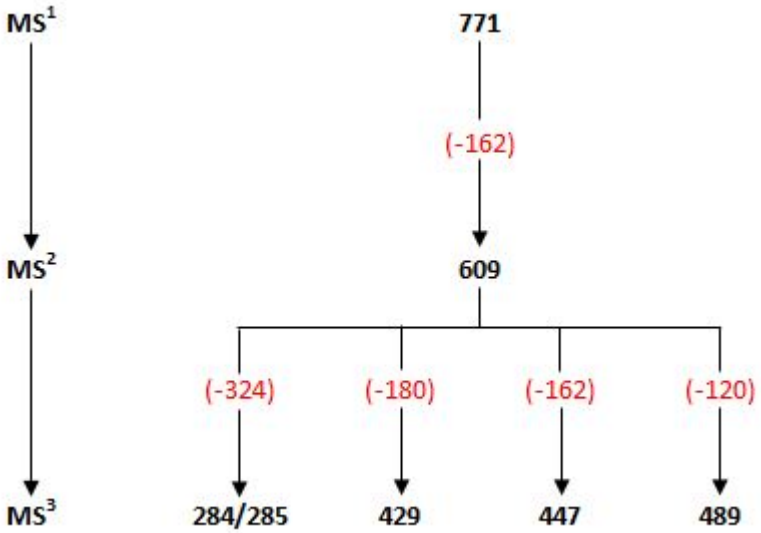


Figure 5.26 Breakdown of the MS^n fragmentation of ions in peak 2 of the alkaline hydrolysate bok choy extract, as selected by the data dependent protocol.

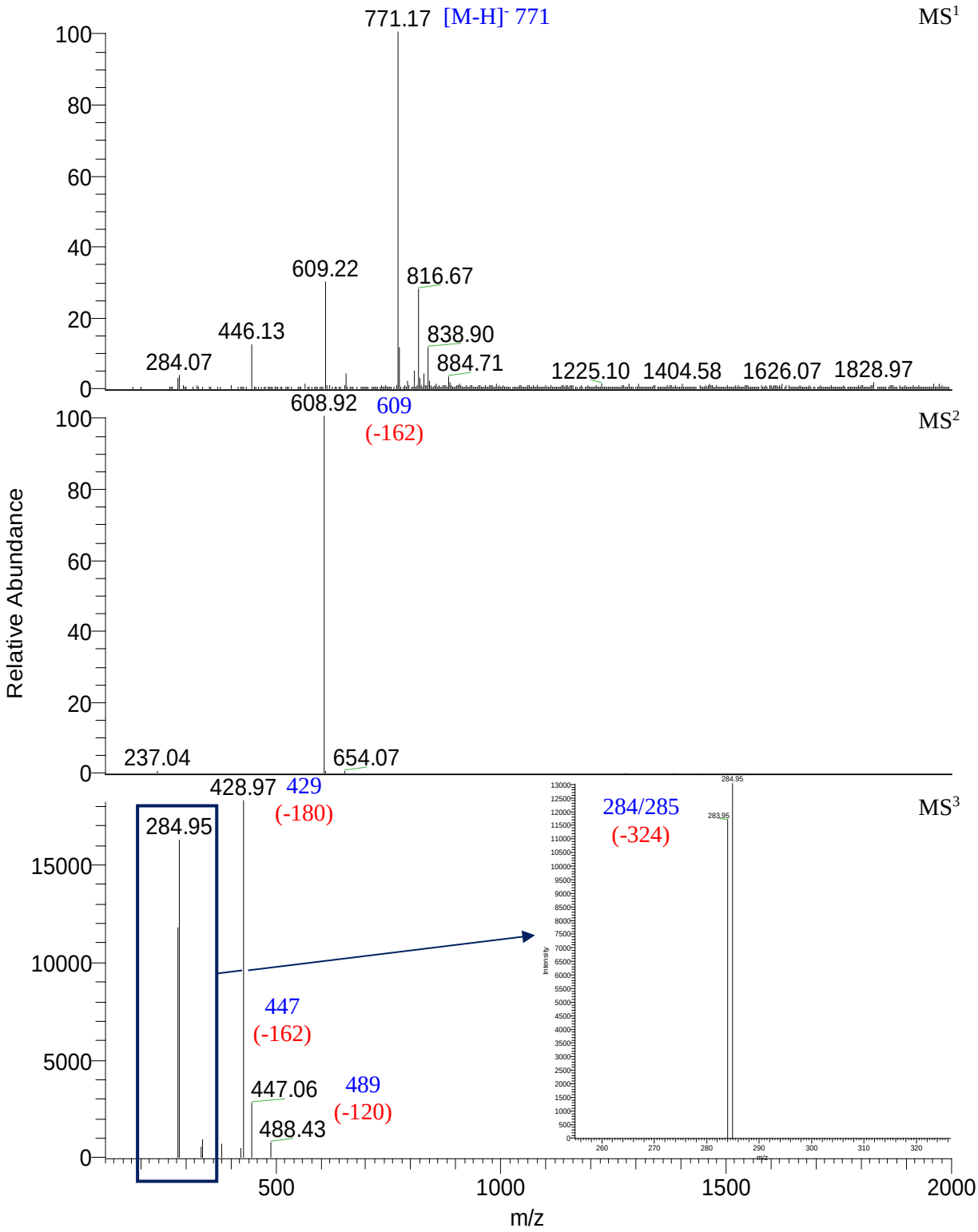


Figure 5.27 MS^n spectra collected during data dependent studies on peak 2 of the alkaline hydrolysate bok choy extract.

Peak 2 displayed an ion at *m/z* 285 ($Y^7_0\ Y^3_0{}^-$ = [M-H-162-324]$^-$) in the MS^3 spectrum, which indicated that the core structure of the flavonol compound was kaempferol, as shown in the insert of Figure 5.27. The MS^2 spectrum showed a single product ion Y^{7-}_0 (*m/z* 609) and the MS^3 spectrum consisted of a total of four product ions, including the aglycone $Y^7_0\ Y^3_0{}^-$ (*m/z* 285) and three others: $Y^7_0\ {}^{0,2}X^3_1{}^-$ (*m/z* 489), $Y^7_0\ Y^3_1{}^-$ (*m/z* 447), and $Y^7_0\ Z^3_1{}^-$ (*m/z* 429). The MS^n fragmentation of peak 2 matched that of peak 1, and therefore the same reasoning that was used to identify peak 1 was also applied to peak 2. It was once again determined that there was a single glucose moiety attached to the C7 position and that there were two sugar moieties conjugated at the C3 position of the flavonol core. Furthermore, the UV-Vis spectrum of peak 2 (Figure 5.28) supported this determination, where the broad absorbance maximum at approximately 350 nm indicated that there was glycosylation at the C3 position.

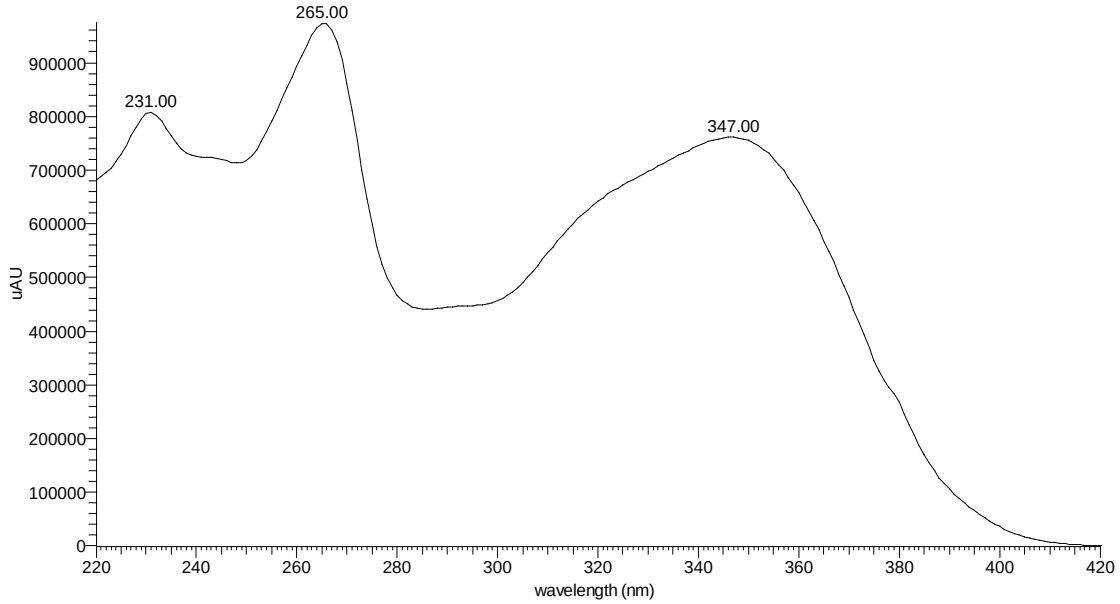


Figure 5.28 UV-Vis spectrum of peak 2 from the alkaline hydrolysate bok choy extract.

The ions present in the MS^3 spectrum and their relative abundances were typical of a sophoroside, hence the diglycoside conjugated at the C3 position was identified as a sophoroside. Peak 2 was therefore identified as kaempferol-3-sophoroside-7-glucoside and the fragmentation of this compound as observed in the data dependent studies is shown in Figure 5.29. A summary of the above findings is displayed in Table 5.16 at the end of this section.

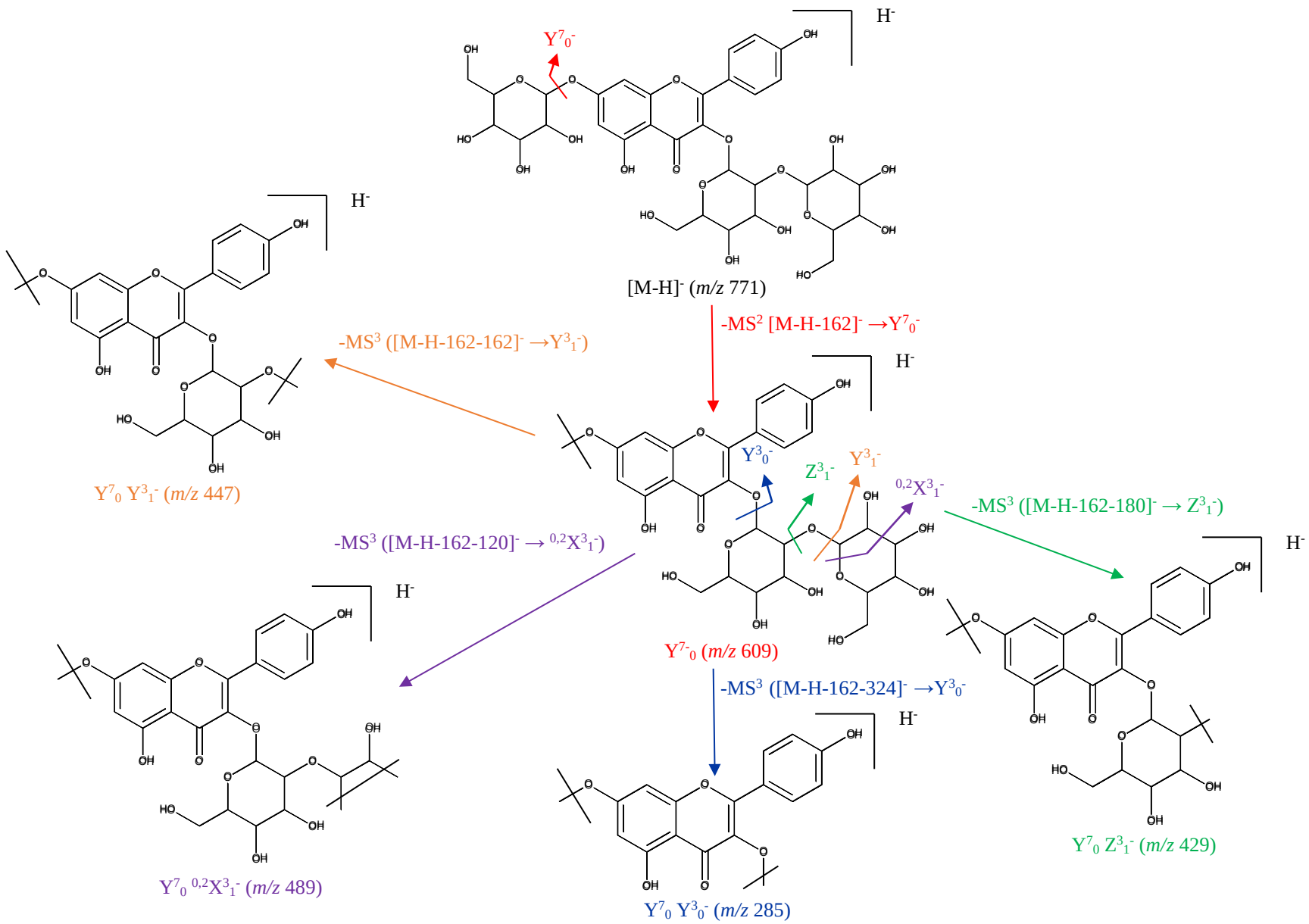


Figure 5.29 Flow diagram showing the chemical structure elucidation and fragmentation pattern of peak 2, identified as kaempferol-3-sophoroside-7-glucoside.

5.3.2.3 Identification and Structure Elucidation of Peak Three in the Alkaline Hydrolysate Bok Choy Extracts

Peak 3 had a retention time of 21.80 min in the HPLC-ESI-MS TIC of the alkaline hydrolysate Sumo bok choy extract shown in Figure 5.21, and was identified as quercetin-3,7-diglucoside. The flow diagram shown in Figure 5.30 below depicts the product ion spectra selected from the data dependent information obtained, whereby only the most intense ions were investigated. The MS^n spectra for these ions are shown in Figure 5.31. The MS^1 spectrum displayed significant noise due to the fact that peak 3 was only slightly more intense than the baseline noise (refer to Figure 5.21).

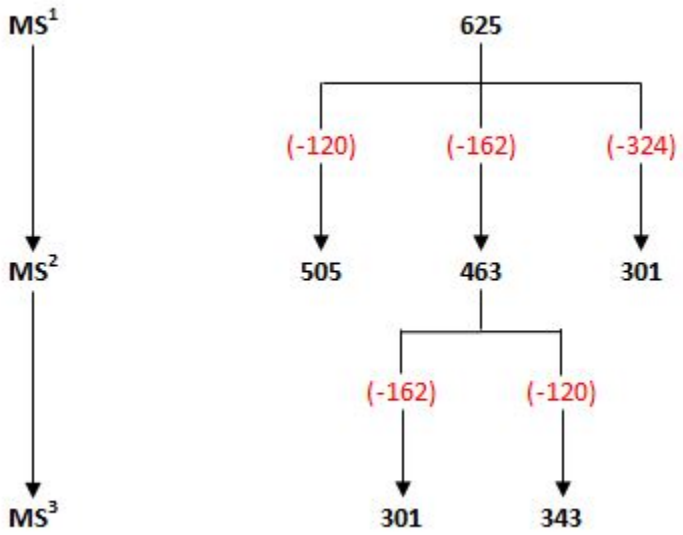


Figure 5.30 Breakdown of the MS^n fragmentation of ions in peak 3 of the alkaline hydrolysate bok choy extract, as selected by the data dependent protocol.

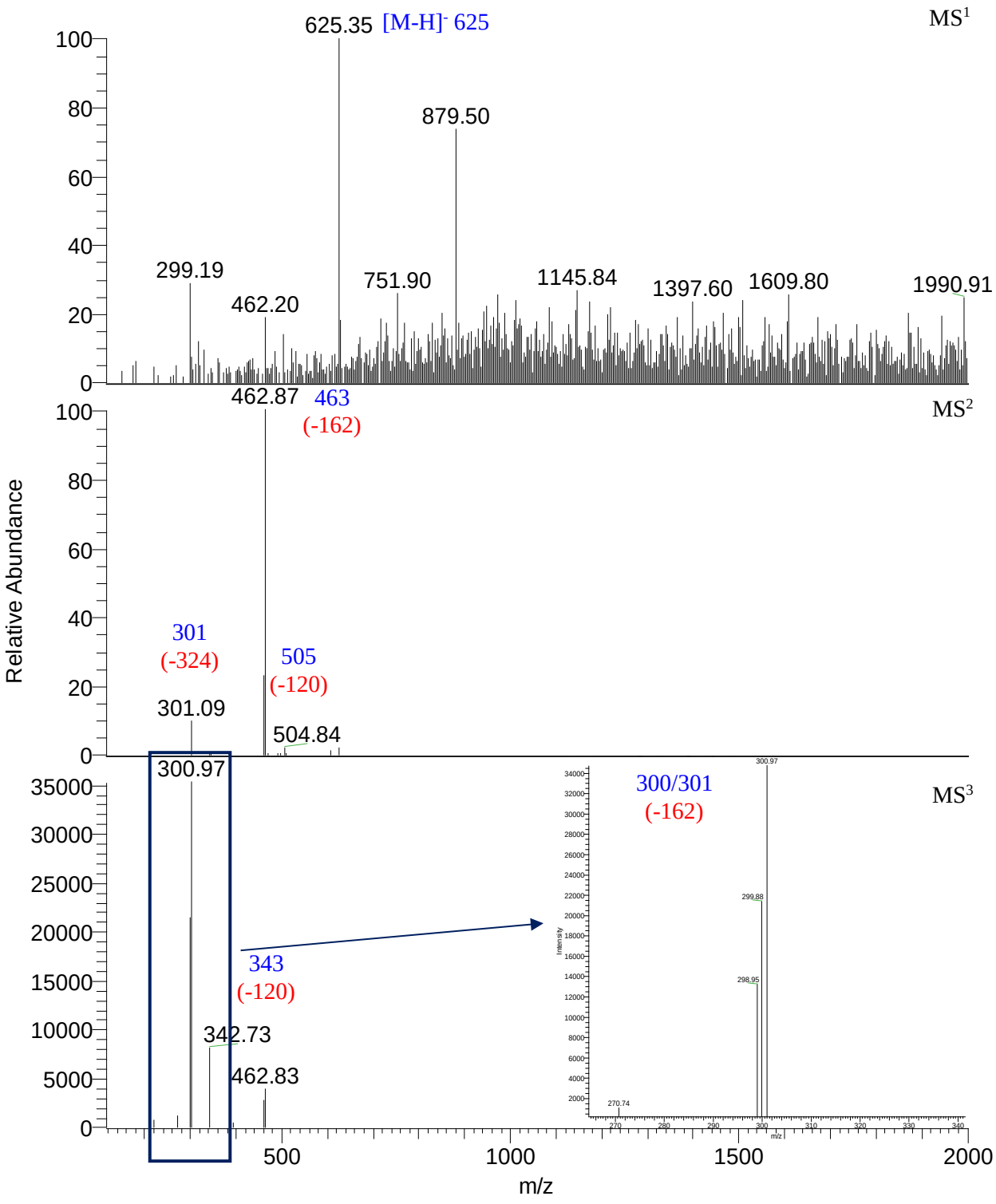


Figure 5.31 MS^n spectra collected during data dependent studies on peak 3 of the alkaline hydrolysate bok choy extract.

Peak 3 displayed an ion at *m/z* 301 ($Y^7_0\ Y^3_0{}^-$ = [M-H-162-162]$^-$) in the MS^3 spectrum, which indicated that the core structure of the flavonol compound was quercetin, as shown in the insert of Figure 5.31. The MS^2 spectrum showed multiple ions, unlike the first two peaks in the alkaline hydrolysate bok choy extract, which only produced a single product ion Y^{7-}_0 = [M-H-162]$^-$. In this case, the ions displayed in the MS^2 spectrum consisted of the following: $^{0,2}X^3_0{}^-$ (*m/z* 505), Y^{7-}_0 (*m/z* 463), and $Y^7_0\ Y^3_0{}^-$ (*m/z* 301) resulting from losses of 120 amu, 162 amu, and 324 amu from the molecular ion (*m/z* 625) respectively. This type of MS^2 fragmentation pattern indicates that the flavonol compound is either a 3,7-diglucoside, or has no sugar substituted at the C7 position. The base peak in the MS^2 spectrum was Y^{7-}_0 (*m/z* 463), which resulted from the loss of a 162 amu fragment (glucose unit without the glycosidic oxygen). This signified the fragmentation of the flavonol aglycone core from the C7 position, therefore, it was determined that the compound is a 3,7-diglucoside. The three ions observed in the MS^2 spectrum represented the fragmentation through the sugar ring ($^{0,2}X^3_0{}^-$ = [M-H-120]$^-$), the loss of one glucose unit (Y^{7-}_0 = [M-H-162]$^-$), and the loss of two glucose units ($Y^7_0\ Y^3_0{}^-$ = [M-H-324]$^-$). Glycosylation at the C3 position was supported by the UV-Vis spectrum shown in Figure 5.32, which displayed the typical broad absorbance maximum at approximately 350 nm.

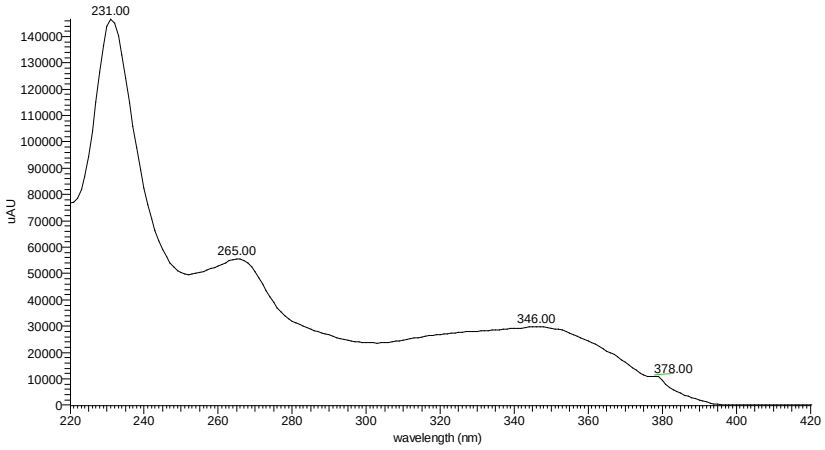


Figure 5.32 UV-Vis spectrum of peak 3 from the alkaline hydrolysate bok choy extract.

The MS^3 spectrum for peak 3 showed two product ions: these were the aglycone $Y^7_0\ Y^3_0{}^-$ (*m/z* 301), and one other product ion $Y^7_0\ ^{0,2}X^3_0{}^-$ (*m/z* 343) which resulted from losses of 162 amu and 120 amu from the Y^{7-}_0 (*m/z* 463) product ion respectively. These two ions represented the fragmentation through the sugar ring ($Y^7_0\ ^{0,2}X^3_0{}^-$ = [M-H-162-120]$^-$) and the loss of the glucose unit conjugated at the C3 position ($Y^7_0\ Y^3_0{}^-$ = [M-H-162-162]$^-$). Peak 3 was therefore identified as quercetin-3,7-diglucoside and the fragmentation of this compound as observed in the data dependent studies is shown in Figure 5.33. A summary of the above findings is displayed in Table 5.16 at the end of this section.

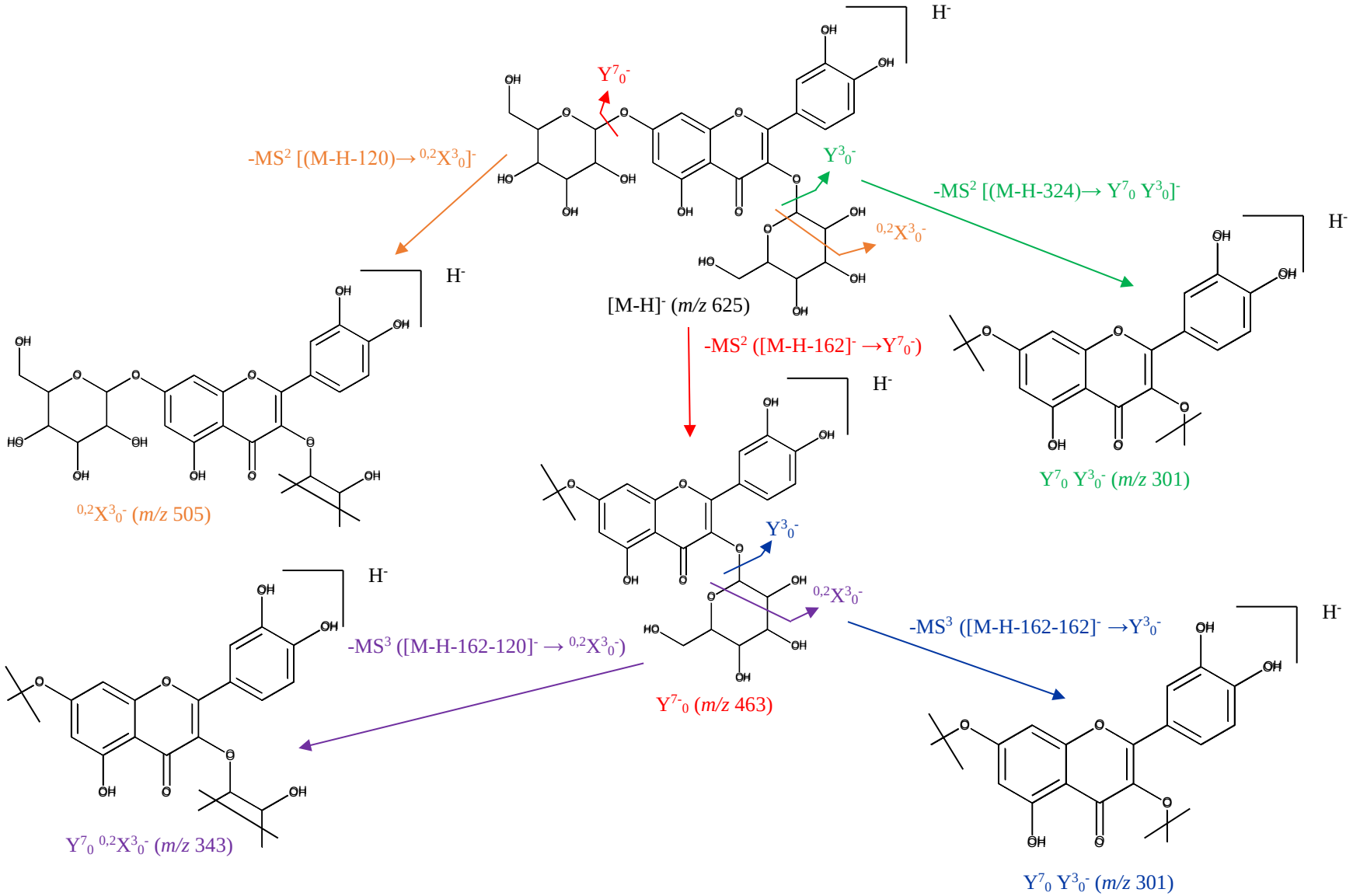


Figure 5.33 Flow diagram showing the chemical structure elucidation and fragmentation pattern of peak 3, identified as quercetin-3,7-diglucoside.

5.3.2.4 Identification and Structure Elucidation of Peak Four in the Alkaline Hydrolysate Bok Choy Extracts

Peak 4 had a retention time of 24.20 min in the HPLC-ESI-MS TIC of the alkaline hydrolysate Sumo bok choy extract shown in Figure 5.21, and was identified as kaempferol-3,7-diglucoside. Peak 4 appeared as a shoulder on peak 5, which had a greater abundance in the TIC. The flow diagram shown in Figure 5.34 below depicts the product ion spectra selected from the data dependent information obtained, whereby only the most intense ions were investigated. The MS^n spectra for these ions are shown in Figure 5.35.

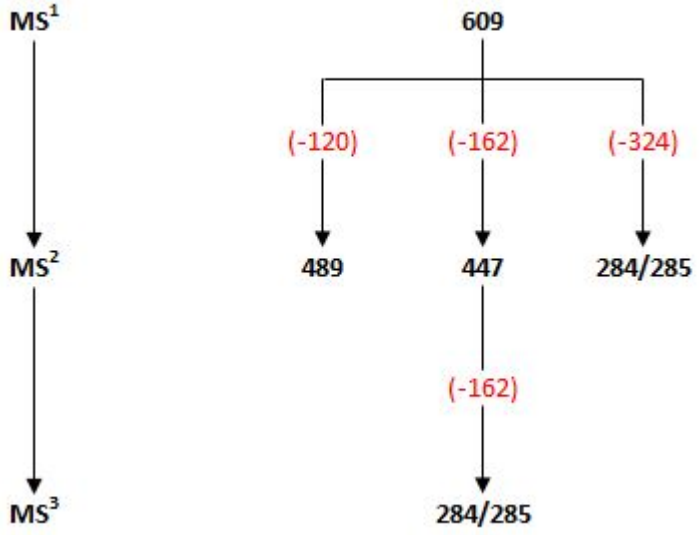


Figure 5.34 Breakdown of the MS^n fragmentation of ions in peak 4 of the alkaline hydrolysate bok choy extract, as selected by the data dependent protocol.

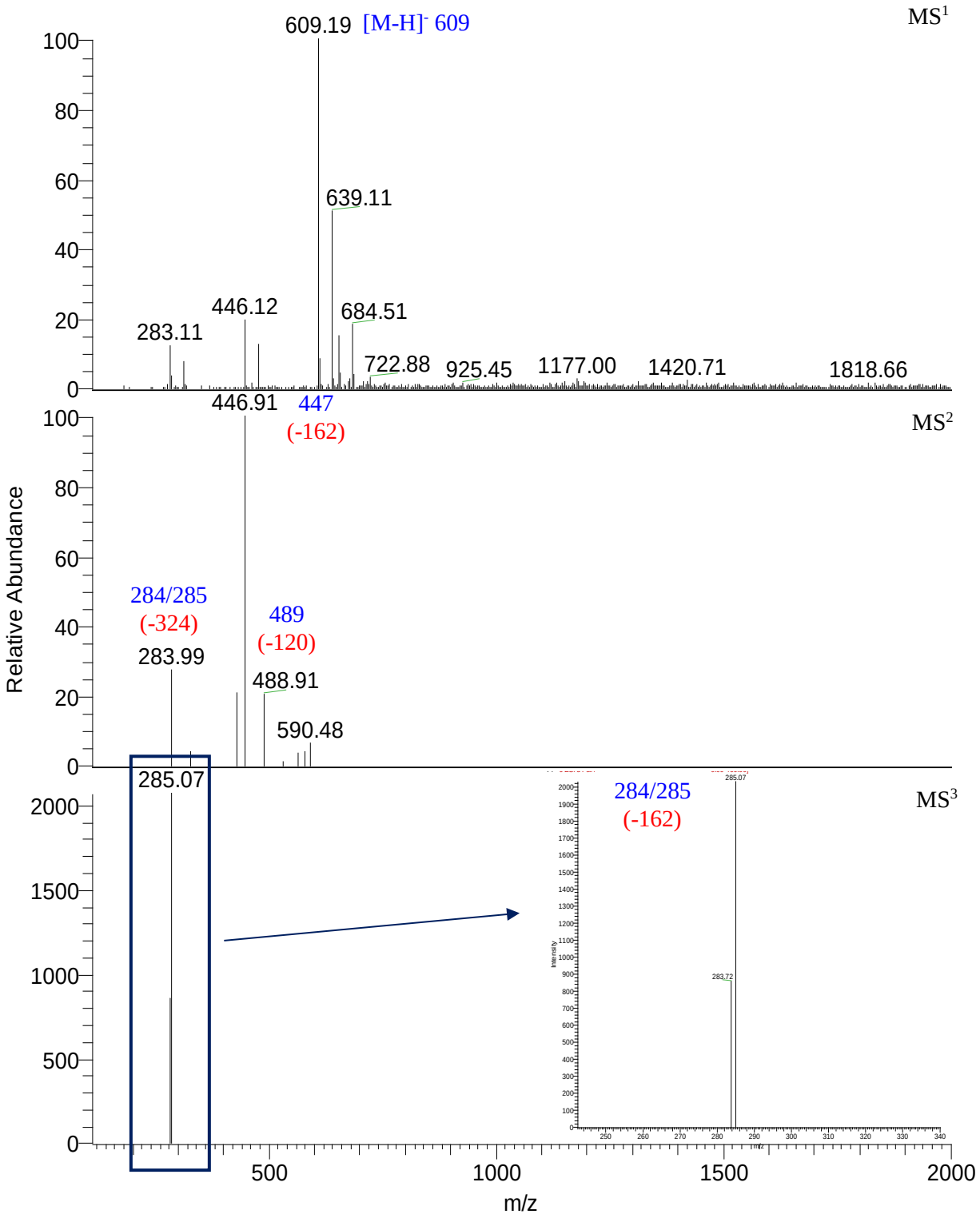


Figure 5.35 MS^n spectra collected during data dependent studies on peak 4 of the alkaline hydrolysate bok choy extract.

Peak 4 displayed an ion at *m/z* 285 ($Y^7_0\ Y^3_0{}^-$ = [M-H-162-162]$^-$) in the MS^3 spectrum, which indicated that the core structure of the flavonol compound was kaempferol, as shown in the insert of Figure 5.35. This was also the only ion that was displayed in the MS^3 spectrum. The MS^2 spectrum showed multiple ions and displayed the same fragmentation pattern as peak 3. These consisted of the following: $^{0,2}X^3_0{}^-$ (*m/z* 489), Y^{7-}_0 (*m/z* 447), and $Y^7_0\ Y^3_0{}^-$ (*m/z* 285) which resulted from losses of 120 amu, 162 amu, and 324 amu from the molecular ion (*m/z* 609) respectively. Once again (as seen for peak 3), the base peak in the MS^2 spectrum was the Y^{7-}_0 (*m/z* 447) ion. This, along with the broad absorbance maximum at 350 nm observed in the UV-Vis spectrum shown in Figure 5.36, indicated that positions C3 and C7 of the flavonol core were glycosylated, and hence the compound was a 3,7-diglucoside. It must also be noted, however, that peaks 4 and 5 co-eluted in the HPLC-PDA chromatogram, which might affect the purity of the UV-Vis spectra for these two compounds.

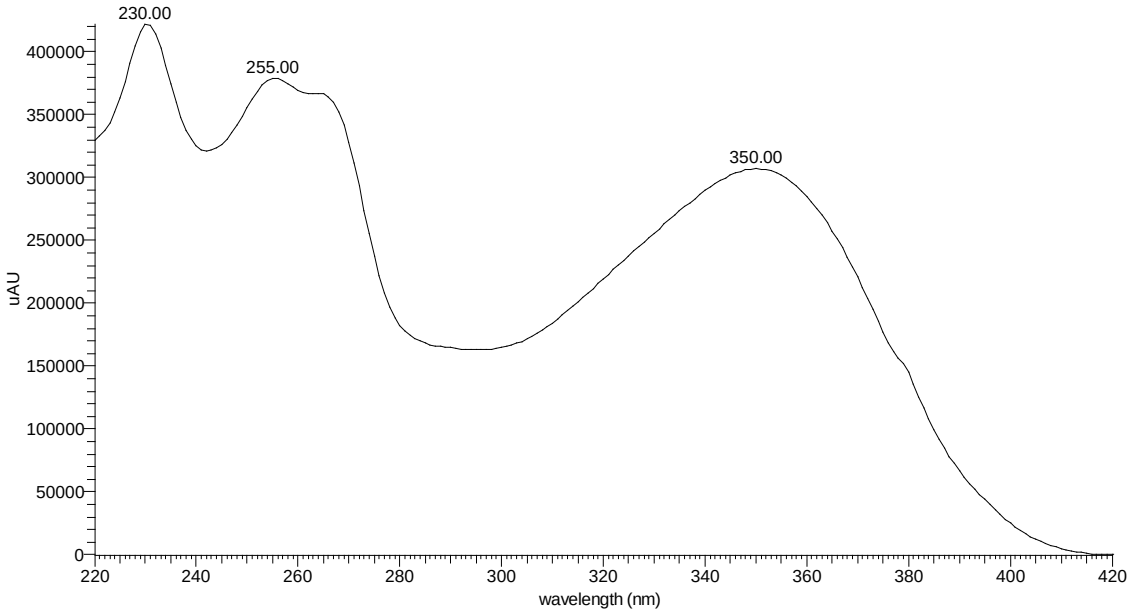


Figure 5.36 UV-Vis spectrum of peak 4 from the alkaline hydrolysate bok choy extract.

The MS^n fragmentation of peak 4 matched that of peak 3, apart from one less ion in the MS^3 spectrum ($Y^7_0\ ^{0,2}X^3_0{}^-$ = [M-H-162-120]$^-$), and the same reasoning that was used to identify peak 3 was also applied to peak 4. Peak 4 was therefore identified as kaempferol-3,7-diglucoside and the fragmentation of this compound as observed in the data dependent studies is shown in Figure 5.37. A summary of the above findings is displayed in Table 5.16 at the end of this section.

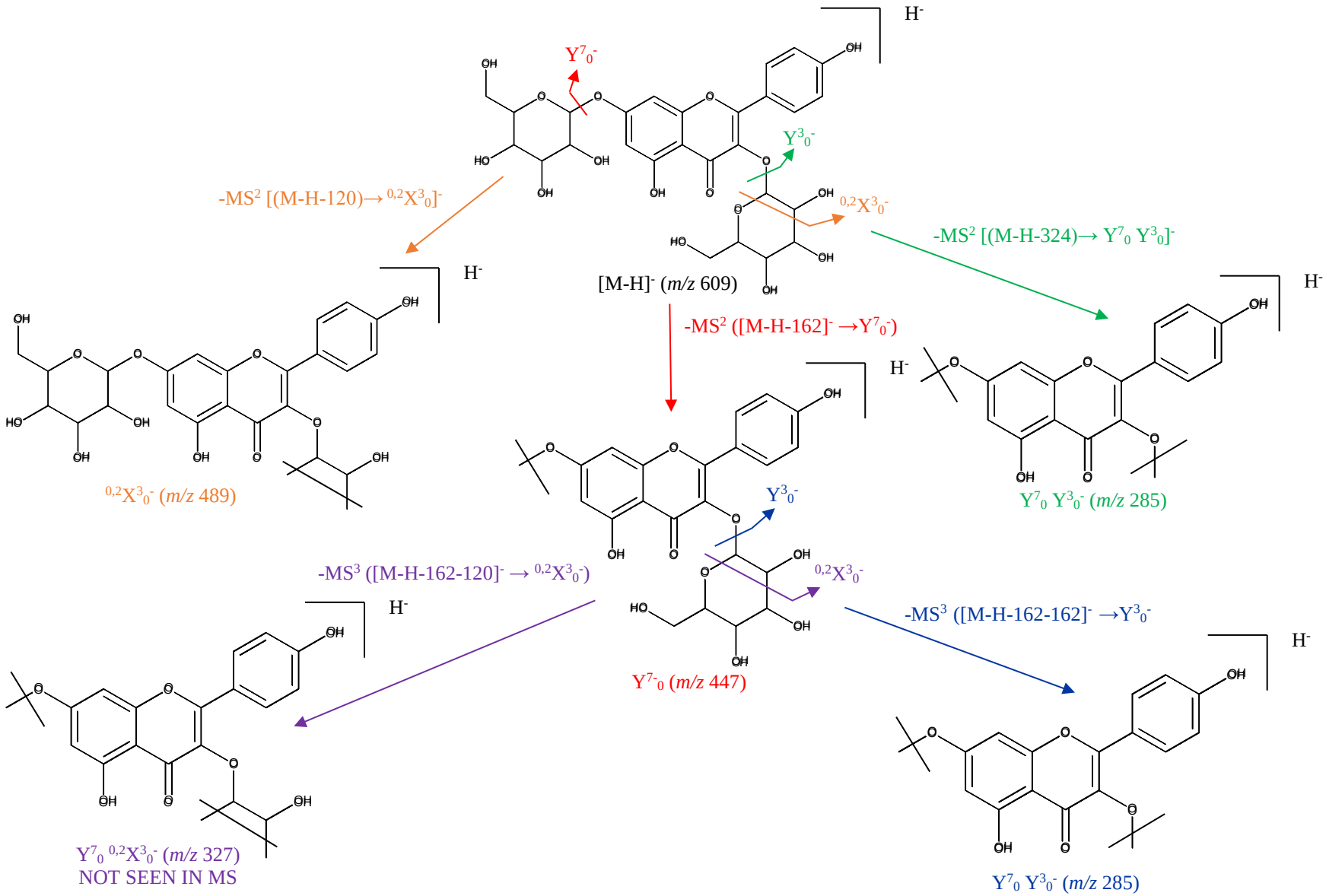


Figure 5.37 Flow diagram showing the chemical structure elucidation and fragmentation pattern of peak 4, identified as kaempferol-3,7-diglucoside.

5.3.2.5 Identification and Structure Elucidation of Peak Five in the Alkaline Hydrolysate Bok Choy Extracts

Peak 5 had a retention time of 24.40 min in the HPLC-ESI-MS TIC of the alkaline hydrolysate Sumo bok choy extract shown in Figure 5.21, and was identified as isorhamnetin-3,7-diglucoside. Peak 5 co-eluted with peak 4 in the TIC but was of slightly greater abundance than peak 4. The flow diagram shown in Figure 5.38 below depicts the product ion spectra selected from the data dependent information obtained, whereby only the most intense ions were investigated. The MS^n spectra for these ions are shown in Figure 5.39.

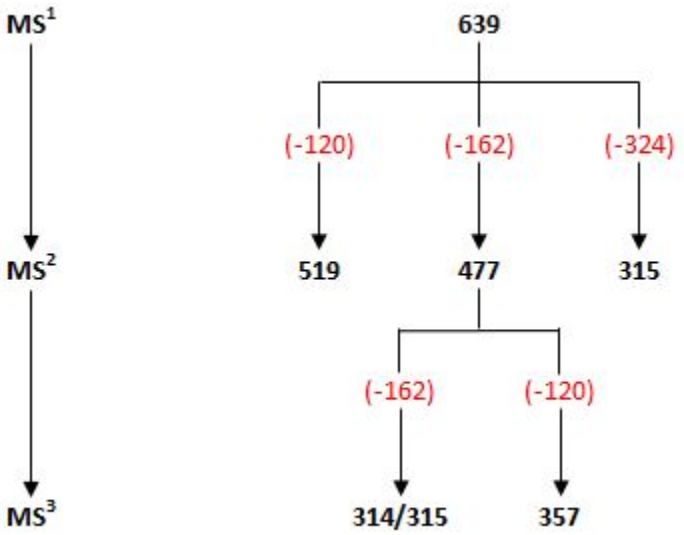


Figure 5.38 Breakdown of the MS^n fragmentation of ions in peak 5 of the alkaline hydrolysate bok choy extract, as selected by the data dependent protocol.

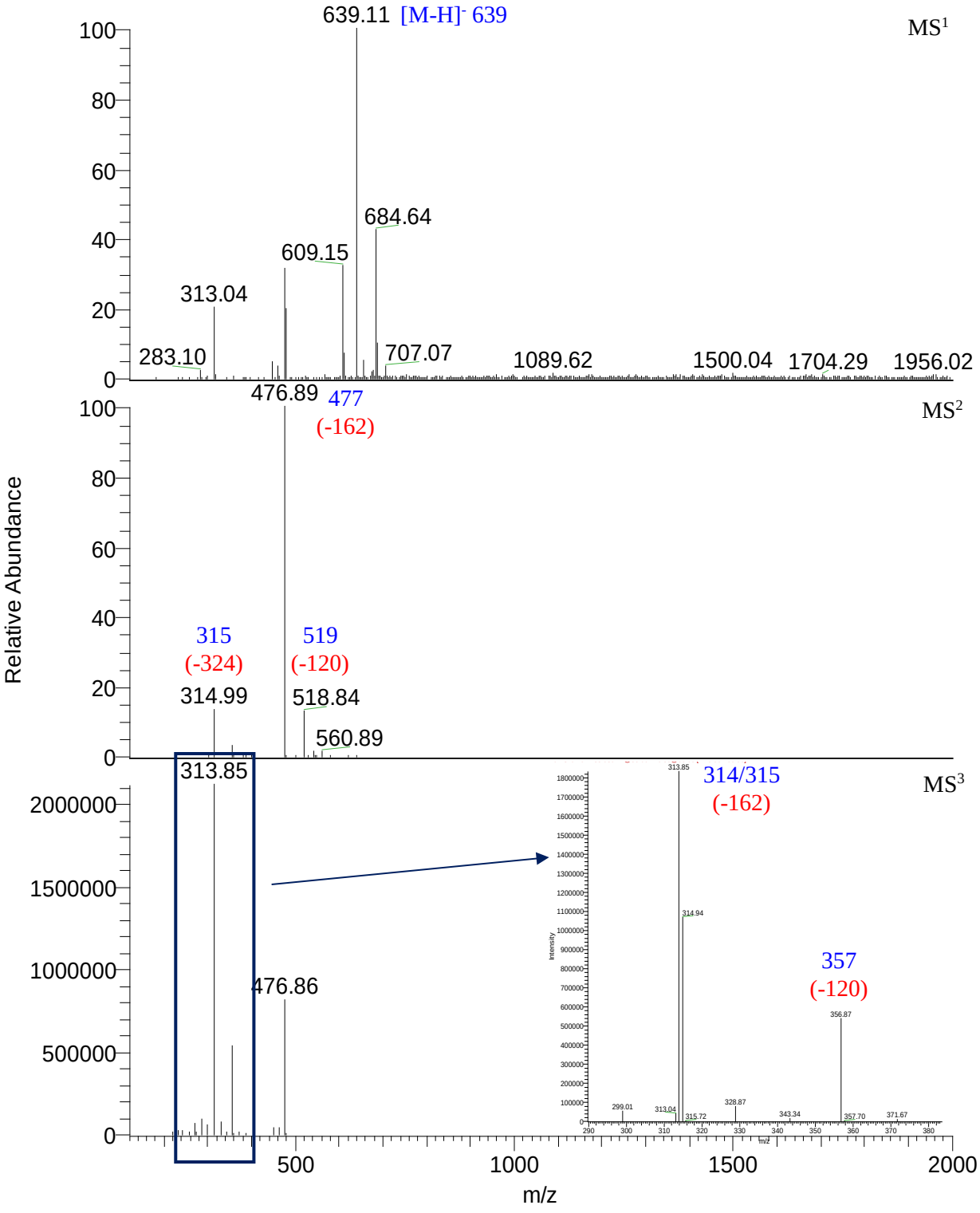


Figure 5.39 MS^n spectra collected during data dependent studies on peak 5 of the alkaline hydrolysate bok choy extract.

Peak 5 displayed an ion at *m/z* 315 ($Y^7_0\ Y^3_0{}^-$ = [M-H-162-162]$^-$) in the MS^3 spectrum, which indicated that the core structure of the flavonol compound was isorhamnetin, as shown in the insert of Figure 5.39. The MS^2 spectrum showed multiple ions, and displayed the same fragmentation pattern as peaks 3 and 4. These ions consisted of the following: $^{0,2}X^3_0{}^-$ (*m/z* 519), Y^{7-}_0 (*m/z* 477), and $Y^7_0\ Y^3_0{}^-$ (*m/z* 315) which resulted from losses of 120 amu, 162 amu, and 324 amu from the molecular ion (*m/z* 639) respectively. Once again (as seen for peaks 3 and 4), the base peak in the MS^2 spectrum was the Y^{7-}_0 (*m/z* 477) ion. This, along with the broad absorbance maximum at approximately 350 nm observed in the UV-Vis spectrum shown in Figure 5.40, indicated that positions C3 and C7 of the flavonol core were glycosylated, and hence the compound was a 3,7-diglucoside. It must also be noted, however, that peaks 4 and 5 co-eluted in the HPLC-PDA chromatogram, which might affect the purity of the UV-Vis spectra for these two compounds.

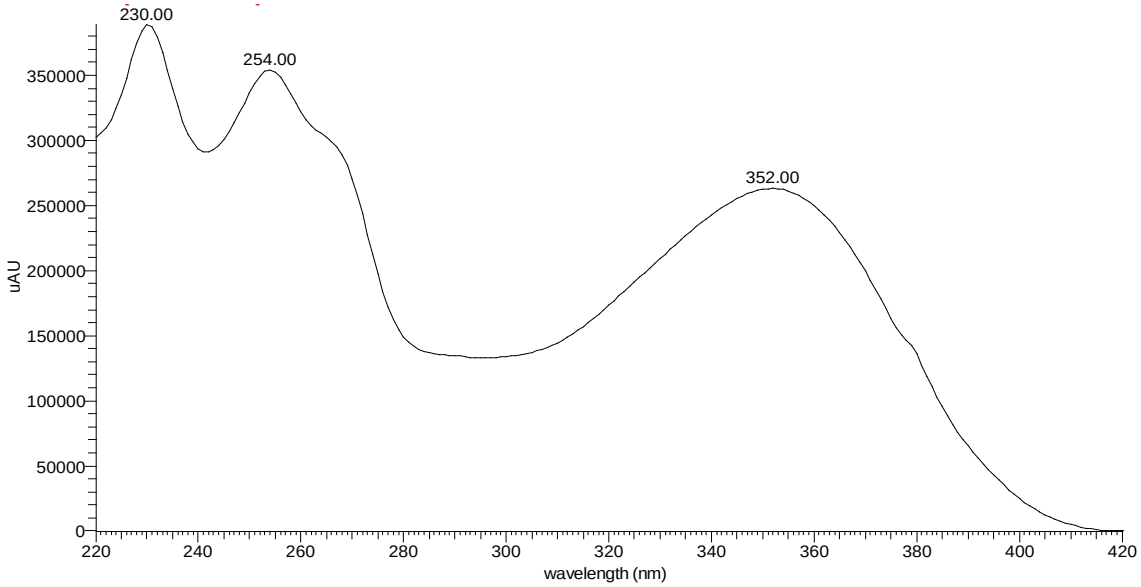


Figure 5.40 UV-Vis spectrum of peak 5 from the alkaline hydrolysate bok choy extract.

The MS^3 spectrum for peak 5 displayed two product ions that matched that of peak 3. These included the aglycone $Y^7_0\ Y^3_0{}^-$ (*m/z* 315), and one other product ion $Y^7_0\ ^{0,2}X^3_0{}^-$ (*m/z* 357) which resulted from losses of 162 amu and 120 amu from the Y^{7-}_0 (*m/z* 477) product ion respectively. Peak 5 was therefore identified as isorhamnetin-3,7-diglucoside, based on the same reasoning as peaks 3 and 4, and the fragmentation of this compound as observed in the data dependent studies is shown in Figure 5.41. A summary of the above findings is displayed in Table 5.16 at the end of this section.

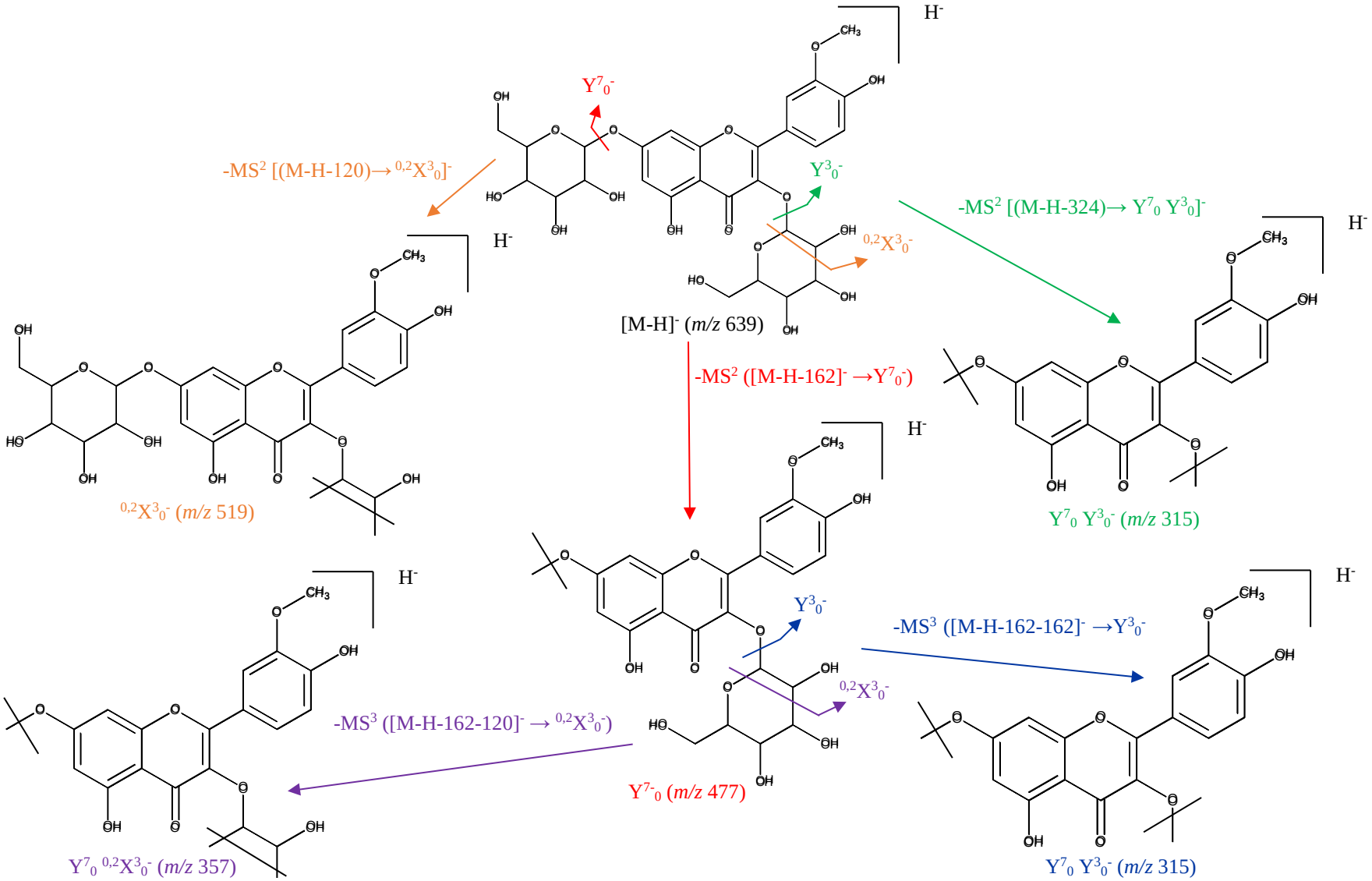
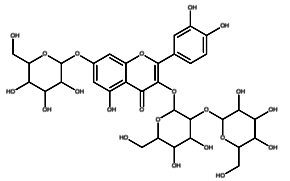
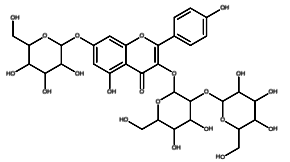
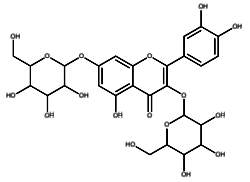
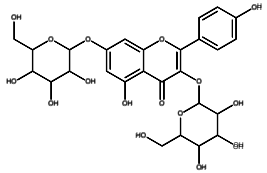
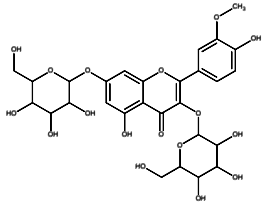


Figure 5.41 Flow diagram showing the chemical structure elucidation and fragmentation pattern of peak 5, identified as isorhamnetin-3,7-diglucoside.

Table 5.16 Characterisation and structure elucidation of flavonol glycoside conjugates in alkaline hydrolysate bok choy extracts. Percent abundance in parentheses. *peaks 4 and 5 co-eluted.

					MS^2 $[M-H]^-$ (*m/z*) (%)			MS^3 $[MS^2$ (100%)$]^-$ (*m/z*) (%)				
Peak	**Compound Identity**	**t_R (min)**	**λ_{max} (nm)**	**MS^1 $[M-H]^-$ (*m/z*)**	**$Y^7_0{}^-$ [M-H-162]$^-$**	**$^{0,2}X^3_0{}^-$ [M-H-120]$^-$**	**$Y^3_0{}^-$ [M-H-324]$^-$**	**$Y^7_0\,Y^3_1{}^-$ [M-H-162-162]$^-$**	**$Y^7_0\,{}^{0,2}X^3_1{}^-$ [M-H-162-120]$^-$**	**$Y^7_0\,Z^3_1{}^-$ [M-H-162-180]$^-$**	**$Y^7_0\,Y^3_0{}^-$ [M-H-162-324]$^-$**	**Structure**
1	Quercetin-3-sophoroside-7-glucoside	19.95	231, 266, 353	787	625 (100)			463 (28)	505 (9)	445 (74)	300/301 (100)	
2	Kaempferol-3-sophoroside-7-glucoside	20.79	231, 265, 347	771	609 (100)			447 (16)	489 (5)	429 (100)	285 (89)	
3	Quercetin-3,7-diglucoside	21.80	231, 265, 346	625	463 (100)	505 (4)	301 (11)		343 (23)		300/301 (100)	
4	Kaempferol-3,7-diglucoside	24.20	230, 255, 350*	609	447 (100)	489 (21)	284/285 (29)				284/285 (100)	
5	Isorhamnetin-3,7-diglucoside	24.40	230, 254, 352*	639	477 (100)	519 (13)	315 (14)		357 (34)		314/315 (100)	

5.3.3 Isolation of Flavonol Glycoside Conjugates in Alkaline Hydrolysate Bok Choy Extracts by Preparative HPLC

Preparative HPLC was used to isolate and collect flavonol glycoside conjugates from an alkaline hydrolysate bok choy extract for further characterisation using NMR, as well as for studies on antiproliferative activity on human colon cancer cells *in vitro*. Preparative HPLC conditions are outlined in 3.7 and fraction collection methodology is detailed in 3.7.1. The optimisation of preparative HPLC conditions was discussed in 4.3 and Figure 5.42 shows a chromatogram of an alkaline hydrolysate Sumo bok choy extract using those conditions. The flavonol glycoside conjugates previously identified in the data dependent MS^n studies are labelled with their corresponding retention times.

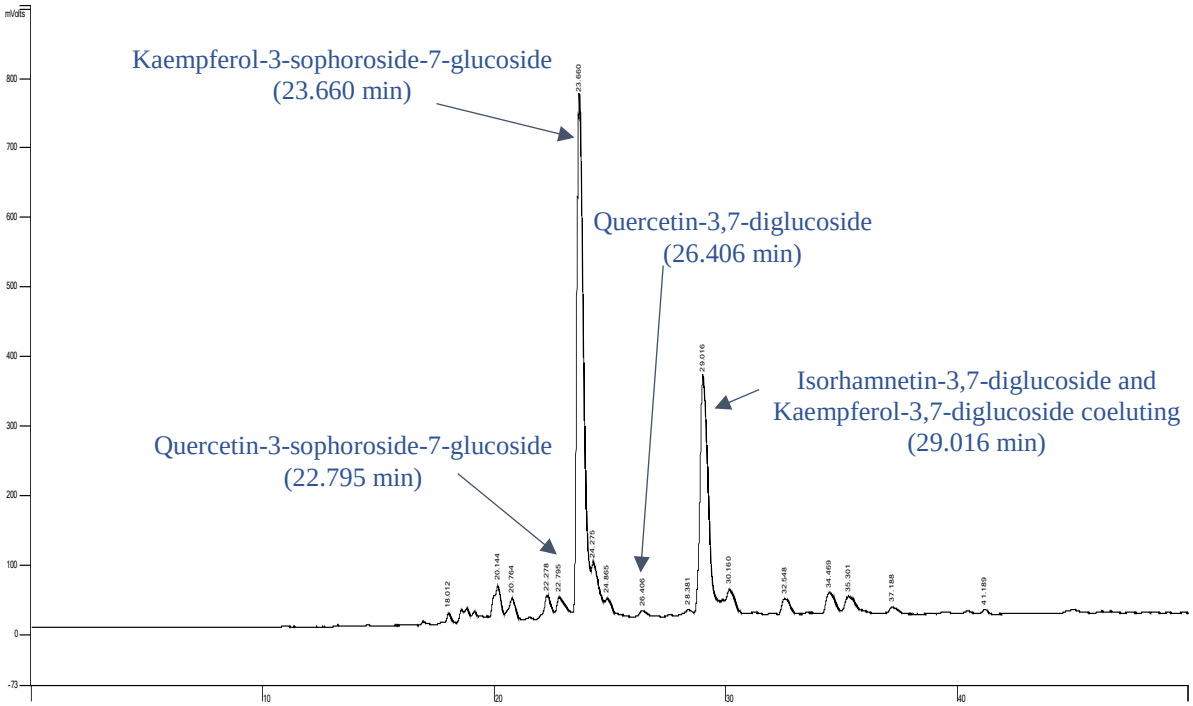


Figure 5.42 Preparative HPLC chromatogram of an alkaline hydrolysate Sumo bok choy extract monitored at 350 nm. Mobile phase and gradient program are outlined in Table 4.1.

The separation obtained on the preparative HPLC is similar to the analytical HPLC, although the third large peak observed in the analytical HPLC-PDA chromatogram (Figure 5.16) was not detected by the UV-Vis detector on the preparative HPLC. Peak 3 in the HPLC-PDA chromatogram displayed a λ_{max} of 322 nm, and, as the UV-Vis detector on the preparative HPLC was set to 350 nm, the compound was not detected. Isorhamnetin-3,7-diglucoside and kaempferol-3,7-diglucoside co-eluted with the preparative HPLC conditions used for this separation, as they did with the analytical HPLC conditions.

Various fractions were collected from the alkaline hydrolysate extract and the corresponding components confirmed by HPLC-PDA/ESI-MS^n. The two major peaks in the extract were collected as fraction 1 and fraction 2 (Figure 5.43) for further analysis and studies. For all fractions, collection began and ended at half of the peak height to ensure highest purity. Fraction 1 was expected to contain pure kaempferol-3-sophoroside-7-glucoside, whereas fraction 2 was expected to contain isorhamnetin-3,7-diglucoside and kaempferol-3,7-diglucoside. Fraction 2 was collected, however, further attempts to obtain a purified sample of isorhamnetin-3,7-diglucoside, which was shown to be the most prominent compound present in that peak, were unsuccessful.

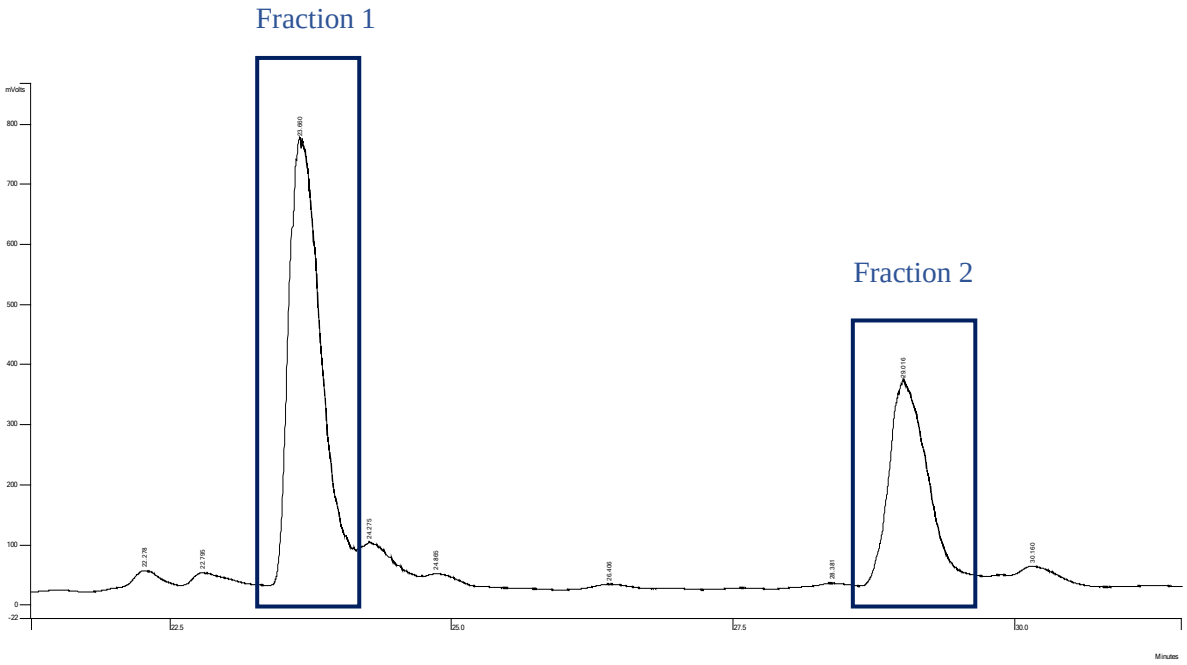


Figure 5.43 Preparative HPLC chromatogram of an alkaline hydrolysate Sumo bok choy extract monitored at 350 nm, showing the two fractions that were collected for further studies.

Kaempferol-3-sophoroside-7-glucoside, on the other hand, was successfully purified from fraction 1 and the structure was confirmed by NMR after a second preparative HPLC fractionation, followed by SPE clean up. Both fractions were also tested for antiproliferative activity on HT-29 human colon cancer cells *in vitro* and results are presented in Chapter VI.

5.3.4 NMR Analysis of Fraction One from an Alkaline Hydrolysate Bok Choy Extract

Fraction 1 from the alkaline hydrolysate bok choy extract was subjected to 1D and 2D NMR for further structure elucidation information and confirmation. All 1D and 2D NMR parameters are detailed in 3.10, including ^{1}H NMR as well as 2D COSY, HSQC, and HMBC experiments. The purified compound (kaempferol-3-*O*-sophoroside-7-*O*-glucoside) was re-constituted in methanol-d_4 and the ^{1}H and ^{13}C NMR spectral data are displayed in Table 5.17. NMR data were consistent with the data reported by Markham *et al.* [233], Nielsen *et al.* [132], and Schliemann *et al.* [135]. The chemical structure of kaempferol-3-*O*-sophoroside-7-*O*-glucoside is shown in Figure 5.44 with the carbon positions labelled. Protons attached to the sugar moieties were assigned using a combination of COSY and HMBC. The HMBC spectrum confirmed that the sugar moiety conjugated at the carbon 3 position was a sophoroside (i.e. 1→2 glycosidic link) and not a gentiobioside.

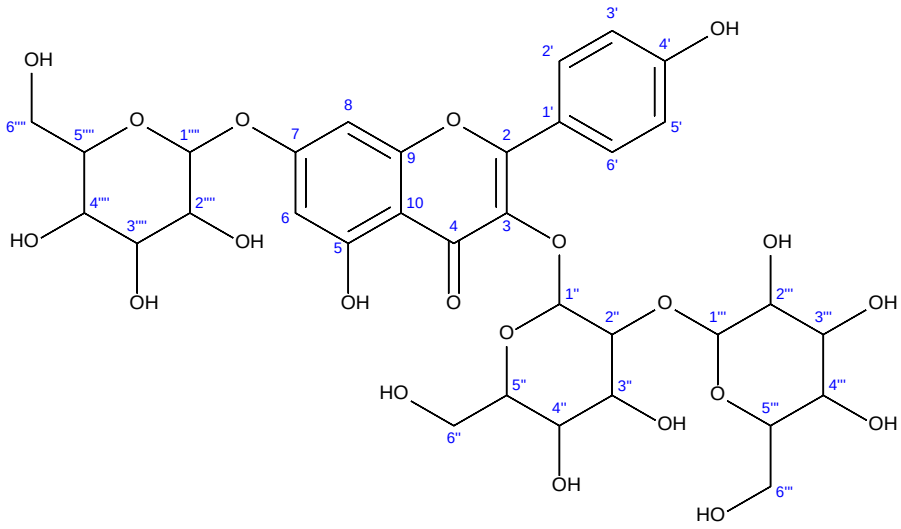


Figure 5.44 Chemical structure of the flavonol compound purified in fraction 1 from an alkaline hydrolysate bok choy extract, as elucidated by NMR and MS^n fragmentation. The compound was identified as kaempferol-3-*O*-sophoroside-7-*O*-glucoside.

Table 5.17 NMR data and structure elucidation of fraction 1 from an alkaline hydrolysate bok choy extract, in methanol-d_4. Chemical shifts in parts per million and coupling constants (*J*) in hertz are given in parentheses. For structure numbering refer to Figure 5.44. The compound was identified as kaempferol-3-*O*-sophoroside-7-*O*-glucoside.

Carbon Position	^{13}C shift	Proton Position	^{1}H shift
Aglycone			
2	157.8 s		
3	133.7		
4	178.4		
5	163.0		
6	100.6 d	6	6.53 d (2.0)
7	164.9		
8	95.6	8	6.81 d (2.0)
9	156.5		
10	106.0		
1'	121.1		
2'	132.6	2'	8.12 d (8.9)
3'	116.1	3'	6.96 d (8.9)
4'	160.3		
5'	116.1	5'	6.96 d (8.9)
6'	132.6	6'	8.12 d (8.9)
Sugar at C-3			
1"	100.7	1"	5.50 d (7.5)
2"	82.7	2"	3.79 dd (7.7, 7.6)
3"	77.8	3"	3.66 m
4"	71.2	4"	3.39 m
5"	78.2	5"	3.26 m
6"	62.3 t	6a"	3.53 m
		6b"	3.74 m
1"'	104.7	1"'	4.80 d (7.5)
2"'	77.8	2"'	3.41 m
3"'	75.4	3"'	3.39 m
4"'^{A}	71.4	4"'	3.43 m
5"'^{B}	78.2	5"'	3.33 m
6"'^{C}	62.3 t	6a"'	3.83 dd (12.1, 2.5)
		6b"'	3.74 m
Sugar at C-7			
1""	101.4	1""	5.09 d (7.7)
2""	77.4	2""	3.54 m
3""	74.5	3""	3.53 m
4""^{A}	71.4	4""	3.40 m
5""^{B}	78.2	5""	3.58 m
6""^{C}	62.4	6a""	3.73 m
		6b""	3.96 dd (12.2, 2.3)

$^{A, B, C}$ assignments are interchangeable

5.4 IDENTIFICATION OF FLAVONOL GLYCOSIDE HYDROXY-CINNAMIC ACID CONJUGATES IN HYDROALCOHOLIC EXTRACTS OF THREE VARIETIES OF BOK CHOY

This section discusses the results of the HPLC-PDA/ESI-MS^n separation of flavonol glycoside-hydroxycinnamic acid conjugates that naturally occur in three different cultivars of bok choy. Freeze-dried, powdered bok choy samples were subjected to a hydroalcoholic solvent extraction using methanol and water and analysed via HPLC-PDA/ESI-MS^n. Bok choy cultivar information and growing conditions are described in 3.2, with the hydroalcoholic extraction procedure and sample preparation described in 3.4.4. HPLC-PDA/ESI-MS^n separation conditions are outlined in 3.6.3 and structure elucidation of these compounds was achieved using a data dependent protocol on the MS^n (3.9.2). Preparative HPLC was also used to collect fractions from a hydroalcoholic bok choy extract for further characterisation using NMR and cancer cell studies. Preparative HPLC conditions and fraction collection are described in 3.7, and NMR conditions are outlined in 3.10.

5.4.1 HPLC-PDA/ESI-MS^n Analysis of Flavonol Glycoside-Hydroxycinnamic Acid Conjugates in Three Bok Choy Cultivars

Flavonol glycoside-hydroxycinnamic acid conjugate standards were not available for this work, therefore the identification of the flavonol glycoside-hydroxycinnamic acid conjugates present in the hydroalcoholic methanol/water extracts of the bok choy cultivars was achieved using a combination of PDA and MS^n data, as well as published data in literature. The previously optimised HPLC-PDA/ESI-MS^n conditions in 4.2.2 for the separation of complex flavonol conjugates in hydroalcoholic extracts were used and the HPLC-PDA chromatograms, recorded at 330 nm, for the Sumo, Karate, and Miyako bok choy hydroalcoholic extracts are shown in Figure 5.45. The ten major peaks are labelled 1-10 and their respective retention times are given in Table 5.18 for each cultivar. The three varieties had similar chromatographic profiles displaying the same ten major peaks, although the peaks observed for the Miyako variety were notably smaller.

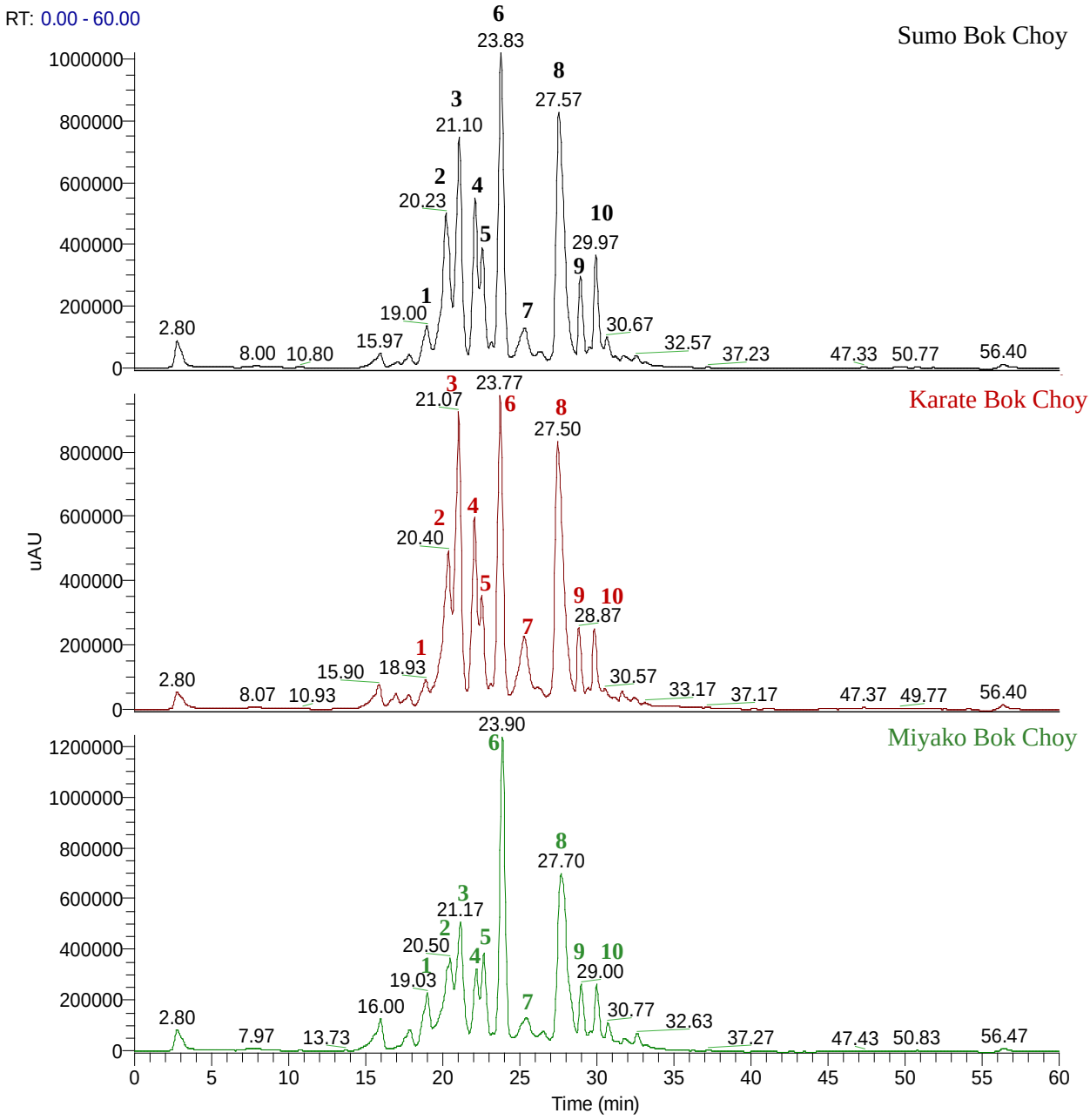


Figure 5.45 HPLC-PDA chromatograms, recorded at 330 nm, of the hydroalcoholic extracts of three bok choy cultivars: Sumo, Karate, and Miyako. The ten major peaks are labelled 1-10.

Table 5.18 HPLC-PDA retention times (t_R) of the ten major peaks present in the hydroalcoholic extracts of three bok choy cultivars.

Bok Choy Cultivar	Peak 1 t_R (min)	Peak 2 t_R (min)	Peak 3 t_R (min)	Peak 4 t_R (min)	Peak 5 t_R (min)	Peak 6 t_R (min)	Peak 7 t_R (min)	Peak 8 t_R (min)	Peak 9 t_R (min)	Peak 10 t_R (min)
Sumo	19.00	20.23	21.10	22.13	22.60	23.83	25.33	27.57	28.97	29.97
Karate	18.93	20.40	21.07	22.10	22.57	23.77	25.33	27.50	28.87	29.87
Miyako	19.03	20.50	21.17	22.20	22.70	23.90	25.47	27.70	29.00	30.00

The UV-Visible spectra for the ten peaks in each variety of bok choy are overlayed and displayed in Figure 5.46. The blue, green, and orange spectra represent the Sumo, Karate, and Miyako bok choy hydroalcoholic extracts respectively. The spectra were identical between the three cultivars, suggesting that the same ten compounds are present in the hydroalcoholic extracts of each bok choy variety. Analysis of the UV-Visible spectra suggested that many of the compounds present were flavonol compounds acylated with hydroxycinnamic acid derivatives. Flavonol glycoside-hydroxycinnamic acid derivatives typically display a maximum with a high absorption between 310-335 nm due to the hydroxycinnamic acid, and a small maximum that coincides with the flavonol band II between 255-268 nm [57]. Peak 1, however, did not exhibit a typical flavonol profile with a broad absorbance maximum at approximately 310 nm, and was not studied further. Peaks 2-5 showed typical flavonol glycoside-hydroxycinnamic acid derivative profiles (see 4.2.5.3), with a distinct broad absorbance band at approximately 330 nm, as well as two other bands at about 235 nm and 268 nm of similar intensity. These peaks were later identified as flavonol glycoside-hydroxycinnamic acid derivatives in MS^n studies. MS^n analysis also showed that peak 2 contained two co-eluting compounds. The UV-Vis spectra for peak 6 displayed a typical flavonol glycoside profile and was identified as isorhamnetin-3,7-diglucoside. Peaks 7-10 showed comparable UV-Vis spectra, consisting of a broad absorbance band at approximately 330 nm and a smaller, sharper band at approximately 235 nm. This suggested that these compounds could be similarly conjugated or could possibly be poly-acylated flavonol glycosides.

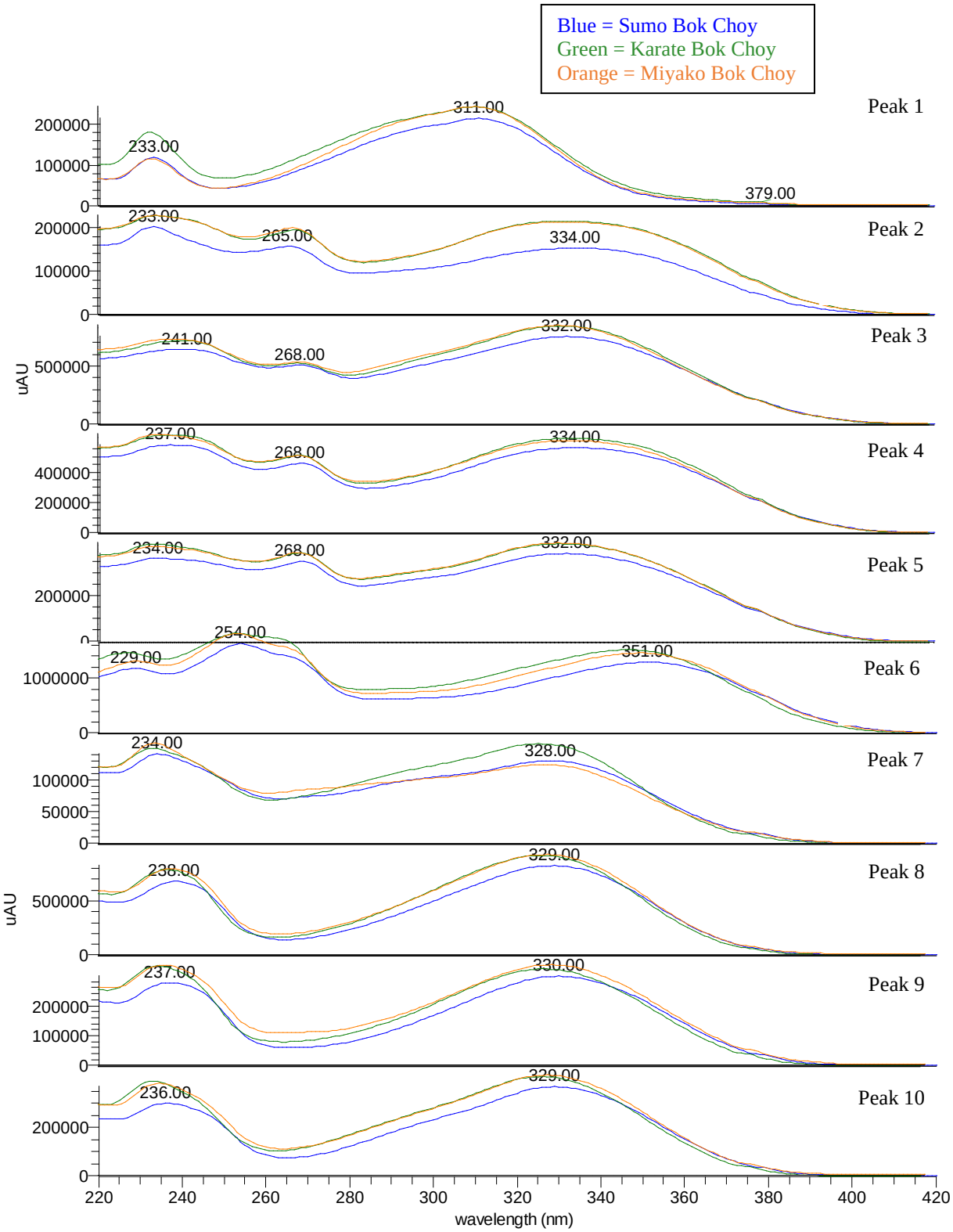


Figure 5.46 UV-Vis spectra (220 to 420 nm) of the ten major peaks present in the hydroalcoholic extracts of each bok choy cultivar: peaks 1 - 10.

It has previously been reported that the UV maximum at 310-335 nm increases, and the contribution from the band I and band II absorptions from the flavonol decrease, when there are two, or three, hydroxycinnamate residues conjugated to the flavonol glycoside [57]. However, it was later shown that peak 7 consisted of two flavonol compounds: a singularly acylated flavonol glycoside, as well as a flavonol mono-glucoside. Furthermore, peaks 8, 9, and 10 could not be ionised under the ESI-MS^n conditions used in this study and were therefore not identifiable. Table 5.19 shows the retention time and UV-Visible data for peaks 1-10 in each of the three bok choy varieties.

Table 5.19 HPLC-PDA retention time (t_R) and UV-Vis spectral data for the ten major peaks present in the hydroalcoholic extracts of each bok choy cultivar.

Peak Data		Sumo	Karate	Miyako
Peak 1	t_R (min)	19.00	18.93	19.03
	UV-Vis (nm)	233,311	233,311	233,311
Peak 2	t_R (min)	20.23	20.40	20.50
	UV-Vis (nm)	232,266,343	235,268,332	233,267,334
Peak 3	t_R (min)	21.10	21.07	21.17
	UV-Vis (nm)	241,268,332	241,268,332	237,268,331
Peak 4	t_R (min)	22.13	22.10	22.20
	UV-Vis (nm)	237,268,334	238,268,334	234,268,334
Peak 5	t_R (min)	22.60	22.57	22.70
	UV-Vis (nm)	234,268,331	233,268,331	234,268,331
Peak 6	t_R (min)	23.83	23.77	23.90
	UV-Vis (nm)	229,254,351	229,254,351	230,254,351
Peak 7	t_R (min)	25.33	25.33	25.47
	UV-Vis (nm)	235,328	236,328	234,328
Peak 8	t_R (min)	27.57	27.50	27.70
	UV-Vis (nm)	239,329	239,329	238,328
Peak 9	t_R (min)	28.97	28.87	29.00
	UV-Vis (nm)	237,330	236,329	236,330
Peak 10	t_R (min)	29.97	29.87	30.00
	UV-Vis (nm)	236,329	235,329	235,329

The HPLC-ESI-MS total ion chromatograms (TIC) for the Sumo, Karate, and Miyako bok choy hydroalcoholic extracts are shown in Figure 5.47, with the thirteen major peaks labelled. This includes two co-eluting compounds in peak 7 (labelled 'a & b') that were also identified in the alkaline hydrolysate bok choy extracts. From the ten major peaks observed in the PDA chromatograms of each bok choy cultivar (Figure 5.45), peaks 2-7 are also present in the TIC for each variety. However, as mentioned above, peak 1, as well as peaks 8-10 are absent in the TIC's, suggesting that these compounds are not flavonols. Corresponding to what was observed in the alkaline hydrolysate extracts, both Sumo and Karate displayed comparable chromatograms with very similar profiles, whereas Miyako displayed a profile with slightly different abundances for some peaks, including peaks 4, 5, and 7.

The negative ion ESI-MS^1 spectra for each of the thirteen peaks, including the two co-eluting compounds in peak 7, are shown in Figure 5.48 for the Sumo bok choy cultivar and the spectra for the Karate and Miyako cultivars are displayed in Appendix G. The MS spectra confirmed, along with the UV-Visible spectra discussed above, that all three varieties contain the same fourteen compounds. Nine compounds were positively identified as flavonol derivatives from the MS^n data dependant studies presented in the next section of the chapter. The remaining five compounds (peaks 9-13) were not characterised.

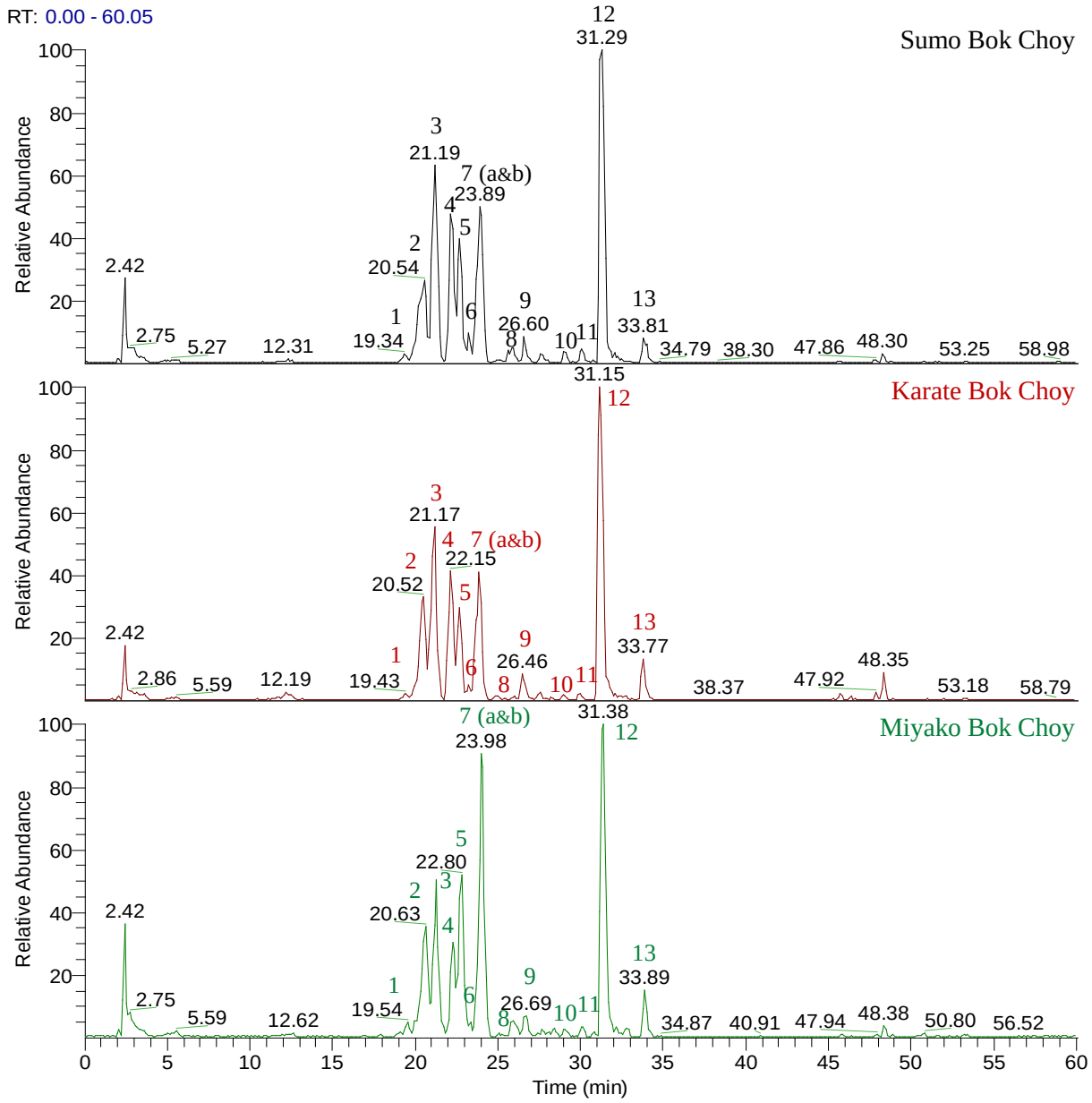


Figure 5.47 HPLC-ESI-MS TIC (scanning mass range 120-2000 amu) of the hydroalcoholic extracts of three bok choy cultivars: Sumo, Karate, and Miyako. The thirteen major peaks are labelled 1-13.

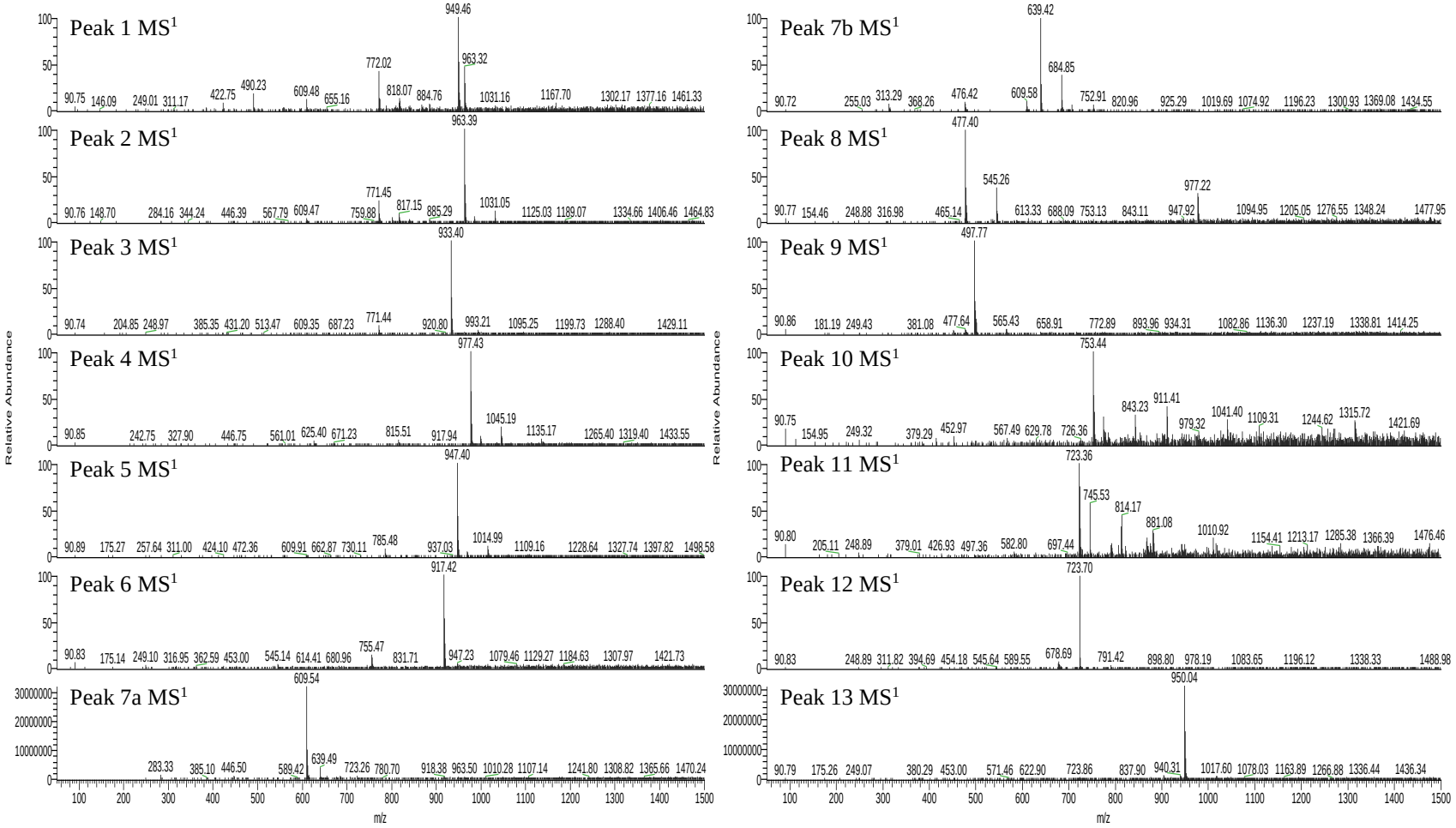


Figure 5.48 ESI-MS[1] spectra in negative ion mode of peaks 1-13 in the hydroalcoholic extract of the Sumo bok choy cultivar.

The retention times and parent/deprotonated molecular ions ($[M-H]^-$) observed in the MS^1 spectra for peaks 1-13 in each of the three bok choy varieties, including the two co-eluting compounds in peak 7, are shown in Table 5.20.

Table 5.20 HPLC-ESI-MS TIC retention time (t_R) and negative parent ion ($[M-H]^-$) data for peaks 1-13 present in the hydroalcoholic extract of each bok choy cultivar. *Two compounds were present in peak 7.

Peak Data		Sumo	Karate	Miyako
Peak 1	t_R (min)	19.34	19.43	19.54
	$[M-H]^-$ (*m/z*)	949.46	949.38	949.36
Peak 2	t_R (min)	20.54	20.52	20.63
	$[M-H]^-$ (*m/z*)	963.39	963.46	963.35
Peak 3	t_R (min)	21.19	21.17	21.28
	$[M-H]^-$ (*m/z*)	933.40	933.38	933.39
Peak 4	t_R (min)	22.17	22.15	22.26
	$[M-H]^-$ (*m/z*)	977.43	977.44	977.50
Peak 5	t_R (min)	22.71	22.69	22.80
	$[M-H]^-$ (*m/z*)	947.40	947.40	947.35
Peak 6	t_R (min)	23.25	23.23	23.35
	$[M-H]^-$ (*m/z*)	917.42	917.32	917.30
Peak 7a	t_R (min)	23.69*	23.66*	23.67*
	$[M-H]^-$ (*m/z*)	609.54	609.58	609.35
Peak 7b	t_R (min)	23.89*	23.87*	23.98*
	$[M-H]^-$ (*m/z*)	639.42	639.42	639.45
Peak 8	t_R (min)	25.84	26.02	25.92
	$[M-H]^-$ (*m/z*)	477.40	477.39	477.40
Peak 9	t_R (min)	26.60	26.46	26.69
	$[M-H]^-$ (*m/z*)	497.77	497.78	497.76
Peak 10	t_R (min)	29.11	28.97	28.98
	$[M-H]^-$ (*m/z*)	753.44	753.37	753.48
Peak 11	t_R (min)	30.09	29.95	30.07
	$[M-H]^-$ (*m/z*)	723.36	723.42	723.54
Peak 12	t_R (min)	31.29	31.15	31.38
	$[M-H]^-$ (*m/z*)	723.70	723.63	723.63
Peak 13	t_R (min)	33.81	33.77	33.89
	$[M-H]^-$ (*m/z*)	950.04	949.98	949.92

5.4.2 Characterisation and Structure Elucidation of Flavonol Glycoside Hydroxycinnamic Acid Derivatives in Hydroalcoholic Bok Choy Extracts using Data Dependent MS^n

Structural information on the flavonol compounds present in the hydroalcoholic extracts of bok choy was obtained by multi-stage mass analysis using the Data Dependent MS^n technique on the LCQ Deca XP Max mass spectrometer. The data dependent acquisition parameters are described in 3.9.2. In the centroid scan mode, the LCQ Deca XP Max performs data dependent scans for up to 25 ions in a given spectrum.

The analysis of the MS^n data and the subsequent structure elucidation of the naturally occurring flavonol conjugates in the hydroalcoholic extracts of three bok choy cultivars was carried out using the same rationale as for the flavonol glycosides present in the alkaline hydrolysate extracts (5.3.2), with reference to several earlier, detailed studies on the fragmentation of flavonols and carbohydrates. As expected from the previous analysis of acid hydrolysate and alkaline hydrolysate extracts of bok choy, kaempferol conjugates were found to be the most abundant flavonol compounds in the hydroalcoholic extracts. The identification/structure elucidation of the acyl portion of the compounds was deduced by MS^n fragmentation patterns and by comparison to the flavonoid acylated derivatives identified in broccoli [57], cauliflower [128], pak choi [60, 136], and kale[67], however, the possibility of regioisomers in the subunits cannot be eliminated. The acyl fragments were identified as the commonly occurring hydroxycinnamic acid moieties including coumaroyl (-146 amu), caffeoyl (-162 amu), feruloyl (-176 amu), and sinapoyl (-206 amu), as well as a less common moiety resulting from a loss of 192 amu. This was previously identified as dihydroxy methoxycinnamic acid (OMeCaffeoyl) by Rochfort *et al.*[60] and Schmidt *et al.* [67] identified the same acyl moiety as hydroxy feruloyl.

Figure 5.49 shows the TIC generated from the data dependent MS^n study on the Sumo bok choy variety. The nine flavonol compounds that were identified are labelled peaks 1-9, along with their corresponding retention times. The same nine compounds were present in all three bok choy cultivars.

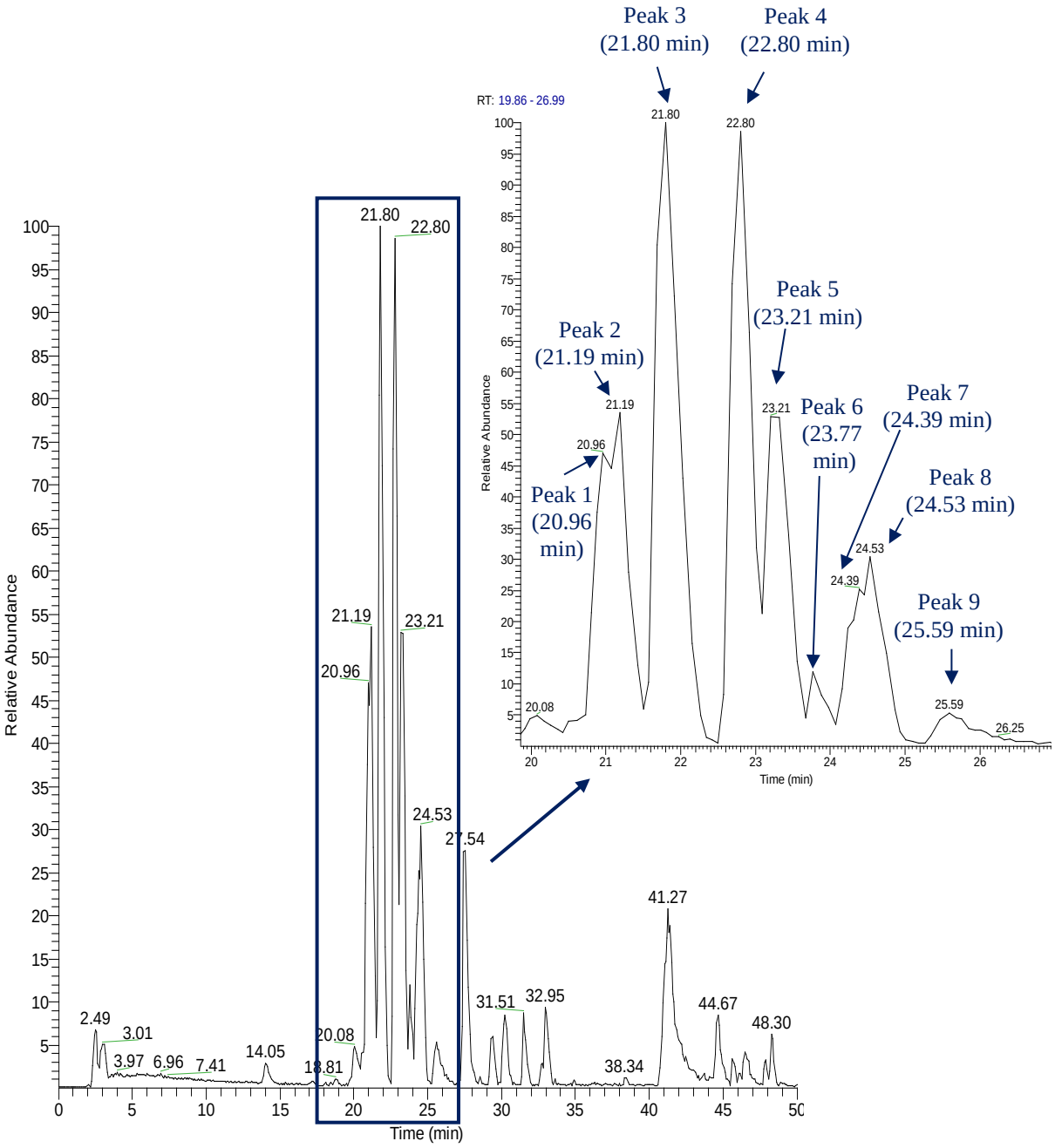


Figure 5.49 HPLC-ESI-MS TIC (scanning mass range 120-2000 amu) of the hydroalcoholic Sumo bok choy extract. The nine flavonol compounds that were identified in the data dependent MS^n studies are labelled peaks 1-9 in the insert.

Nine compounds in total were identified in the hydroalcoholic bok choy extracts: six were identified as flavonol glycoside-hydroxycinnamic acid conjugates, two were flavonol di-glucosides, and the ninth compound was a simple flavonol mono-glucoside. All nine compounds were previously identified by Rochfort *et al.* [60] in the analysis of a cultivar of pak choi known as 'Shanghai.' The two flavonol di-glucosides were also previously identified and structures characterised in the data dependent MS^n studies on the alkaline hydrolysate bok choy extracts. This study, in addition to previous studies on pak choi [60, 136], suggest that the overall complexity of the flavonol compounds found in bok choy is a lot less than those reported in broccoli [57], cauliflower [128], and kale [67]. Vallejo *et al*. [57], for example, reported that some flavonols present in broccoli were substituted with up to five sugars, including compounds such as 3-sophorotriosides-7-sophorosides.

The nine flavonol compounds (labelled 1-9 in Figure 5.49) that were identified in the hydroalcoholic extracts of the bok choy cultivars contained one of the three flavonol aglycone ions in either the MS^4, MS^3, or MS^2 spectra, depending on whether the compound was a flavonol glycoside-hydroxycinnamic acid derivative, a flavonol di-glycoside, or a flavonol mono-glucoside. Structure elucidation was then based on the particular aglycone ion observed in the final order spectrum (i.e. *m/z* 301, 315, or 285). Only the data for the Sumo cultivar are presented in this section as the same nine compounds were present in all three varieties.

Analysis of the UV-Vis spectra obtained from the PDA chromatograms of the hydroalcoholic bok choy extracts suggested that many of the compounds present were flavonol compounds acylated with hydroxycinnamic acid derivatives through the presence of a broad maximum at approximately 330 nm [57] (5.4.1). Peaks 1-6 all displayed typical spectra of flavonol glycoside-hydroxycinnamic acid derivatives. Peaks 7-9, however, displayed typical spectra of flavonol glycosides through the presence of a broad maximum at approximately 350 nm. This suggested that there was a mixture of both glycosylated and acylated, as well as simply glycosylated flavonol compounds that occurred naturally within the bok choy plant.

The MS^n data dependent protocol obtained spectra to the fourth order (MS^4) for peaks 1-6, whereas peaks 7 and 8 (the di-glycosides) displayed fragmentation up to MS^3, and peak 9 (the mono-glucoside) only MS^2. Analysis of the MS^n data for peak 1 suggested that the compound was a hydroxycinnamic acid derivative of quercetin-3-sophoroside-7-glucoside (peak 1 in the alkaline hydrolysate extract) as it displayed the exact same fragmentation pattern, except for the additional loss of the acyl moiety in the MS^3. Furthermore, peaks 2-6 were identified as hydroxycinnamic acid derivatives of kaempferol-3-sophoroside-7-glucoside (peak 2 in the alkaline hydrolysate extract). Once again, their MS^2, MS^3,

and MS^4 spectra contained the characteristic fragments that were also observed for peak 2 in the alkaline hydrolysate extract. The fragments observed in the MS^2, MS^3, and MS^4 spectra for peaks 1-6 in the hydroalcoholic extract corresponded to those observed in the MS^1, MS^2, and MS^3 spectra for the relevant compounds in the alkaline hydrolysate extract. Peaks 7 and 8 were identified as the same two 3,7-diglycosides that were previously found and identified in the alkaline hydrolysate extract. They exhibited the exact same characteristic fragmentation patterns as those discussed in 5.3.2. Lastly, peak 9, was identified as a flavonol mono-glucoside and displayed a simple fragmentation pattern that consisted of a MS^1 and MS^2 spectra only.

The characterisation and structure elucidation data for each of the nine peaks, except for peaks 7 and 8 for which the data was previously presented in the analysis of the alkaline hydrolysate extract, are presented in the subsequent sections as follows:

- A flow diagram showing the fragmentation of ions selected by the data dependent MS^n protocol
- The ESI-MS^n spectra
- The UV-Visible spectrum
- The chemical structure elucidation and fragmentation pattern flow diagram

5.4.2.1 Identification and Structure Elucidation of Peak One in the Hydroalcoholic Bok Choy Extracts

Peak 1 had a retention time of 20.96 min in the HPLC-ESI-MS TIC of the hydroalcoholic Sumo bok choy extract shown in Figure 5.49 and was identified as quercetin-3-sophoroside(caffeoyl)-7-glucoside. The flow diagram shown in Figure 5.50 below depicts the product ion spectra selected from the data dependent information obtained, whereby only the most intense ions were investigated. The MS^n spectra for these ions are shown in Figure 5.51.

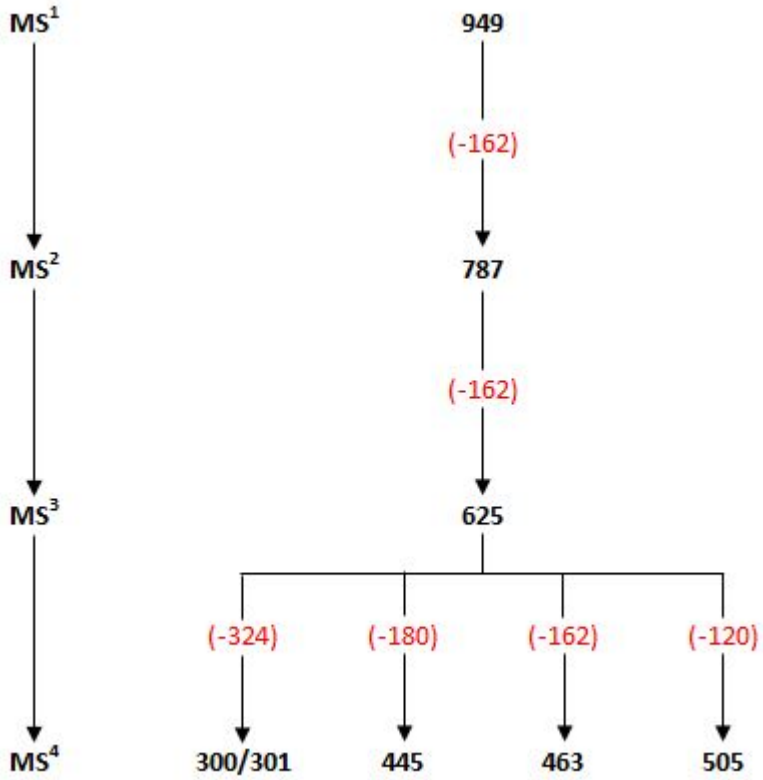


Figure 5.50 Breakdown of the MS^n fragmentation of ions in peak 1 of the hydroalcoholic bok choy extract, as selected by the data dependent protocol.

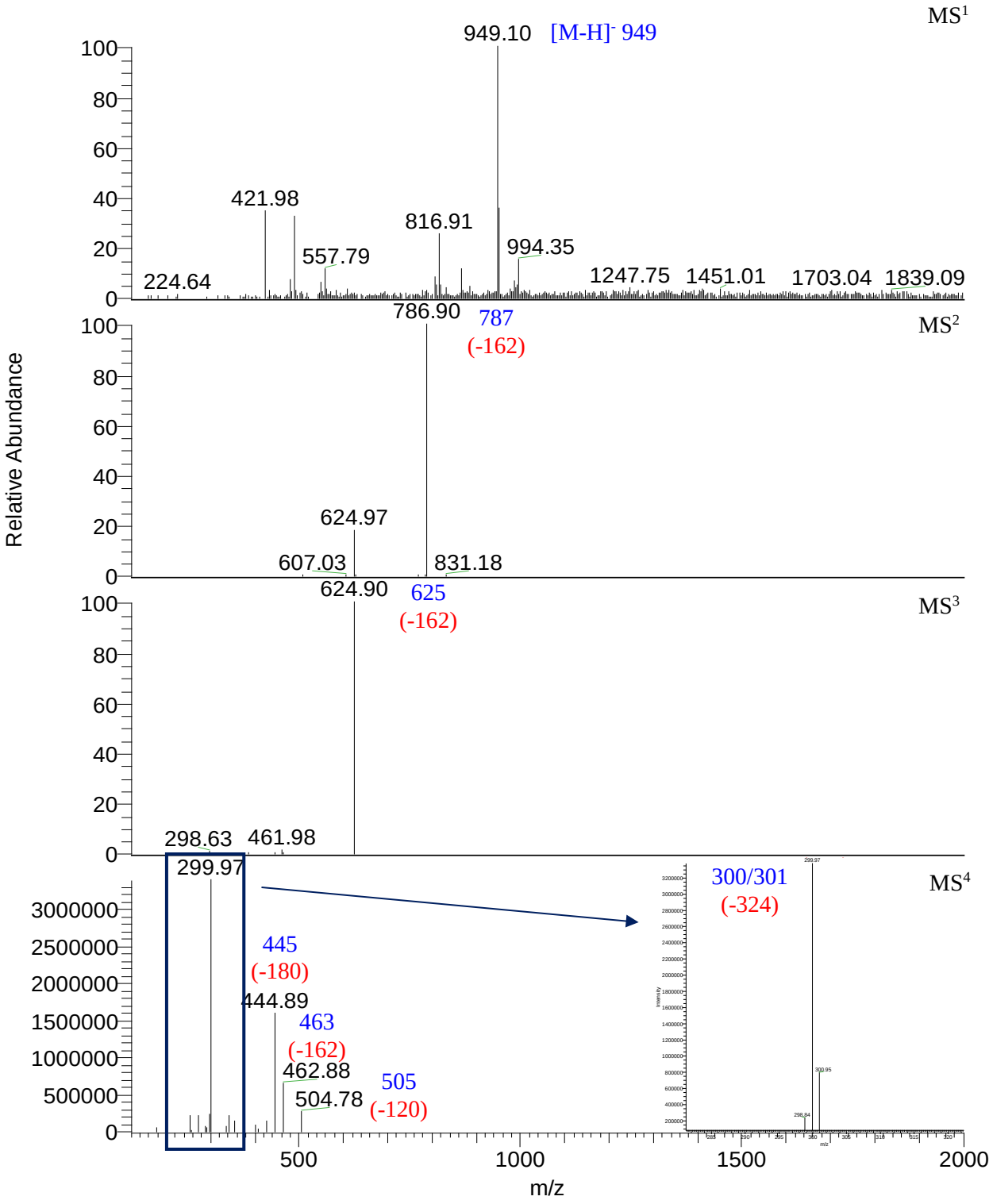


Figure 5.51 MS^n spectra collected during data dependent studies on peak 1 of the hydroalcoholic bok choy extract.

Peak 1 displayed a 301 amu product ion ($Y^7_0\,Y^3_2\,Y^3_0{}^-$ = [M-H-162-162-324]$^-$) in the MS^4 spectrum, as shown in the insert of Figure 5.51, which showed that it was a quercetin based flavonol. The MS^2 spectrum showed a single product ion $Y^7_0{}^-$ (*m/z* 787) which resulted from the loss of a 162 amu fragment (glucose unit without the glycosidic oxygen); this signified a loss from the C7 position of the aglycone. The MS^3 spectrum showed another single product ion $Y^7_0\,Y^3_2{}^-$ (*m/z* 625) which resulted from the loss of another 162 amu fragment. This fragmentation suggested the loss of another glucose unit without the glycosidic oxygen, however, this could also be the loss of a hydroxycinnamic acid moiety. The MS^4 spectrum showed four product ions, including the aglycone $Y^7_0\,Y^3_2\,Y^3_0{}^-$ (*m/z* 301) and three others: $Y^7_0\,Y^3_2\,{}^{0,2}X^3_1{}^-$ (*m/z* 505), $Y^7_0\,Y^3_2\,Y^3_1{}^-$ (*m/z* 463), and $Y^7_0\,Y^3_2\,Z^3_1{}^-$ (*m/z* 445), which resulted from losses of 324 amu, 120 amu, 162 amu and 180 amu from the $Y^7_0\,Y^3_2{}^-$ (*m/z* 625) product ion respectively. This MS^4 fragmentation pattern is typical of a sophoroside (Glucose 1→2 Glucose) where the 324 amu fragment represents two sugars attached to the C3 position of the aglycone. The high abundance of the [M-H-162-162-162]$^-$ (*m/z* 463) and [M-H-162-162-180]$^-$ (*m/z* 445) ions also supported the sophoroside identity. The -120 amu (fragmentation through the sugar ring), -162 amu (loss of glucose without the glycosidic oxygen), and -180 amu (loss of glucose with the glycosidic oxygen) represented losses from the sophoroside moiety.

The fragmentation pattern of peak 1 in the hydroalcoholic extract matched that of peak 1 in the alkaline hydrolysate extract, with the additional loss of a second 162 amu fragment in the MS^3 spectrum. This additional fragment could easily be interpreted as the loss of another glucose unit without the glycosidic oxygen, however, if there were two glucose units attached at the C7 position then a loss of 324 amu [M-H-324]$^-$ would have been observed in the MS^2 spectrum [225]. It is more likely that the fragment observed in the MS^3 is due to the loss of a hydroxycinnamic acid moiety. A common feature of the flavonol 3-sophoroside(hydroxycinnamic acid)-7-glucosides found in bok choy is the loss of a single glucose unit at the C7 position of the aglycone in the MS^2 spectrum, followed by the loss of the hydroxycinnamic moiety in the MS^3 spectrum [60], after which, the MS^4 spectrum contains the product ions that are typical of sophoroside fragmentation. This behaviour is displayed by the MS^n spectra of peak 1 and suggested that the loss of 162 amu in the MS^3 spectra is a hydroxycinnamic acid moiety.

This is further supported by the UV-Vis spectrum (Figure 5.52), where the UV-Vis absorbance maximum at approximately 330 nm indicated further substitution at the C3 position. This hypsochromic shift of 20 nm from the 350 nm absorbance maximum observed for quercetin-3-sophoroside-7-glucoside, and the other glycosylated flavonol compounds, is typical of hydroxycinnamic acid derivatives [57].

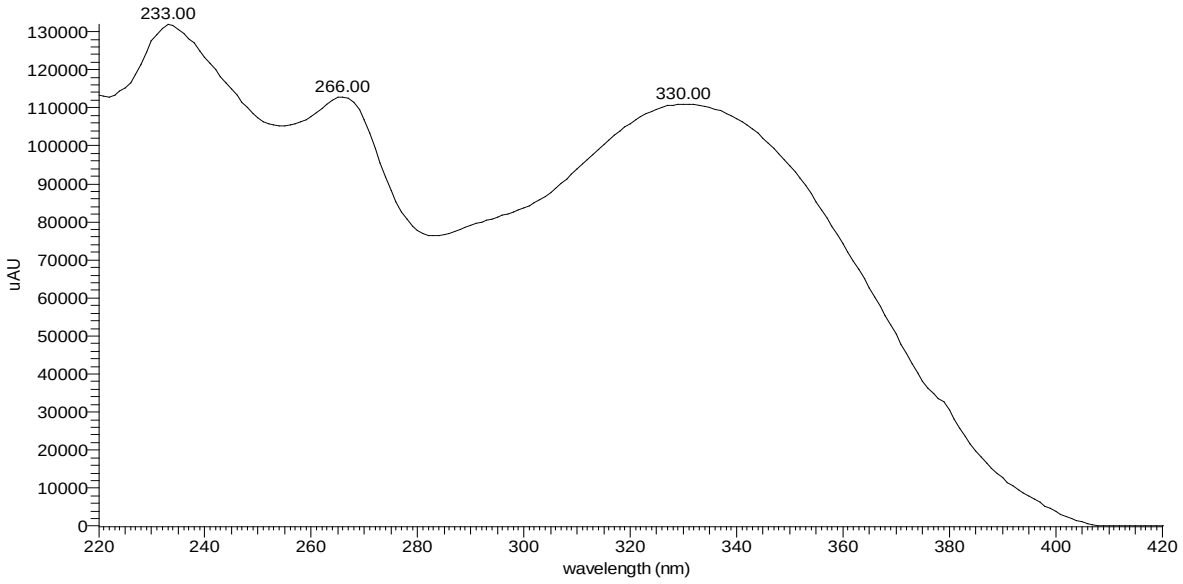


Figure 5.52 UV-Vis spectrum of peak 1 from the hydroalcoholic bok choy extract.

Both the UV-Vis and MS^n data inferred that the compound was a hydroxycinnamic derivative of quercetin-3-sophoroside-7-glucoside. As the loss of 162 amu in the MS^3 spectra was attributed to the cleavage of the hydroxycinnamic acid moiety, the hydroxycinnamic acid was identified as caffeoyl (the univalent radical derived from caffeic acid) which has a mass of 163 amu ($[M-H]^-$ = 162) [60]. Peak 1 was therefore identified as quercetin-3-sophoroside(caffeoyl)-7-glucoside and the fragmentation of this compound as observed in the data dependent studies is shown in Figure 5.53. A summary of the above findings is displayed in Table 5.21 at the end of this section.

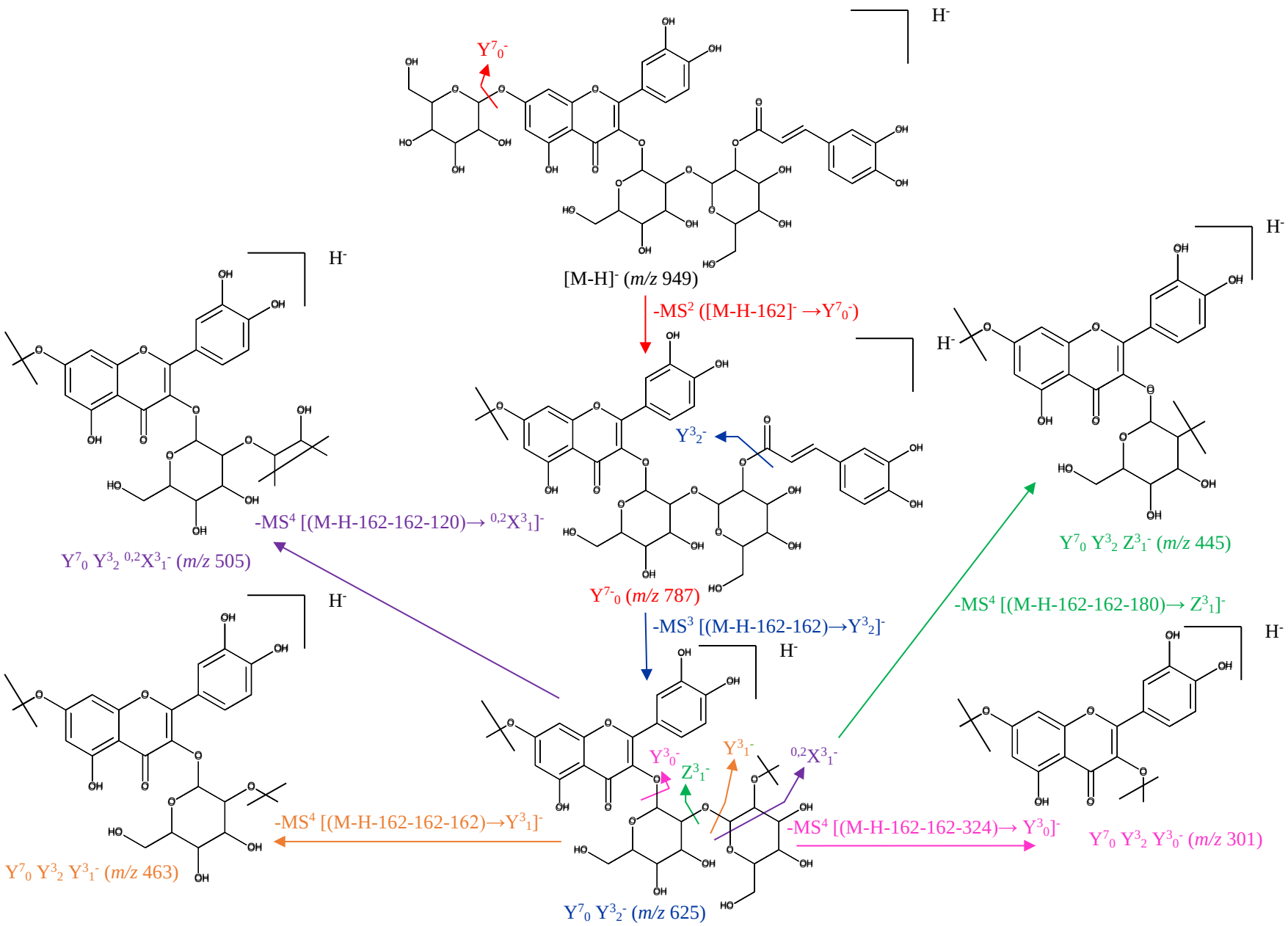


Figure 5.53 Flow diagram showing the chemical structure elucidation and fragmentation pattern of peak 1, identified as quercetin-3-sophoroside(caffeoyl)-7-glucoside.

5.4.2.2 Identification and Structure Elucidation of Peak Two in the Hydroalcoholic Bok Choy Extracts

Peak 2 had a retention time of 21.19 min in the HPLC-ESI-MS TIC of the hydroalcoholic Sumo bok choy extract shown in Figure 5.49 and was identified as kaempferol-3-sophoroside (hydroxy feruloyl)-7-glucoside. The flow diagram shown in Figure 5.54 below depicts the product ion spectra selected from the data dependent information obtained, whereby only the most intense ions were investigated. The MS^n spectra for these ions are shown in Figure 5.55.

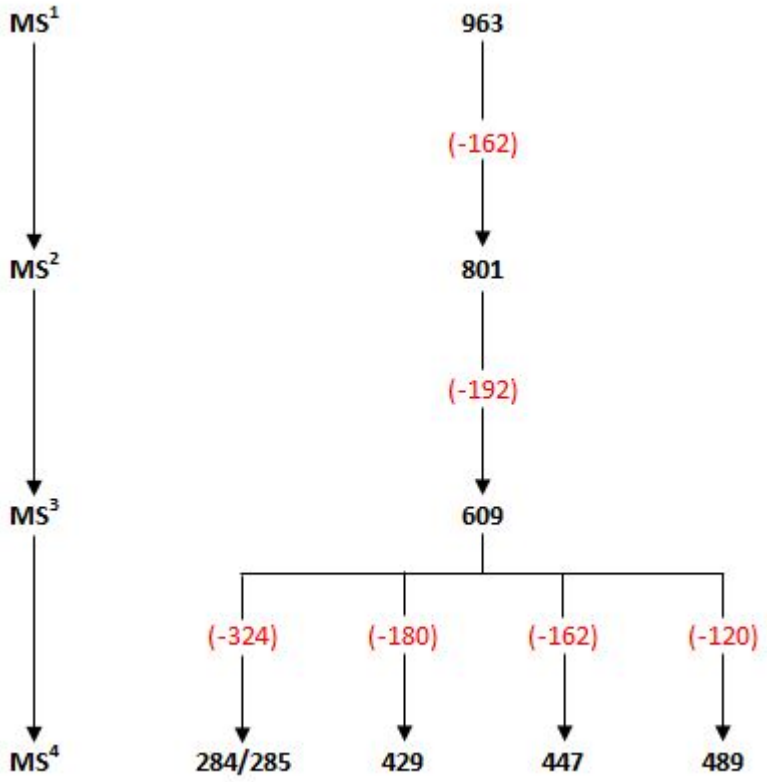


Figure 5.54 Breakdown of the MS^n fragmentation of ions in peak 2 of the hydroalcoholic bok choy extract, as selected by the data dependent protocol.

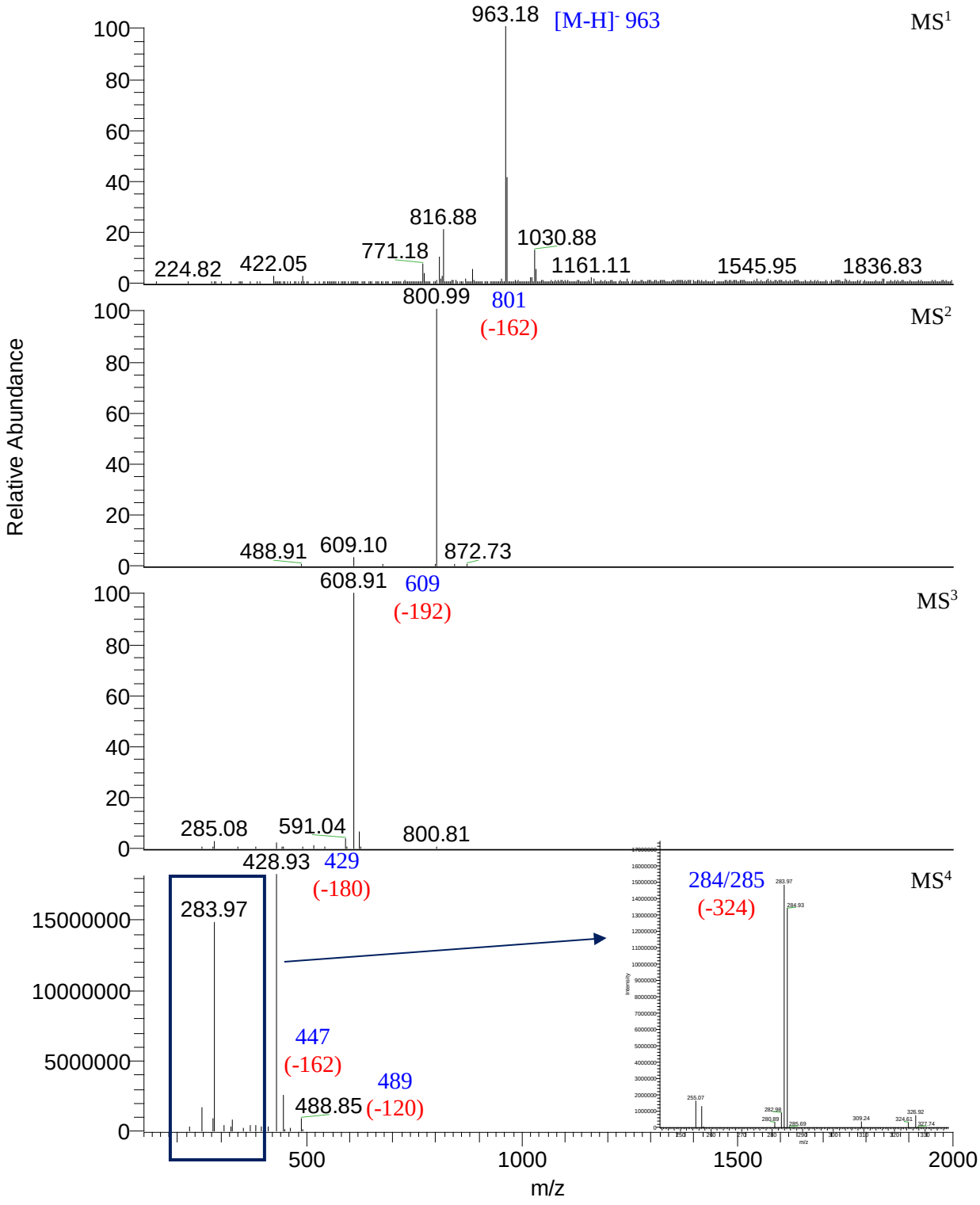


Figure 5.55 MS^n spectra collected during data dependent studies on peak 2 of the hydroalcoholic bok choy extract.

The *m/z* 285 ion ($Y^7_0\,Y^3_2\,Y^3_0{}^-$ = [M-H-162-192-324]$^-$) in the MS^4 spectrum for peak 2, as shown in the insert of Figure 5.55, showed that it was a kaempferol based flavonol. The MS^2 spectrum showed a single product ion $Y^7_0{}^-$ (*m/z* 801) which resulted from the loss of a 162 amu fragment (glucose unit without the glycosidic oxygen); this signified a loss from the C7 position of the aglycone. The MS^3 spectrum showed another single product ion $Y^7_0\,Y^3_2{}^-$ (*m/z* 609) which resulted from the loss of a 192 amu fragment. Based on the fragmentation pattern of peak 1, and the fact that peak 2 followed a similar pattern, it was deduced that the loss of the 192 amu fragment in the MS^3 spectrum was due to the cleavage of a hydroxycinnamic acid moiety. The fragments observed in the MS^4 spectrum coincided with those observed in peak 1, consisting of four product ions. This included the aglycone $Y^7_0\,Y^3_2\,Y^3_0{}^-$ (*m/z* 285) and three others: $Y^7_0\,Y^3_2\,{}^{0,2}X^3_1{}^-$ (*m/z* 489), $Y^7_0\,Y^3_2\,Y^3_1{}^-$ (*m/z* 447), and $Y^7_0\,Y^3_2\,Z^3_1{}^-$ (*m/z* 429), which resulted from losses of 324 amu, 120 amu, 162 amu and 180 amu from the $Y^7_0\,Y^3_2{}^-$ (*m/z* 609) product ion respectively. Once again, this fragmentation pattern is typical of a sophoroside where the -324 amu fragment represents the loss of two sugars attached to the C3 position of the aglycone, the -120 amu represents the fragmentation through the sugar ring, the -162 amu represents the loss of glucose without the glycosidic oxygen, and the -180 amu represents the loss of glucose with the glycosidic oxygen from the sophoroside moiety.

The fragments observed in the MS^n spectra for peak 2 matched those of peak 1, except for the MS^3 spectrum which showed a loss of 192 amu as opposed to 162 amu. Furthermore, peak 1 was a quercetin based compound, whereas peak 2 consisted of a kaempferol core. Hence, it was deduced that peak 2 was also a hydroxycinnamic acid derivative such as peak 1, however, in this case a kaempferol-3-sophoroside-7-glucoside. The fragmentation pattern of peak 2 in the hydroalcoholic extract also matched that of peak 2 in the alkaline hydrolysate extract (previously identified as kaempferol-3-sophoroside-7-glucoside), with the additional loss of a 192 amu fragment in the MS^3 spectrum attributed to the hydroxycinnamic acid moiety. Furthermore, this is supported by the UV-Vis spectrum (Figure 5.56), where the UV-Vis absorbance maximum at approximately 330 nm indicated further substitution at the C3 position, most likely due to the conjugation of a hydroxycinnamic acid.

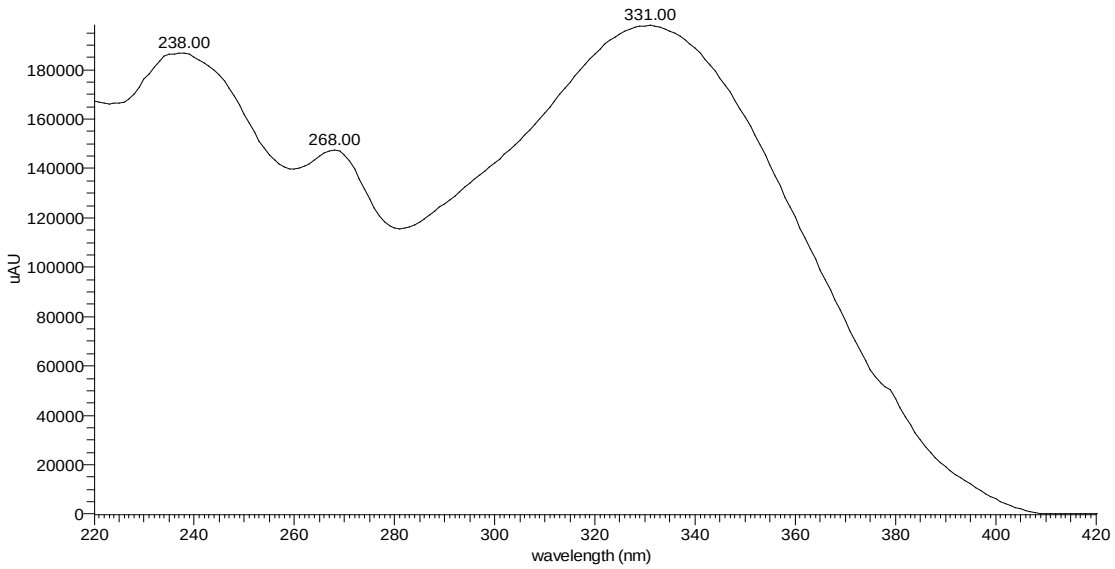


Figure 5.56 UV-Vis spectrum of peak 2 from the hydroalcoholic bok choy extract.

As seen with peak 1 in the hydroalcoholic extract, both the UV-Vis and MS^n data obtained for peak 2 identified the compound as a hydroxycinnamic derivative of kaempferol-3-sophoroside-7-glucoside. The hydroxycinnamic acid moiety was identified as hydroxy feruloyl ($[M-H]^-$ = 192) by Schmidt *et al.* [67], and dihydroxy methoxycinnamic acid by Rochfort *et al*. [60]. Peak 2 was therefore identified as kaempferol-3-sophoroside(hydroxyferuloyl)-7-glucoside and the fragmentation of this compound as observed in the data dependent studies is shown in Figure 5.57. A summary of the above findings is displayed in Table 5.21 at the end of this section.

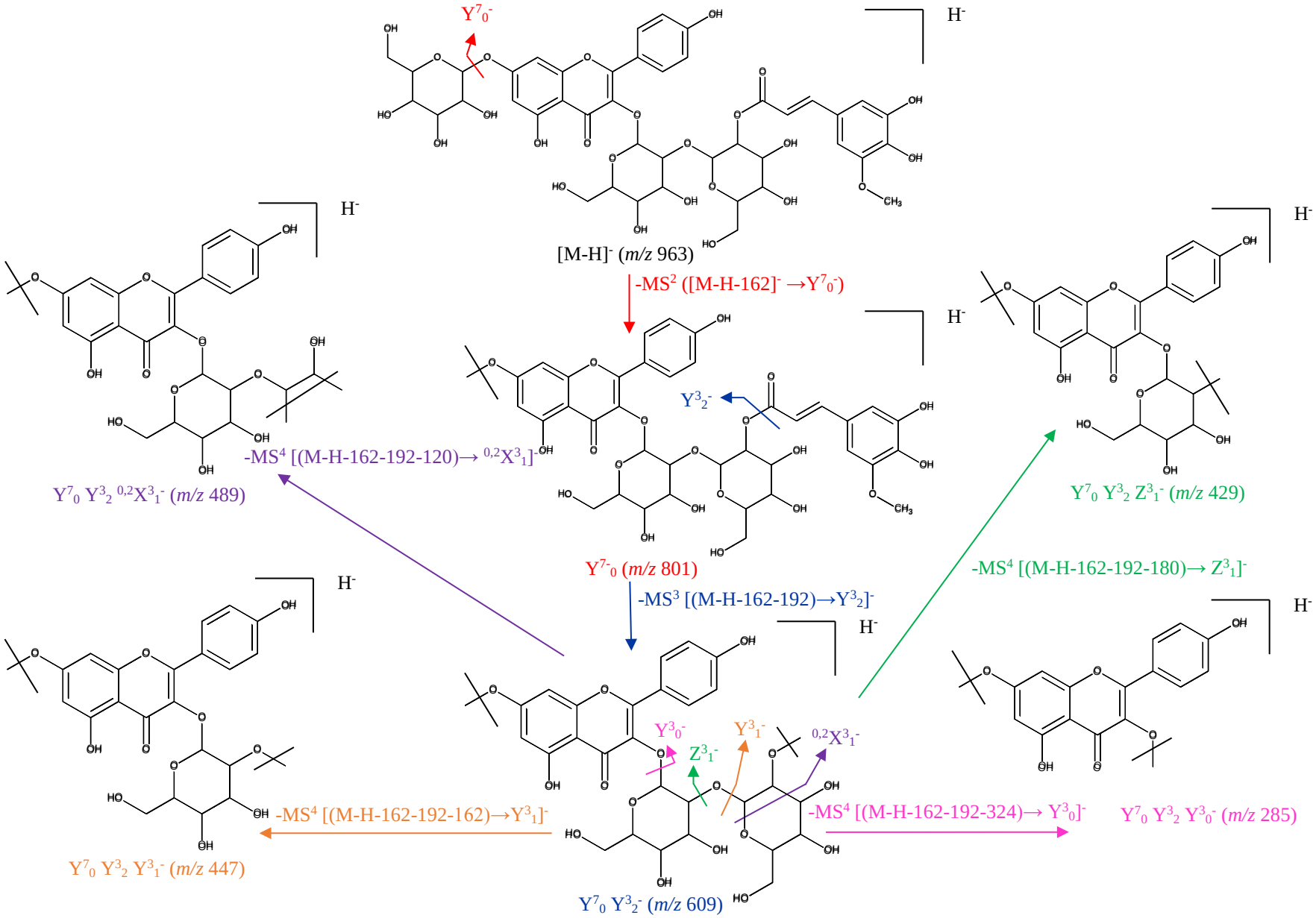


Figure 5.57 Flow diagram showing the chemical structure elucidation and fragmentation pattern of peak 2, identified as kaempferol-3-sophoroside(hydroxyferuloyl)-7-glucoside.

5.4.2.3 Identification and Structure Elucidation of Peak Three in the Hydroalcoholic Bok Choy Extracts

Peak 3 had a retention time of 21.80 min in the HPLC-ESI-MS TIC of the hydroalcoholic Sumo bok choy extract shown in Figure 5.49 and was identified as kaempferol-3-sophoroside(caffeoyl)-7-glucoside. The flow diagram shown in Figure 5.58 below depicts the product ion spectra selected from the data dependent information obtained, whereby only the most intense ions were investigated. The MS^n spectra for these ions are shown in Figure 5.59.

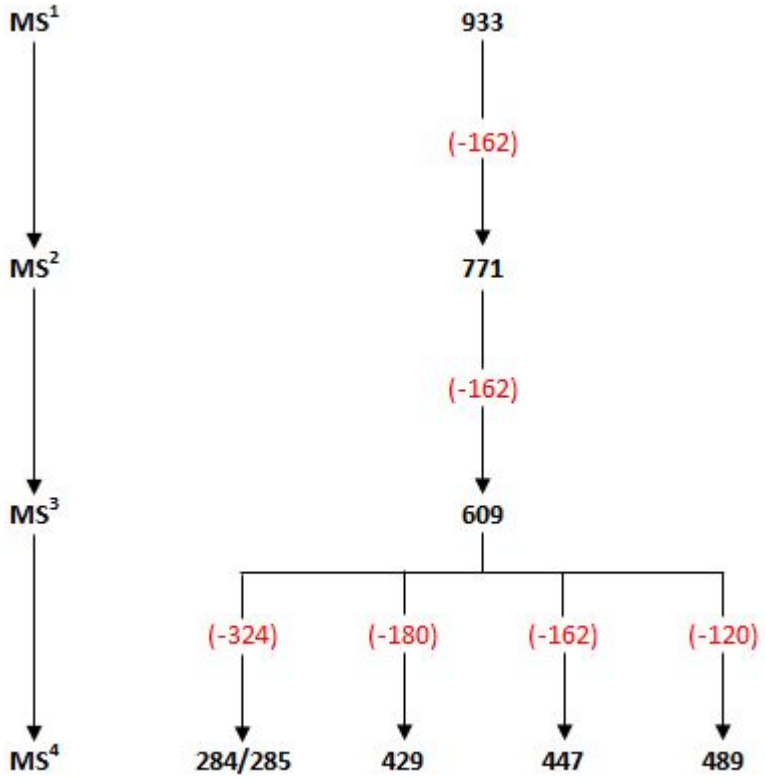


Figure 5.58 Breakdown of the MS^n fragmentation of ions in peak 3 of the hydroalcoholic bok choy extract, as selected by the data dependent protocol.

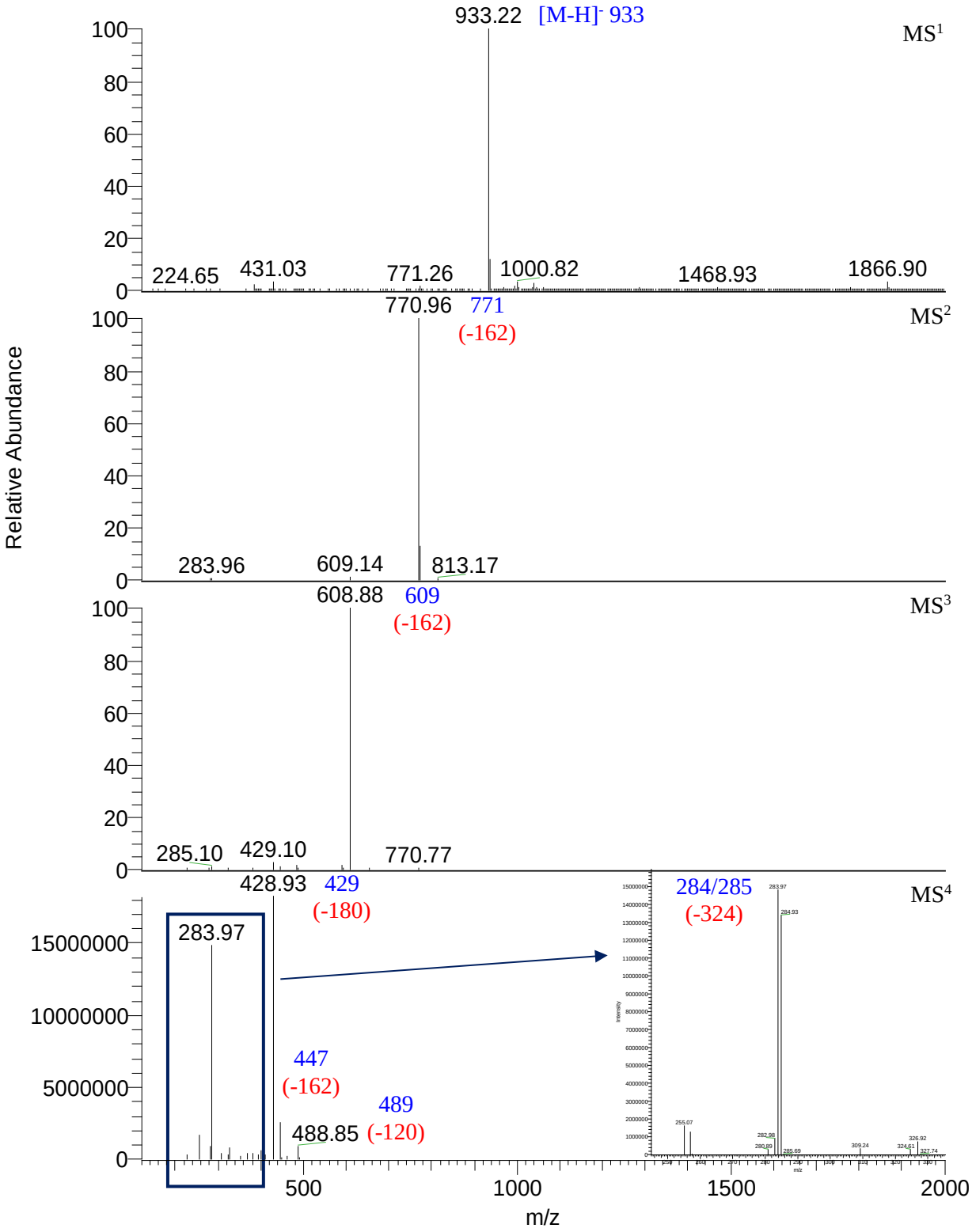


Figure 5.59 MS^n spectra collected during data dependent studies on peak 3 of the hydroalcoholic bok choy extract.

Peak 3 displayed a 285 amu product ion ($Y^7_0\,Y^3_2\,Y^3_0{}^-$ = [M-H-162-162-324]$^-$) in the MS^4 spectrum, as shown in the insert of Figure 5.59, which showed that it was a kaempferol based flavonol. The MS^2 spectrum showed a single product ion $Y^7_0{}^-$ (*m/z* 771) which resulted from the loss of a 162 amu fragment. This signified the loss of a glucose unit without the glycosidic oxygen from the C7 position of the aglycone. The MS^3 spectrum showed another single product ion $Y^7_0\ Y^3_2{}^-$ (*m/z* 609) which resulted from the loss of another 162 amu fragment. The fragmentation pattern matched that of peaks 1 and 2, therefore, the loss of the 162 amu fragment in the MS^3 spectrum was due to the cleavage of a hydroxycinnamic acid moiety. The fragments observed in the MS^4 spectrum were the same as those observed in peak 2, consisting of four product ions. These were the aglycone $Y^7_0\,Y^3_2\,Y^3_0{}^-$ (*m/z* 285), $Y^7_0\,Y^3_2{}^{0,2}X^3_1{}^-$ (*m/z* 489), $Y^7_0\,Y^3_2\,Y^3_1{}^-$ (*m/z* 447), and $Y^7_0\,Y^3_2\,Z^3_1{}^-$ (*m/z* 429), which resulted from losses of 324 amu, 120 amu, 162 amu and 180 amu from the $Y^7_0\ Y^3_2{}^-$ (*m/z* 609) product ion respectively.

The fragmentation patterns seen in the MS^2 and MS^4 spectra were identical to those for peak 2 and were consistent with the compound being a hydroxycinnamic acid derivative of kaempferol-3-sophoroside-7-glucoside. This was supported by the UV-Vis spectrum (Figure 5.60), where the UV-Vis absorbance maximum at approximately 330 nm indicated further substitution at the C3 position, most likely due to the conjugation of a hydroxycinnamic acid.

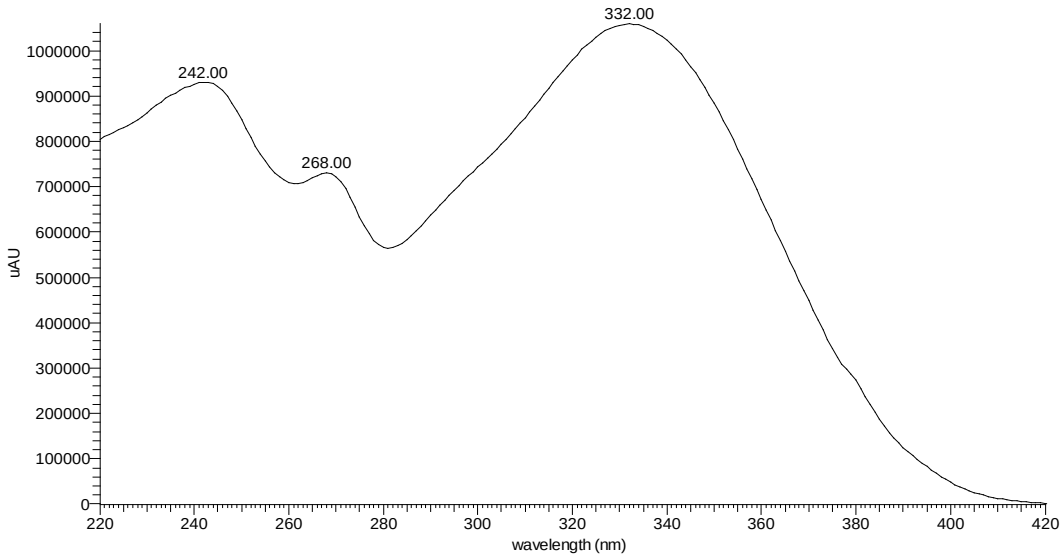


Figure 5.60 UV-Vis spectrum of peak 3 from the hydroalcoholic bok choy extract.

The only difference in the MS^n spectra for peak 3, as compared to peak 2, was a loss of 162 amu in the MS^3 spectrum instead of a loss of 192 amu, indicating that these compounds differ only in their hydroxycinnamic acid moiety. The loss of the 162 amu fragment corresponded to the same loss that was observed in the MS^3 spectrum for peak 1, i.e. a caffeoyl moiety ($[M-H]^-$ = 162). Peak 3 was therefore identified as kaempferol-3-sophoroside(caffeoyl)-7-glucoside and the fragmentation of this compound as observed in the data dependent studies is shown in Figure 5.61. A summary of the above findings is displayed in Table 5.21 at the end of this section.

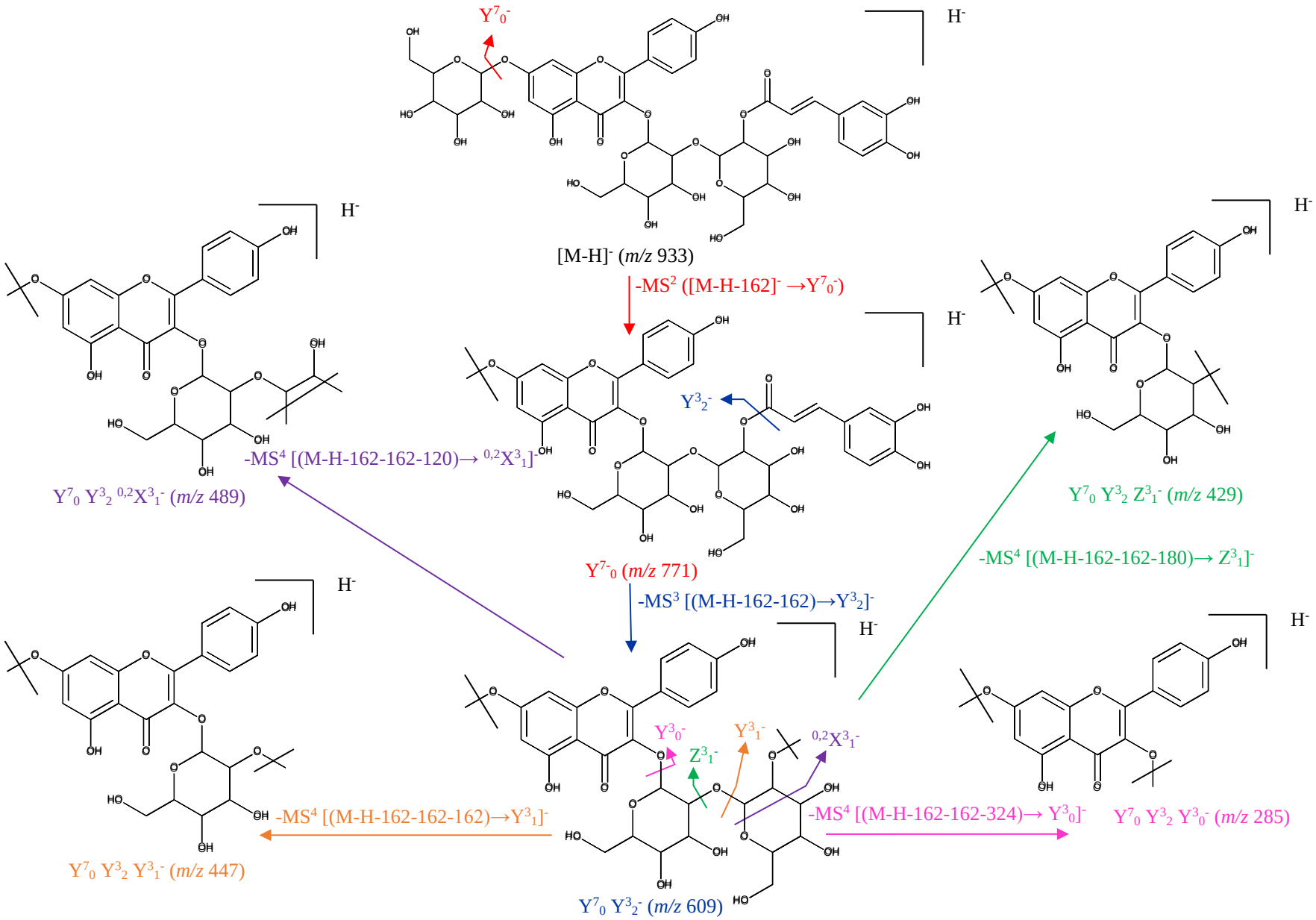


Figure 5.61 Flow diagram showing the chemical structure elucidation and fragmentation pattern of peak 3, identified as kaempferol-3-sophoroside(caffeoyl)-7-glucoside.

5.4.2.4 Identification and Structure Elucidation of Peak Four in the Hydroalcoholic Bok Choy Extracts

Peak 4 had a retention time of 22.80 min in the HPLC-ESI-MS TIC of the hydroalcoholic Sumo bok choy extract shown in Figure 5.49 and was identified as kaempferol-3-sophoroside(sinapoyl)-7-glucoside. The flow diagram shown in Figure 5.62 below depicts the product ion spectra selected from the data dependent information obtained, whereby only the most intense ions were investigated. The MS^n spectra for these ions are shown in Figure 5.63.

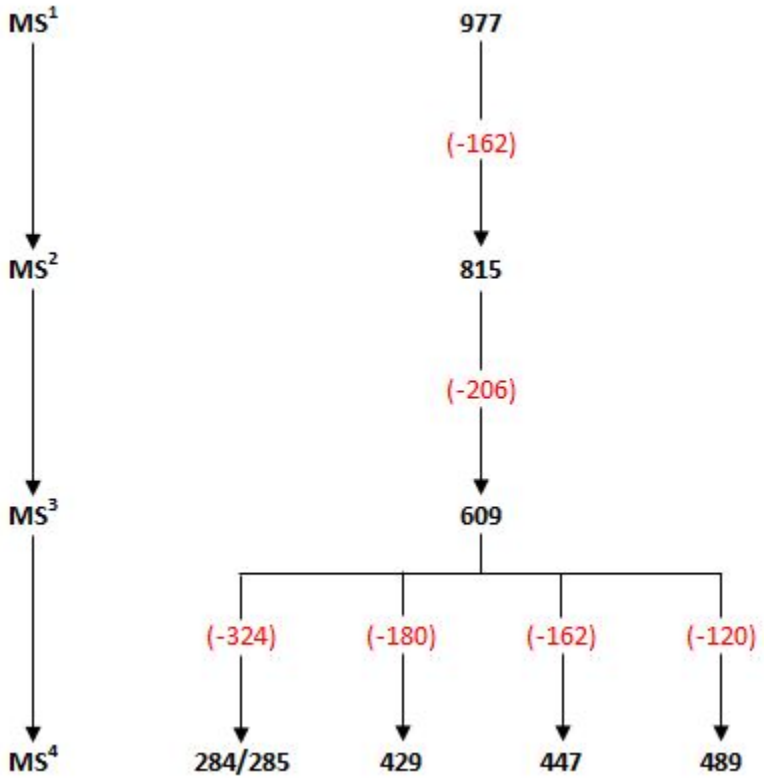


Figure 5.62 Breakdown of the MS^n fragmentation of ions in peak 4 of the hydroalcoholic bok choy extract, as selected by the data dependent protocol.

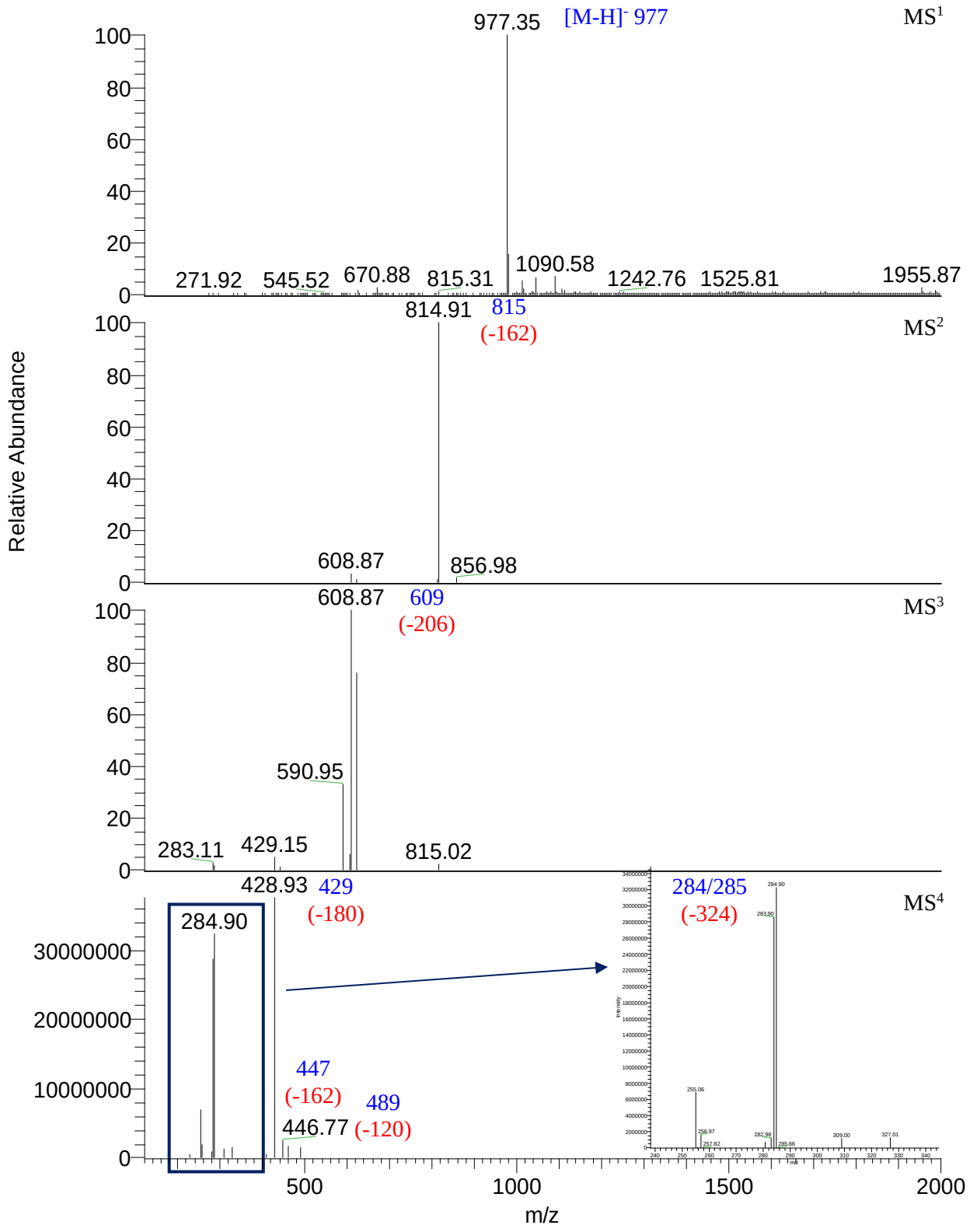


Figure 5.63 MS^n spectra collected during data dependent studies on peak 4 of the hydroalcoholic bok choy extract.

The MS^n spectra for peak 4 displayed very similar fragmentation patterns as peaks 2 and 3 where the only difference was seen in the MS^3 spectrum. The 285 amu product ion ($Y^7_0\,Y^3_2\,Y^3_0{}^-$ = [M-H-162-162-324]$^-$) in the MS^4 spectrum, as shown in the insert of Figure 5.63, showed that the compound also consisted of a kaempferol core. The MS^2 spectrum showed a single product ion $Y^7_0{}^-$ (*m/z* 815) which resulted from the loss of a 162 amu fragment and the MS^3 spectrum showed another single product ion $Y^7_0\,Y^3_2{}^-$ (*m/z* 609) which resulted from the loss of a 206 amu fragment. The fragments observed in the MS^4 spectrum were the same as those observed in peaks 2 and 3, consisting of four product ions. These were the aglycone $Y^7_0\,Y^3_2\,Y^3_0{}^-$ (*m/z* 285), $Y^7_0\,Y^3_2{}^{0,2}X^3_1{}^-$ (*m/z* 489), $Y^7_0\,Y^3_2\,Y^3_1{}^-$ (*m/z* 447), and $Y^7_0\,Y^3_2\,Z^3_1{}^-$ (*m/z* 429), which resulted from losses of 324 amu, 120 amu, 162 amu and 180 amu from the $Y^7_0\,Y^3_2{}^-$ (*m/z* 609) product ion respectively. The MS^n spectra for peak 4 corresponded to those for peaks 2 and 3, except for the MS^3 spectrum, hence it was deduced that peak 4 was also a hydroxycinnamic acid derivative of kaempferol-3-sophoroside-7-glucoside. This was further supported by the UV-Vis spectrum (Figure 5.64), where the UV-Vis absorbance maximum at approximately 330 nm indicated further substitution at the C3 position, most likely due to the conjugation of a hydroxycinnamic acid.

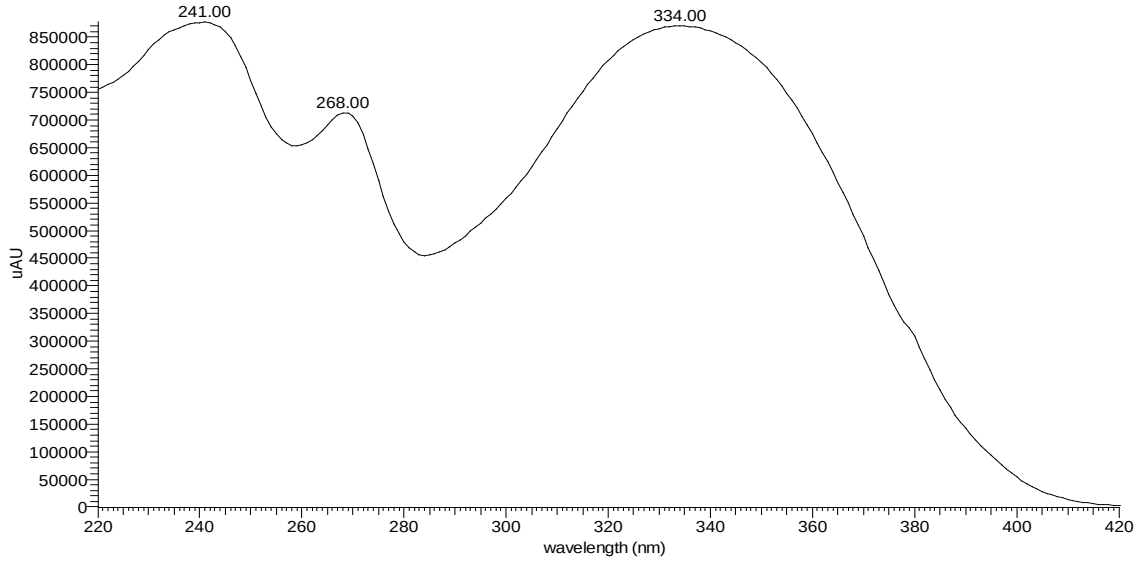


Figure 5.64 UV-Vis spectrum of peak 4 from the hydroalcoholic bok choy extract.

The loss of the 206 amu fragment attributed to the hydroxycinnamic acid in the MS^3 spectrum was identified as sinapoyl ([M-H]$^-$ = 206). Therefore, peak 4 was identified as kaempferol-3-sophoroside(sinapoyl)-7-glucoside and the fragmentation of this compound as observed in the data dependent studies is shown in Figure 5.65. A summary of the above findings is displayed in Table 5.21 at the end of this section.

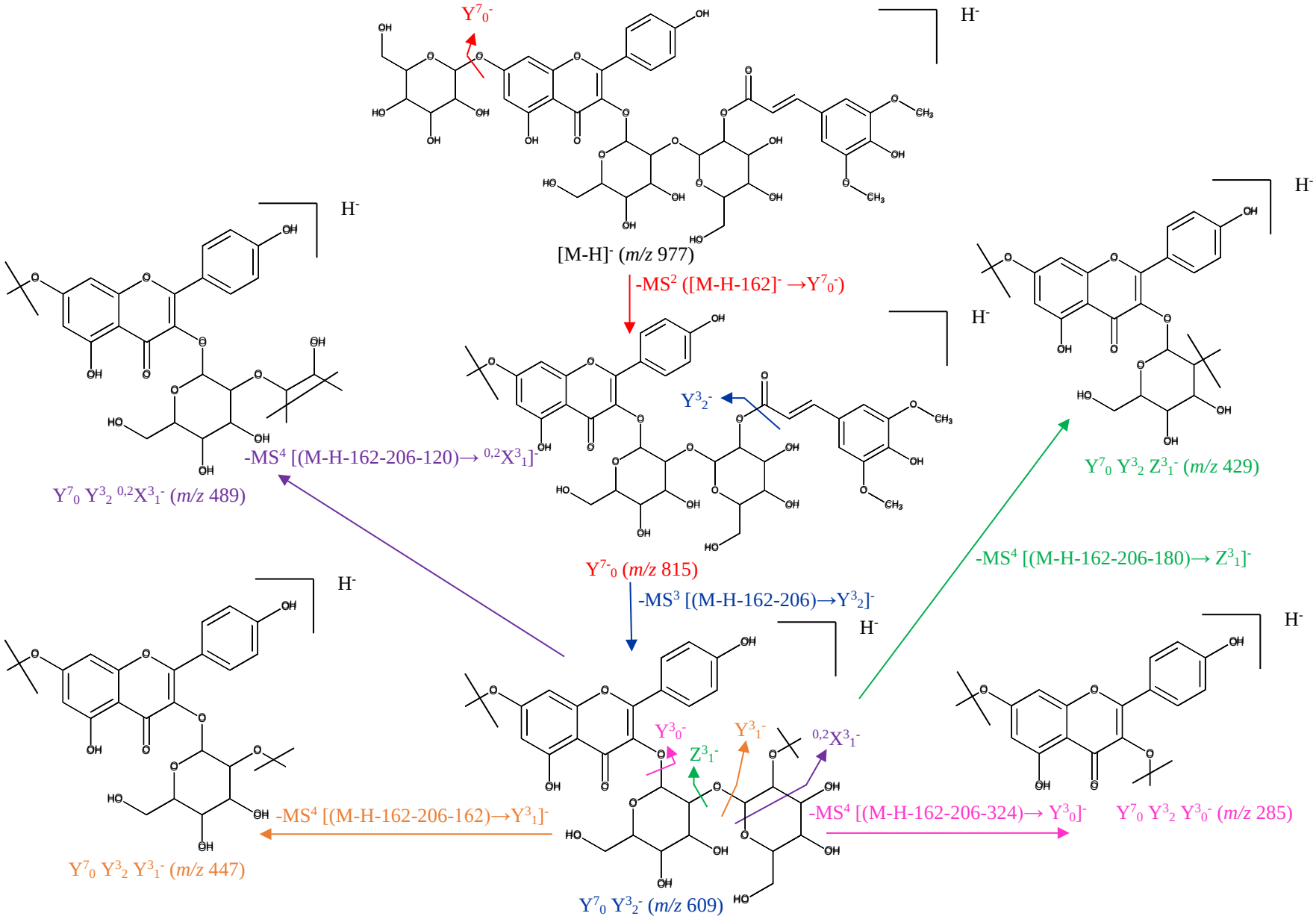


Figure 5.65 Flow diagram showing the chemical structure elucidation and fragmentation pattern of peak 4, identified as kaempferol-3-sophoroside(sinapoyl)-7-glucoside.

5.4.2.5 Identification and Structure Elucidation of Peak Five in the Hydroalcoholic Bok Choy Extracts

Peak 5 had a retention time of 23.21 min in the HPLC-ESI-MS TIC of the hydroalcoholic Sumo bok choy extract shown in Figure 5.49 and was identified as kaempferol-3-sophoroside(feruloyl)-7-glucoside. The flow diagram shown in Figure 5.66 below depicts the product ion spectra selected from the data dependent information obtained, whereby only the most intense ions were investigated. The MS^n spectra for these ions are shown in Figure 5.67.

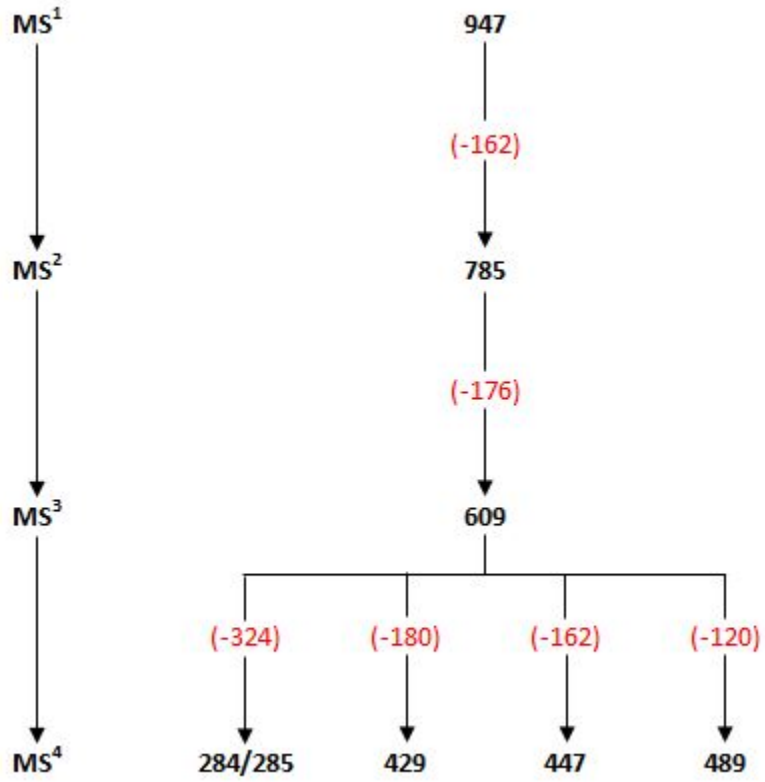


Figure 5.66 Breakdown of the MS^n fragmentation of ions in peak 5 of the hydroalcoholic bok choy extract, as selected by the data dependent protocol.

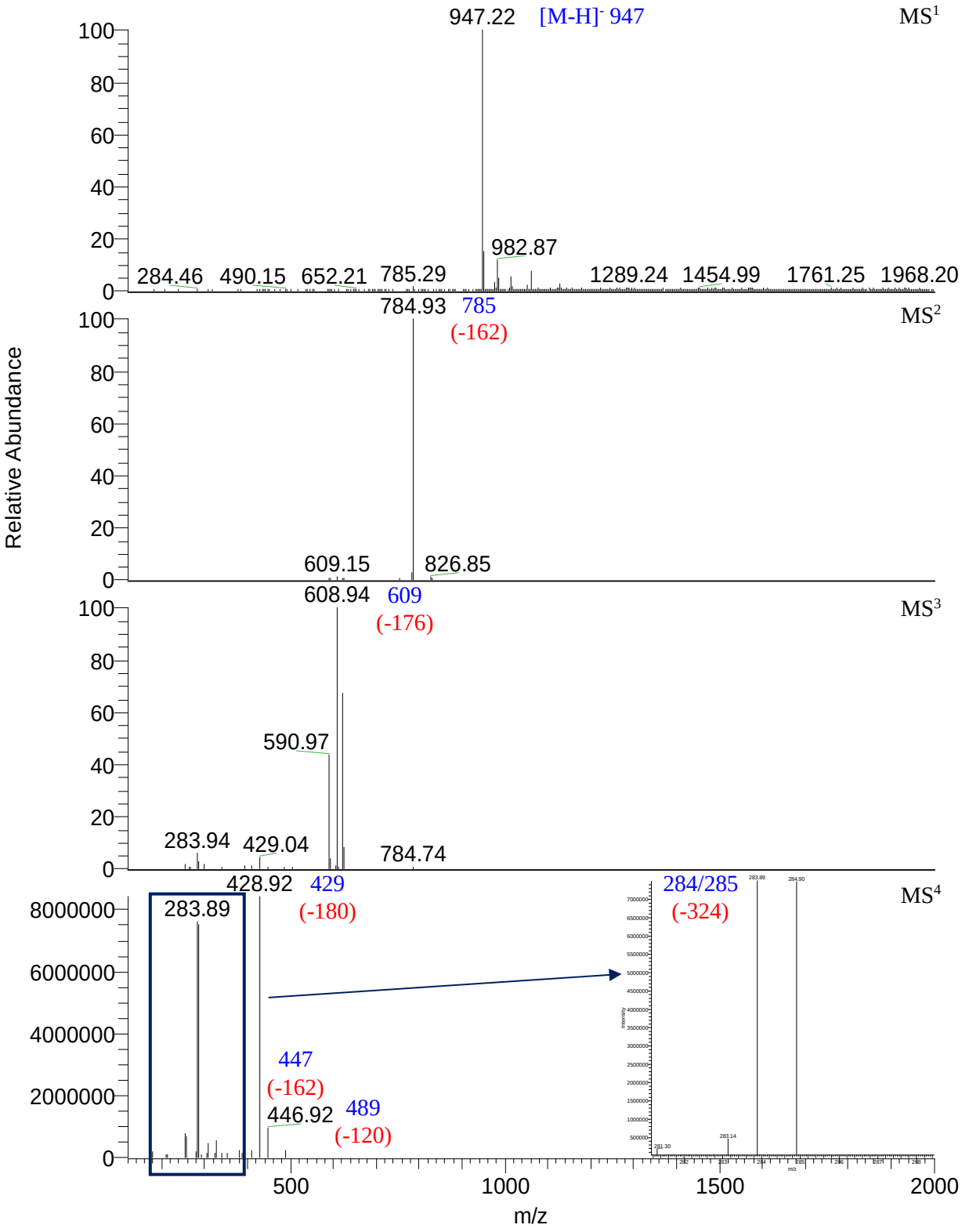


Figure 5.67 MS^n spectra collected during data dependent studies on peak 5 of the hydroalcoholic bok choy extract.

The MS^n spectra for peak 5 displayed very similar fragmentation patterns as peaks 2, 3 and 4 where the only difference was seen in the MS^3 spectrum, indicating that these compounds differ only in their hydroxycinnamic acid moiety. There was a 285 amu product ion ($Y^7_0\,Y^3_2\,Y^3_0{}^-$ = [M-H-162-162-324]$^-$) in the MS^4 spectrum, as seen in the insert of Figure 5.67, which signified that the compound consisted of a kaempferol core. The MS^2 spectrum showed a single product ion $Y^7_0{}^-$ (*m/z* 785) which resulted from the loss of a 162 amu fragment and the MS^3 spectrum showed another single product ion $Y^7_0\,Y^3_2{}^-$ (*m/z* 609) which resulted from the loss of a 176 amu fragment. The fragments observed in the MS^4 spectrum were the same as those observed for peaks 2, 3 and 4. These were the aglycone $Y^7_0\,Y^3_2\,Y^3_0{}^-$ (*m/z* 285), $Y^7_0\,Y^3_2\,{}^{0,2}X^3_1{}^-$ (*m/z* 489), $Y^7_0\,Y^3_2\,Y^3_1{}^-$ (*m/z* 447), and $Y^7_0\,Y^3_2\,Z^3_1{}^-$ (*m/z* 429), which resulted from losses of 324 amu, 120 amu, 162 amu and 180 amu from the $Y^7_0\,Y^3_2{}^-$ (*m/z* 609) product ion respectively. The UV-Vis spectrum shown in Figure 5.68 had a similar absorbance maximum at approximately 330 nm indicating that the compound was a hydroxycinnamic acid derivative.

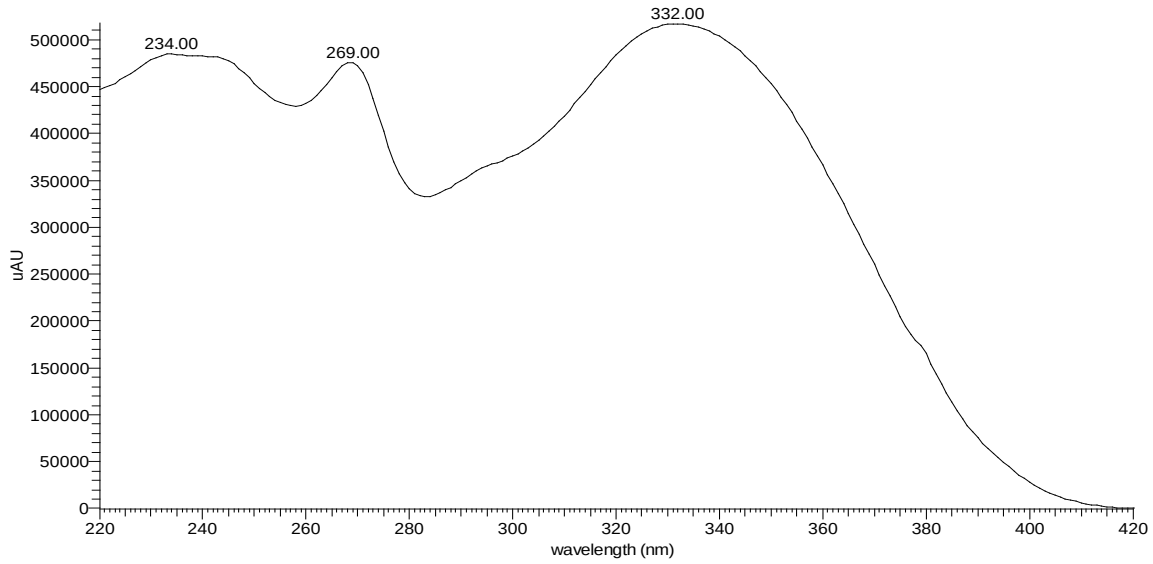


Figure 5.68 UV-Vis spectrum of peak 5 from the hydroalcoholic bok choy extract.

The loss of the 176 amu fragment in the MS^3 spectrum was attributed to the hydroxycinnamic acid moiety and identified as feruloyl ([M-H]$^-$ = 176). Therefore, using the same reasoning as for peaks 2, 3, and 4, peak 5 was identified as kaempferol-3-sophoroside(feruloyl)-7-glucoside and the fragmentation of this compound as observed in the data dependent studies is shown in Figure 5.69. A summary of the above findings is displayed in Table 5.21 at the end of this section.

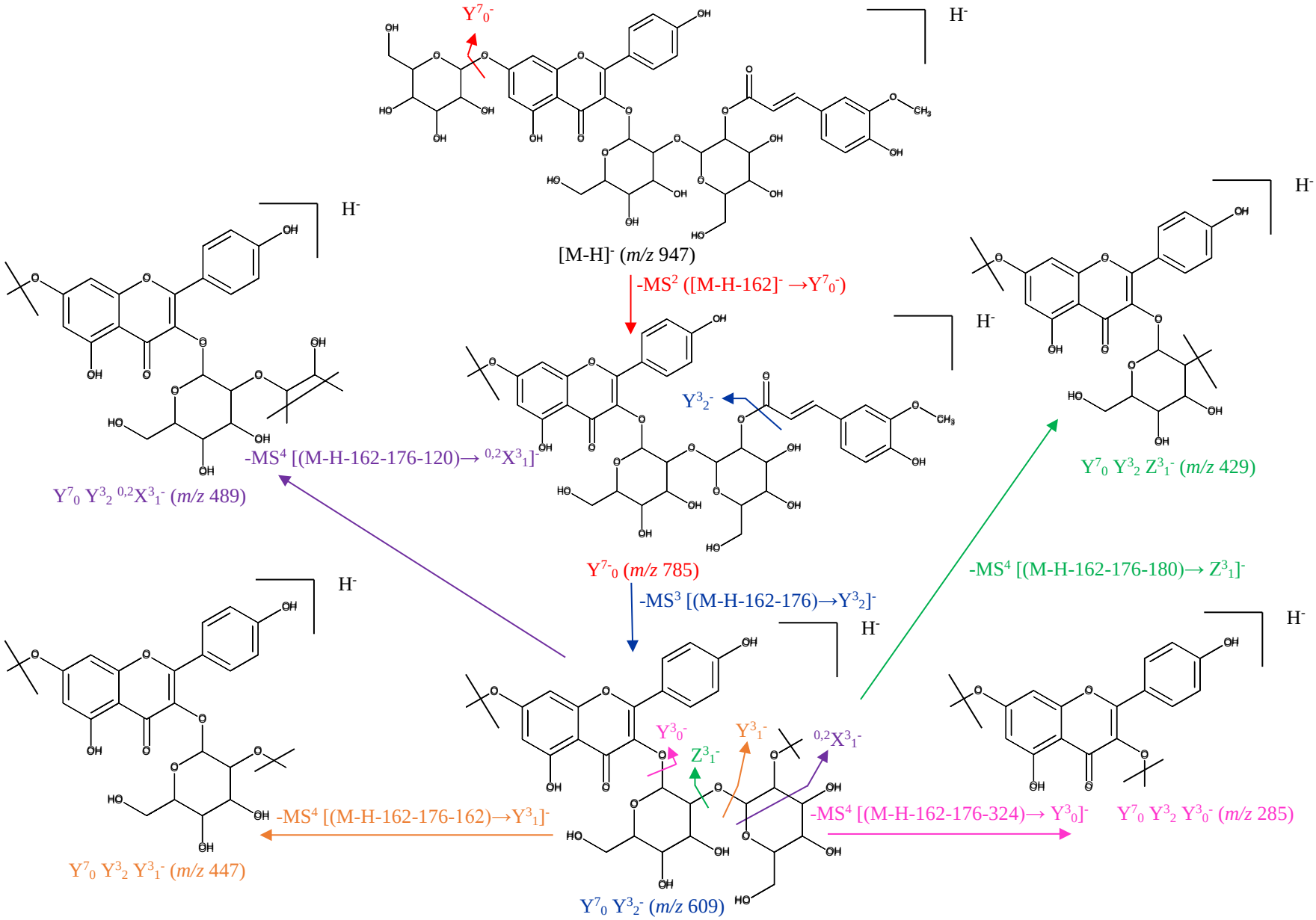


Figure 5.69 Flow diagram showing the chemical structure elucidation and fragmentation pattern of peak 5, identified as kaempferol-3-sophoroside(feruloyl)-7-glucoside.

5.4.2.6 Identification and Structure Elucidation of Peak Six in the Hydroalcoholic Bok Choy Extracts

Peak 6 had a retention time of 23.77 min in the HPLC-ESI-MS TIC of the hydroalcoholic Sumo bok choy extract shown in Figure 5.49 and was identified as kaempferol-3-sophoroside(coumaroyl)-7-glucoside. The flow diagram shown in Figure 5.70 below depicts the product ion spectra selected from the data dependent information obtained, whereby only the most intense ions were investigated. The MS^n spectra for these ions are shown in Figure 5.71.

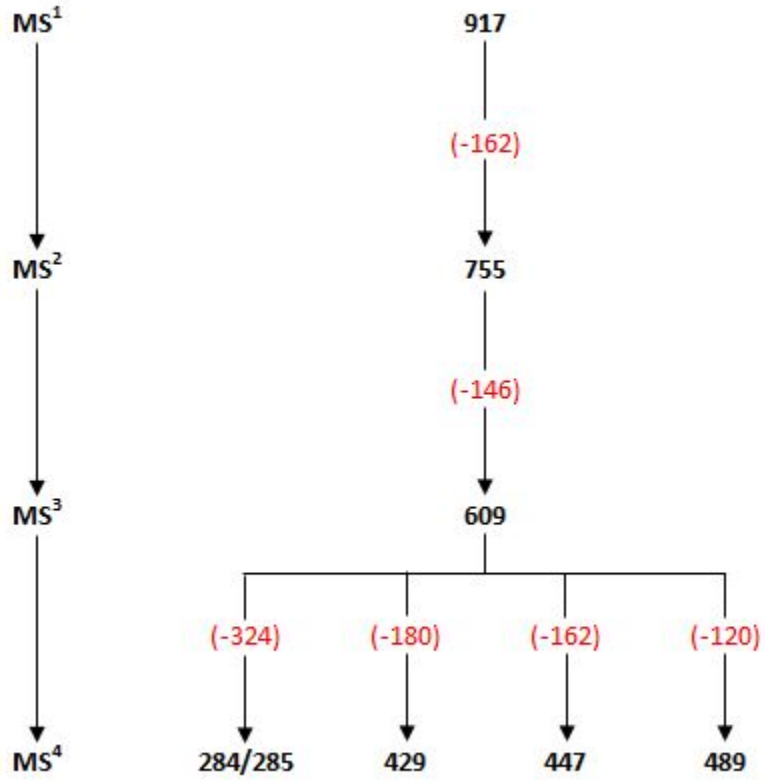


Figure 5.70 Breakdown of the MS^n fragmentation of ions in peak 6 of the hydroalcoholic bok choy extract, as selected by the data dependent protocol.

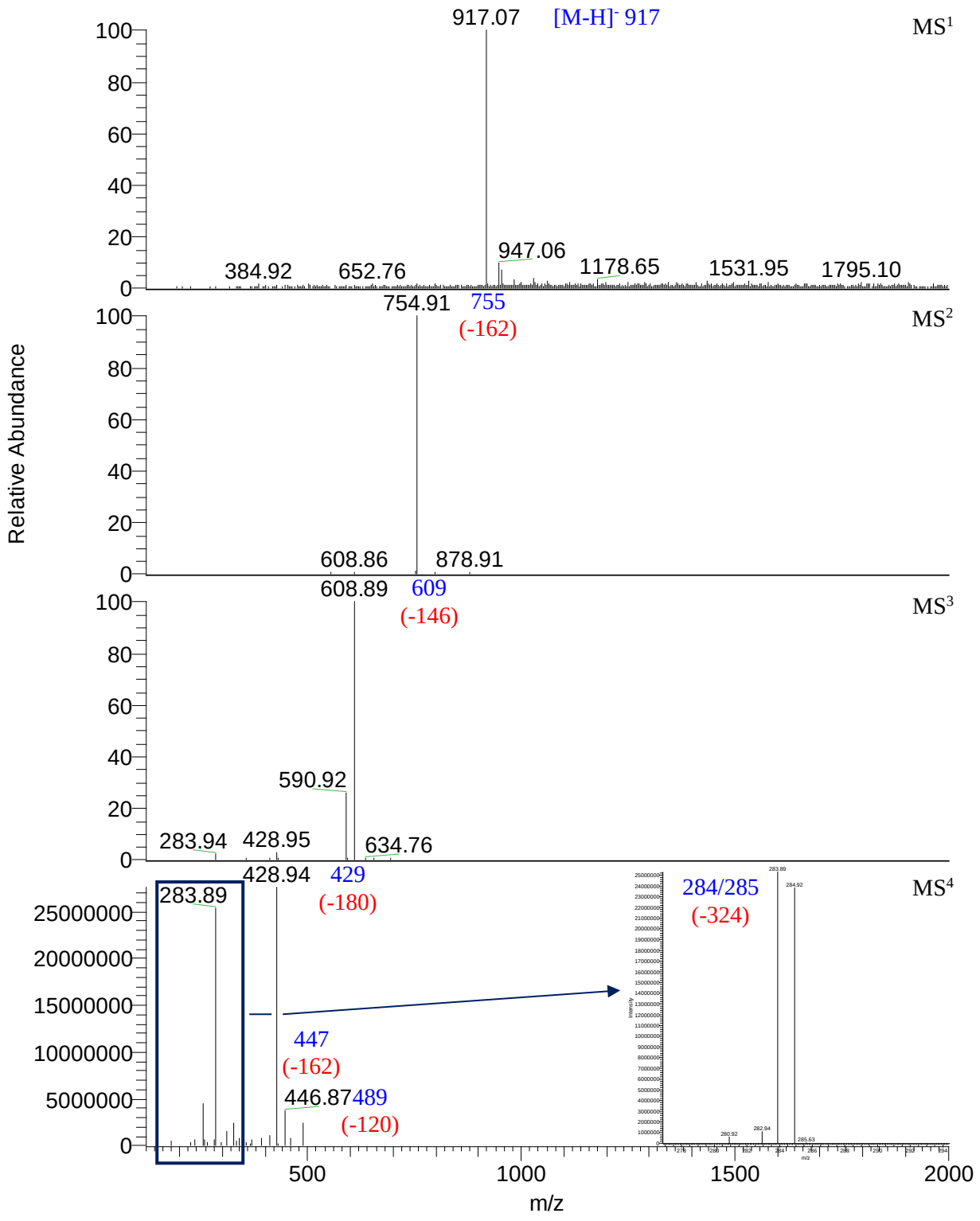


Figure 5.71 MS^n spectra collected during data dependent studies on peak 6 of the hydroalcoholic bok choy extract.

The MS^n spectra for peak 6 displayed very similar fragmentation patterns as peaks 2, 3, 4, and 5 where the only difference was seen in the MS^3 spectrum, indicating that these compounds differ only in their hydroxycinnamic acid moiety. There was a 285 amu product ion ($Y^7_0\,Y^3_2\,Y^3_0{}^-$ = [M-H-162-162-324]$^-$) in the MS^4 spectrum, as seen in the insert of Figure 5.71, which signified that the compound consisted of a kaempferol core. The MS^2 spectrum showed a single product ion $Y^7_0{}^-$ (*m/z* 755) which resulted from the loss of a 162 amu fragment and the MS^3 spectrum showed another single product ion $Y^7_0\,Y^3_2{}^-$ (*m/z* 609) which resulted from the loss of a 146 amu fragment. The fragments observed in the MS^4 spectrum were the same as those observed for peaks 2, 3, 4, and 5. These were the aglycone $Y^7_0\,Y^3_2\,Y^3_0{}^-$ (*m/z* 285), $Y^7_0\,Y^3_2{}^{0,2}X^3_1{}^-$ (*m/z* 489), $Y^7_0\,Y^3_2\,Y^3_1{}^-$ (*m/z* 447), and $Y^7_0\,Y^3_2\,Z^3_1{}^-$ (*m/z* 429), which resulted from losses of 324 amu, 120 amu, 162 amu and 180 amu from the $Y^7_0\,Y^3_2{}^-$ (*m/z* 609) product ion respectively. The UV-Vis spectrum shown in Figure 5.72 showed an absorbance maximum at approximately 330 nm indicating that the compound was a hydroxycinnamic acid derivative.

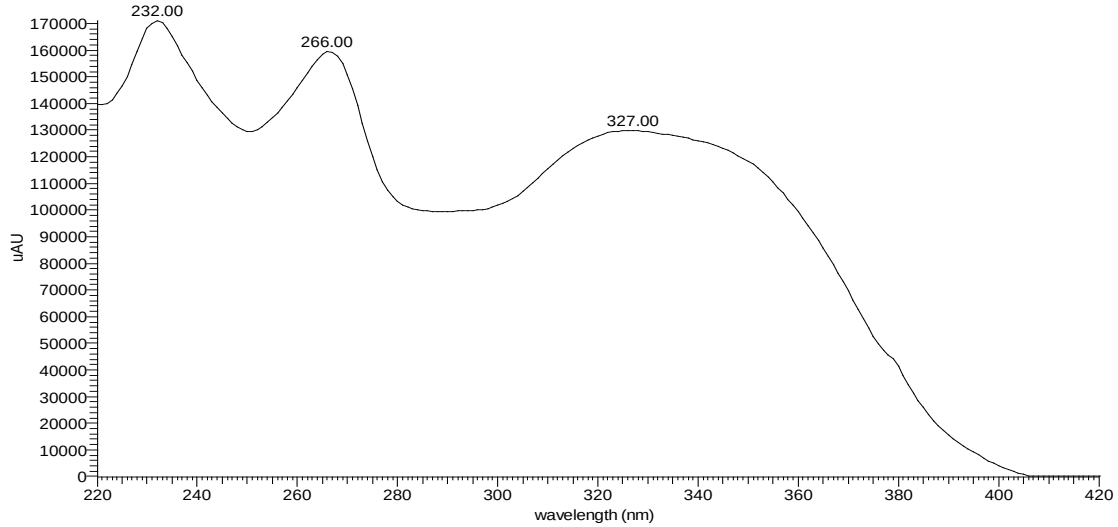


Figure 5.72 UV-Vis spectrum of peak 6 from the hydroalcoholic bok choy extract.

The loss of the 146 amu fragment in the MS^3 spectrum was attributed to the hydroxycinnamic acid moiety and identified as coumaroyl ([M-H]$^-$ = 146). Therefore, using the same reasoning as for peaks 2, 3, 4, and 5, peak 6 was identified as kaempferol-3-sophoroside(coumaroyl)-7-glucoside and the fragmentation of this compound as observed in the data dependent studies is shown in Figure 5.73. A summary of the above findings is displayed in Table 5.21 at the end of this section.

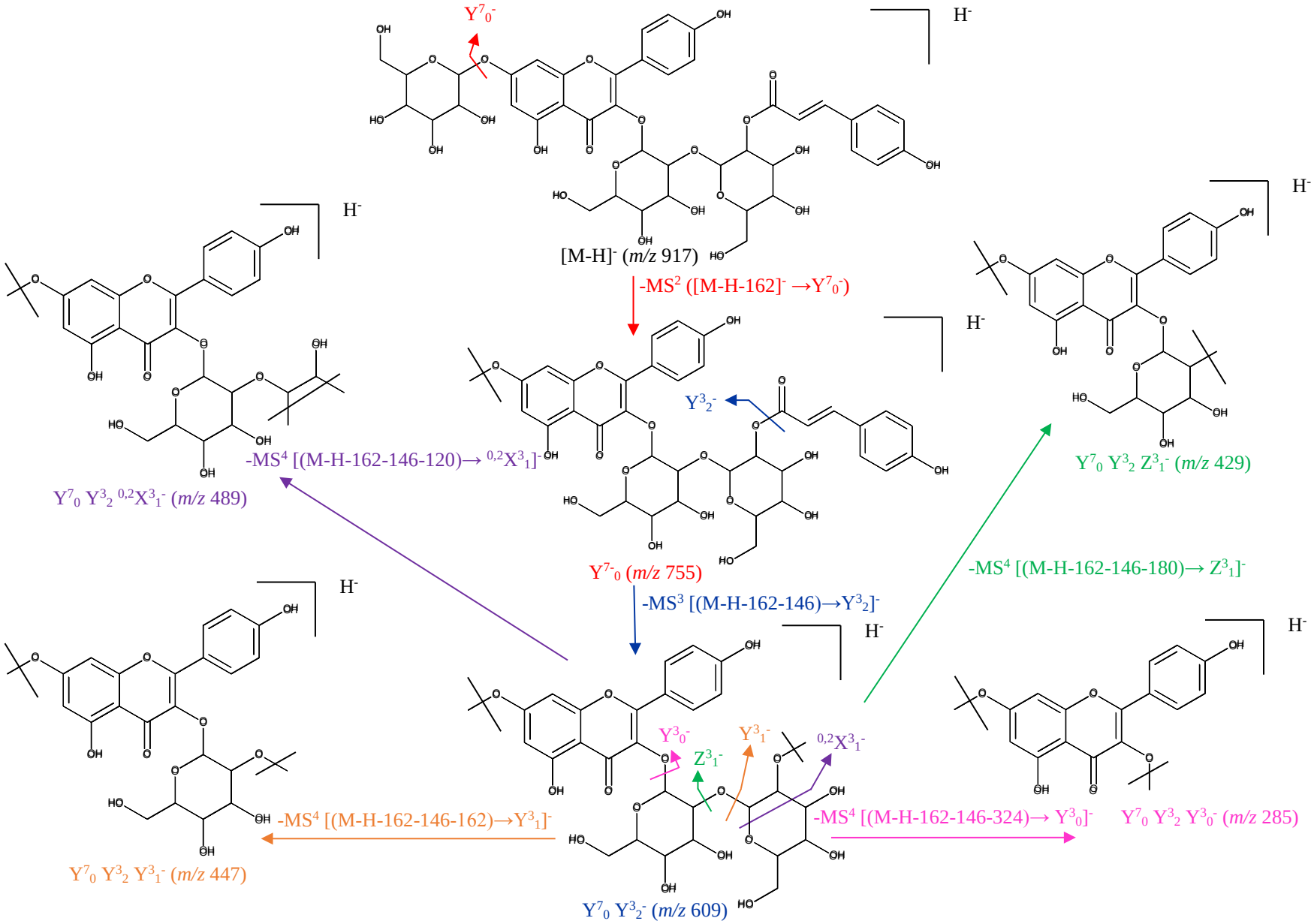


Figure 5.73 Flow diagram showing the chemical structure elucidation and fragmentation pattern of peak 6, identified as kaempferol-3-sophoroside(coumaroyl)-7-glucoside.

5.4.2.7 Identification and Structure Elucidation of Peak Seven in the Hydroalcoholic Bok Choy Extracts

Peak 7 had a retention time of 24.39 min in the HPLC-ESI-MS TIC of the hydroalcoholic Sumo bok choy extract shown in Figure 5.49, and was identified as kaempferol-3,7-diglucoside. This compound was previously identified as peak 4 in the alkaline hydrolysate bok choy extracts and all data including MS^n spectra and the UV-Vis spectrum, as well as the chemical structure elucidation and fragmentation pattern flow diagram, was presented in Section 5.3.2.4.

5.4.2.8 Identification and Structure Elucidation of Peak Eight in the Hydroalcoholic Bok Choy Extracts

Peak 8 had a retention time of 24.53 min in the HPLC-ESI-MS TIC of the hydroalcoholic Sumo bok choy extract shown in Figure 5.49, and was identified as isorhamnetin-3,7-diglucoside. This compound was previously identified as peak 5 in the alkaline hydrolysate bok choy extracts and all data including MS^n spectra and the UV-Vis spectrum, as well as the chemical structure elucidation and fragmentation pattern flow diagram, was presented in Section 5.3.2.5.

5.4.2.9 Identification and Structure Elucidation of Peak Nine in the Hydroalcoholic Bok Choy Extracts

Peak 9 had a retention time of 25.59 min in the HPLC-ESI-MS TIC of the hydroalcoholic Sumo bok choy extract shown in Figure 5.49 and was identified as isorhamnetin-3-glucoside. The flow diagram shown in Figure 5.74 below depicts the product ion spectra selected from the data dependent information obtained, whereby only the most intense ions were investigated. The MS^n spectra for these ions are shown in Figure 5.75.

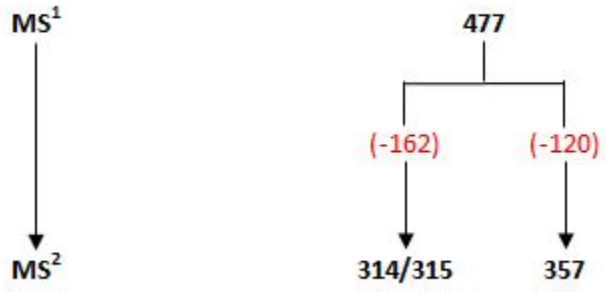


Figure 5.74 Breakdown of the MS^n fragmentation of ions in peak 9 of the hydroalcoholic bok choy extract, as selected by the data dependent protocol.

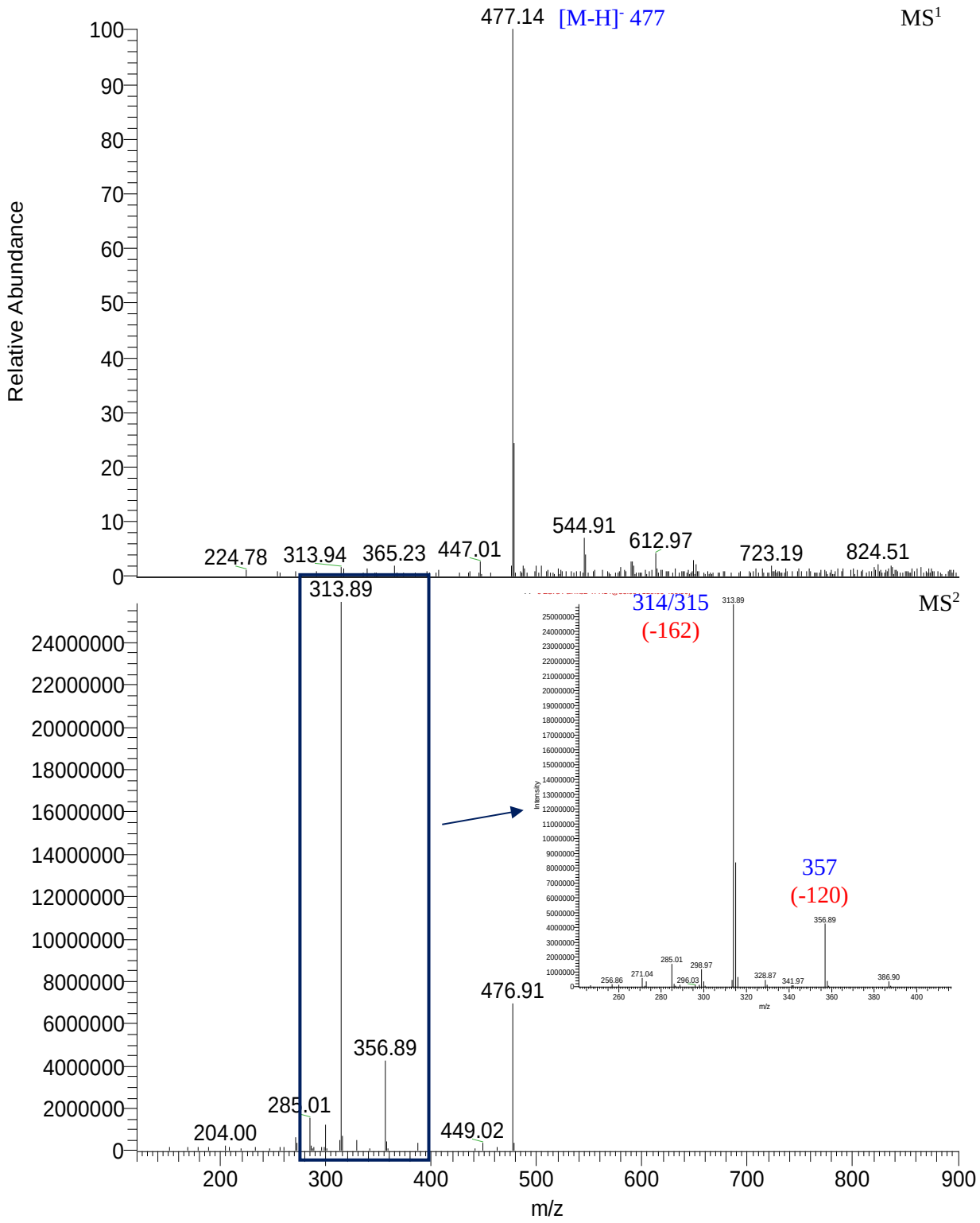


Figure 5.75 MS^n spectra collected during data dependent studies on peak 9 of the hydroalcoholic bok choy extract.

The data dependent protocol only collected MS spectra to the second order for peak 9. The MS^2 spectrum consisted of two major ions, including a 315 product ion ($Y^3_0{}^-$ = [M-H-162]$^-$) and a 357 product ion ($^{0,2}X^3_0{}^-$ = [M-H-120]$^-$), as shown in the insert of Figure 5.75. The *m/z* 315 ion suggested that the compound was an isorhamnetin based flavonol and the production of the aglycone from a loss of 162 amu from the *m/z* 477 parent ion indicated that the compound was a mono-glucoside of isorhamnetin. Given that there is a second product ion $^{0,2}X^3_0{}^-$ (*m/z* 357) displayed in the MS^2 spectrum resulting from a loss of 120 amu (fragmentation through the sugar ring) infers that the isorhamnetin compound is un-substituted at the C7 position and hence the glucose unit is attached at the C3 position. This, along with the broad absorbance maximum at approximately 350 nm observed in the UV-Vis spectrum shown in Figure 5.76, indicated that the flavonol core was glycosylated at position C3.

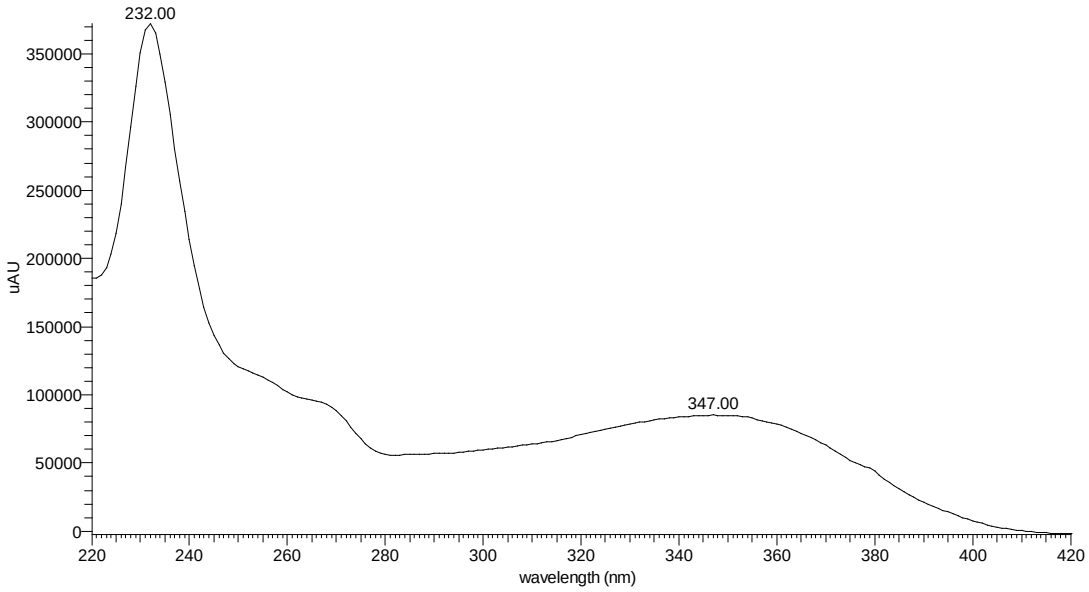


Figure 5.76 UV-Vis spectrum of peak 9 from the hydroalcoholic bok choy extract.

Peak 9 was therefore identified as isorhamnetin-3-glucoside and the fragmentation of this compound as observed in the data dependent studies is shown in Figure 5.77. A summary of the above findings is displayed in Table 5.21 at the end of this section.

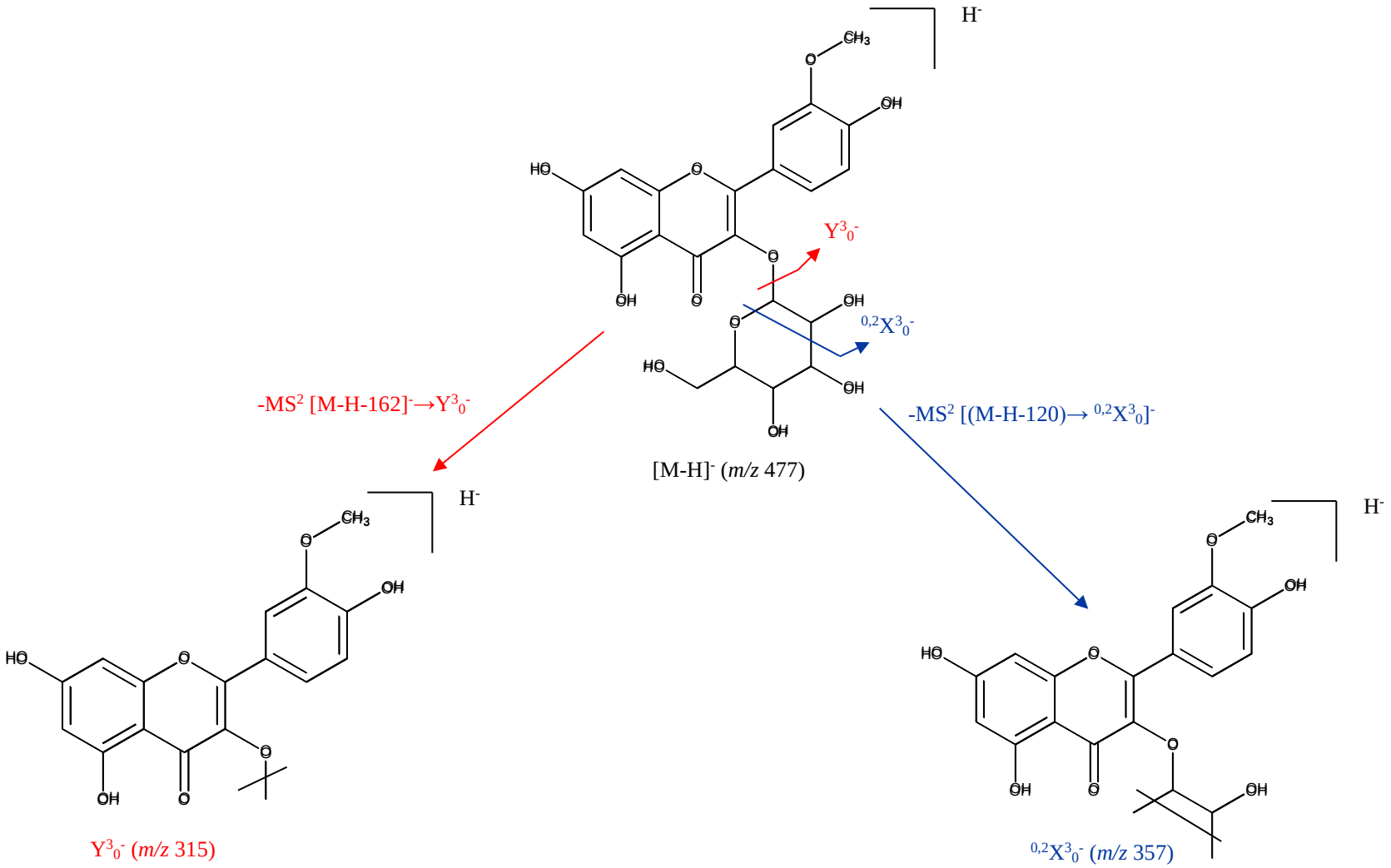
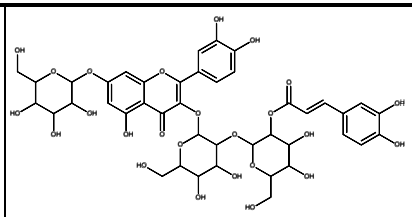
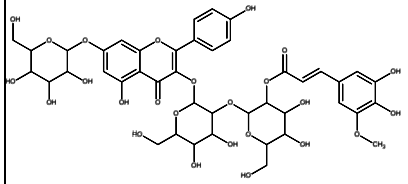
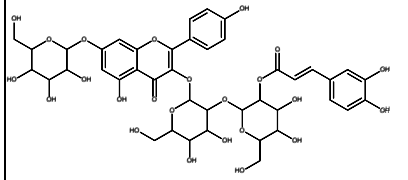
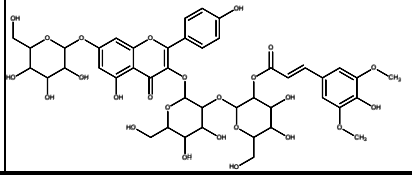
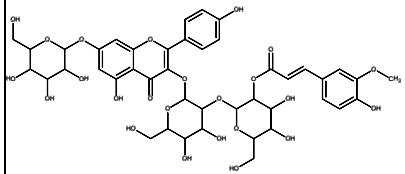
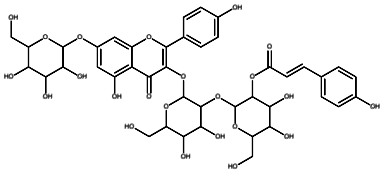
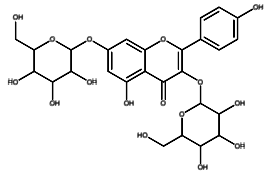
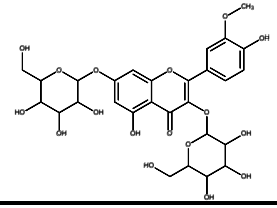
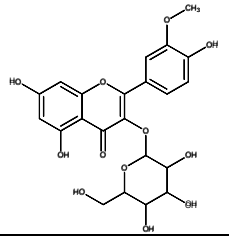


Figure 5.77 Flow diagram showing the chemical structure elucidation and fragmentation pattern of peak 9, identified as isorhamnetin-3-glucoside.

Table 5.21 Characterisation and structure elucidation of flavonol compounds in hydroalcoholic bok choy extracts. Percent abundance in parentheses.

Peak	Compound Identity	t_R (min)	λ_{max} (nm)	MS^1 $[M-H]^-$ (*m/z*)	MS^2 $[M-H]^-$ (*m/z*) (%)	MS^3 $[MS^2(100\%)]^-$ (*m/z*) (%)	MS^4 $[MS^3(100\%)]^-$ (*m/z*) (%)				Structure
					$Y^7_0{}^-$	$Y^7_0\ Y^3_2{}^-$	$Y^7_0\ Y^3_2\ Y^3_1{}^-$	$Y^7_0\ Y^3_2\ {}^{0,2}X^3_1{}^-$	$Y^7_0\ Y^3_2\ Z^3_1{}^-$	$Y^7_0\ Y^3_2\ Y^3_0{}^-$	
					$[M-H-162]^-$	$[M-H-162-R^*]^-$	$[M-H-162-R-162]^-$	$[M-H-162-R-120]^-$	$[M-H-162-R-180]^-$	$[M-H-162-R-324]^-$	
1	Quercetin-3-sophoroside (caffeoyl) -7-glucoside	20.96	233, 266, 330	949	787 (100)	625 (100) *R = 162	463 (20)	505 (10)	445 (48)	300/301 (100)	
2	Kaempferol-3-sophoroside (hydroxy feruloyl) -7-glucoside	21.19	238, 268, 331	963	801 (100)	609 (100) *R = 192	447 (16)	489 (7)	429 (100)	284/285 (80)	
3	Kaempferol-3-sophoroside (caffeoyl) -7-glucoside	21.80	242, 268, 332	933	771 (100)	609 (100) *R = 162	447 (14)	489 (5)	429 (100)	284/285 (82)	
4	Kaempferol-3-sophoroside (sinapoyl) -7-glucoside	22.80	241, 268, 334	977	815 (100)	609 (100) *R = 206	447 (7)	489 (5)	429 (100)	284/285 (86)	

Peak	Compound Identity	t_R (min)	λ_{max} (nm)	MS^1 $[M-H]^-$ (*m/z*)	MS^2 $[M-H]^-$ (*m/z*) (%)	MS^3 $[MS^2(100\%)]^-$ (*m/z*) (%)	MS^4 $[MS^3$ (100%)$]^-$ (*m/z*) (%)				Structure
					Y^7_0 $[M-H-162]^-$	$Y^7_0\ Y^3_2$ $[M-H-162-R^*]^-$	$Y^7_0\ Y^3_2\ Y^3_1$ $[M-H-162-R-162]^-$	$Y^7_0\ Y^3_2\ {}^{0,2}X^3_1$ $[M-H-162-R-120]^-$	$Y^7_0\ Y^3_2\ Z^3_1$ $[M-H-162-R-180]^-$	$Y^7_0\ Y^3_2\ Y^3_0$ $[M-H-162-R-324]^-$	
5	Kaempferol-3-sophoroside (feruloyl) -7-glucoside	23.21	234, 269, 332	947	785 (100)	609 (100) *R = 176	447 (12)	489 (3)	429 (100)	284/285 (91)	
6	Kaempferol-3-sophoroside (coumaroyl) -7-glucoside	23.77	232, 266, 327	917	755 (100)	609 (100) *R = 146	447 (14)	489 (9)	429 (100)	284/285 (93)	
					MS^2 $[M-H]^-$ (*m/z*) (%)			MS^3 $[MS^2(100\%)]^-$ (*m/z*) (%)			
					Y^7_0 $[M-H-162]^-$	${}^{0,2}X^3_0$ $[M-H-120]^-$	Y^3_0 $[M-H-324]^-$	$Y^7_0\ Y^3_0$ $[M-H-162-324]^-$			
7	Kaempferol-3,7-diglucoside	24.39	230, 255, 350*	609	447 (100)	489 (21)	284/285 (29)	284/285 (100)			
8	Isorhamnetin-3,7-diglucoside	24.53	230, 254, 352*	639	477 (100)	519 (13)	315 (14)	314/315 (100)			

Peak	Compound Identity	t_R (min)	λ_{max} (nm)	MS^1 $[M-H]^-$ (*m/z*)	MS^2 $[M-H]^-$ (*m/z*) (%)				Structure
						$Y^3_0{}^-$ $[M-H-162]^-$	$^{0,2}X^3_0{}^-$ $[M-H-120]^-$		
9	Isorhamnetin-3-glucoside	25.59	232, 268, 347	477		314/315 (100)	357 (16)		

5.4.3 Isolation of Flavonol Glycoside-Hydroxycinnamic Acid Conjugates in Hydroalcoholic Bok Choy Extracts by Preparative HPLC

Preparative HPLC was used to isolate and collect some of the acylated and glycosylated flavonol compounds from a hydroalcoholic methanol/water bok choy extract for further characterisation using NMR, as well as for studies on antiproliferative activity on human colon cancer cells *in vitro*. Preparative HPLC conditions are outlined in 3.7 and fraction collection methodology is detailed in 3.7.1. The optimisation of preparative HPLC conditions was discussed in 4.3 and Figure 5.78 shows a chromatogram of a hydroalcoholic bok choy extract of the Sumo variety using the optimised conditions. The nine flavonol compounds previously identified in the data dependent MS^n studies are labelled with their corresponding retention times.

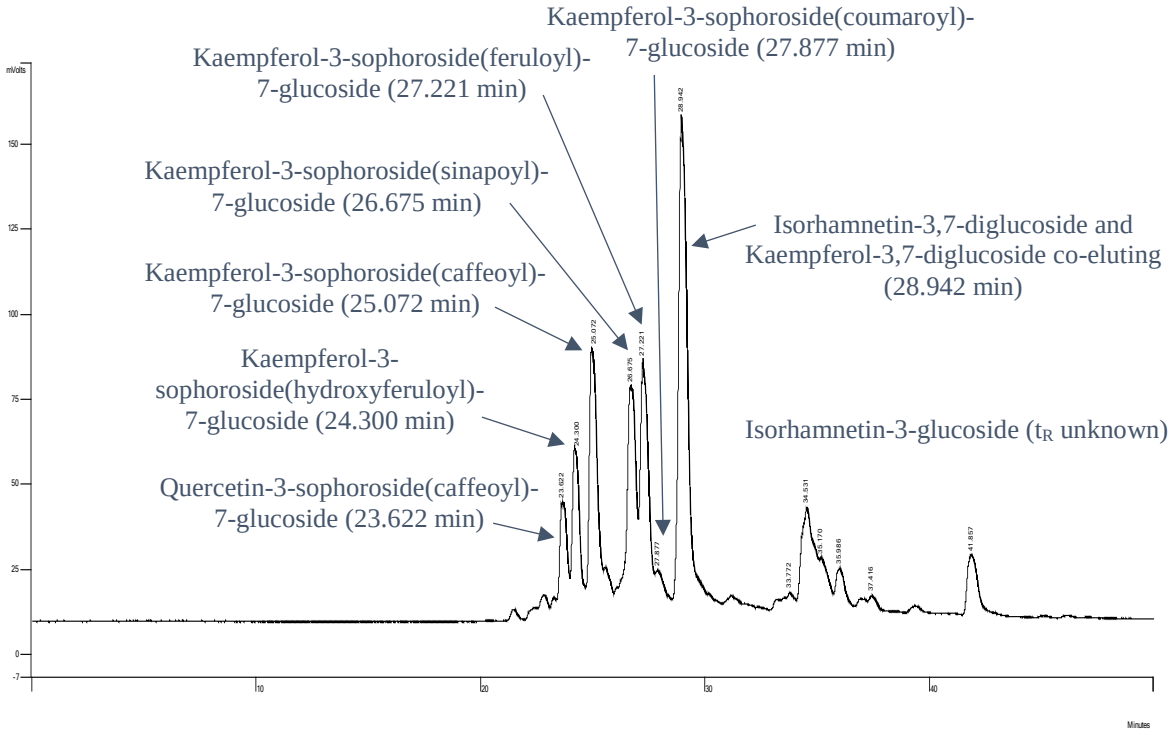


Figure 5.78 Preparative HPLC chromatogram of a hydroalcoholic Sumo bok choy extract monitored at 330 nm. Mobile phase and gradient program are outlined in Table 4.1.

The separation obtained on the preparative HPLC was similar to the analytical HPLC, although the abundances of the peaks varied slightly. Isorhamnetin-3,7-diglucoside and kaempferol-3,7-diglucoside co-eluted in the preparative HPLC separation and many of the peaks were not completely resolved. Isorhamnetin-3-glucoside could not be identified in the preparative HPLC chromatogram and may be co-eluting with the Isorhamnetin-3,7-diglucoside and kaempferol-3,7-diglucoside peak.

Various fractions were collected, and the corresponding components confirmed by HPLC-PDA/ESI-MS^n. The six major peaks in the extract were collected as fractions 1-6 (Figure 5.79) for further analysis and studies. Fractions 1-5 were expected to contain individual flavonol glycoside-hydroxycinnamic acid derivatives: one quercetin-3-sophoroside-7-glucoside derivative and four kaempferol-3-sophoroside-7-glucoside derivatives, whereas fraction 6 was expected to contain the two flavonol diglycosides that were previously identified in the alkaline hydrolysate extract as isorhamnetin-3,7-diglucoside and kaempferol-3,7-diglucoside.

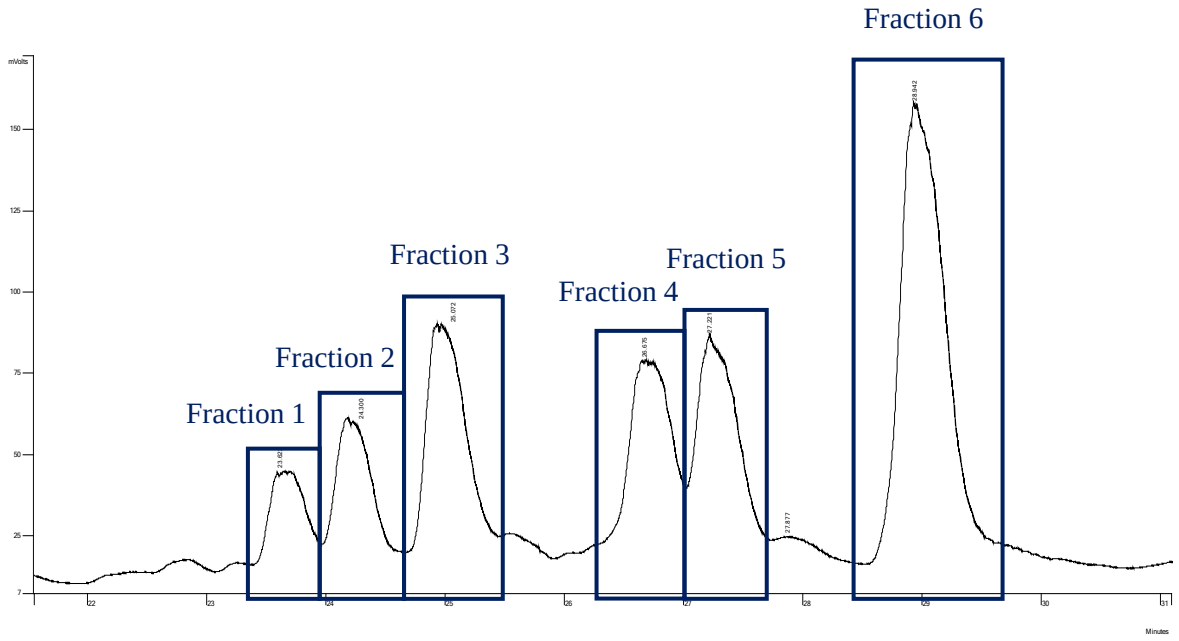


Figure 5.79 Preparative HPLC chromatogram of a hydroalcoholic Sumo bok choy extract monitored at 350 nm, showing the six fractions that were collected for further studies.

Six fractions were collected, however, HPLC-PDA/ESI-MS^n analysis showed that fractions 1, 2, 4, 5, and 6 were not pure compounds, and that only fraction 3 was pure enough for further studies. Fraction 3 was identified as kaempferol-3-sophoroside(caffeoyl)-7-glucoside. Further characterisation and structure elucidation was conducted on fraction 3 using NMR, and its antiproliferative activity on HT-29 human colon cancer cells *in vitro* was examined. The results for the antiproliferative effect of this compound are presented in Chapter VI.

5.4.4 NMR Analysis of Fraction Three from a Hydroalcoholic Bok Choy Extract

Fraction 3 from the hydroalcoholic bok choy extract was subjected to 1D and 2D NMR for further structure elucidation information and confirmation. All 1D and 2D NMR parameters are detailed in 3.10, including ^{1}H NMR as well as 2D COSY, HSQC, and HMBC experiments. The purified compound (kaempferol-3-sophoroside(caffeoyl)-7-glucoside) was re-constituted in methanol-d_4 and subjected to ^{1}H NMR as well as the 2D NMR experiments, however, the NMR data were inconclusive due to the small amount of material collected.

5.5 CONCLUSION

This chapter presented and discussed the results of the chemical analysis and structure elucidation of flavonol compounds present in three bok choy cultivars: Sumo, Karate, and Miyako, using HPLC-PDA/ESI-MS^n. Quercetin, isorhamnetin, and kaempferol were identified as the three main flavonols present in the bok choy cultivars and the aglycone levels were quantified after acid hydrolysis. Kaempferol was the major constituent (85.5 – 122 mg/100 g DW), followed by isorhamnetin (38.3 – 66.7 mg/100 g DW), and then quercetin (10 – 20.6 mg/100 g DW). The Miyako variety contained the highest levels of both quercetin and isorhamnetin, however, kaempferol was comparable in all three cultivars of bok choy. Although Miyako contained higher levels of quercetin and isorhamnetin, all three bok choy cultivars contained similar total aglycone levels (Sumo 183.3 mg/100 g DW, Karate 159.9 mg/100 g DW, and Miyako 197.3 mg/100 g DW bok choy). Therefore, the differences in flavonol aglycone content between the three bok choy cultivars were considered insignificant and no firm conclusions could be made as to whether one bok choy cultivar contained more health-promoting flavonols than another.

The HPLC-PDA/ESI-MS^n method used for the separation and quantification of flavonol aglycones was based on a previously published method and was verified through peak area and retention time reproducibility, detector linearity, and recovery data. Data from both the PDA detector and the MS in SIM mode were used for quantification. The MS SIM mode allowed for highly selective and specific separation, and provided more accurate quantification of the flavonols, as isorhamnetin and kaempferol were not completely resolved in the PDA chromatograms.

Five flavonol glycoside conjugates were identified and characterised in the bok choy cultivars after alkaline hydrolysis. Structure elucidation was achieved using a combination of UV-Vis spectral data, MS^n fragmentation patterns obtained from data dependent studies, and previously published data in literature. The five flavonol glycosides consisted of two flavonol-3-sophoroside-7-glucosides, and three flavonol-3,7-diglucosides. The names of the compounds are as follows:

- quercetin-3-sophoroside-7-glucoside,
- kaempferol-3-sophoroside-7-glucoside,
- quercetin-3,7-diglucoside,
- kaempferol-3,7-diglucoside, and
- isorhamnetin-3,7-diglucoside.

Kaempferol-3-sophoroside-7-glucoside was isolated and purified from an alkaline hydrolysate bok choy extract using preparative HPLC and the structure confirmed by 1D and 2D NMR, in addition to the MS^n and UV-Vis data. The purified compound, along with another fraction (containing kaempferol-3,7-diglucoside and isorhamnetin-3,7-diglucoside) collected from the alkaline hydrolysate using preparative HPLC were also examined for antiproliferative activity on HT-29 cells *in vitro* (results presented in Chapter VI).

Nine naturally occurring flavonol derivatives were identified in the bok choy cultivars after hydroalcoholic extraction. Structure elucidation was determined in the same manner as for the flavonol glycosides in the alkaline hydrolysate extracts. The nine flavonol compounds consisted of six complex flavonol glycoside-hydroxycinnamic acid derivatives, two flavonol di-glycosides (previously identified in the alkaline hydrolysate extracts), and one flavonol mono-glucoside. The majority of the compounds were conjugates of kaempferol. The names of the compounds are as follows:

- quercetin-3-sophoroside(caffeoyl)-7-glucoside,
- kaempferol-3-sophoroside(hydroxy feruloyl)-7-glucoside,
- kaempferol-3-sophoroside(caffeoyl)-7-glucoside,
- kaempferol-3-sophoroside(sinapoyl)-7-glucoside,
- kaempferol-3-sophoroside(feruloyl)-7-glucoside,
- kaempferol-3-sophoroside(coumaroyl)-7-glucoside,
- kaempferol-3,7-diglucoside,
- isorhamnetin-3,7-diglucoside, and
- isorhamnetin-3-glucoside.

Six fractions from a hydroalcoholic bok choy extract were collected via preparative HPLC, however, only one fraction (fraction 3) was pure enough for NMR analysis. Fraction 3 was identified as kaempferol-3-sophoroside(caffeoyl)-7-glucoside in MS^n data dependent studies and was subjected to 1D and 2D NMR for further structure elucidation and confirmation, however, insufficient data were obtained. The isolated compound was, however, used in the cancer cell studies and its antiproliferative activity on HT-29 cells was assessed in the following chapter.

Based on the findings reported in this chapter, no firm conclusions could be drawn as to whether one bok choy cultivar may be more beneficial to human health than another, based on their flavonol content. This will be further explored in Chapter VI by assessing the antiproliferative activity of the three cultivars on human colon cancer cells *in vitro*.

CHAPTER VI
CANCER CELL STUDIES

6.1 INTRODUCTION

There have been numerous effective anti-cancer drugs developed over the years, such as cisplatin, methotrexate, 5-fluorouracil, Taxol etc., that are currently being used to treat cancer patients. However, most of these drugs still have serious side effects on the human body, for example fatigue, muscle aches and pain, hair loss, fertility issues, among others. Hence, there is a call for the development of new drugs that possess low toxicity, have a minor environmental impact, and preferably originate from a natural source. In addition to the treatment of cancer, there is also a general call for the investigation of the potential of fruits and vegetables, and the naturally occurring chemicals within them, in preventing the onset of cancer.

Many studies have investigated the use of medicinal plants in the treatment and prevention of cancer, which has also expanded to include fruits, vegetables, and other plant foods (refer to Table 2.11). The general view of fruits and vegetables being merely sources of food and nutrition has changed over the past several decades as more and more studies on the medical and/or bioactive properties of phytochemicals reveal their powerful attributes. Phytochemicals possess a range of bioactivities and health promoting properties for humans (as discussed in Section 2.2.2), and it is thought that a combination of these bioactivities play an important role in their ability to act as anti-cancer agents. They have many complementary and overlapping mechanisms of action, including antioxidant activity and the ability to scavenge free radicals, neuroprotective effects on antiaging, antibacterial and antiviral effects, anti-inflammatory properties by stimulation of the immune system, regulation of hormone metabolism, antiatherosclerosis by inhibition, proliferation and migration of vascular cells, modulation of carcinogen metabolism, regulation of gene expression on oncogenes and tumour suppressor genes in cell proliferation and differentiation, induction of cell cycle arrest and apoptosis, suppression of angiogenesis, inhibition of signal transduction pathways, modulation of enzyme activities in detoxification, oxidation and reduction, and action on other possible targets [234, 17, 23, 32, 12, 25, 8, 6]. Compelling data obtained from laboratory studies, epidemiological investigations, as well as human clinical trials demonstrate that flavonoids can induce cancer chemoprevention and chemotherapy [238]. The bioactivities of these compounds, as well as their plant food extracts, on colorectal cancer has become a major research area due to the direct contact that plant foods and their constituents have with the gastrointestinal tract.

The most accepted view of carcinogenesis is that cancer is caused by DNA alterations and that the formation of a malignant tumour requires these cells with altered DNA to acquire several capabilities, the so-called hallmarks of cancer. These include apoptosis resistance (i.e. programmed cell death is switched off in the damaged cells and they continue to proliferate without any mediation), increased angiogenesis (i.e. the generation of new blood vessels), and the capacity to invade and metastasise (i.e. spread to other parts of the body). This study investigated the antiproliferative activities of bok choy extracts, selected fractions, and individual flavonol compounds, on the proliferation of HT-29 human colorectal adenocarcinoma cells *in vitro*, using the MTT assay. The experiments assessed the potential of bok choy extracts, and several flavonol compounds, to positively influence human health, both from a chemopreventive perspective as well as a chemotherapeutic. By assessing the inhibitory effects of the crude bok choy extracts on the cancerous cells, the study aimed to determine whether there is the possibility of preventing the onset of colorectal cancer by regular consumption of bok choy in the habitual diet. The investigation of the antiproliferative effects of standard flavonol compounds looked at the potential of these compounds to be used as chemotherapy drugs in the treatment of colorectal cancer, or perhaps as purified supplements in the prevention of colorectal cancer. Information regarding the methodology employed for these experiments is outlined in 3.10.

The specific aims that will be addressed in this chapter include the following:

1. Assess the antiproliferative effects of three different cultivars of crude bok choy extracts on HT-29 cells *in vitro* and identify any differences in their inhibitory abilities.
2. Assess the antiproliferative effects of kaempferol, isorhamnetin, and quercetin aglycones and mono-glucosides, as well as two purified flavonol derivatives and a further fraction containing two compounds isolated from bok choy, on HT-29 cells *in vitro.* Identify any differences in their inhibitory abilities, and determine any synergistic effects when cells are treated with these compounds in various combinations.
3. Compare the effects observed for the individual flavonol compounds, and the fraction containing the isolated compounds, to that of the crude bok choy extracts.
4. Identify any differences between aglycones, mono-glucosides, di-glycosides, and acylated di-glycosides in their abilities to inhibit cell proliferation in HT-29 cells *in vitro*.

6.2 ANTIPROLIFERATIVE EFFECTS OF BOK CHOY EXTRACTS ON HT-29 CELLS

Research has demonstrated that various fruit and vegetable phytochemical extracts exhibit strong antiproliferative activities against cancer cells *in vitro*, and several epidemiological studies have also indicated that a diet rich in fruits and vegetables can reduce the risk of developing cancer. Of interest to this study is the vegetable bok choy, a member of the *Brassica* genus, which has shown to be associated with a reduced risk of cancer when consumed regularly in the diet [93]. These vegetables are known to contain a complex mixture of health-related phytochemicals, including phenolic compounds, carotenoids, and glucosinolates, as well as vitamins, fibre, soluble sugars, and minerals [93-94]. It is not well understood which constituents are responsible for the observed anti-cancer effects, or whether the effects may result because of the synergistic action of several constituents. It is hypothesised, however, that the anti-cancer properties are attributed to the complex mixture of phytochemicals present in whole foods, not just attributed to individual constituents within the plant. This hypothesis may explain why no single antioxidant can replace the combination of natural phytochemicals in fruits and vegetables to achieve the same health benefits [16]. For example, two studies conducted by Veeriah *et al.* [306, 262] reported that flavonoids from an apple extract inhibited colon cancer (HT-29) cell growth more potently than a synthetic mixture comprising eight flavonoids that mimicked the composition of the natural apple extract [262]. In another study by Seeram *et al.* [256], the antiproliferative effects of cranberry extracts were shown to be more effective against human oral (KB, CAL27), prostate (RWPE-1, RWPE-2, 22Rv1), and colon cancer (HT-29, HCT116, SW480, SW620) cell lines than the individual phytochemicals and fractions of the extracts. These studies suggested that the whole fruit extracts are more effective than the individual phytochemicals for inhibiting cancer cell growth *in vitro*.

6.2.1 Antiproliferative Effects of the Sumo Bok Choy Cultivar on HT-29 Cells Over Time

Initially, MTT cell proliferation assays (3.11.5) were conducted on a hydroalcoholic extract of the Sumo bok choy cultivar to determine if a bok choy extract could inhibit cell proliferation of HT-29 cells *in vitro*. Dry plant matter was prepared in complete media (McCoy's 5A) containing 0.1% v/v DMSO. The test concentrations ranged from 0.2 to 10.0 mg/L and were incubated with HT-29 cells over 96 hours. The MTT assay was performed at 24 hour time intervals (24, 48, 72, and 96 hours) during the exponential growth phase of the HT-29 cells (4.6.2). Figure 6.1 shows the cell proliferation of the HT-29 cells (as % control values, refer to 3.11.5.1) when treated with the Sumo hydroalcoholic bok choy extract at the four time points. All MTT cell proliferation assays were repeated independently twice, with each concentration of test

compound/fraction/extract and controls assayed in triplicate within each independent experiment. The errors bars on the graph indicate the standard deviation between these assays.

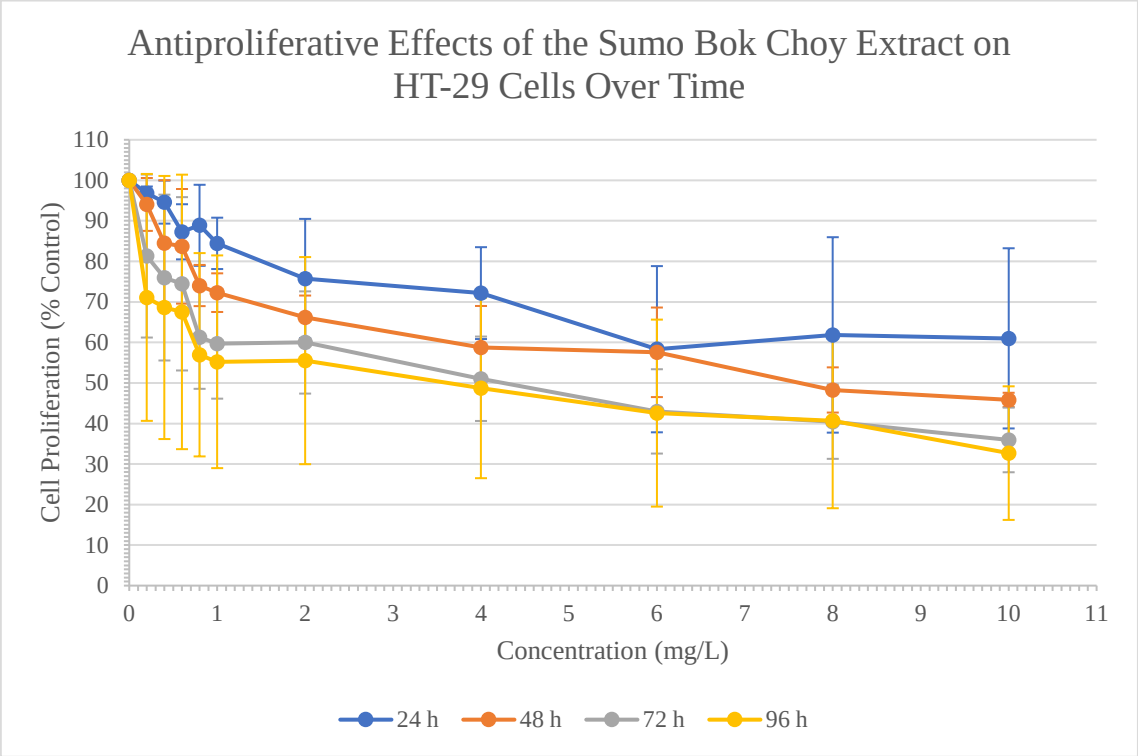


Figure 6.1 Proliferation of HT-29 cells treated with a hydroalcoholic extract of the Sumo bok choy cultivar after 24, 48, 72, and 96 hours of exposure. Data represents the mean of two separate experiments (n = 3 in each experiment), with error bars indicating ± SD.

The bok choy extract had an antiproliferative effect on the HT-29 cells (Figure 6.1). The effects were concentration dependent, as evidenced by the downward trend as concentration increased. There also appeared to be a time dependent effect, whereby the inhibitory effects at the 24 hour time point were lower, and an increase in the effects were observed as the incubation time increased. However, no firm conclusions could be drawn as to whether the antiproliferative effects are time dependent due to the large variance between the two independent experiments. Although statistically, the experiments in this part of the study were not repeated sufficiently to draw any firm conclusions, the purpose of the MTT cell proliferation assays was to provide some preliminary results and discuss any possible trends that may be observed.

Graphs displaying the antiproliferative effects over the 96-hour time period for some of the flavonol compounds examined as part of this study are shown in Appendix H. These include kaempferol (aglycone), quercetin-3-*O*-glucoside (quercetin mono-glucoside), and kaempferol-3-sophoroside(caffeoyl)-7-glucoside (flavonol glycoside-hydroxycinnamic acid derivative) purified from a hydroalcoholic bok choy extract. The 72-hour time point was chosen for the

remainder of the assays on the other bok choy cultivars, selected fractions, and flavonol compounds, as it had the least amount of variance between experiments.

6.2.2 Antiproliferative Effects of Bok Choy Cultivars on HT-29 Cells After 72 Hours of Exposure

Preliminary studies confirmed that the Sumo bok choy extract exhibited quite potent antiproliferative activity against HT-29 cells and could therefore have potential dietary benefits against the prevention of colon cancer. Hence, it was of interest to establish whether there were any variations in the magnitude of inhibitory effects between the different cultivars. If different cultivars of the same fruit or vegetable possess varying potency on cancer cell proliferation, then this information could potentially be used to select cultivars for health-promoting properties. Several studies have reported that different cultivars of the same fruit or vegetable inhibit cell proliferation of cancer cells *in vitro* to different extents. For example, Olsen *et al*. [258]and Meyers *et al.* [305], reported that different cultivars of strawberries displayed varying extents of antiproliferative activity on cancer cell proliferation *in vitro*. Similarly, a study on the antiproliferative activities of nine different apple varieties against human colon (HT-29) and stomach (MKN45) cancer cells by Serra *et al.* [307] showed that all nine apple cultivars exerted antiproliferative activities against both cell lines, however, these activities differed greatly within cultivars. A recent study by Guo *et al.* [277] on the antiproliferative activities of four varieties of Sea buckthorn berries against human liver cancer (HepG2) cells also demonstrated that different varieties exhibit different levels of antiproliferative ability. However, while some varieties displayed significant differences, there were some varieties where no significant variation was observed.

The antiproliferative effects of three bok choy cultivars: Sumo, Karate, and Miyako, were investigated on HT-29 cells after treatment for 72 hours. The antiproliferative effects of the hydroalcoholic extracts of each bok choy cultivar are shown in Figure 6.2. Data are displayed as concentration (mg/L) vs cell proliferation (% control values).

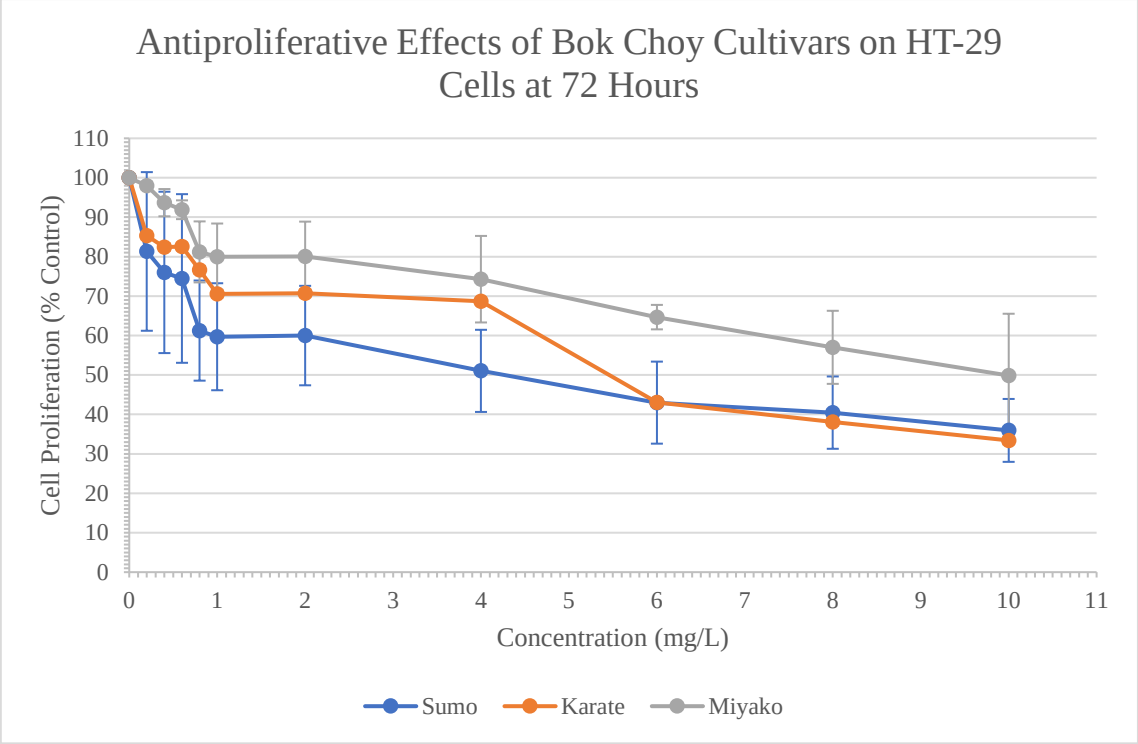


Figure 6.2 Proliferation of HT-29 cells treated with hydroalcoholic bok choy extracts of three cultivars: Sumo, Karate, and Miyako, after 72 hours of exposure. Data represents the mean of two separate experiments (n = 3 in each experiment), with error bars indicating ± SD.

Inhibition of HT-29 cell proliferation occurred in a dose dependent manner after treatment with each of the bok choy cultivars. The Sumo variety was more effective than the other two varieties, with Miyako being the least bioactive. However, the variances in antiproliferative activity were not considered to be significantly different.

The IC_{50} (i.e. concentration at which 50% inhibition is reached) was also calculated for each bok choy cultivar as a further comparison of antiproliferative abilities of the different varieties. All IC_{50} values were calculated using a nonlinear regression (curve fit), i.e. a dose-response inhibition curve of log(inhibitor) vs. response – variable slope (four parameters), on GraphPad Prism 7.03 (3.11.5.1). The initial unit of concentration was mg/L of dried bok choy extract, however, this was also converted to total flavonol aglycone equivalents in µM, based on the quantification data obtained in Chapter V (5.2.3). This allowed for direct comparisons to be made to the other MTT cell proliferation assays using flavonol standards and purified compounds. The IC_{50} values, along with the quantification data extracted from Chapter V, are displayed in Table 6.1.

Table 6.1 IC_{50} values of three different bok choy cultivar hydroalcoholic extracts after 72 hours of exposure on HT-29 cells. IC_{50} values were calculated using a nonlinear regression (curve fit) on GraphPad Prism 7.03. Flavonol aglycone quantification data obtained from 5.2.3 (i.e. PDA data from Table 5.9) was used to calculate IC_{50} values in µM equivalents as per total flavonol aglycone content. Q = quercetin, I = isorhamnetin, K = kaempferol. DW = Dry Weight.

Bok Choy Cultivar	IC_{50} (mg/L)	Q (mg/100 g DW)	I (mg/100 g DW)	K (mg/100 g DW)	Total Aglycone Levels (mg/100 g DW)	IC_{50} as Total Flavonol Aglycone Equivalents (µM)
Sumo	1.58	13.9	47.4	122	183	0.0098
Karate	3.18	11.6	38.3	110	160	0.017
Miyako	4.01	20.6	66.7	110	197	0.027

The differences between the IC_{50} values of the three bok choy cultivars were not considered to be significant. This corresponded to the findings in the quantification studies, whereby the differences in the total flavonol aglycone content between the three cultivars were also not considered to be significant. It was, however, interesting to note the substantially low IC_{50} values obtained for the bok choy extracts. This indicated that the constituents within bok choy could potentially be potent anti-cancer agents and that the vegetable could theoretically play a role in colon cancer prevention. The low IC_{50} values also supported the hypothesis that the observed anti-cancer effects of the bok choy extract could be due to the synergistic action of the complex mixture of phytochemicals present within the vegetable, and not just attributed to individual constituents within the plant, as the subsequent studies on individual flavonol compounds all had significantly higher IC_{50} values (refer to Tables 6.2, 6.3, and 6.4).

6.3 ANTIPROLIFERATIVE EFFECTS OF FLAVONOL COMPOUNDS ON HT-29 CELLS

The antiproliferative effects of the aglycones kaempferol, isorhamnetin, and quercetin, as well as their mono-glucoside forms, were assessed on HT-29 cells. The anti-cancer properties of flavonol compounds are mostly studied in the aglycone form, including antiproliferative effects on cancer cells *in vitro* as well as *in vivo* studies [7, 23, 235-238, 37, 239]. There have also been several studies on mono-glucoside forms *in vitro*, including both quercetin-3-*O*-glucoside [303, 252, 277] and isorhamnetin-3-*O*-glucoside [277]. Even though flavonols in the aglycone form do not occur naturally within the bok choy plant, it is possible that the aglycone form, as well as the mono-glucoside form, may potentially be present in the colon following digestion of the vegetable.

The prediction and extrapolation of *in vitro* and animal studies can become quite difficult due to the contradictory data on the absorption, metabolism, and excretion of dietary flavonols in the

gut. Whether flavonol glycosides, or only aglycones, are able to pass the gut wall continues to be a subject of dispute [328-330]. Glycosylated flavonols are generally thought to be cleaved by lactase phlorizin hydrolase (LPD) or β-glucosidase in the colon prior to absorption [331], however, some studies have shown that glycosides can be absorbed without hydrolysis [332, 55]. Either way, flavonol compounds that are not absorbed in the small intestine, as well as compounds that are absorbed and metabolised in the liver and then excreted in bile, enter the colon. Flavonols are then absorbed via active transportation or passive diffusion into enterocytes (i.e. the epithelial cells that line the intestine and are responsible for absorbing nutrients from the digestive tract) [330]. Furthermore, flavonols can undergo methylation, glucuronidation, and sulfation, or a combination of these processes, via the phase II detoxification pathway. This conjugation primarily occurs in enterocytes, the liver, and the kidney [25, 119]. Any unabsorbed flavonols in the colon may be degraded into simpler phenolic acids by colonic microflora, or their heterocyclic oxygen-containing ring can be split. These conjugated forms of flavonols, some phenolic compounds produced by the colon microflora, flavonol aglycones, and some flavonol glycosides can reach systemic circulation and tissues and are then excreted in urine. Therefore, it may in fact be possible for flavonols in their aglycone and mono-glucoside forms to be present in the colon and have direct contact with potential cancerous cells.

In addition to the flavonol aglycone and mono-glucoside compounds that were purchased commercially and evaluated for antiproliferative effects on HT-29 cells, two flavonol conjugates and a fraction containing two compounds were also isolated and purified from bok choy extracts. This included a purified flavonol di-glycoside: kaempferol-3-sophoroside-7-glucoside, isolated from an alkaline hydrolysate extract, and a flavonol glycoside-hydroxycinnamic acid derivative: kaempferol-3-sophoroside(caffeoyl)-7-glucoside, isolated from a hydroalcoholic bok choy extract. A fraction consisting of two flavonol di-glycosides: kaempferol-3,7-diglucoside and isorhamnetin-3,7-diglucoside (with isorhamnetin-3,7-diglucoside being the predominant compound in the fraction), was also isolated from an alkaline hydrolysate extract.

The flavonol mono-glucosides and further glycosylated and acylated flavonol derivatives (isolated from bok choy) were investigated, not only to determine if they had any antiproliferative activity on HT-29 cells, but to provide some insight as to what affect conjugation might have on the antiproliferative abilities of flavonol compounds. It was also of interest to examine how the antiproliferative abilities of the purified flavonol compounds and fraction would compare to that of the crude bok choy extracts, considering that they were some of the major constituents of the alkaline and hydroalcoholic extracts. Furthermore, the flavonol compounds were examined in various combinations to assess for any synergistic effects.

The test concentrations for the flavonol compounds ranged from 0.1 to 200 μM, made up in complete media (McCoy's 5A) containing 0.1% v/v DMSO. The concentration of the fraction was calculated based on the predominant compound present: isorhamentin-3,7-diglucoside. The range of concentrations used was chosen to reflect a low concentration spectrum relative to what may be seen at a dietary level, as well as a high concentration spectrum which could represent the effects of potential supplements or chemotherapeutic treatments.

6.3.1 Antiproliferative Effects of Flavonol Aglycones on HT-29 Cells After 72 Hours of Exposure

The antiproliferative effects of quercetin, isorhamnetin, and kaempferol were assessed with HT-29 cells to determine if there were any differences between the three flavonols. Recently, Guo *et al.* [277] treated human liver cancer (HepG2) cells with several flavonols identified in Sea buckthorn berry extracts, including quercetin, isorhamnetin and kaempferol. Isorhamnetin showed the highest antiproliferative effect with the lowest IC_{50} concentration of 29.0 ± 4.1 μM, followed by kaempferol and quercetin with IC_{50} concentrations of 57.3 ± 6.1 μM and 80.0 ± 3.4 μM respectively. Another study by Li *et al.* [278] examined the cytotoxicity activity of several compounds, including quercetin and isorhamnetin, isolated from seeds of *Carex folliculata* L. (a forage prevalent in the northern United States) against two human colon cancer (HCT116 and HT-29), and two human breast cancer (MCF-7 and MDA-MB-231) cell lines. Isorhamnetin had a stronger antiproliferative effect compared to quercetin, with IC_{50} concentrations of 71 μM, 72 μM, 97 μM, and >100 μM for MDA-MB-231, HCT116, MCF-7, and HT-29 respectively. Quercetin had IC_{50} values greater than 100 μM for each cell line. Isorhamnetin is a metabolite of quercetin in the human body, therefore the anti-cancer effects that are observed from quercetin may in part be mediated through isorhamnetin [290].

The HT-29 cells were also treated with various combinations of the three flavonol aglycones to determine if there were any synergistic effects. The combinations involved two or three of the flavonol aglycones in equal portions at a total concentration similar to that of individual aglycone test solutions (0.1 to 200 μM). These assays were conducted to mimic what may be available in the gut after ingestion of bok choy, as well as to observe whether there were any increased inhibitory actions due to the combination of two or more compounds as compared to the individual compounds at equal concentrations. Previous i*n vitro* studies by Ackland *et al.* [68] and Liu [16] have demonstrated that flavonoids act synergistically. For example, Ackland *et al.* [68] reported that the combined treatments of quercetin and kaempferol on human gut and breast cancer cells were more effective than the additive effects of each individual flavonol. There have also been a number of studies that have investigated the synergistic effects of quercetin in

combination with commonly used chemotherapy drugs on cancer cells *in vitro*, all of which reported an enhanced antiproliferative effect [295, 61].

The results of the antiproliferative effects of the three flavonol aglycones, both individually and in various combinations to examine synergistic effects, are shown in Figure 6.3. Data were obtained after 72 hours of exposure and are displayed as concentration (μM) vs cell proliferation (% control values).

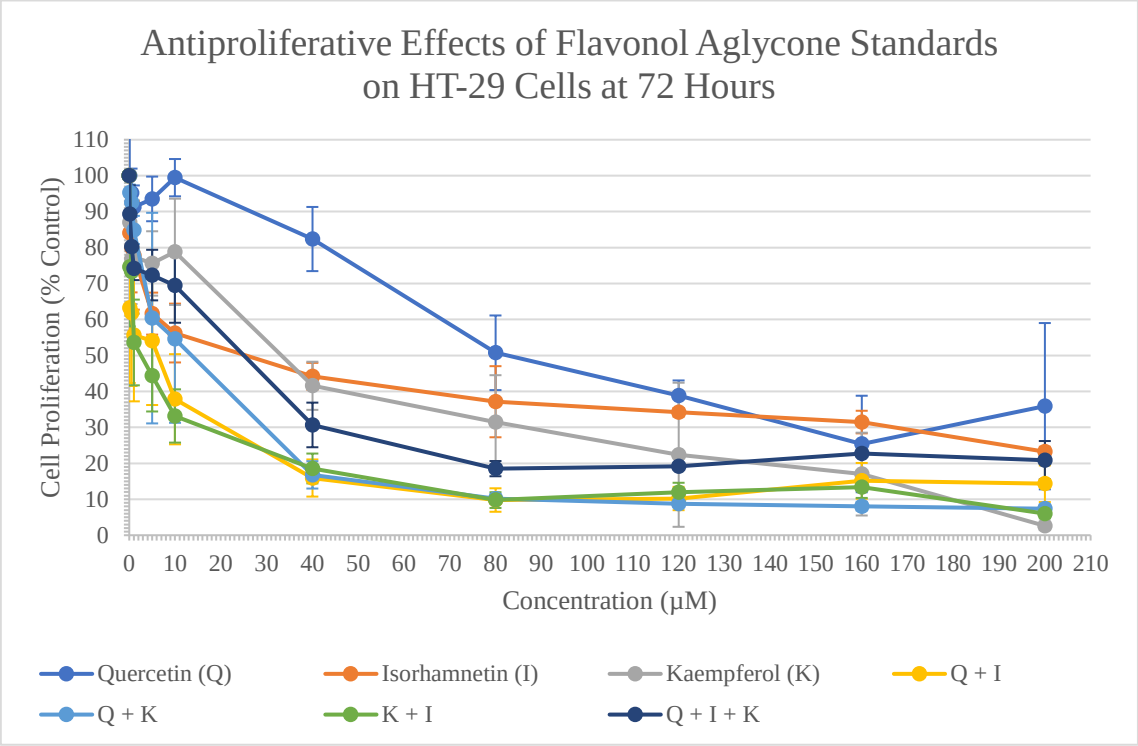


Figure 6.3 Proliferation of HT-29 cells treated with quercetin (Q), isorhamnetin (I), and kaempferol (K), individually, in pairs, and all three together, after 72 hours of exposure. Data represents the mean of two separate experiments (n = 3 in each experiment), with error bars indicating ± SD.

All three flavonol aglycones exhibited antiproliferative activity against the HT-29 cells. Quercetin displayed the least antiproliferative effect, especially at the lower concentration spectrum. There was a clear enhancement of inhibitory action on HT-29 cells when the cells were treated with mixtures of the aglycones in various combinations, especially in pairs (i.e. Q + I, Q + K, and K + I). It was interesting to note, however, that when the cells were treated with all three of the flavonol aglycones together (Q + I + K), the antiproliferative effect did not increase when compared to the effects displayed by the aglycones in pairs. This could be due to the reduced concentration of each individual flavonol aglycone within the mixed test solution. Nonetheless, the antiproliferative activity exhibited by the three aglycones tested together was marginally

stronger than that of the individual flavonol aglycones and therefore some synergistic action was observed.

There was a slight increase in HT-29 cell proliferation observed with the treatment of quercetin at lower concentrations. This observation was concurrent with the findings reported by van der Woude *et al.* [251] who investigated the effect of quercetin on proliferation of two colon carcinoma cell lines (HCT-116 and HT-29), as well as a breast cancer cell line (MCF-7). The authors reported a significant decrease in cell proliferation for the colon carcinoma cell lines at relatively high concentrations (above 30 mM for HCT-116 and 80 mM for HT-29), however, a subtle but significant increase in cell proliferation was observed upon exposure of all cell types to relatively low concentrations of quercetin (up to 20% for colon cell lines and up to 100% for MCF-7). These results suggested that there is a dualistic influence of quercetin on cell proliferation of cancerous cells.

The IC_{50} values that were calculated for the flavonol aglycones individually, and in their various combinations, after 72 hours of exposure are displayed in Table 6.2.

Table 6.2 IC_{50} values of flavonol aglycone standards, quercetin, isorhamnetin, and kaempferol, individually, in pairs, and all three together, after 72 hours of exposure on HT-29 cells. IC_{50} values were calculated using a nonlinear regression (curve fit) on GraphPad Prism 7.03.

Flavonol Aglycone	IC_{50} (μM)
Quercetin (Q)	25.2
Isorhamnetin (I)	15.0
Kaempferol (K)	35.8
Quercetin + Isorhamnetin (Q + I)	11.2
Quercetin + Kaempferol (Q + K)	9.88
Kaempferol + Isorhamnetin (K + I)	5.80
Quercetin + Isorhamnetin + Kaempferol (Q + I + K)	16.5

Isorhamnetin displayed the greatest inhibitory effect on HT-29 cell proliferation, with the lowest IC_{50} of 15.0 μM, followed by quercetin (25.2 μM), and kaempferol (35.8 μM). The IC_{50} obtained for isorhamnetin in this study was much lower than those reported by some previous studies on the same cell line. Li *et al.* [278] reported an IC_{50} value of greater than 100 μM after 72 hours of exposure to HT-29 cells, and Antunes-Ricardo *et al.*[255] reported an IC_{50} of 75.25 μM after 48 hours of isorhamnetin exposure. This is also the case for quercetin, whereby previous studies reported IC_{50} values on the HT-29 cell line after 72 hours of exposure as >80 μM [252] and >100 μM [278]. The IC_{50} value obtained for quercetin was, however, comparable to that reported by Zhang *et al.* [62] (30.8 μM) after 48 hours of exposure to a breast cancer cell line (MCF-7). There were no previous studies that had documented the IC_{50} value for kaempferol on the HT-29 cell line. The value obtained in this study was similar, however, to previous research on the human lung cancer

cell line A549, which reported an IC_{50} of 35 μM after 48 hours of exposure [281], and a rat liver cancer cell line (H4IIE), which reported an IC_{50} of 41 μM after 24 hours of exposure [279].

In general, the IC_{50} values confirm a synergistic effect between the flavonol aglycones when they were incubated in pairs, as well as in a trio combination. The various combined treatments of quercetin, isorhamnetin, and kaempferol on the colon cancer cells were clearly more effective than the additive effects of each individual flavonol. The combination that proved to be the most potent, i.e. exhibited the strongest antiproliferative activity, was kaempferol and isorhamnetin with an IC_{50} value of 5.80 μM, followed by quercetin and kaempferol (9.88 μM), and quercetin and isorhamnetin (11.2 μM). Interestingly, the antiproliferative effects were not further enhanced when all three of the flavonol aglycones were tested together; the IC_{50} value increased to 16.5 μM, which was similar to that of isorhamnetin alone, however still lower than quercetin and kaempferol alone. This could possibly be due to the fact that each individual flavonol within the trio combination test solutions is one third of the final concentration, and therefore they may not display as powerful antiproliferative activities.

6.3.2 Antiproliferative Effects of Flavonol Mono-Glucosides on HT-29 Cells After 72 Hours of Exposure

The antiproliferative effects of quercetin-3-*O*-glucoside, isorhamnetin-3-*O*-glucoside, and kaempferol-3-*O*-glucoside were assessed with HT-29 cells after 72 hours of exposure to determine if there were any differences between the three compounds, as well as between the aglycones and their corresponding mono-glucosides. A study by Yang *et al.* [303] demonstrated that quercetin-3-*O*-glucoside exhibited significant antiproliferative activity against human breast cancer cells (MCF-7) *in vitro*. Moreover, a study by You *et al.* [252] compared the antiproliferative activities of quercetin, quercetin-3-*O*-glucoside, and rutin, on six different cancer cell lines including colon, breast, hepatocellular, and lung cancer. Quercetin-3-*O*-glucoside was the most potent anti-cancer agent, followed by the quercetin, and finally rutin. More recently, in a study by Guo *et al.* [277], quercetin-3-*O*-glucoside and isorhamnetin-3-*O*-glucoside were ranked the 5th and 6th strongest inhibitors out of the 15 compounds that were assessed for antiproliferative activities on human liver cancer cells (HepG2).

The HT-29 cells were also exposed to all three flavonol mono-glucosides together to assess potential synergistic effects between the flavonols in the glycosylated form.

The results of the antiproliferative effects of the three flavonol mono-glucosides, both individually and all three together, are shown in Figure 6.4. Data were obtained after 72 hours of exposure and is displayed as concentration (μM) vs cell proliferation (% control values).

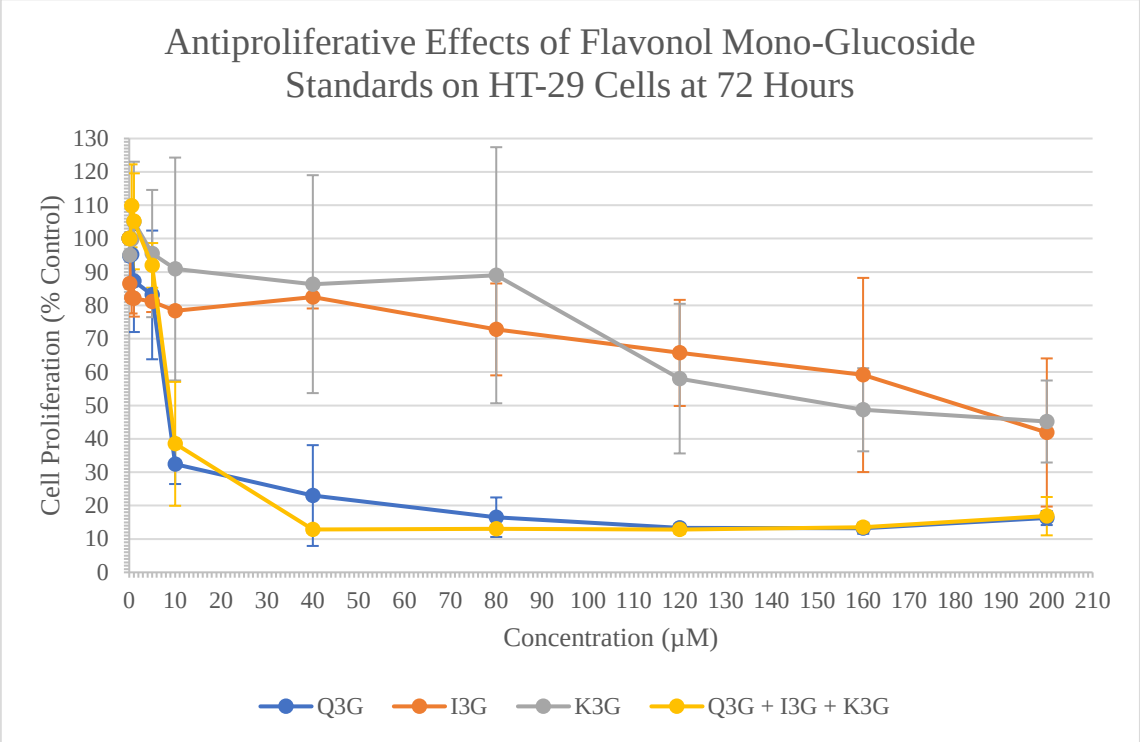


Figure 6.4 Proliferation of HT-29 cells treated with flavonol mono-glucoside standards: quercetin-3-*O*-glucoside (Q3G), isorhamnetin-3-*O*-glucoside (I3G), and kaempferol-3-*O*-glucoside (K3G), individually, and all three together, after 72 hours of exposure. Data represents the mean of two separate experiments (n = 3 in each experiment), with error bars indicating ± SD.

The graph displayed antiproliferative effects on HT-29 cells after 72 hours of exposure for all three of the flavonol mono-glucosides. Isorhamnetin-3-*O*-glucoside and kaempferol-3-*O*-glucoside exhibited similar antiproliferative abilities, whereas quercetin-3-*O*-glucoside displayed significantly stronger inhibitory action on the HT-29 cells. When all three of the flavonol mono-glucosides were combined and incubated in unison, the antiproliferative effect matched that of the quercetin-3-*O*-glucoside standard.

The IC_{50} values for the flavonol mono-glucosides after 72 hours of exposure to the HT-29 cells are shown in Table 6.3.

Table 6.3 IC_{50} values of flavonol mono-glucoside standards: quercetin-3-*O*-glucoside, isorhamnetin-3-*O*-glucoside, and kaempferol-3-*O*-glucoside, individually, and all three together, after 72 hours of exposure on HT-29 cells. IC_{50} values were calculated using a nonlinear regression (curve fit) on GraphPad Prism 7.03.

Flavonol Mono-Glucoside	IC_{50} (μM)
Quercetin-3-*O*-Glucoside (Q3G)	7.568
Isorhamnetin-3-*O*-Glucoside (I3G)	116.0
Kaempferol-3-*O*-Glucoside (K3G)	50.86
Quercetin-3-*O*-Glucoside + Isorhamnetin-3-*O*-Glucoside + Kaempferol-3-*O*-Glucoside (Q3G + I3G + K3G)	7.346

Quercetin-3-*O*-glucoside displayed the strongest antiproliferative effect with an IC_{50} value of 7.568 μM, followed by kaempferol-3-*O*-glucoside and isorhamnetin-3-*O*-glucoside with IC_{50} concentrations of 50.86 μM and 116 μM respectively. The IC_{50} for quercetin-3-*O*-glucoside has been previously reported as 46.4 μM on MCF-7 (breast cancer) cells after 96 hours of exposure [303] and 209 μM on HepG2 (liver cancer) cells after 72 hours of exposure [277]. Guo *et al.* [277] also reported an IC_{50} value for isorhamnetin-3-*O*-glucoside on HepG2 (liver cancer) cells after 72 hours of exposure as 244 μM. All of the IC_{50} values reported in literature are substantially higher than those obtained in this study, however, these were all reported for different cell lines.

A synergistic effect was observed between the flavonol compounds. When the HT-29 cells were treated with all three of the flavonol mono-glucosides in combination, the IC_{50} value that was obtained (7.346 μM) was substantially lower than that of kaempferol-3-*O*-glucoside and isorhamnetin-3-*O*-glucoside alone, however, it was very similar to the IC_{50} for quercetin-3-*O*-glucoside.

6.3.3 Antiproliferative Effects of Purified Flavonol Compounds and Fractions from Bok Choy on HT-29 Cells After 72 Hours of Exposure

The antiproliferative effects of the two purified flavonol compounds, and the isolated fraction, from bok choy extracts were assessed on HT-29 cells after 72 hours of exposure. Kaempferol-3-sophoroside-7-glucoside, and the two di-glycosides (isorhamnetin-3,7-diglucoside and kaempferol-3,7-diglucoside), were the main constituents of the alkaline hydrolysate bok choy extract, and kaempferol-3-sophoroside(caffeoyl)-7-glucoside was present in large amounts in the hydroalcoholic bok choy extract. Differences in inhibitory action on HT-29 cell proliferation between the two purified flavonol compounds and the collected fraction was examined, in order to determine if one particular flavonol compound was more potent than the other. A synergistic assay was also carried out, whereby kaempferol-3-sophoroside-7-glucoside and the fraction from the alkaline hydrolysate were incubated together to examine if antiproliferative activity would increase when the cells were treated with the two simultaneously.

The antiproliferative effects of the isolated compounds and fraction from bok choy extracts on HT-29 cells are displayed in Figure 6.5. Data were obtained after 72 hours of exposure and are displayed as concentration (μM) vs cell proliferation (% control values).

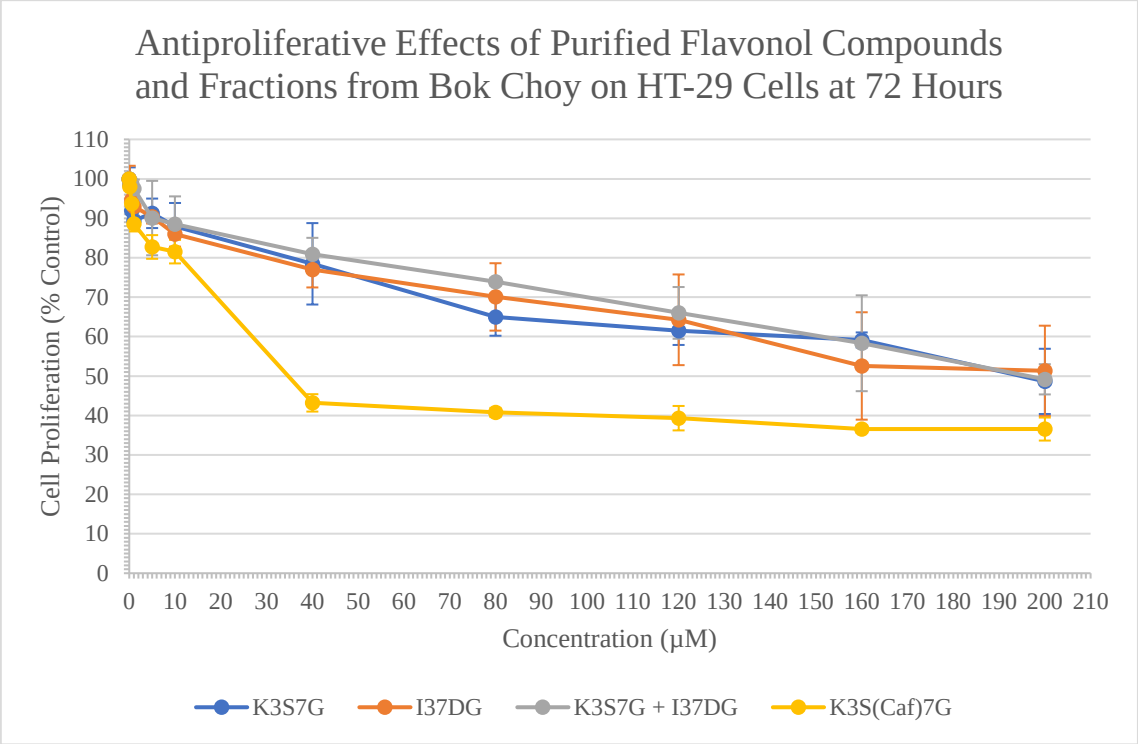


Figure 6.5 Proliferation of HT-29 cells treated with flavonol compounds/fractions isolated from bok choy extracts: kaempferol-3-sophoroside-7-glucoside (K3S7G), fraction containing isorhamnetin-3,7-diglucoside and kaempferol-3,7-diglucoside (I37DG), and kaempferol-3-sophoroside(caffeoyl)-7-glucoside (K3S(Caf)7G), individually, and K3S7G and I37DG together, after 72 hours of exposure. Data represents the mean of two separate experiments (n = 3 in each experiment), with error bars indicating ± SD.

The purified compounds, as well as the isolated fraction, exhibited antiproliferative effects on the HT-29 cells in a concentration dependent manner, however, kaempferol-3-sophoroside(caffeoyl)-7-glucoside displayed a considerably stronger response. The antiproliferative ability of both kaempferol-3-sophoroside-7-glucoside and the isorhamnetin-3,7-diglucoside fraction, as well as the combination of these two, was comparable. Contrary to the previous experiments on flavonol aglycones and mono-glucosides, there were no synergistic effects observed between kaempferol-3-sophoroside-7-glucoside and the isorhamnetin-3,7-diglucoside fraction.

The IC_{50} values for the purified flavonol compounds and isolated fraction after 72 hours of exposure to the HT-29 cells are shown in Table 6.4.

Table 6.4 IC_{50} values of flavonol compounds purified from bok choy extracts: kaempferol-3-sophoroside-7-glucoside (K3S7G), fraction containing isorhamnetin-3,7-diglucoside and kaempferol-3,7-diglucoside (I37DG), and kaempferol-3-sophoroside(caffeoyl)-7-glucoside (K3S(Caf)7G), individually, and K3S7G and I37DG together, after 72 hours of exposure on HT-29 cells. IC_{50} values were calculated using a nonlinear regression (curve fit) on GraphPad Prism 7.03.

Purified Flavonol Compound/Fraction	IC_{50} (μM)
Kaempferol-3-sophoroside-7-glucoside (K3S7G)	37.14
Isorhamnetin-3,7-diglucoside Fraction (I37DG)	31.71
Kaempferol-3-sophoroside-7-glucoside + Isorhamnetin-3,7-diglucoside Fraction (K3S7G + I37DG)	47.08
Kaempferol-3-sophoroside(Caffeoyl)-7-glucoside (K3S(Caf)7G)	14.06

Kaempferol-3-sophoroside(caffeoyl)-7-glucoside exhibited the most potent antiproliferative effect with an IC_{50} of 14.06 μM. Kaempferol-3-sophoroside-7-glucoside, and the fraction containing isorhamnetin-3,7-diglucoside and kaempferol-3,7-diglucoside, were less effective at inhibiting HT-29 cell proliferation with IC_{50} values of 37.14 μM and 31.71 μM respectively. In contrast to previous findings, there were no clear synergistic effects observed when the cells were treated with the combination of the flavonol di-glycosides. The IC_{50} calculated for the combination of kaempferol-3-sophoroside-7-glucoside and the collected fraction was 47.08 μM, which was higher than that of the test solutions individually.

The antiproliferative activity of the crude bok choy extracts were significantly stronger than any of the purified flavonol compounds, fractions, or aglycone and mono-glucoside standards, possibly due to the combination of the many different naturally occurring chemicals within the vegetable.

6.4 EFFECT OF CONJUGATION ON ANTIPROLIFERATIVE ABILITIES OF FLAVONOL COMPOUNDS

The IC_{50} values for all the individual flavonols, including standards and purified compounds from bok choy extracts (the isolated fraction is also included, representing isorhamnetin-3,7-diglucoside), obtained from this study are tabulated in Table 6.5. They are grouped according to their flavonol core, to observe trends that may be present as a result of conjugation on antiproliferative activity.

Table 6.5 IC_{50} values of flavonol compounds in different conjugated forms after 72 hours of exposure on HT-29 cells. IC_{50} values were calculated using a nonlinear regression (curve fit) on GraphPad Prism 7.03.

Flavonol	IC_{50} (µM)
Quercetin Compounds	
Quercetin aglycone (Q)	25.21
Quercetin-3-*O*-glucoside (Q3G)	7.568
Isorhamnetin Compounds	
Isorhamnetin aglycone (I)	15.00
Isorhamnetin -3-*O*-glucoside (I3G)	116.0
Isorhamnetin-3,7-diglucoside (I37DG)	31.71
Kaempferol Compounds	
Kaempferol aglycone (K)	35.80
Kaempferol -3-*O*-glucoside (K3G)	50.86
Kaempferol-3-sophoroside-7-glucoside (K3S7G)	37.14
Kaempferol-3-sophoroside(caffeoyl)-7-glucoside (K3S(Caf)7G)	14.06

No general trend was observed between the three major flavonols and results were different for each individual flavonol. For quercetin, the mono-glucoside form was significantly stronger in its antiproliferative effect against HT-29 cells compared to the aglycone. These results were similar to the findings of You *et al.* [252].

The opposite was observed for isorhamnetin, whereby the mono-glucoside form was determined to exhibit the weakest antiproliferative ability. Three forms of isorhamnetin were examined: the aglycone, a mono-glucoside, and a di-glycoside (present in the isolated fraction as the major compound). The IC_{50} values indicated that the compounds were ranked isorhamnetin > isorhamnetin di-glycoside > isorhamnetin mono-glucoside, in order of greatest inhibitory activity. Kaempferol-3,7-diglucoside was also present in the isorhamnetin-3,7-diglucoside fraction and this may have skewed the results.

A total of four kaempferol compounds were tested for antiproliferative activity on the HT-29 cells, including the aglycone and mono-glucoside standards, and a di-glycoside and di-glycoside-hydroxycinnamic acid conjugate purified from the bok choy extracts. The kaempferol compounds were ranked in the following order, according to strongest antiproliferative activity: kaempferol di-glycoside-hydroxycinnamic acid derivative > kaempferol aglycone > kaempferol di-glycoside > kaempferol mono-glucoside. The most highly conjugated kaempferol compound displayed the strongest inhibitory action, as opposed to the aglycone, and the kaempferol mono-glucoside exhibited the least potency against the HT-29 cells. The IC_{50} values for the kaempferol aglycone and kaempferol di-glycoside were similar.

Although these results do not confirm a clear effect of glycosylation and acylation on the antiproliferative abilities of flavonol compounds, the study does demonstrate that the conjugation pattern of flavonols can affect their bioactivity and mechanisms of action, and hence their antiproliferative activity against cancer cells *in vitro*. Further work is required to provide more conclusive data.

6.5 CONCLUSION

The ability of crude bok choy extracts, selected fractions, and individual flavonol compounds; including both commercially purchased flavonol aglycone and mono-glucoside standards, as well as purified flavonol derivatives from bok choy, to inhibit proliferation of HT-29 cells *in vitro* were evaluated using the MTT assay. The antiproliferative activity of all three of the bok choy cultivars, as well as the various flavonol compounds and fractions tested, on HT-29 cells was evident. The antiproliferative effects, in all cases, were shown to be concentration dependent, and preliminary results also indicated a potential time dependent effect. The crude bok choy extracts were found to be the most potent anti-cancer agents, in terms of displaying the strongest antiproliferative activity on the cells. All three cultivars of bok choy displayed similar inhibitory effects, with the variation between the three not being considered significant. Among the three flavonol aglycone standards tested, isorhamnetin (IC_{50} = 15.0 μM) exhibited the strongest antiproliferative activity, followed by quercetin (IC_{50} = 25.2 μM), and then kaempferol (IC_{50} = 35.8 μM). However, the flavonol mono-glucoside standards displayed a different trend, whereby quercetin-3-*O*-glucoside (IC_{50} = 7.568 μM) was found to be the strongest inhibitor, followed by kaempferol-3-*O*-glucoside (IC_{50} = 50.86 μM) and isorhamnetin-3-*O*-glucoside (IC_{50} = 50.86 μM).

The flavonol glycoside-hydroxycinnamic acid derivative: kaempferol-3-sophoroside(caffeoyl)-7-glucoside, that was isolated from a hydroalcoholic bok choy extract displayed the strongest antiproliferative effect (IC_{50} = 14.06 μM) of the purified compounds and fractions. Kaempferol-3-sophoroside-7-glucoside, purified from the alkaline hydrolysate extract, and the fraction containing kaempferol-3,7-diglucoside and isorhamnetin-3,7-diglucoside, showed similar activity with IC_{50} values of 37.14 μM and 31.71 μM respectively.

The antiproliferative activity against HT-29 cells in order of greatest activity is as follows: Sumo bok choy extract > Karate bok choy extract > Miyako bok choy extract > quercetin-3-*O*-glucoside > kaempferol-3-sophoroside(caffeoyl)-7-glucoside > isorhamnetin > quercetin > isorhamnetin-3,7-diglucoside fraction > kaempferol > kaempferol-3-sophoroside-7-glucoside > kaempferol-3-*O*-glucoside > isorhamnetin-3-*O*-glucoside.

In general, the flavonol compounds acted synergistically, enhancing their antiproliferative effects. When the flavonol aglycones and mono-glucoside conjugates were assessed in combinations of two or three, an increased effect was observed, as compared to the individual compounds at equal concentrations. The only exception was the combination of the purified compound kaempferol-3-sophoroside-7-glucoside and the isorhamnetin-3,7-diglucoside fraction whereby the IC_{50} increased slightly as compared to the individual experiments.

There was no clear effect or trend observed regarding conjugation, such as glycosylation and acylation, on the antiproliferative abilities of the flavonol compounds that were assessed.

In summary, the results obtained in this study suggested that bok choy has the potential to positively influence human health, both from a chemopreventive and possibly chemotherapeutic perspective. The regular consumption of bok choy in the habitual diet could possibly prevent the onset of colorectal cancer, and some of the flavonol compounds present in bok choy could potentially be used as chemotherapy drugs in the treatment of colorectal cancer, or as purified supplements in the prevention of colorectal cancer. The results presented in this study are considered preliminary as it lacks enough independent replicates to enable statistical analysis of the data, and hence no firm conclusions can be drawn. Further work is required to attain more conclusive data and the mechanisms of action (e.g. mechanisms of cell death via the comet assay) need to be further explored.

CHAPTER VII
GENERAL DISCUSSION, CONCLUSION AND FUTURE DIRECTIONS

7.1 INTRODUCTION

Research on flavonols in fruits, vegetables and other plant foods, can be divided into two main areas: chemical analysis via various analytical techniques, and the analysis of their bioactivities and mechanisms of action both *in vitro* and *in vivo*. The accurate identification and quantification of flavonols in food is crucial to demonstrating the health benefits of these foods. This can be achieved within a matter of minutes using "state of the art" analytical equipment, i.e. HPLC-PDA/ESI-MS^n. Cancer therapy using natural substances and prevention through dietary intervention have become an important issue for health management. Studies on anticarcinogenic activity *in vitro* have focussed on both the direct and indirect actions of flavonols on cancer cells and have reported a variety of anti-cancer effects, in addition to *in vivo* experiments which have displayed anti-tumour activities.

The main aims of this research were:

1. To measure the levels of the major flavonols present in three bok choy cultivars and to identify the naturally occurring glycosylated and acylated flavonol derivatives by HPLC-PDA/ESI-MS^n and NMR, and
2. To assess the anti-cancer properties of these compounds, as well as hydroalcoholic extracts of bok choy, by investigating their antiproliferative activity on a human colon cancer cell line (HT-29) *in vitro*.

7.2 SUMMARY OF MAJOR FINDINGS

7.2.1 Method Optimisation and Other Preliminary Studies

Several parameters for HPLC-PDA/ESI-MS^n analysis such as the HPLC column, the composition and gradient of the mobile phase, the optimum PDA detector wavelength, and the ESI-MS^n ionisation conditions were optimised prior to the separation and identification of the flavonol compounds present in the three different extracts (i.e. acid hydrolysate, alkaline hydrolysate and hydroalcoholic extract). The preparative HPLC method was scaled up from the optimised HPLC-PDA/ESI-MS^n method and adjusted. Furthermore, the optimum hydrolysis time for the acid hydrolysate was determined, as well as the most appropriate syringe filter for each of the extracts. Analysis of the flavonol compounds present in the different bok choy extracts via HPLC-PDA/ESI-MS^n was conducted in the negative ionisation mode using formic acid as the mobile

phase additive. The capillary temperature and capillary voltage were optimised to 350ºC and -48.50 V respectively using rutin as the tune compound.

The passage dilution factor for routine maintenance of HT-29 cells, in addition to the cell density and time range for the MTT cell proliferation assays were optimised for the cancer cell studies.

7.2.2 Quantification and Characterisation of Flavonols in Bok Choy Cultivars

Three locally grown bok choy cultivars: Sumo, Karate, and Miyako were grown under glasshouse conditions in Victoria, Australia, and the flavonol aglycone content was quantified after acid hydrolysis using HPLC-PDA/ESI-MS^n. Data from both the PDA detector and the MS in SIM mode were used for quantification. A highly selective and specific separation was achieved using the MS SIM mode which provided more accurate quantification of the flavonols, as isorhamnetin and kaempferol were not completely resolved in the PDA chromatograms. Kaempferol was present in the highest amount (85.5 – 122 mg/100 g DW), followed by isorhamnetin (38.3 – 66.7 mg/100 g DW), and quercetin (10 – 20.6 mg/100 g DW). The Miyako variety contained the highest levels of both quercetin and isorhamnetin, however, kaempferol was comparable in all three cultivars of bok choy. Although Miyako contained higher levels of quercetin and isorhamnetin, all three bok choy cultivars contained similar total aglycone levels (Sumo: 183.3 mg/100 g DW, Karate: 159.9 mg/100 g DW, and Miyako: 197.3 mg/100 g DW bok choy). Therefore, the differences in flavonol aglycone content between the three bok choy cultivars were considered insignificant and no firm conclusions could be made as to whether one bok choy cultivar may contain more health-promoting flavonols than another.

Five flavonol glycoside conjugates were identified and characterised in the bok choy cultivars after alkaline hydrolysis using a combination of UV-Vis spectral data, MS^n fragmentation patterns obtained from data dependent studies, and previously published data in literature. The flavonol glycosides consisted of two flavonol-3-sophoroside-7-glucosides, and three flavonol-3,7-diglucosides. One of the glycosides: kaempferol-3-sophoroside-7-glucoside, was isolated and purified using preparative HPLC and the structure confirmed by 1D and 2D NMR. The purified compound, along with another fraction containing kaempferol-3,7-diglucoside and isorhamnetin-3,7-diglucoside, were examined for antiproliferative activity on HT-29 cells *in vitro*.

Nine naturally occurring flavonol derivatives were identified in the bok choy cultivars after hydroalcoholic extraction. Structure elucidation was determined in the same manner as for the flavonol glycosides in the alkaline hydrolysate extracts. The nine flavonol compounds consisted of six complex flavonol glycoside-hydroxycinnamic acid derivatives, two flavonol di-glycosides (previously identified in the alkaline hydrolysate extracts), and one flavonol mono-glucoside. Several fractions were isolated from the hydroalcoholic extract using preparative HPLC,

however, only one was determined to be pure enough for further studies. Kaempferol-3-sophoroside(caffeoyl)-7-glucoside was collected from one of the fractions and subjected to 1D and 2D NMR, however, insufficient data were obtained for complete structure identification. The compound was, however, used in the cancer cell studies and evaluated for antiproliferative activity on HT-29 cells *in vitro*.

7.2.3 Cancer Cell Studies

The antiproliferative activities of crude bok choy extracts, selected fractions, and individual flavonol compounds on HT-29 human colorectal adenocarcinoma cells *in vitro* were evaluated using the MTT cell proliferation assay. The antiproliferative activity of the bok choy cultivars, as well as the various flavonol compounds and fractions tested, on HT-29 cells was evident. The antiproliferative effects, in all cases, were shown to be concentration dependent after 72 hours of exposure and could possibly be time dependent according to preliminary results over a 96-hour time range.

The crude bok choy extracts were found to be the most potent anti-cancer agents, in terms of displaying the strongest antiproliferative activity on the cells. All three cultivars of bok choy displayed similar inhibitory effects, with the variation between the three not being considered significant. Antiproliferative activity against the HT-29 cells for all the bok choy extracts, fractions, and flavonol compounds assessed were ranked in order of greatest activity as follows: Sumo bok choy extract > Karate bok choy extract > Miyako bok choy extract > quercetin-3-*O*-glucoside > kaempferol-3-sophoroside(caffeoyl)-7-glucoside > isorhamnetin > quercetin > isorhamnetin-3,7-diglucoside fraction > kaempferol > kaempferol-3-sophoroside-7-glucoside > kaempferol-3-*O*-glucoside > isorhamnetin-3-*O*-glucoside.

In general, the flavonol compounds acted synergistically, enhancing their antiproliferative effects. An increased effect was observed, as compared to the individual compounds at equal concentrations, when the flavonol aglycones and mono-glucoside conjugates were assessed in combinations of two or three. The only exception was the combination of the purified compound kaempferol-3-sophoroside-7-glucoside and the isorhamnetin-3,7-diglucoside fraction whereby the IC_{50} increased slightly as compared to the individual experiments. There was no clear association observed between the level of conjugation (i.e. glycosylation and acylation) of the flavonol compounds and antiproliferative activity.

The results presented in this study were preliminary and no firm conclusions could be drawn, however, the results do suggest that bok choy has the potential to positively influence human health, both from a chemopreventive and possibly chemotherapeutic perspective.

7.3 GENERAL CONCLUSION

The chemical analysis of three bok choy cultivars investigated in this study confirmed that bok choy is an important source of dietary flavonols, in particular, kaempferol, isorhamnetin, and quercetin. Preliminary results from the *in vitro* cancer cell studies suggested that bok choy has the potential to positively influence human health. The regular consumption of bok choy in the habitual diet could possibly prevent the onset of colorectal cancer, due to the potent antiproliferative activity exhibited by the extracts on HT-29 cells, and the flavonol compounds found within bok choy could possibly be used to design novel chemotherapeutic agents. To the best of our knowledge, this was the first report of antiproliferative effects of bok choy extracts on cancer cells *in vitro*.

7.4 FUTURE DIRECTIONS

The continual development of analytical methods that provide improved sensitivity, resolution, and through-put, in conjunction with automated processes for sample extraction, clean-up and concentration is crucial to facilitate the mass screening of phytochemicals in plants and metabolites in biological samples. The ready availability of standard reference compounds is also fundamental to the success of this work. In most cases, analytical standards are not commercially available and therefore need to be isolated and purified from plant material using SPE, preparative HPLC, and other techniques. Improved purification methods to produce these compounds are required to be developed to further these studies.

Accumulating evidence from epidemiological studies (case-control studies, prospective cohort, and others) has demonstrated that fruit and vegetable consumption has health-promoting properties due to the rich sources of diverse phytochemicals present in these plant foods. However, much of the evidence linking fruit and vegetable intake to health continues to be observational, and data in some areas are still contradictory. Therefore, there is still a need for more controlled, clinical intervention trials to investigate and confirm the effect of the consumption of fruits and vegetables on various diseases such as cancer. Further studies are also required to reveal the mechanisms behind the anti-cancer effects of different phytochemical components of fruits and vegetables.

It was evident in all the cell culture experiments conducted on the antiproliferative activities of flavonol compounds, fractions, and bok choy extracts in this study that an inhibitory effect on HT-29 cells was observed. Therefore, it would be of interest to determine whether the antiproliferative effects were a result of apoptosis or necrosis, as well as to examine which flavonols may be preferentially used and metabolised by the cells.

Flavonols such as quercetin, isorhamnetin and kaempferol might be valuable agents in anti-cancer strategies and studies of their clinical use for development of novel drugs should be continued. Beneficial effects have also been reported when these flavonols are combined with standard chemotherapeutic drugs, leading to a decrease in the dosage and associated toxicity while targeting specific resistance mechanisms. By combining the two, the genotoxic damage caused by the standard chemotherapeutic drug to normal cells can be diminished, thereby reducing the chance of secondary cancers developing. Further work is needed to develop and produce novel drugs from natural sources and the potential to design novel chemotherapy drugs from flavonols would be an area of interest for future research. It may be possible to introduce structural variations into the backbone of flavonol compounds, and to modify their structures to further improve biological activity and exhibit more potent anti-cancer effects.

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APPENDIX A

Table A.1 Foods containing the highest content of flavonols that are of interest to this study; quercetin, kaempferol, and isorhamnetin (aglycones). Information extracted from the USDA database for the flavonoid content of selected foods (release 3.1) [29]. FW = Fresh Weight.

Flavonol	Food	Content (mg/100 g FW)
Quercetin	Capers, raw	233.84
	Lovage, leaves, raw	170.00
	Elderberry, juice concentrate	108.16
	Radish leaves, raw	70.37
	Chokeberry, juice concentrate	68.17
	Rocket, wild, raw	66.19
	Carob fiber	58.13
	Dill weed, fresh	55.15
	Coriander leaves, raw	52.90
	Yellow peppers, hot, raw	50.63
	Fennel, leaves, raw	48.80
	Juniper berries, ripe	46.61
	Oregano, Mexican, dried	42.00
	Onions, red, raw	39.21
Kaempferol	Capers, raw	259.19
	Saffron	205.48
	Kale, raw	46.80
	Mustard greens, raw	38.30
	Arugula, raw	34.89
	Ginger, wild	33.60
	Beans, common, raw	26.00
	Onions, welsh, raw	24.95
	Watercress, raw	23.03
	Chinese cabbage, raw	22.51
	Radish seeds, sprouted, raw	21.85
	Garden cress, raw	13.00
	Chia seeds, raw	12.30
	Turnip greens, raw	11.87
	Endive, raw	10.10
	Chives, raw	10.00
	Broccoli, raw	7.84
Isorhamnetin	Parsley, dried	331.24
	Dill weed, fresh	43.50
	Sea buckthorn berry, raw	38.29
	Kale, raw	23.60
	Mustard greens, raw	16.20
	Fennel, leaves, raw	9.30
	Chives, raw	6.75
	Cherries, sour, dry, unsweetened	8.91
	Asparagus, raw	5.70
	Elderberries, raw	5.42
	Onions, raw	5.01

Table A.2 Flavonol (quercetin, kaempferol, and isorhamnetin aglycones) content in *Brassica* species. Information extracted from the USDA database for the flavonoid content of selected foods (release 3.1) [29]. FW = Fresh Weight.

***Brassica* species**	**Flavonol**	**Content (mg/100 g FW)**
Kale (*Brassica oleracea* (Acephala Group))	Quercetin Kaempferol Isorhamnetin	22.58 46.80 23.60
Mustard greens (*Brassica juncea*)	Quercetin Kaempferol Isorhamnetin	8.80 38.30 16.20
Broccoli (*Brassica oleracea* var. *italica*)	Quercetin Kaempferol	3.26 7.84
Collards (*Brassica oleracea* var. *viridis*)	Quercetin Kaempferol	2.57 8.74
Broccoli raab (*Brassica ruvo*)	Quercetin	2.25
Cabbage, Chinese (pak choi) (*Brassica rapa* (Chinensis Group))	Quercetin Kaempferol	2.06 4.33
Brussels sprouts (*Brassica oleracea* (Gemmifera Group))	Quercetin Kaempferol	1.92 0.86
Turnip greens (*Brassica rapa* (Rapifera Group)	Quercetin Kaempferol	0.73 11.87
Cauliflower (*Brassica oleracea* (Botrytis Group))	Quercetin Kaempferol	0.54 0.36
Kohlrabi (*Brassica oleracea* (Gongylodes Group))	Quercetin Kaempferol	0.40 2.43
Cabbage, savoy (*Brassica oleracea* (Capitata Group))	Quercetin Kaempferol	0.36 0.79
Cabbage, red (*Brassica oleracea* (Capitata Group))	Quercetin Kaempferol	0.36 0.00
Cabbage (*Brassica oleracea* (Capitata Group))	Quercetin Kaempferol	0.28 0.18
Rutabagas (*Brassica napus* var. *napobrassica*)	Quercetin Kaempferol	0.05 0.32
Cabbage, Chinese (pe-tsai) (*Brassica rapa* (Pekinensis Group))	Quercetin Kaempferol	0.01 0.10

APPENDIX B

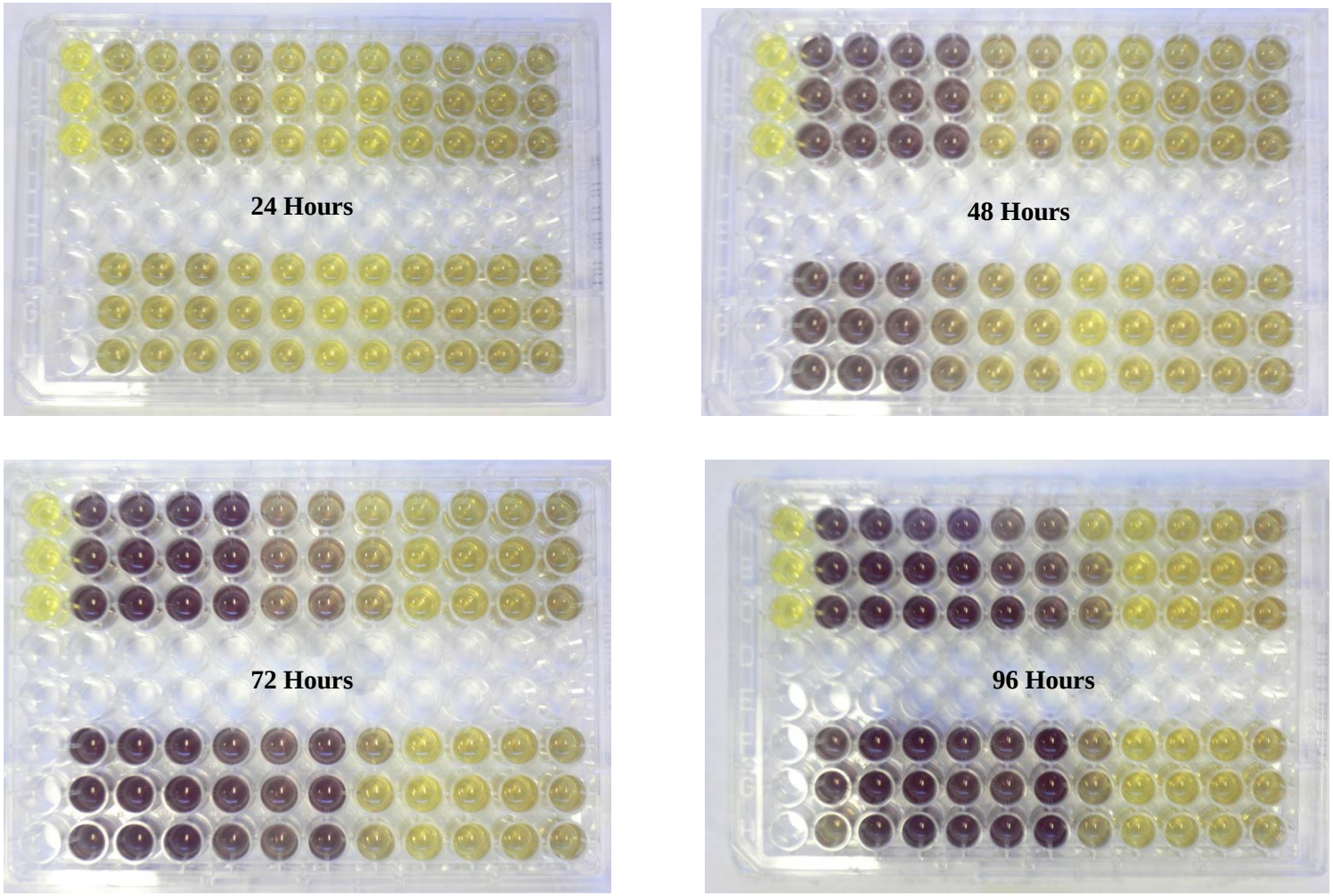

Figure B.1 Photographs of a MTT proliferation assay showing colour change of MTT salt over time. The intensity of the purple colour produced is a measure of cell viability, i.e. the more intense the purple colour, the more metabolically active cells are present.

APPENDIX C

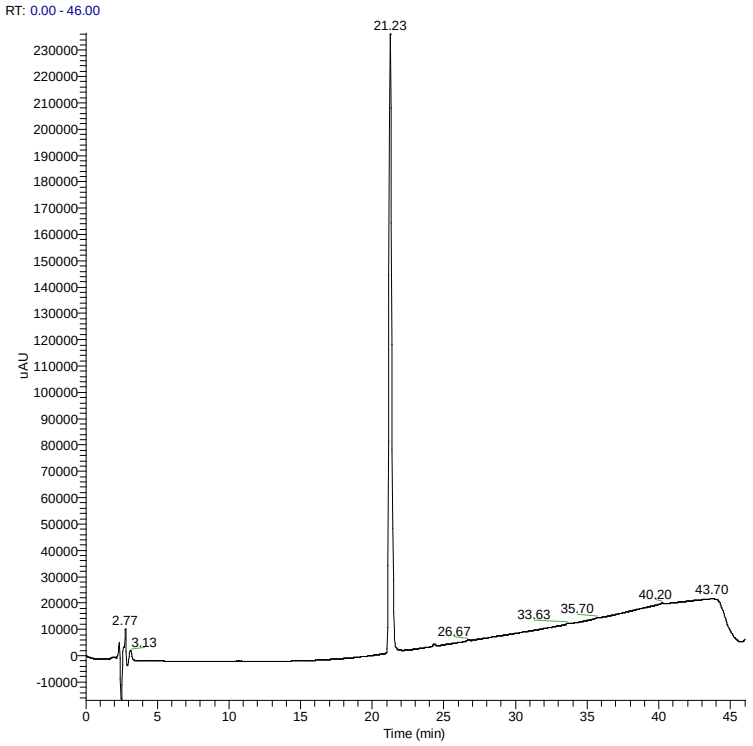

Figure C.2 HPLC-PDA chromatogram, recorded at 370 nm, of the quercetin aglycone standard. Used to identify peak 1 in the acid hydrolysate extracts of the bok choy cultivars.

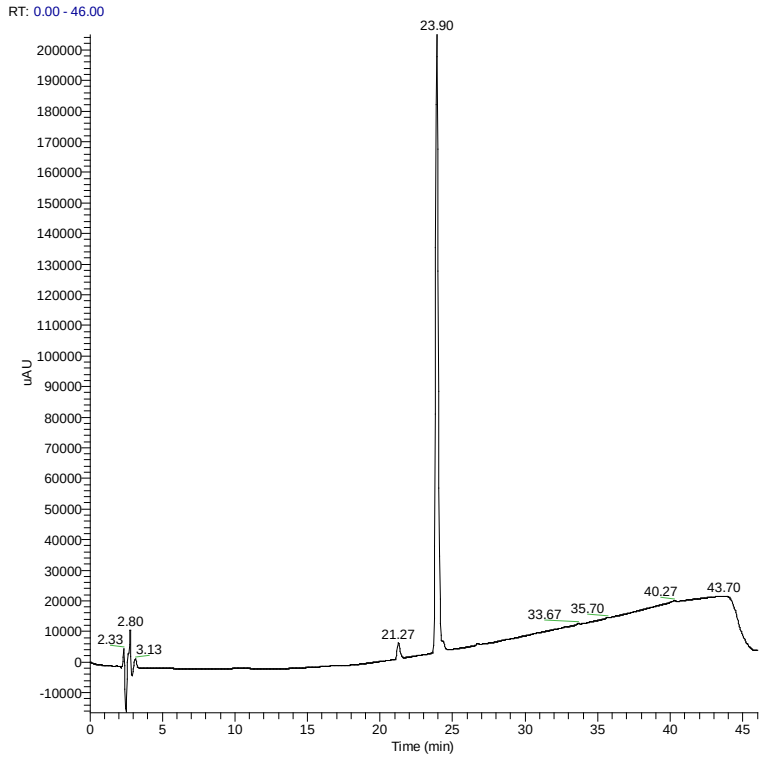

Figure C.3 HPLC-PDA chromatogram, recorded at 370 nm, of the isorhamnetin aglycone standard. Used to identify peak 2 in the acid hydrolysate extracts of the bok choy cultivars.

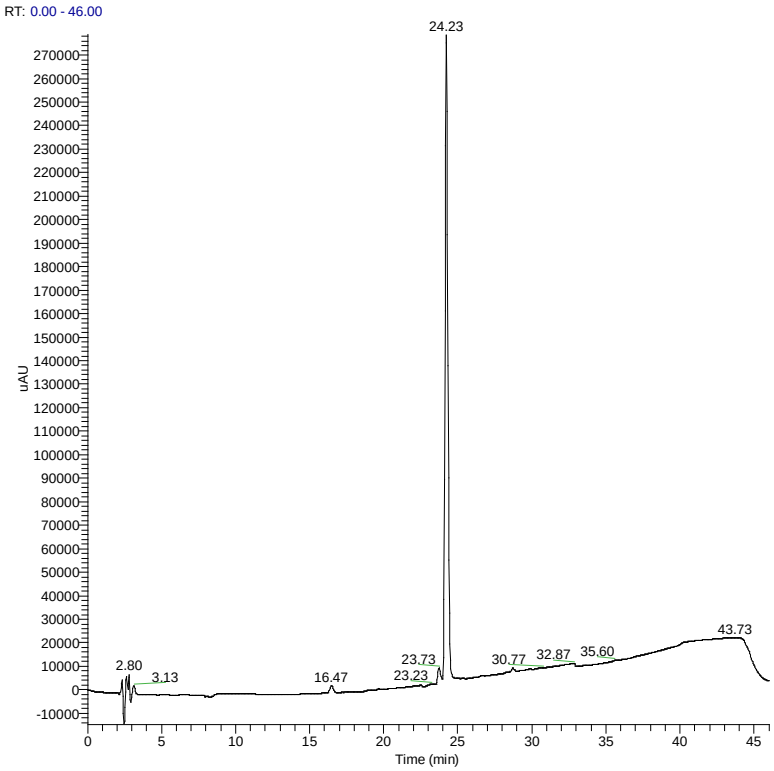

Figure C.4 HPLC-PDA chromatogram, recorded at 370 nm, of the kaempferol aglycone standard. Used to identify peak 3 in the acid hydrolysate extracts of the bok choy cultivars.

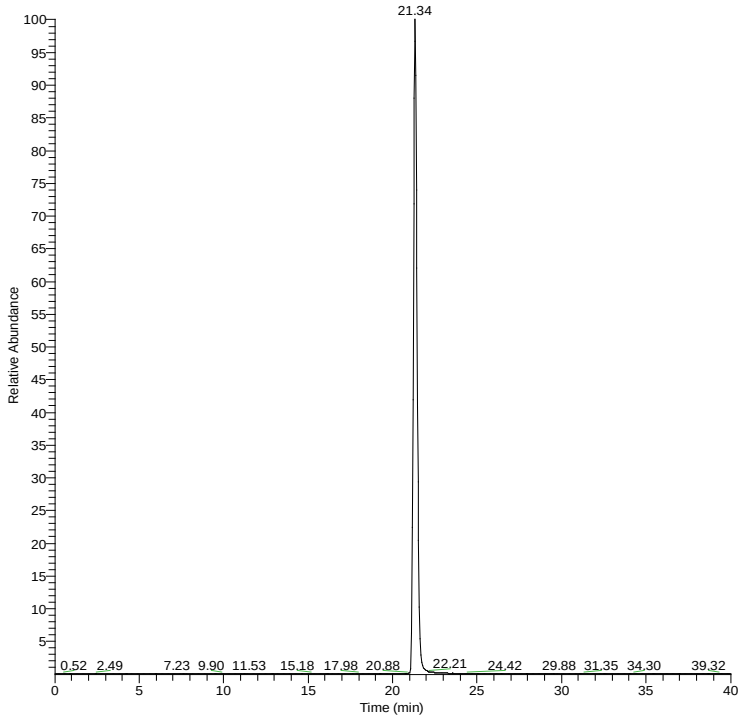

Figure C.5 HPLC-MS TIC of the quercetin aglycone standard. Used to identify peak 1 in the acid hydrolysate extracts of the bok choy cultivars.

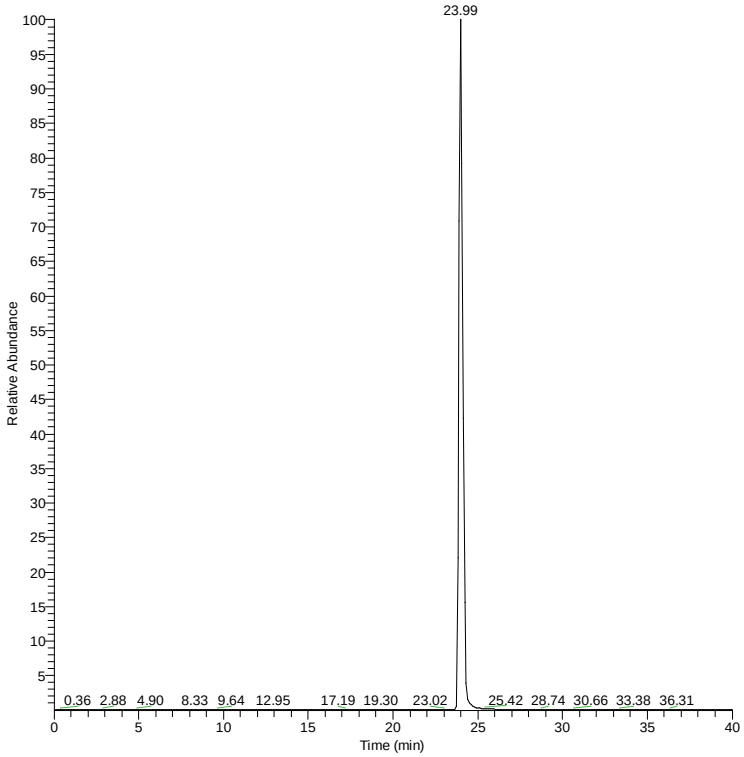

Figure C.6 HPLC-MS TIC of the isorhamnetin aglycone standard. Used to identify peak 2 in the acid hydrolysate extracts of the bok choy cultivars.

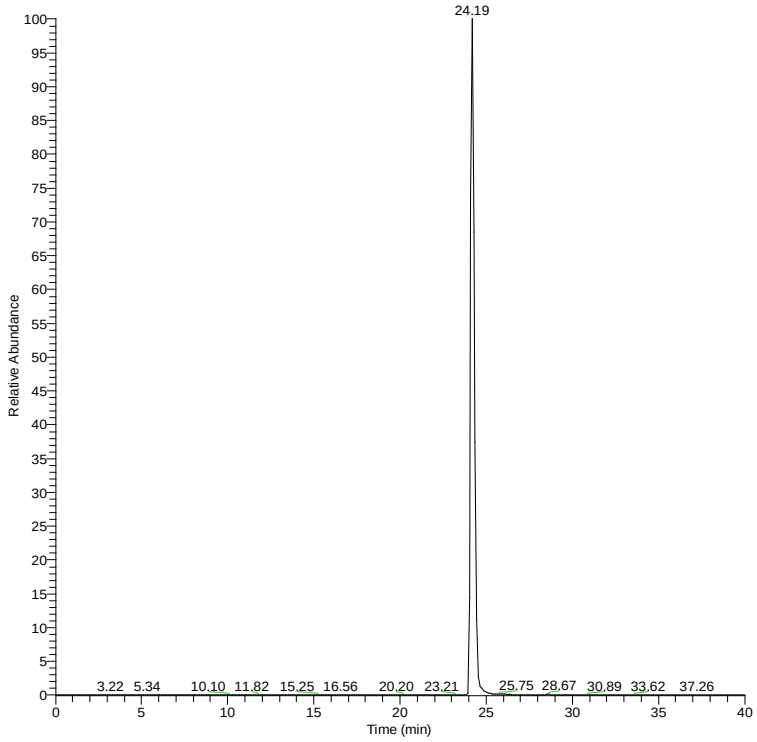

Figure C.7 HPLC-MS TIC of the kaempferol aglycone standard. Used to identify peak 3 in the acid hydrolysate extracts of the bok choy cultivars.

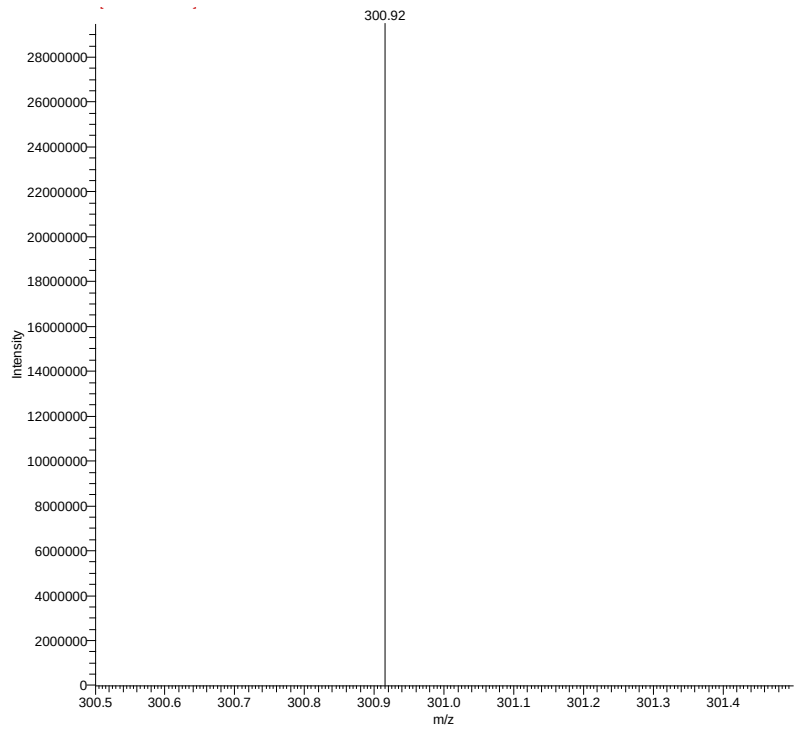

Figure C.8 MS^1 spectra in negative ion mode of the quercetin aglycone standard. Used to identify peak 1 in the acid hydrolysate extracts of the bok choy cultivars.

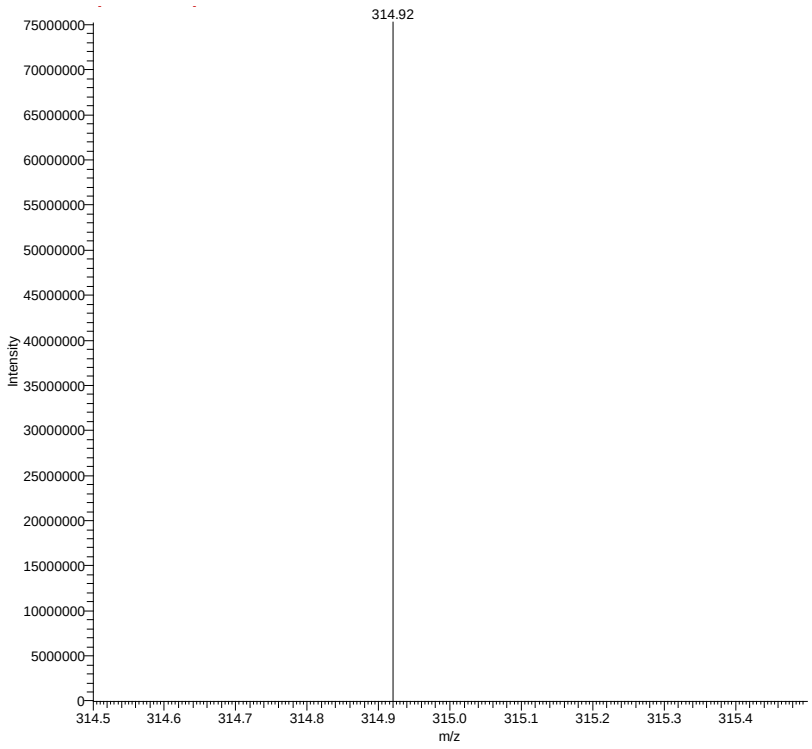

Figure C.9 MS^1 spectra in negative ion mode of the isorhamnetin aglycone standard. Used to identify peak 2 in the acid hydrolysate extracts of the bok choy cultivars.

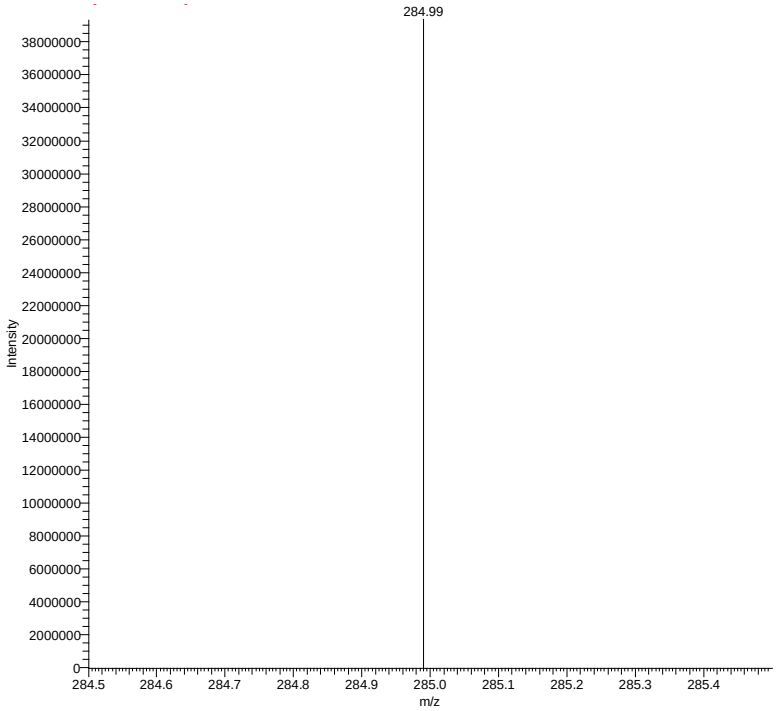

Figure C.10 MS^1 spectra in negative ion mode of the kaempferol aglycone standard. Used to identify peak 3 in the acid hydrolysate extracts of the bok choy cultivars.

APPENDIX D

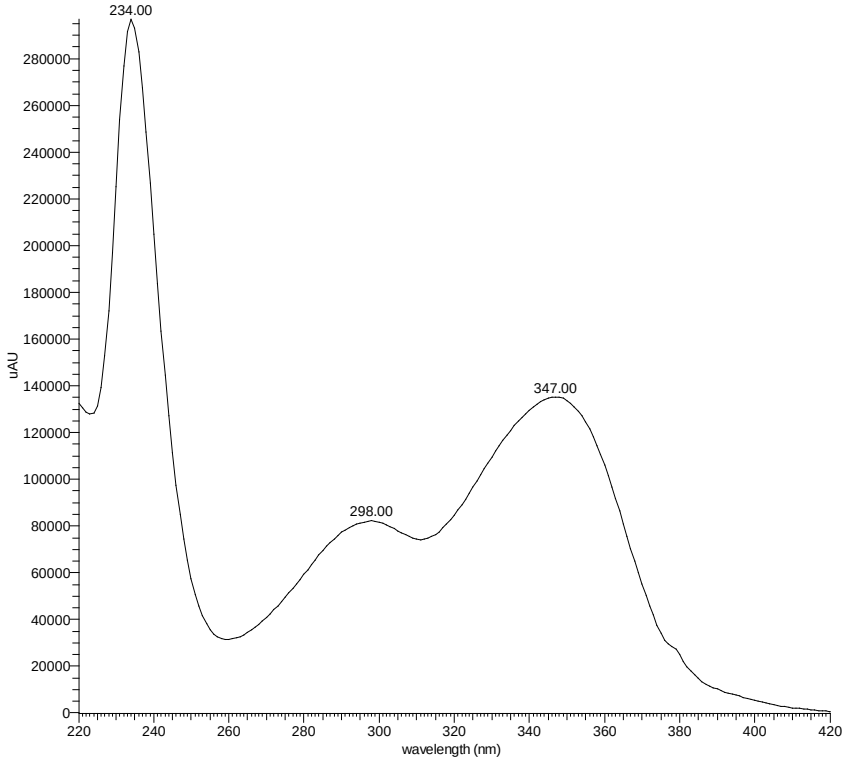

Figure D.1 UV-Vis spectrum (220 to 420 nm) of peak 4 from a Sumo bok choy acid hydrolysate extract.

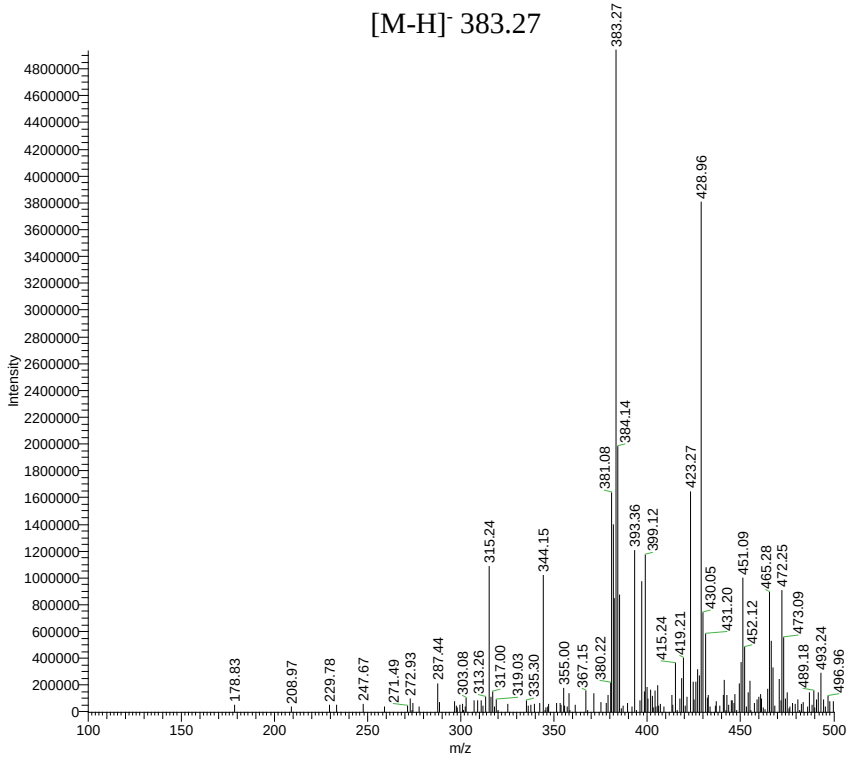

Figure D.7 MS^1 spectra in negative ion mode of peak 4 from a Sumo bok choy acid hydrolysate extract.

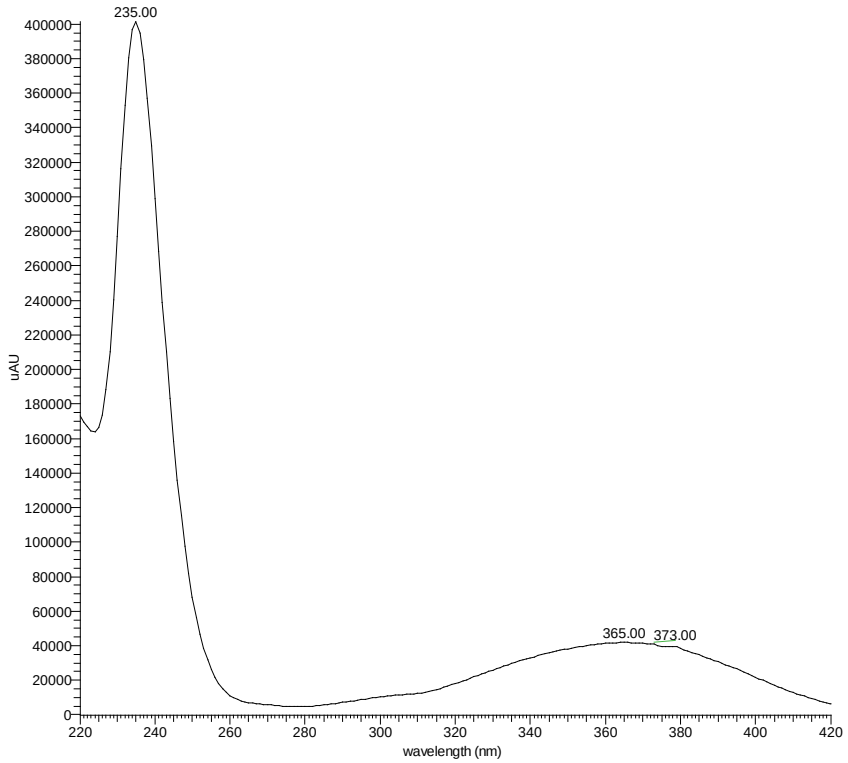

Figure D.8 UV-Vis spectrum (220 to 420 nm) of peak 5 from a Sumo bok choy acid hydrolysate extract.

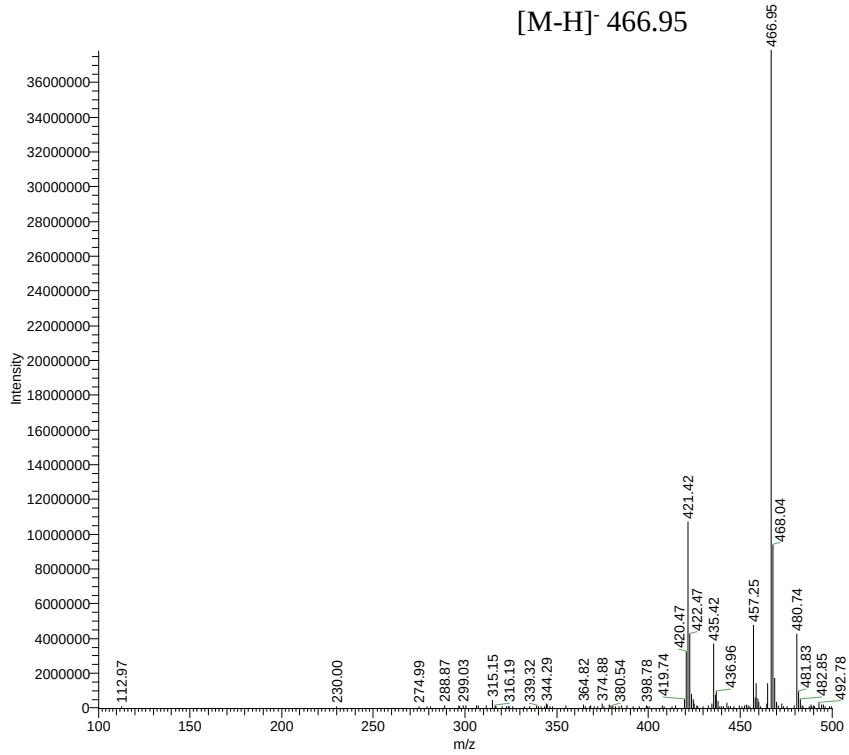

Figure D.13 MS^1 spectra in negative ion mode of peak 5 from a Sumo bok choy acid hydrolysate extract.

APPENDIX E

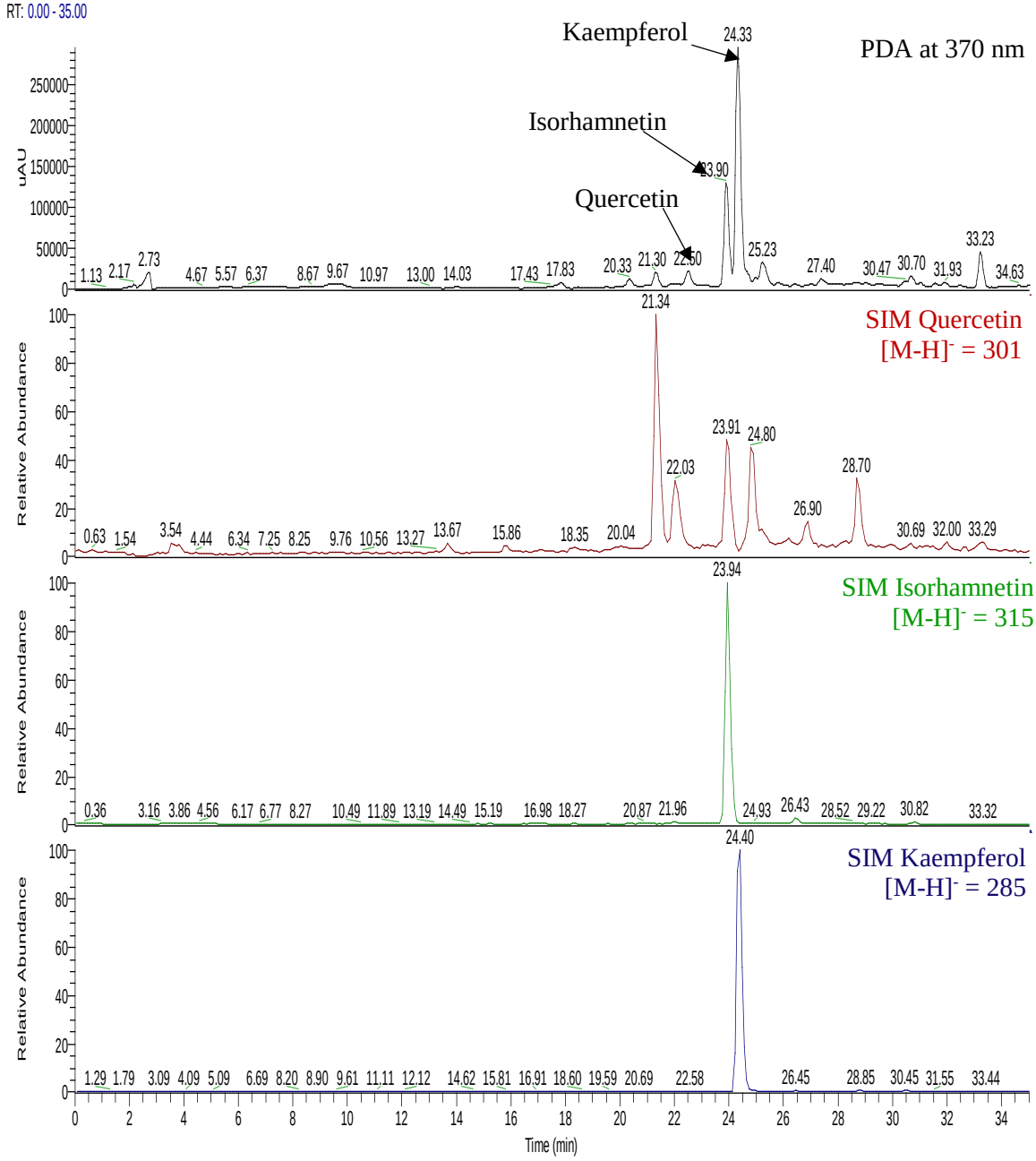

Figure E.2 HPLC-PDA and HPLC-ESI-MS SIM chromatograms of a Karate bok choy acid hydrolysate extract used for quantification.

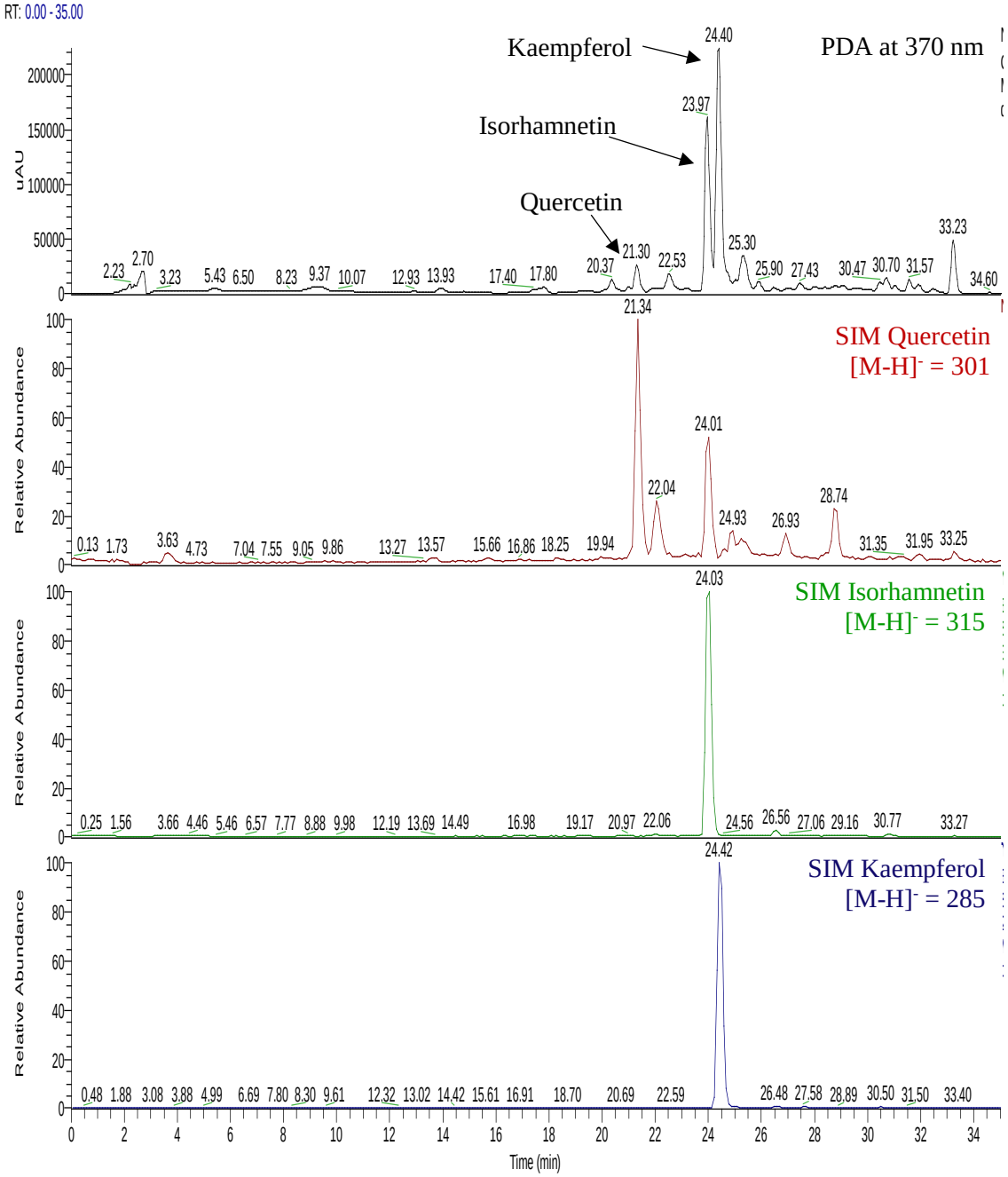

Figure E.3 HPLC-PDA and HPLC-ESI-MS SIM chromatograms of a Miyako bok choy acid hydrolysate extract used for quantification.

APPENDIX F

Table F.1 HPLC-PDA retention time (t_R) and peak area reproducibility data from 20 successive injections of a Sumo bok choy acid hydrolysate extract. Used for method verification studies.

PDA Data (λ = 370 nm)						
	Quercetin		Isorhamnetin		Kaempferol	
Run	t_R (min)	Peak Area	t_R (min)	Peak Area	t_R (min)	Peak Area
1	21.10	297537	23.60	1950501	24.00	4167220
2	20.57	286726	23.20	2085053	23.63	4173023
3	20.67	275774	23.33	1966489	23.73	4127358
4	20.50	292693	23.17	1954304	23.57	4243006
5	20.67	272983	23.33	1984839	23.77	4075448
6	20.47	271151	23.20	1926356	23.63	4112959
7	20.67	274790	23.33	2014168	23.77	4165007
8	20.50	273562	23.20	1952313	23.63	4121192
9	20.70	292317	23.37	1900800	23.77	4195386
10	20.73	287111	23.40	1940313	23.80	4163794
11	20.63	298197	23.33	1954748	23.77	4086215
12	20.53	287475	23.23	1964020	23.67	4141198
13	20.67	278981	23.33	1915371	23.77	4144380
14	20.50	270489	23.23	1917166	23.67	4139734
15	20.67	279998	23.33	1995471	23.77	4069048
16	20.53	272938	23.20	2008982	23.63	4143100
17	20.67	275670	23.33	1945900	23.77	4171132
18	20.53	270168	23.23	1982600	23.67	4196642
19	20.67	281858	23.37	1931661	23.80	4143501
20	20.50	273836	23.17	1957230	23.60	4171749
Mean	20.62	280713	23.29	1962414	23.72	4147555
STD	0.14	9192	0.10	41908	0.10	42381
% CV	0.68	3.274	0.44	2.1355	0.42	1.0218

Table F.2 HPLC-ESI-MS SIM retention time (t_R) and peak area reproducibility data from 20 successive injections of a Sumo bok choy acid hydrolysate extract. Used for method verification studies.

MS Data (SIM mode)						
	Quercetin (301)		Isorhamnetin (315)		Kaempferol (285)	
Run	t_R (min)	Peak Area	t_R (min)	Peak Area	t_R (min)	Peak Area
1	21.14	40756021	23.62	548551136	24.03	353473168
2	20.61	40881070	23.27	544267296	23.67	372952408
3	20.72	43757983	23.38	543569534	23.78	353641255
4	20.55	43097090	23.20	541719091	23.60	340418954
5	20.72	41790341	23.38	527504774	23.79	348023917
6	20.50	44629946	23.25	520866276	23.66	341017347
7	20.69	43495934	23.35	506790720	23.83	345245614
8	20.51	44932452	23.25	502703478	23.66	338726370
9	20.73	42680605	23.32	487250740	23.80	327585596
10	20.74	44136843	23.41	492343363	23.89	322034166
11	20.72	41669945	23.39	495744172	23.79	319906234
12	20.56	42016358	23.30	498621758	23.79	339608744
13	20.77	43267867	23.43	485841365	23.83	318709615
14	20.56	40448631	23.32	487038455	23.73	322068629
15	20.69	41442516	23.37	493194669	23.85	335092317
16	20.59	40460147	23.27	493944930	23.67	316458420
17	20.69	41656364	23.37	487610747	23.78	311276401
18	20.58	41618075	23.33	493441142	23.74	315911354
19	20.70	41924170	23.38	490260946	23.87	314698737
20	20.58	41890404	23.25	490341636	23.66	317277011
Mean	20.67	42327638	23.34	506580311	23.77	332706313
STD	0.14	1354539	0.09	22257098	0.10	16670095
% CV	0.67	3.200129	0.39	4.3935971	0.43	5.0104535

Table F.3 HPLC-PDA retention time (t_R) and peak area reproducibility data from 20 successive injections of a 6 mg/L mixed standard containing quercetin, isorhamnetin and kaempferol. Used for method verification studies.

PDA Data (λ = 370 nm)						
	Quercetin		Isorhamnetin		Kaempferol	
Run	t_R (min)	Peak Area	t_R (min)	Peak Area	t_R (min)	Peak Area
1	21.10	1048262	23.70	1432251	24.00	3465659
2	21.07	1062311	23.70	1456868	24.13	3488959
3	21.20	1048754	23.83	1442680	24.00	3477990
4	21.13	1042961	23.77	1440895	24.13	3474791
5	21.27	1053269	23.87	1451203	24.00	3488341
6	21.17	1047238	23.80	1424287	24.13	3468413
7	21.33	1072645	23.93	1420819	24.03	3443378
8	21.10	1050656	23.70	1492254	24.20	3493537
9	21.23	1049941	23.87	1498577	24.07	3494042
10	21.03	1092640	23.63	1502481	24.23	3514230
11	21.20	1083357	23.83	1487266	24.07	3496547
12	21.07	1056552	23.67	1499528	24.27	3507893
13	21.20	1086872	23.80	1452553	24.10	3481280
14	21.03	1062333	23.67	1451844	24.23	3490546
15	21.17	1051271	23.80	1420445	24.13	3499827
16	21.03	1093201	23.63	1460402	24.20	3442609
17	21.20	1072897	23.80	1458677	24.30	3493724
18	21.03	1062554	23.67	1441523	24.13	3501373
19	21.23	1042300	23.87	1438962	24.03	3493320
20	21.00	1072581	23.63	1450118	24.20	3497731
Mean	21.14	1062630	23.76	1456182	24.13	3485710
STD	0.09	16466	0.09	26408	0.09	18995
% CV	0.44	1.5496	0.39	1.8135	0.38	0.54493

Table F.4 HPLC-ESI-MS SIM retention time (t_R) and peak area reproducibility data from 20 successive injections of a 6 mg/L mixed standard containing quercetin, isorhamnetin and kaempferol. Used for method verification studies.

MS Data (SIM mode)						
	Quercetin (301)		Isorhamnetin (315)		Kaempferol (285)	
Run	t_R (min)	Peak Area	t_R (min)	Peak Area	t_R (min)	Peak Area
1	21.13	126538779	23.73	689252891	24.09	148794274
2	21.15	128450121	23.71	662384510	24.19	146968932
3	21.18	130552381	23.82	672531884	24.10	140658348
4	21.20	127454452	23.79	667498545	24.17	145019294
5	21.30	131285446	23.91	690142338	24.09	145849709
6	21.23	127548615	23.87	678744132	24.20	142822778
7	21.30	129646520	23.91	691213580	24.11	143391200
8	21.15	126789989	23.75	681322207	24.24	144543395
9	21.28	131489745	23.97	675986422	24.12	145054669
10	21.15	127845653	23.74	704485267	24.33	139126688
11	21.28	128485523	23.97	673685649	24.14	136434651
12	21.16	126743012	23.73	685450123	24.33	132079844
13	21.28	131482250	23.94	712683559	24.14	132688320
14	21.15	128464468	23.71	662529037	24.30	138084034
15	21.25	127998377	23.91	675774940	24.20	138616324
16	21.13	130448037	23.68	706287409	24.22	132270709
17	21.26	127578965	23.91	677485234	24.39	139991005
18	21.15	132848852	23.80	696852474	24.22	129311939
19	21.36	127485862	23.90	676523358	24.04	133765980
20	21.13	130596238	23.77	695036338	24.26	130423608
Mean	21.21	128986664	23.83	683793495	24.19	139294785
STD	0.07	1890405	0.10	14231460	0.09	5987976
% CV	0.34	1.465582	0.40	2.0812511	0.39	4.298780

APPENDIX G

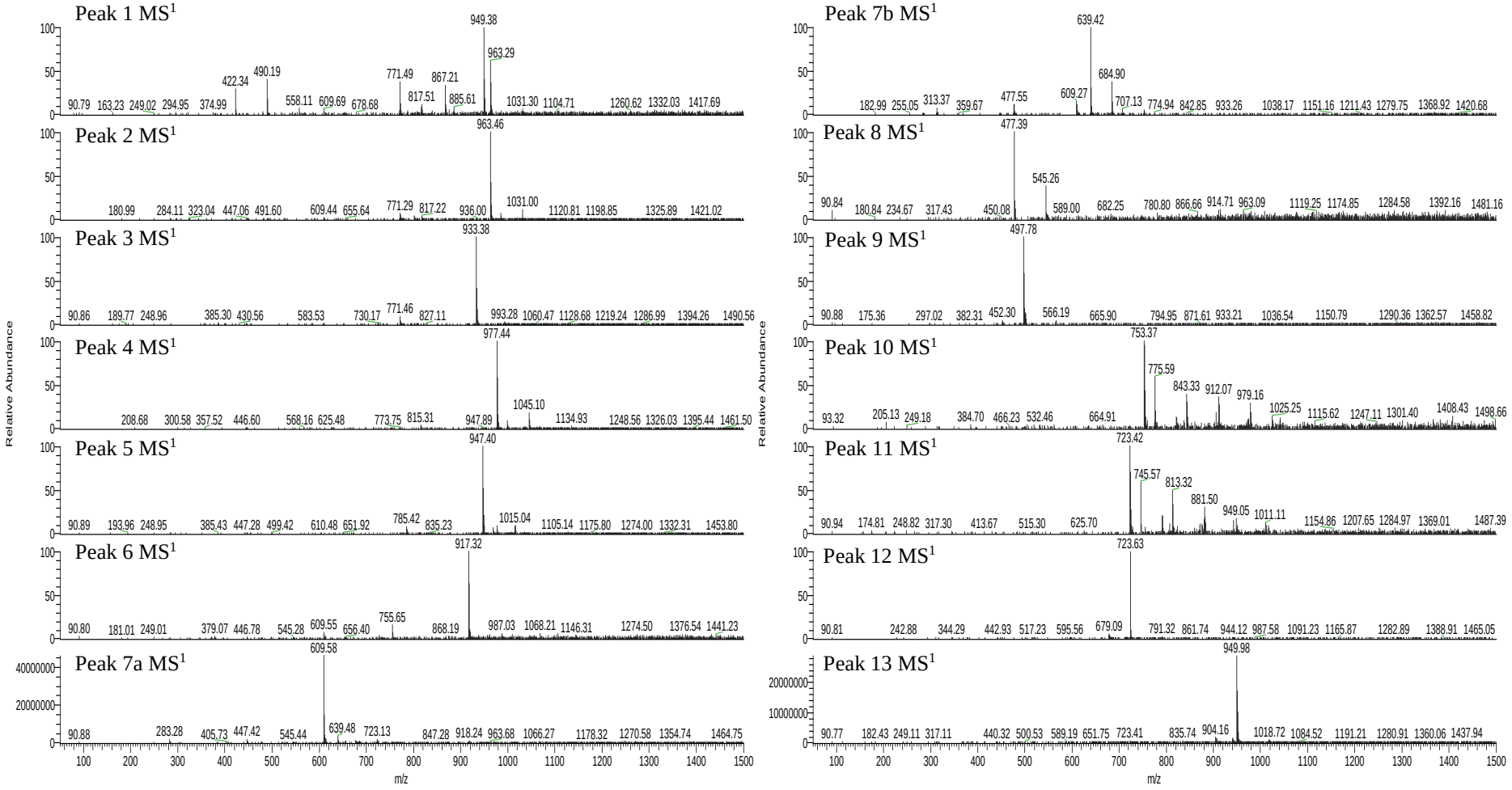

Figure G.2 ESI-MS[1] spectra in negative ion mode of peaks 1-13 in the hydroalcoholic extract of the Karate bok choy cultivar.

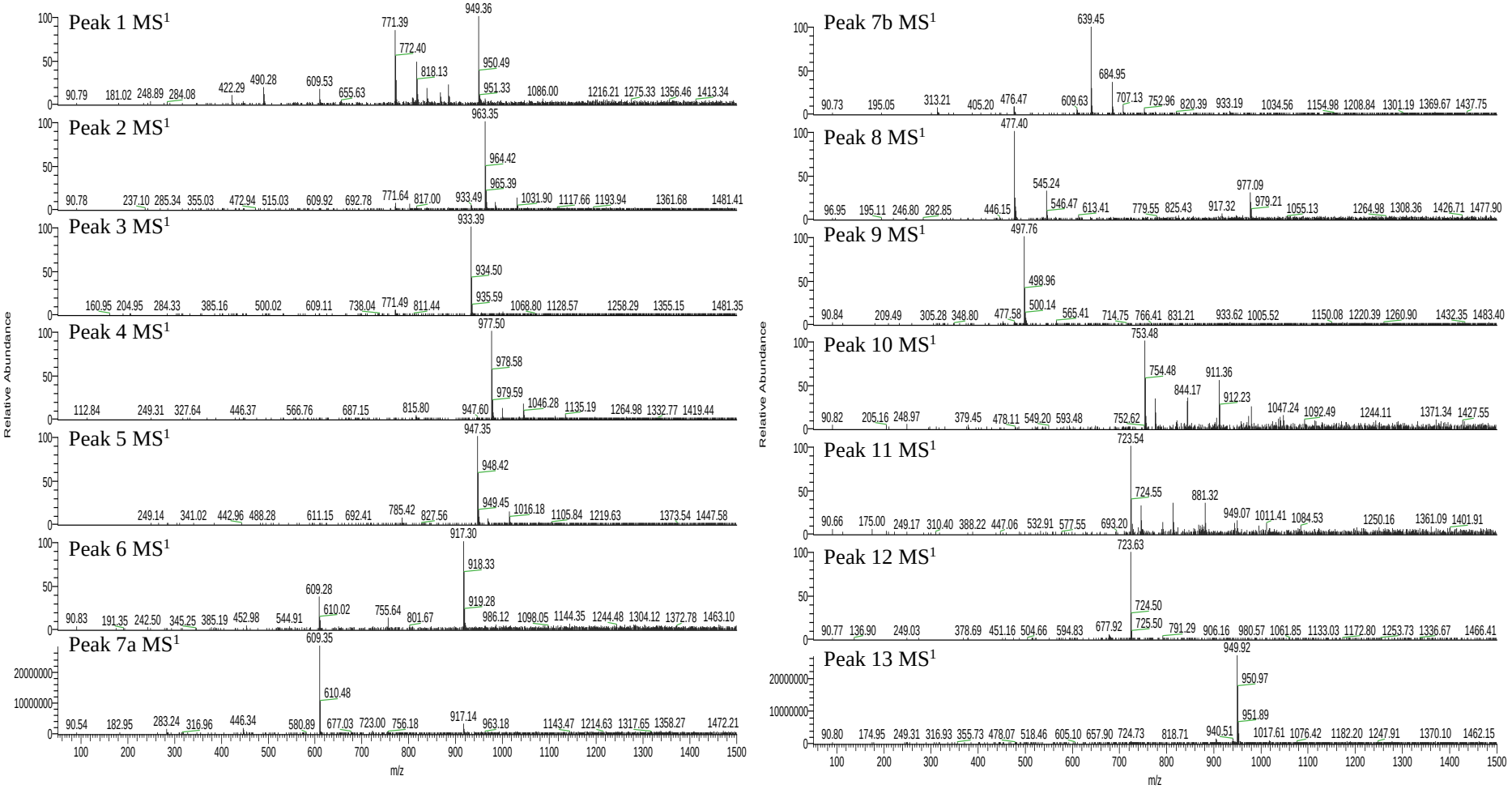

Figure G.4 ESI-MS[1] spectra in negative ion mode of peaks 1-13 in the hydroalcoholic extract of the Miyako bok choy cultivar.

APPENDIX H

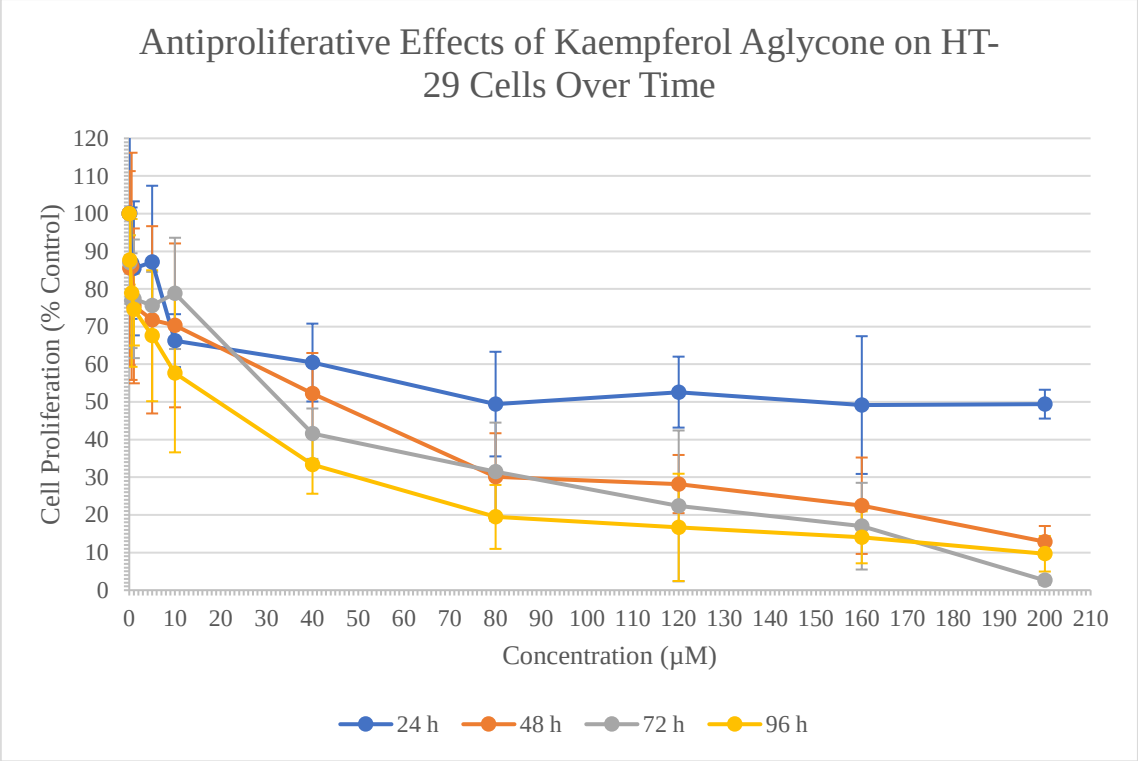

Figure H.2 Proliferation of HT-29 cells treated with kaempferol aglycone after 24, 48, 72, and 96 hours of exposure. Data represents the mean of two separate experiments (*n* = 3 in each experiment), with error bars indicating ± SD.

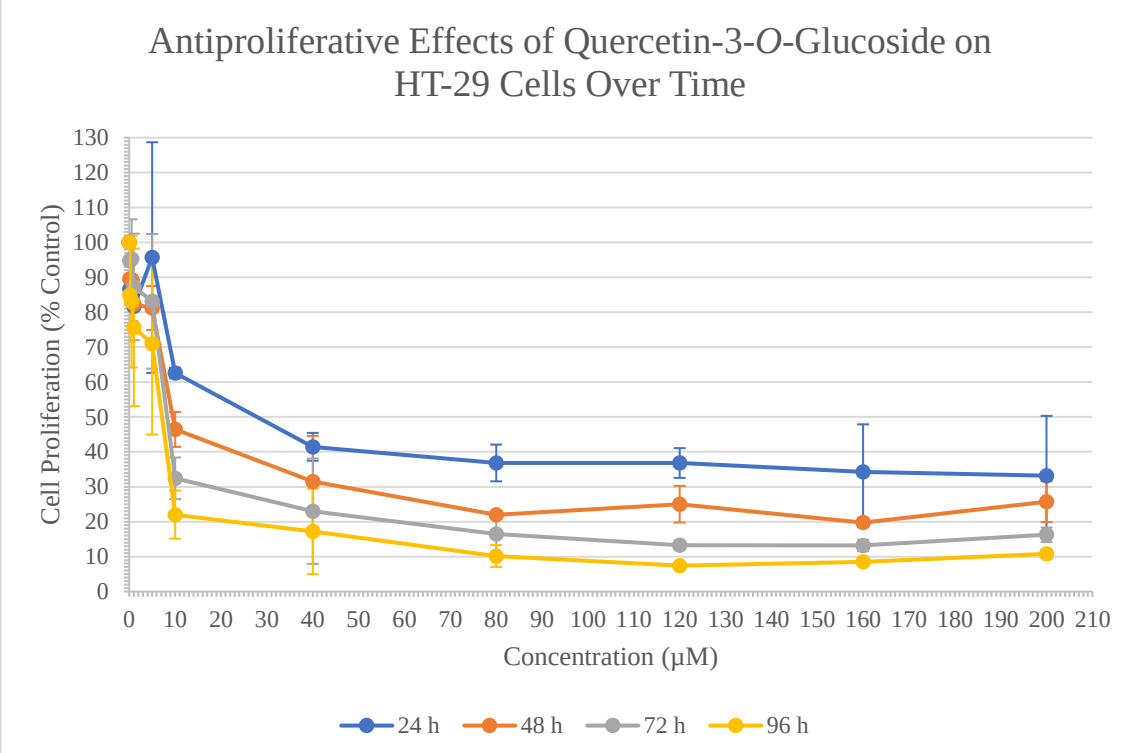

Figure H.3 Proliferation of HT-29 cells treated with quercetin-3-*O*-glucoside after 24, 48, 72, and 96 hours of exposure. Data represents the mean of two separate experiments (*n* = 3 in each experiment), with error bars indicating ± SD.

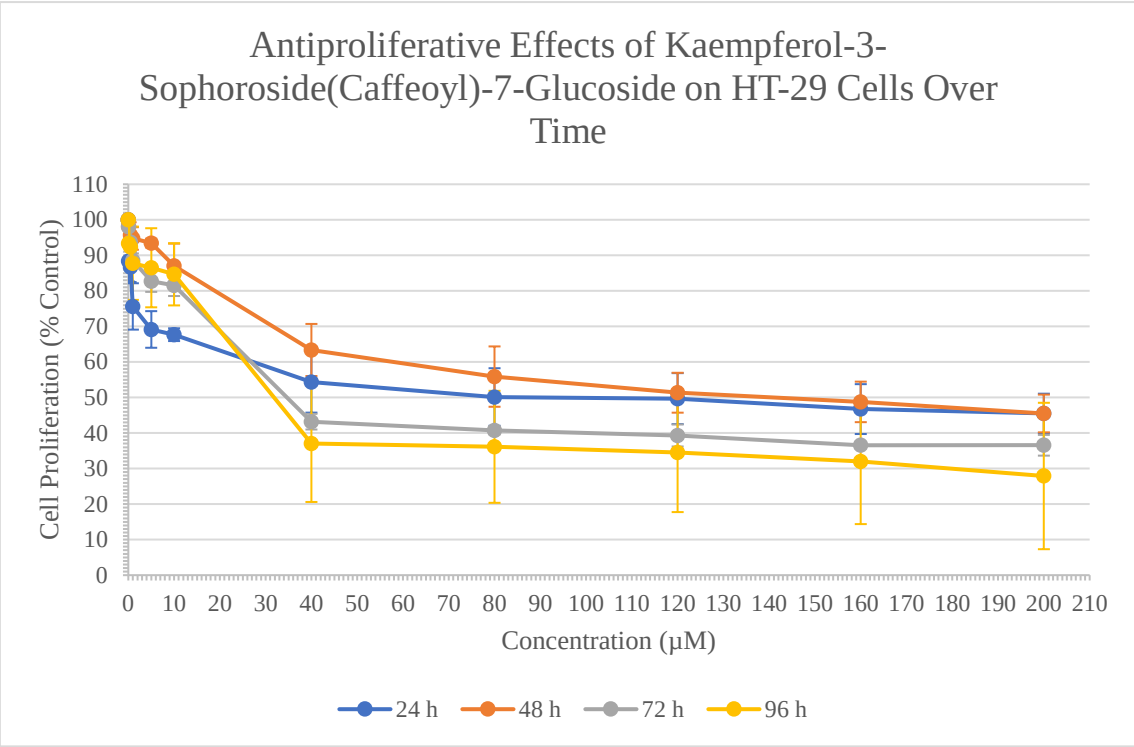

Figure H.4 Proliferation of HT-29 cells treated with kaempferol-3-sophoroside(caffeoyl)-7-glucoside after 24, 48, 72, and 96 hours of exposure. Data represents the mean of two separate experiments (n = 3 in each experiment), with error bars indicating ± SD.