T.R.N.C.

NEAR EAST UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

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DECLARATION

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

Name, Last Name **:** MANASSEH TACHIA BWANKWOT

Signature **:**

Date **:** 05 June 2017

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To my father, Lord Jesus and my dear mum Hannatu Bwankwot

ABSTRACT

Bwankwot, M. T. Determination of Isoorientin in *Asphodelus Ramosus L.* by High-Performance Liquid Chromatography. Near East University, Institute of Health Sciences, Analytical Chemistry Program, Master of Science Thesis, Nicosia, 2017.

Reversed-phase high-performance liquid chromatography with a photo-diode array detector (RP-HPLC-DAD) was used for the determination of isoorientin in *Asphodelus ramosus L.*. Four similar flavonoids [i.e., (+)-catechin, isovitexin, luteolin and apigenin] which are commonly found in some *Asphodelus* species were also separated within the same run. Optimum chromatographic conditions were achieved on a Zorbax SB-Aq column (4.6 mm × 150 mm, 5 μm), a linear gradient system starting with 5:95 to 90:10 (v/v) ACN/H_2O in 12 min, at a flow rate of 1.0 mL min^{-1}, 25°C, and a sample injection volume of 5 μL. Isoorientin was monitored at 280 nm maximum wavelength. Ultrasound-assisted solid-liquid extraction was performed using 1.0 g of the dried leave samples with 10.0 mL of 50/50 (v/v) MeOH/H_2O within 20 min. The extract was filtered and diluted three times with 45/55 (v/v) ACN/H_2O before being injected into the HPLC. Isoorientin could be quantified while the other four flavonoids were not found in the studied plant leaves. Calculations showed that the sample contained 1.24±0.05% of isoorientin. Limit of detection (LOD) and limit of quantitation (LOQ) were found as 0.10 and 0.34%, respectively. The calibration curve was linear over the dynamic range of 0.34–5.0%, %RSD ($n = 7$) was lower than 3.3 and coefficient of determination (R^2) was 0.9974. The method was proven to be fast, cheap, inexhaustible and efficient for the extraction of isoorientin from *Asphodelus ramosus L.*

Keywords: *Asphodelus ramosus L.*, Gradient elution, High-performance liquid chromatography, Isoorientin.

ÖZET

Bwankwot, M. T. *Asphodelus Ramosus L.* numunelerinde Isoorientin'in Yüksek Performanslı Sıvı Kromatografisi ile Tayini. Yakın Doğu Üniversitesi, Sağlık Bilimleri Enstitüsü, Analitik Kimya Programı, Yüksek Lisans Tezi, Lefkoşa, 2017.

Asphodelus Ramosus L. bitkisindeki izoorientin diyot serili dedektörlü ters faz yüksek performanslı sıvı kromatografisi (RP-HPLC-DAD) ile tayin edilmiştir. *Aspholedus* türlerinde sıklıkla bulunan benzer yapıdaki dört flavonoidin de [(+)-kateşin, izoviteksin, luteolin ve apigenin] aynı koşullarda kromatografik ayırımı gerçekleştirilmiştir. Optimum kromatografik çalışma koşulları: Zorbax SB-Aq kolonu (4,6 mm × 150 mm, 5 µm), 12 dakika içinde hacimce 5:95'ten 90:10 ACN:H_2O oranlarına değişen doğrusal gradient elisyon, dakikada 1.0 mL akış hızı; 25°C ve enjeksiyon hacmi 5 µL olarak belirlenmiştir. İzoorientin piki maksimum absorbladığı 280 nm dalga boyunda kaydedilmiştir. 1,0 g kurutulmuş yaprak örnekleri, 10,0 mL 50:50 (h/h) MeOH:H_2O ile 20 dakika boyunca sonike edilerek katı-sıvı ekstraksiyon gerçekleştirilmiştir. Süzülen ekstre, HPLC'ye enjekte edilmeden önce, 45:55 (h/h) ACN:H_2O ile üç kat seyreltilmiştir. Çalışılan bitki yapraklarında dört flavonoit tespit edilemezken izoorientinin kantitatif tayini yapılabilmiştir. Hesaplamalar örnekte % 1,24±0,05 izoorientin olduğunu göstermektedir. Teşhis sınırı (LOD) 0,10, tayin sınırı (LOQ) ise % 0,34 olarak bulunmuştur. Kalibrasyon doğrusu % 0,34–5,0 derişimleri arasında doğrusal, bağıl standart sapma %RSD ($n = 7$) cinsinden 3,3'ten daha küçük ve determinasyon katsayısı (R^2) 0,9974'tür. Geliştirilen metodun hızlı, ekonomik, kolay ve verimli olduğu ispatlanmıştır.

Anahtar sözcükler: *Asphodelus ramosus L.,* Gradient elisyon, yüksek performanslı sıvı kromatokrafisi, izoorientin.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
ACN	Acetonitrile
ASE	Accelerated solvent Extraction
CCC	Counter current chromatography
CPC	Centrifugal partition chromatography
DAD	Diode-array detector
EGC	Epigallo-catechin
EGCG	Epigallocatechin-3-gallate
GC	Gas chromatography
HPLC	High performance liquid chromatography
LC	Liquid chromatography
LDR	Linear dynamic range
LOD	Limit of detection
LOQ	Limit of quantitation
MAE	Microwave-assisted Extraction
MeOH	Methanol
NEU	Near East University
NMR	Nuclear magnetic resonance
NP	Normal phase
PFE	Pressurized Fluid Extraction
PLE	Pressurized liquid Extraction
Ppm	Parts Per Million
PRC	People Republic of China
RP	Reversed phase
RP-HPLC	Reversed-phase high-performance liquid chromatography
RSD	Relative standard deviation
RT	Room temperature
SB–Aq	Stable bond – Aqueous
SFE	Subcritical Fluid Extraction

SPE	Solid phase Extraction
SWE	Subcritical water Extraction
SWNIR	Short-wave near-infrared
TLC	Thin-layer chromatography
TRNC	Turkish Republic of Northern Cyprus
UAE	Ultrasound-assisted Extraction
UV	Ultraviolet
VIS	Visible

CHAPTER 1
INTRODUCTION

1.1 Phenolics

Phenolics are compounds possessing one or more aromatic rings with one or more hydroxyl groups attached to these rings. A wide range of phenolic structures are presently available, over 8000 spanning from light weight molecules to highly polymerized ones like phenolic acids and tannins respectively. These classes of substances are mostly used for inhibiting parasites, predators and shield against ultraviolet radiation or belligerence by pathogens, also for manifesting the colors of plants. These compounds are prevalent in fruits and vegetables, incorporated in human diet [1].

Phenolic compounds are ubiquitous parts of plant foods like olive, chocolate, vegetables, legumes, cereals, tea, and coffee, among others. They contribute to the general organoleptic properties of foods. As a major compound found in coffee, caffeic acid is mostly esterified with chlorogenic and quinic acids in many fruits and vegetables. Ferulic acid esterified to hemicellulose located in the cell, is often found in cereals. Phenolic compounds can be found free, although very commonly in free and/or bound forms, their corresponding methyl and ethyl esters and glycosides occur. Phenolic acids can be categorized as: derivatives of benzoic acid such as gallic acid, and derivatives of cinnamic acid such as coumaric, ferulic and caffeic acid [2].

With their abilities to scavenge free radicals, donate hydrogen, chelate metals, break radical chain reactions and react with singlet oxygen *in vitro* and *in vivo*, phenolic compounds have strong antioxidant activities associated with these properties [3]. The synthetic antioxidants have restricted use in food as they are suspected to be carcinogenic. Therefore, natural antioxidant sources of plant origin are of great interest

to the food industry [4].

1.2 Flavonoids

As a class of polyphenol, flavonoids, above 6000 in number, have been discovered. A small group is necessary in the human diet. This class of polyphenols is identified for involvement in cardiovascular disease protection, having antioxidation (a mechanism they have proven to exert their health effects, amongst other ways) activities *in vitro*. The bioavailability of flavonoids was limited, including their health effects and metabolic activities, until discovered in present years. Conversely, their bioactive potential for long has been discovered [5].

Flavonoids are sub-grouped, having differences in chemical and biological properties, depending on the sub-group.

1.2.1 Chemistry and Classification

Flavonoids constitute two benzene rings, with an attached oxygen-containing pyrene ring. Flavonoids possessing a hydroxyl group in the C-3 position of the C ring are classified as 3-hydroxyflavonoids (flavonols, leucoantho-cyanidins, anthocyanidins and catechins), and those without it as 3-desoxyflavonoids (flavanones and flavones). Depending on how additional hydroxyl or methyl groups have been introduced to the different positions of the molecule, determines the classification within the two families. When the B ring is bound to C-3 of the C ring instead of C-2, marks the difference of isoflavonoids from the other groups. Equally, anthocyanidins and catechins, lack the carbonyl group on C-4 [6].

In plants, the aglycones, the form lacking the sugar moieties, flavonoids are rare, unlike the glycosidic forms. At least eight monosaccharides or blends with di- or tri-saccharides bound to the various hydroxyl groups of the aglycone can be found [7].

These attachments produce a large number of flavonoids, with the commonest of the sugar moieties being *d*-glucose and *l*-rhamnose.

1.2.2 Occurrence in Food

Flavonoids are present in most edible fruits and vegetables, but there are different types of flavonoids obtained from different dietary sources. Intake estimates for flavonoids on a population level are only available for a few flavonoid sub-classes, such as flavonols, flavanones, and isoflavones [8].

1.2.3 Flavonols

Quercetin, according to Häkkinen et al. [9] was found to be the most prevalent flavonol, as determined in onions, apples, tea, and berries. Onion contains a high amount of quercetin, which is also present in a wide variety of vegetables and fruits. Tea and wine have relatively low content of quercetin than onions, but onion is consumed in little quantities as compared to high quantities of wine and tea in many countries [8].

1.2.4 Flavanones

Almost exclusively, flavanones are present solely in citrus fruits. The fibrous part having high concentrations, with the juice having low concentration [10]. The chief flavanones in oranges, mandarin and grapes are hesperetin-7-rutinoside, naringenin-7-rutinoside, naringenin-7-neohesperoside (70%) and narirutin (20%) [11]. Fresh tomatoes, especially tomato skin, also contain naringenin chalcones. In tomatoes and tomato-based products, low concentrations of naringenin are also found.

1.2.5 (+)-Catechins

The esters of gallic acid with aglycones, characterize the occurrence of catechins.

In a wide variety of plant foods, (+)-catechin and (+)-epicatechin are present in varying concentrations specially higher in tea and red wine and also in fruits and vegetables, such as apples, peaches, grapes and pears [12].

1.2.6 Flavones

Flavones, popularly known to be in high concentrations in the diet are apigenin and luteolin. They are found most predominantly in red pepper and celery, with dietary intake in low concentrations, having a little variety of plant foods which contain them [8].

1.2.7 Anthocyanidins

The obvious red, violet or blue colors as exhibited by edible fruits such as apples among other fruits, is due to the presence of anthocyanins (or anthocyanidin glycosides). Malvidin, pelargonidin, cyanidin and delphinidin are commonly found anthocyanidins [13].

1.2.8 Isoflavonoids

Soybean and soy products are the major sources of isoflavonoids, with genistein and daidzein as the prevalent isoflavonoids in legumes. They are in relatively lower concentrations in other food products and legumes, than in soybean and soy products [14].

1.2.9 Isoorientin

Isoorientin a C-glycosylflavones (6-C-glucosyl luteolin) has a wide range of activity profile, which has made it to attract attention within the scientific community in drug discovery and development, and also in the development of new and potent

therapeutic agents. Its discovery was mostly from natural sources, which is paramount, though there are recent methods to synthesize it in the laboratory [15]. In an *in vivo* bioassay-guided fractionation study, isoorientin was isolated as the main active component responsible for the control of blood sugar in Type II diabetes [16]. Isoorientin is a C-glucosylflavonoid, and the earlier investigations have revealed that isoorientin also displayed *in vivo* and dose-dependent analgesic, anti-inflammatory, antihyperlipidemic, antioxidant and hepatoprotective activities, in addition to its hypoglycemic effects [17, 18].

1.2.10 Isovitexin

Isovitexin (6-C-glucosyl apigenin), which is an isomer of vitexin (8-C-glucosyl apigenin) found alongside in plants like pigeon pea [19, 20] has antioxidant properties [21]. It also possess anti-inflammatory [22], anti-bacteria [23], memory modulation [24], and anti-pathogenic [25] properties. It has been reported that isovitexin has a measurable interaction with the human serum albumin (which carries a wide variety of drugs in blood plasma) [26], as also experimented on rats, which were administered some herbal plants (i.e., *santalum album L.* leaves extract) [27]. There are few reports on the side effects, metabolic process and tissue distribution of isovitexin in rats, when administered as a single substance [28].

1.2.11 Luteolin

Luteolin (2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4H-chromen-4-one), a member of the flavonoid family, is found in many vegetables, fruits and herbs. It is found at high concentrations in thyme, peppermint, parsley, celery, green pepper, perilla leaves and chamomile tea [29]. It is well known that luteolin has antioxidant, anti-inflammatory, anticancer, anti-allergic, antiplatelet and anti-ulcer properties [30]. Luteolin has also been reported to reduce the neurotoxicity induced by neurotoxic agents in cell culture systems. In addition, it has been used for antiviral activities, cataract

prevention, and antithrombotic action [31].

1.2.12 Apigenin

Apigenin is a 4,5,7-trihydroxy flavone, belonging to a less toxic and non-mutagenic flavones subclass of flavonoids. It is derived from *Cosmos bipinnatus* and many other plants. It has a variety of pharmacological activities, including antioxidant [32], anti-tumor [33], anti-inflammatory [34], ant-bacterial [35], and induced apoptosis [36]. It has protective effect on radiation-induced chromosomal damage in the human lymphocytes [37]. Apigenin is one of the very important secondary metabolites produced by *C. bipinnatus*. In traditional herbal medicine, *C. bipinnatus* has been used as a remedy for various diseases such as jaundice and intermittent fever [38]. Apigenin was suggested as a useful anticancer candidate for chemotherapy and cancer prevention [34]. In another study, anti-proliferative effects of apigenin on human breast cancer cell lines have been demonstrated. It strongly inhibited tumor cell invasion in estrogen-insensitive breast tumor cell line [35], reporting that apigenin inhibited the growth of human cervical carcinoma HeLa cells through an apoptotic pathway.

1.2.13 (+)-Catechin

The main flavonoids present in green tea include catechins (flavan-3-ols). Green tea also contains phenolic acids (such as gallic acid, caffeic acid, etc.) and flavonols (such as quercetin and myricetin, etc.). The beneficial effects associated with green tea have been attributed to its polyphenols, particularly catechins such as epigallocatechin-3-gallate (EGCG) and epigallo-catechin (EGC or (+)-catechin), which are the first and second major catechins that are *in vivo* strong antioxidants [39]. It has been shown that green tea catechins enhance the liver functions [40], reduce body fat and decrease body weight after long-term consumption (usually 12 weeks) [39]. Green tea catechins exhibit high-level antioxidant activity, even more than vitamins C and D [41].

In view of these beneficial effects, the development of a simple, inexpensive and rapid analytical method for the simultaneous separation and determination of such compounds in plant material sources is necessary. The chemical structures, logP and M_r values of the studied analytes, i.e., isoorientin, isovitexin, luteolin, apigenin and (+)-catechin are given in **Table 1.1**.

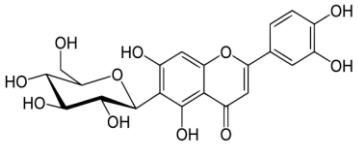
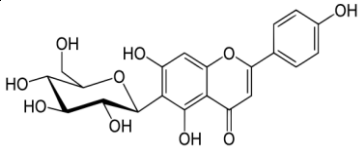
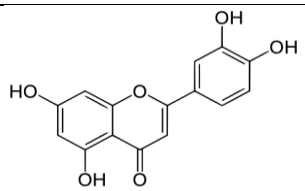
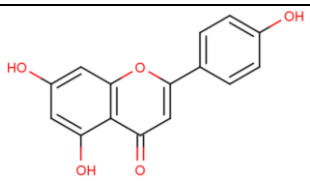
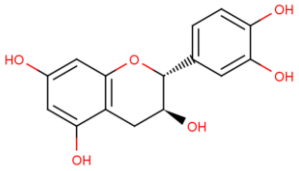
1.3 *Asphodelus ramosus L.*

In this study, isoorientin and four other flavonoids (i.e., catechin isovitexin, luteolin and apigenin) were simultaneously separated using HPLC and their presence in *Asphodelus ramosus* (*A. ramosus*) was investigated. The genus *Asphodelus* comprises 187 genera and 2500 species. It is a circum-mediterranean genus, which includes five sections and is represented by 16 species [42].

A. ramosus is a robust erect perennial plant which can be 1 m high or more. It is rhizome compact, the clustered fleshy roots bearing large fusiform tubers, with strap-shaped leaves; flat, slightly keeled, glabrous, and glaucous about 20–60 cm long (or more), 1-3 cm wide (or more) [43]. In February till the end of March, *A. ramosus* flowers during spring and cold times in the eastern Mediterranean. Its flowers open upwardly, characterizing the ephemeral of *A. ramosus* [44].

To the best of our knowledge, scarce data is still available on the determination of flavonoids in *Asphodelus ramosus* using HPLC.

Table 1.1: Chemical structures of the studied analytes.

Analyte	**Chemical Structure**	$LogP$	$M_r\ (g/mol)$
Isoorientin		-0.35	448.38
Isovitexin		-0.05	432.38
Luteolin		2.40	286.24
Apigenin		2.71	270.24
(+)-Catechin		1.80	290.26

1.4 Sample Preparation and Characterization

1.4.1 Extraction

The first step to get bioactive compounds for the making of dietary nutraceuticals or supplements, cosmetics, and other industrial consumables, is to extract them from the plant materials. The plant materials are milled, ground and homogenized, which is also accompanied by air-drying or freeze-drying before extraction. Care needs to be made on the conditions for drying, not to cause undesirable change of the plant constituents.

Therefore, the physicochemical properties of the analytes must be considered [45].

A widely used method to prepare plant material extracts, which is efficient and easy to perform, is solvent extraction, also known as solid-liquid extraction (SLE). The polarities of different types of solvents are considered, extraction temperature, sample-to-solvent quantities and extraction time. These parameters generally determine the yield of the analyte obtained after extraction. The polarity of the solvents used, as well as the physicochemical nature of the analyte such as its solubility in the given extraction solvent determine how efficient its extraction is. Contained in different quantities are phenolics varying from simple (e.g., phenolic acids, anthocyanins) to highly polymerized substances (e.g., tannins) in plant materials. Some phenolics may be found in their associated form with other components in the plant like carbohydrates and proteins [46] which changes their polarities. In addition, phenolics and other non-phenolic substances with similar polarities may be extracted with the same solvent. Thus, SLE can be considered as a general non-specific method, which makes hydrolysis, sample clean-up procedures and/or more specific extraction protocols necessary to handle these situations in some case.

Popularly, solvents, such as acetonitrile, ethyl acetate, ethanol, acetone, methanol, and their combinations in the same or different volumes, have been used for the extraction of phenolics from plant materials, usually with different proportions of water. Selecting the right solvent plays a major role in the yield of polyphenols extracted and extraction rate [47]. Of these solvents, methanol, ethanol, acetonitrile and acetone were generally found efficient for extraction of phenolic compounds [48, 49]. Numerous factors must be considered while selecting the appropriate extraction conditions, which include extraction solvent, extraction temperature, stirring/agitation rate, toxicity, cost and solubility of the target analytes in the extraction solvent.

Generally, extraction efficiency increases with extraction time and temperature. However, extra care must be taken in order to avoid hydrolysis of the polyphenols at

prolonged extraction time and high temperatures. For example, anthocyanins have been shown to degrade at temperatures above 70 °C and that 20–50 °C to be an optimum temperature for their extraction [48]. Hence, selecting an efficient extraction procedure/method is critical, so as to maintain the stability of phenolic compounds.

Microwave-assisted extraction (MAE) [50], pressurized fluid extraction (PFE) [51], supercritical fluid extraction (SFE) [52], subcritical water extraction (SWE) [53] and accelerated solvent extraction (ASE) [54] have recently been proven to be efficient techniques for the extraction of phenolic compounds. Although Soxhlet extraction is commonly used for this purpose [55-57], the former have the advantage of using lower solvent volumes and providing a good yield within a shorter extraction time.

Ultrasound-assisted extraction (UAE) has recently gained a wide acceptance among scientists as an efficient extraction technique of different compounds from a variety of samples as it offers many advantages over conventional extraction techniques. These advantages include higher extraction efficiency at a significantly reduced time, energy, chemicals consumed, thus less cost and chemical waste produced. The mechanisms for ultrasonic enhancement involve the shear force created by the collapse of cavitation bubbles upon the propagation of the acoustic waves in the kHz range [58]. Bubbles collapse can produce interesting chemical, physical and mechanical effects [59]. Surface peeling, erosion, particle breakdown and fragmentation of the matrix during irradiation and an enhanced hydration of the matrix due to ultrasound are among the mechanisms proposed to explain better extraction efficiencies through the use of ultrasound [60]. The extraction of various phenolic compounds from different parts of plants such as leaves, stalks, fruits and plant seeds has been widely achieved through the use of UAE [48, 61].

Homogenization of plant material into finely divided particles has the ability to improve extraction yield due to larger surface area. Also, affecting the extraction yield is the volume of solvent used for extracting a weighed amount of sample which has to be

optimized [62, 63].

1.4.2 Analysis and Quantification of Phenolics

Various analytical techniques have been attempted for the determination of flavonoids. Capillary electrophoresis (CE) with its different modes [64] and gas chromatography (GC) [65] have been utilized, but the challenge with using GC is the need for derivatization of the flavonoids to turn them into a volatile form which is compactable with the instrument. Nuclear magnetic resonance (NMR) [66] has also been used. Thin-layer chromatography (TLC) chemical screening is fast, simple, and of low cost in identification of chemical ingredient from plant extracts. Many papers about TLC chemical screening have been published in recent years [67]. More recently, the use of countercurrent separation techniques such as countercurrent chromatography (CCC) [68] and centrifugal partition chromatography (CPC) found application primarily as purification methods for natural products including phenolic compounds [69].

Owing to its suitability with many detectors (e.g., UV-VIS, MS, fluorescence, electrochemical, etc.), high reproducibility, high accuracy, suitability with volatile, semi-volatile and non-volatile analytes, suitability for thermally fragile analytes and applicability to substances of prime interest in industry, HPLC remains the first choice for the determination of phenolic compounds. The hyphenation of HPLC with fluorescence [70], MS [71] and electrochemical detectors [72].

DAD is the most commonly used one among these hyphenated techniques for the determination of phenolic compounds [73-75] due to its lower price and availability in many analytical laboratories.

In this study, gradient elution-HPLC with a DAD detector was used to separate five common flavonoids and to determine isoorientin in *Asphodelus ramosus.*

1.4.3 Chromatography

Chromatography, first described by Tswett1in 1906, is a method in which the components of a mixture are separated on an adsorbent material filled in column making use of a “flowing system”. The adsorbent material (basically a solid), or stationary phase has transformed over the years, including paper, thin layers of solids attached to glass plates, immobilized liquids, gels, and solid particles packed in columns. Fluids, either a liquid or a gas are the flowing components of the system, or mobile phase. Concurrent with development of the different adsorbent materials has been the development of methods more specific to particular classes of analytes. In general, however, the trend in development of chromatography has been toward faster, more efficient and environmentally friendly systems [76].

1.4.4 Liquid Chromatography

The principle for the separation of analytes in liquid chromatography is based on the mechanism of retention of the analytes in the column. This mechanism classified into adsorption, partition, ion-exchange and size exclusion defines the different types or modes of liquid chromatography bearing the same principle. Two basic modes that are generally recognized for the elution of analytes from the column are isocratic and gradient elution. Isocratic elution keeps a constant elution composition of the mobile phase, while the gradient elution has a changing composition of the mobile phase with time; this is mostly utilized for the partition chromatography (in the reversed-phase mode), with analytes having varying retention factor values.

Chromatographic conditions have to be optimized for a better separation of the analytes in the column, and also to avoid peak broadening, with the column being able to retain the analytes by varying strength. The factors that dictate a better resolution are the efficiency of the column (N), selectivity factor (α) and the retention factor (k'). The selectivity factor, α, can be altered by changing the column and/or mobile phase type,

which can completely change the interaction of analytes in the column, due to great differences in polarity. The retention factor, k', is influenced by the pH and composition of the mobile phase, the presence of a modifier in the mobile phase and the column temperature. The higher k', the better the resolution. However, k' between 2 and 10 is generally considered ideal for resolution. The efficiency of the column, N, is affected by physical properties such as column length, diameter and particle size, and also the mobile phase flow rate. Resolution, defined as the measure of a method to separate two adjacent components, can be achieved as a result of optimization of the different chromatographic parameters. A resolution greater than 1.5 (baseline resolution) is generally required for good quantitation [76].

1.4.5 High-Performance Liquid Chromatography (HPLC)

HPLC is based upon the principle of distribution or partitioning of the sample mixture between two phases in the chromatographic bed. The stationary phase is the column which is packed with a solid, porous, surface active material in a small-particle nature. While, the mobile phase is a liquid, which is used to elute the sample material, with different interaction that can be detected and quantified with the detector. Each sample component appears as a peak signal as it passes through the detector.

Qualitatively, under similar chromatographic conditions, the retention time of a component is always almost constant. These chromatographic conditions are sample size/volume, mobile phase composition, flow rate, separation temperature, column type, among others. The time interval required by a component to travel through the column from injection to the appearance of the signal is the retention time (t_R).

Quantitatively, a graph called calibration graph is drawn from various concentrations to obtain different peak areas or heights. A comparison of the unknown's peak height/ area can be used for determining the concentration of an analyte with an unknown concentration in the sample [77].

1.5 Modes of HPLC

With frequency in use of partition chromatography, makes it popular amongst the different procedures of liquid chromatographic procedures. Base on relative polarities between the stationary phase and the mobile phase, partition chromatography operates in two modes. Initially used was the normal-phase chromatography (NP), characterized by the usage of highly polar columns, with packing material materials such as silica and alumina, with a relatively non polar elution solvent like hexane. In reversed-phase chromatography (RP), the stationary phase is non-polar, often a C-18 hydrocarbon, and the mobile phase is relatively polar (such as water, methanol, acetonitrile or tetrahydrofuran). The least polar components of the sample are eluted first, being the most soluble in the non-polar mobile phase; increasing the polarity of the mobile phase has the effect of increasing the retention time of the polar components in normal-phase chromatography. In contrast, in the reversed-phase chromatography, the most polar components of the sample are eluted first, and increasing the mobile phase polarity decreases the retention time of these components [78].

Adsorption chromatography has a similar principle to normal-phase chromatography. Adsorption–displacement process characterizes the retention. The adsorption characteristics are based on the polarity of the molecules, the active sites of the stationary phase interact with the solute molecules. The more polar analyte displaces the other in the stationary phase, the adsorption characteristics of the two solute molecules parallel one another. The two stationary phases normally used are silica and alumina. Silica has a higher sample capacity, which makes it the most preferred one [79].

Ion-exchange chromatography utilizes charged surfaces (stationary phase) to separate charged species. There are two modes of ion-exchange chromatography; suppressor-based and single-bond ion-exchange chromatography.

Size-exclusion chromatography is applied for the separation of high-molecular-weight species like proteins and fats in biological samples. Silica or polymer particles of small sizes (~10 μm) are used as the packing material, with a network of uniform pores, through which the mobile phase and solute can diffuse [79].

1.6 Method Development in RP-HPLC

Often, in choosing a column for a partition chromatographic separation, the polarity of the stationary phase is roughly matched with that of the analytes; a mobile phase of a considerably different polarity is then used for elution. In choosing the mobile phase, polarity, UV transparency, purity, inertness toward the sample components, toxicity and price are considered. In a liquid mobile phase, the sample components interact with both the mobile phase and the stationary phase, making method development to be more complex in liquid chromatography than in gas chromatography. **Figure 1.1** describes the steps towards a systematic approach for developing an efficient and fast HPLC method.

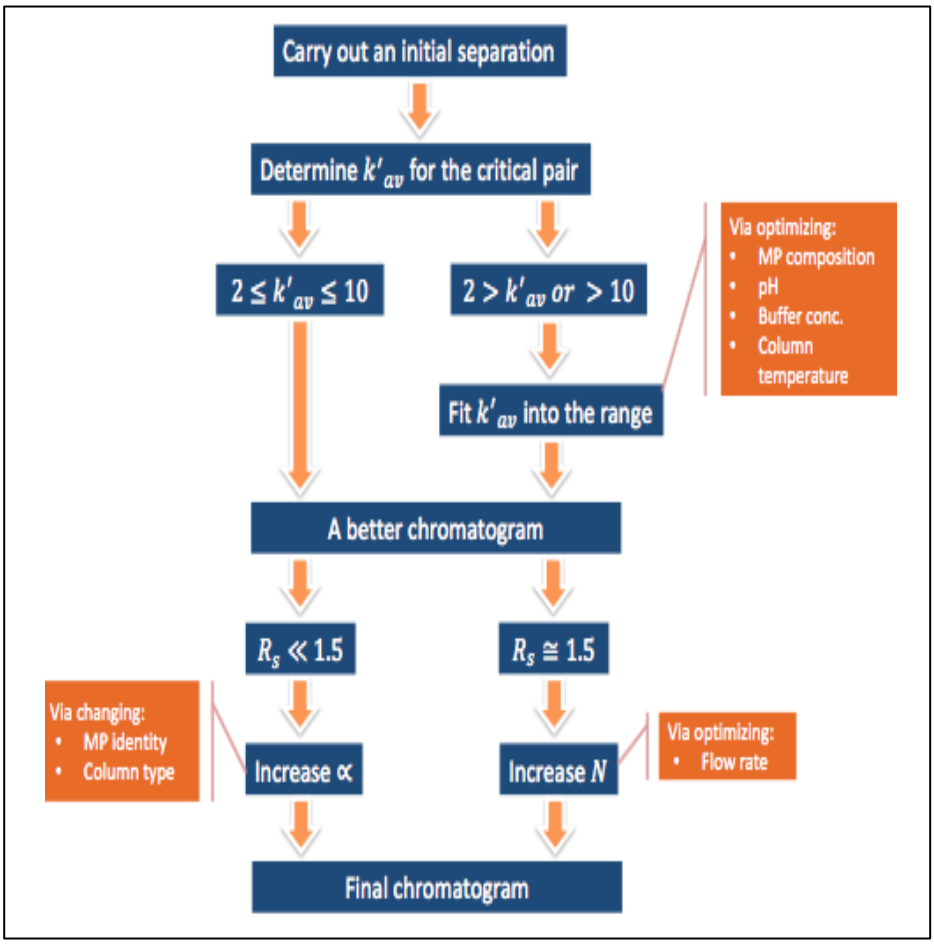


Figure 1.1: Systematic HPLC method development.

Improving the resolution of a chromatographic column is based upon varying one or more of the three parameters, N, k′, and α. Equations **(1.1)** through **(1.4)** in **Table 1.2** are used to calculate each of these parameters from the chromatogram obtained.

Table 1.2. Important relationships in HPLC [79].

Equation	Term	Meaning	Equation No.
$k' = \frac{t_R - t_M}{t_M}$	k' t_R t_M	Retention (capacity) factor Retention time Dead time	**(1.1)**
$N = 16\left(\frac{t_R}{W}\right)^2$	N W	Number of theoretical plate (efficiency) Peak width	**(1.2)**
$\propto = \frac{k'_B}{k'_A} = \frac{(t_R)_B - t_M}{(t_R)_A - t_M}$	$\propto$	Selectivity factor	**(1.3)**
$R_s = \frac{\sqrt{N_{av}}}{4} \times \frac{k'_{av}}{k'_{av} + 1} \times \frac{\propto - 1}{\propto}$	R_s N_{av} k'_{av}	Resolution Average N of two adjacent peaks Average k' of two adjacent peaks	**(1.4)**

1.7 Components of HPLC System

There are different components of the HPLC instrument that work as a single unit for the separation, detection and quantitation of various components. Having a general principle of operation of the main components, the instrument used for this study has the elements as described below.

1.7.1 Quaternary Pump

The four-channel gradient pump comprises a high-speed proportioning valve and a pump assembly. It provides gradient generation by low-pressure mixing.

A two channel system describes the quaternary pump, giving a gradient elution by low pressure mixing. It comprises either a pump or four pumps, giving high pressure which can be up to 400 bar to elute components from the column with less retention time.

In this type of pumps, a process consisting of aspiration, compression and discharge of the mobile phase is continuously repeated. The operation of the step motor is converted, through a cam, to reciprocating motion of the plunger (piston). A material such as sapphire or a special ceramic is usually used for the plunger.

The eluent is aspirated and discharged by the motion of this plunger. The benefit of having a reciprocating pump is that; it has small internal volume (35-400 μL), therefore, low dead volume. It also offers high output pressure (up to 10,000 psi). It is readily adaptable to gradient elution and provides a constant flow rate that is independent of column back-pressure and solvent viscosity.

1.7.2 Degasser

Degassing comprises a 4-channel vacuum container, including 4 tubular plastic membranes, and a vacuum pump. As the eluent passes through the vacuum tubes as pumped from the solvent reservoirs, the dissolved gas will almost be completely removed, upon leaving the vacuum degasser.

Bubble formation while mixing the solvents can lead to a number of problems in HPLC analysis, which can be prevented by degassing of the mobile phase. These problems include unstable and noisy baselines, air bubbles passing through detectors lead to fake peaks, and excessive pressure can develop which can lead to eventual pump failure.

1.7.3 Autosampler

The autosampler ensures that the specified sample is injected onto the column, and that the mixing unit and injection needle are always free from any sample residues before the next sampling sequence begins. All the injection valves have different stator heads and different rotor seals. The volume of each valve is different.

The major advantage of the autosampler entails accurate injected volume; needle clean up after injection to prevent carry over. The software regulates the sequence of samples in the autosampler for injecting from the vials according to arranged positions. However, feeding the vial number correctly on the autosampler rack and listing out the sequence correctly in the software is very important.

1.7.4 Thermostated Column Compartment

The temperature of the column can be controlled to obtain a more robust method. With the control or regulation of the temperature of the column, high separation temperatures can be applied which can, in general, cause the analytes to be eluted faster. As temperature increases, viscosity decreases, which also decrease back-pressure, because diffusion coefficient increases, leading to higher theoretical plate number (N). Notwithstanding, temperature must be controlled to avoid deterioration of the column with temperatures used between 40 to 60 °C.

1.7.5 Diode-Array Detector (DAD)

Its illumination source is a combination of a deuterium-arc-discharge lamp for the ultraviolet (UV) wavelength range and a tungsten lamp for the visible (Vis) and short-wave near-infrared (SWNIR) wavelength range. The achromat (source lens) forms a single, focused beam of light through the flow cell.

Using DAD, light from the lamps is shone directly onto the flow cell; light that passes through the flow cell is dispersed by the diffraction grating, and the amount of the dispersed light is estimated for each wavelength by the photodiode arrays.

1.8 Benefits of This Study

Over the years, determination and characterization of many compounds including flavonoids have improved. In the past, UV/VIS spectrophotometry and thin layer chromatography (TLC) were the main techniques used for their characterization. However, with the advent of more sophisticated techniques like DAD detectors, infrared, mass spectroscopy and nuclear magnetic resonance, more detailed information can be obtained to better characterize these organic compounds.

Some analytical methods have been carried out to determine isoorientin, isovitexin, luteolin, apigenin, and (+)-catechin in species of *Asphodelus* plant materials. (+)-catechin, isoorientin and isovitexin were determined in *A. aestivus* [80, 81] while luteolin and apigenin in were determined in *A. tenuifolius* [82]. It was speculated that the development of an efficient HPLC method by which the five commonly found analytes in species of *asphodelus* simultaneously separated would be beneficial. Such a method would offer several advantages such as cost-effectiveness, rapidness, reduction of toxic chemicals, time, labor and energy.

1.9 Objectives of This Work

The aim of this research is to determine isoorientin and investigate four other flavonoids (isovitexin, luteolin, apigenin and catechin) in *Asphodelus ramosus* leaf extract. The aims can be listed as follows:

- To separate some flavonoids commonly found in *Asphodelus* leaf extract.
- To determine isoorientin in *A. ramosus* leaf extract.
- To establish an efficient and robust methods for determining these compounds within the shortest possible time.

CHAPTER 2
EXPERIMENTAL

2.1 Instrumentation

An Agilent 1200 series HPLC system (Agilent Technologies Co., USA), compacted with G1311A quaternary pump, G1316A column temperature controller, G1329B ALS, G1322A on-line degasser and G1315 B photodiode-array detector, controlled by Agilent ChemStation for LC 3D systems software (Rev. B. 03.01) was used. Separation was performed usıng a Zorbax SB–Aq column (4.6 x 150 mm, 5 µm) from (Agilent Technologies Co., USA).

2.2 Reagents and Solutions

Methanol CHROMASOLV® gradient grade, for HPLC ≥ 99.9% was obtained from Sigma-Aldrich (St. Louis, USA). Acetonitrile CHROMASOLV® gradient grade, for HPLC ≥ 99.9% was from Honeywell (Seelze, Germany). Deionized water DI water (18.2 $M\Omega.cm$) was obtained using a water purification system at Near East University Hospital (Nicosia, TRNC). The five authentic standards were kindly provided by Prof. Dr. İhsan ÇALIŞ previously extracted, purified and characterized by his group (unpublished work).

2.3 Equipment

The centrifuge machine used for centrifugation was from Hettich Zentrifuge D-78532 (Tuttlingen, Germany). A Schwabach vortex with REAX top (Walpersdorfer Str., Germany) was used for shaking and mixing. A Bandelin Sonorex Digitec ultrasonicator

(Germany) was used for ultrasonication. The weighing balance used was from Mettler Toledo classic plus AB204 – S/FACT (Greifensee, Switzerland). The oven used for drying the samples was from Binder (Germany). A kitchen blender with stainless steel blades used for homogenization of the sample into fine particles was from Sinbo (SCM 2927) (P.R.C).

2.4 Sampling and Sample Pre-treatment

The season for *A. ramosus* in Northern Cyprus is between January and April. The samples were collected on 7th of April 2017. The fresh plant of *A. ramosus* (both leaves and tubers) was collected on campus behind the Near East University Hospital.

The fresh plant material was washed with distilled water several times, cut with a stainless steel knife into small sizes and dried in the laboratory at room temperature for two days, then transferred into the oven for 48 hours at 35 °C. It was then blended into finely homogenized particles using the blender, put into glass bottles, sealed tightly and stored in a dry place at room temperature till analysis.

2.5 Preparation of Standards

To prepare stock solutions of the analytes, the solid standards were dissolved in methanol to make 1000 mg L^{-1} each of the various standards. A 50 mg L^{-1} mixed working standard solution of the five analytes was prepared by pipetting 50 µL of each stock solution into an HPLC vial and making up the solution to 1000 µL with 45:55 (%, v/v) of ACN/H_2O. Individual standard solutions were also prepared at 50 mg L^{-1} in the same way. Both individual standard and mixture solutions were injected to the HPLC instrument.

2.6 Procedure

1.0 g of the dry sample was accurately weighed using an electronic balance into a 50-mL beaker and 10 mL of methanol/water (50:50, % v/v) were added to the solid material. The mixture was ultrasonicated for 10 min. The extract was filtered with a cotton wool into a 10 mL volumetric flask and the filtrate was completed to the mark with the same extraction solvent. The sample solution was transferred into a falcon tube and centrifuged for 3 min at 6000 rpm. 50 µL of the sample solution was diluted three, five or ten times with 45:55 (v/v) of the mobile phase and injected into the HPLC instrument.

CHAPTER 3
RESULTS AND DISCUSSION

3.1 Systematic Method Development

The choice of chromatographic method and procedure were done considering the chemistry of the analytes and sample to be analyzed. The following were put into consideration to decide on the chromatographic method to determine these compounds;

- For molecules with $M_r < 10^4$ adsorption, partition or ion-exchange chromatography are preferable.
- For molecules with $M_r > 10^4$ size-exclusion chromatography is preferable.
- For water-insoluble analyte, adsorption or partition chromatography is preferred.
- For water-soluble analytes that are also ionic, ion-exchange chromatography is preferred.
- For water-soluble analytes that are also nonionic, partition (NP- or RP-) chromatography is preferred.

If one of the solvents is water and the other is a non-polar solvent, then the $logP$ value is a measure of lipophilicity or hydrophobicity of the analyte distributed in the solvents. The partition coefficient, P, is defined as the ratio of the concentrations of a solute between two immiscible solvents, and the logarithm of this ratio is thus $logP$. This value is defined in **Equation (3.1)** for the common biphasic system of n-octanol and water.

$$logP_{o/w} = \log [\text{analyte}]_{\text{octanol}}/[\text{analyte}]_{\text{water}} \quad \textbf{(3.1)}$$

Considering that for the analytes $M_r < 10^4$, size-exclusion is not appropriate and since the analytes are water-soluble, adsorption chromatography is not appropriate. $logP$ values for (+)-catechin, isoorientin, isovitexin, luteolin and apigenin are 1.80, -0.35, -0.05, 2.40 and 2.71, respectively, showing high and intermediate polarity. These values indicate that isovitexin and isoorientin are highly polar, while the others have an intermediate polarity. Thus, they may all be extracted into an aqueous solution. This also proposes that the analytes would favor the aqueous mobile phase more than a reversed-phase column. Thus, the separation can be done using partition chromatography.

Looking at its microspecies distribution as shown in the pK_a graph in **Figure 3.1,** isoorientin can be considered as ionizable (with decrease in pH, the hydroxyl groups start to ionize after about pH 3.2) and would be completely in its ionic form at all pH range afterwards. Therefore, ion-exchange chromatography may also be appropriate. Other species also have similar patterns of ionization of the hydroxyl groups based on their similar structures and pK_a graphs as shown in **Appendix 1**. This can be observed better for (+)-catechin (**Appendix 1**)**.** An example for possible ionization at different pH values is given in **Appendix 2** for (+)-catechin.

Based on these criteria, the most appropriate chromatographic mode for separating these analytes is partition (reversed-phase) chromatography. Since they are polar compounds, a non-polar stationary phase was used.

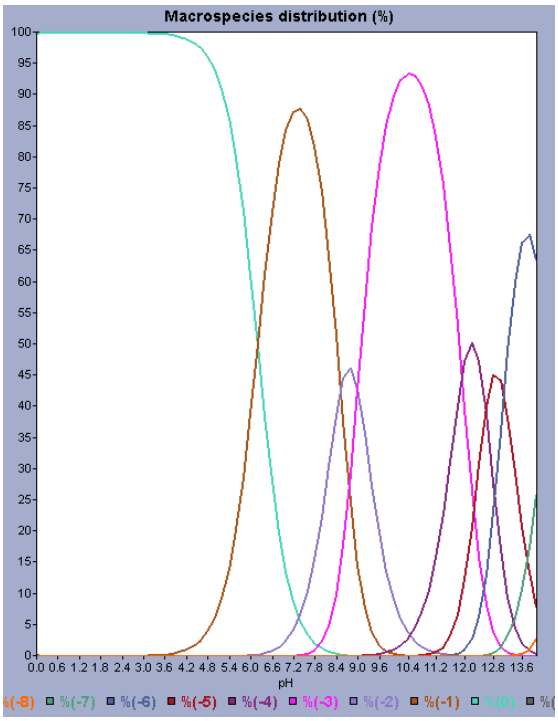


Figure 3.1: Plot of microspecies distribution versus pH for isoorientin.

3.1.1 Initial Chromatographic Conditions

According to Zeraik, M.L. et al. [83] separation of isoorientin, isovitexin and similar flavonoids can be achieved using a gradient system with the elution program shown in **Table 3.1.**

Table 3.1: Gradient elution program [83].

Time/min	A% (Acetonitrile)	B% (Acetic acid/water)
0-2	10-10	90-90
2-6	10-14	90-86
6-16	14-17	86-83
16-23	17-19	83-81
23-28	19-23	81-77

The relatively wide range of $logP$ of the studied analytes and similar studies from the literature led to the preference of gradient elution of these compounds. Therefore, the initial chromatographic conditions started with a gradient scan with ACN/H_2O system. Initial chromatographic conditions were as shown in **Table 3.2**.

Table 3.2: Initial HPLC Conditions.

Physical parameters	Column	ZORBAX SB-Aq, 4.6 mm ID x 150 mm (5 µm)
	Flow Rate	1.0 mL/min
	Temperature	RT
	Detector/wavelength	DAD 225 nm (BW16). Reference: none
	Injection volume	5.0 µL
Chemical parameters	Mobile phase	ACN (C):H_2O (A) Gradient 90:10 A:C from 0 min to 10:90 A:C within 20 min

The resulting chromatogram is shown in **Figure 3.2**. It can be observed that all of the five analytes were separated within 11 minutes.

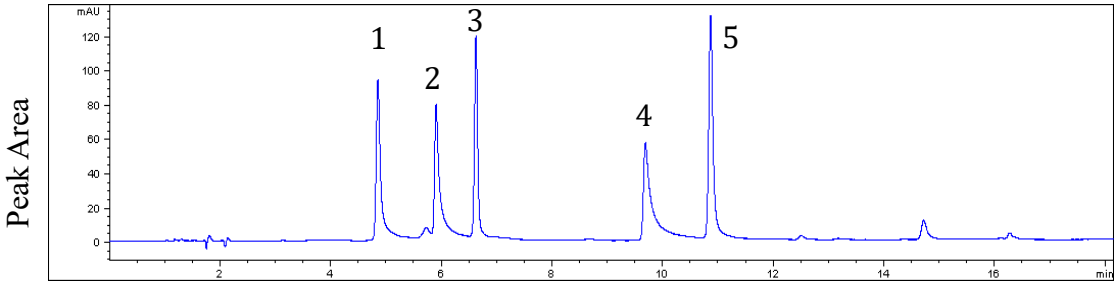


Figure 3.2: Chromatogram showing the separation of five standards; Peaks: 1, (+)-catechin; 2, isoorientin, 3, isovitexin, 4, luteolin and 5, apigenin.

3.2 Selection of Optimum Wavelength

DAD is an important alternative in the absence of a mass detector as it enables the simultaneous scanning of a wide range of wavelengths for efficient and fast selection of the optimum maximum wavelength. From the 3D plots given in **Figure 3.3** and the UV absorption spectra in **Figure 3.4**, it can be seen that the flavonoids absorbed throughout the whole UV region except for isoorientin which absorbed approximately up to 300 nm. It was also observed that each analyte had three absorption maxima. The wavelengths of 225, 280 and 350 nm were considered (**Figure 3.3**). Although absorption at 225 nm was the highest among these values, this value is close to the lowest limit of the UV region and other interferences could easily absorb. At 350 nm, on the other hand, the first peak (i.e., isoorientin) did not absorb. Based on these observations, 280 nm was chosen as optimum.

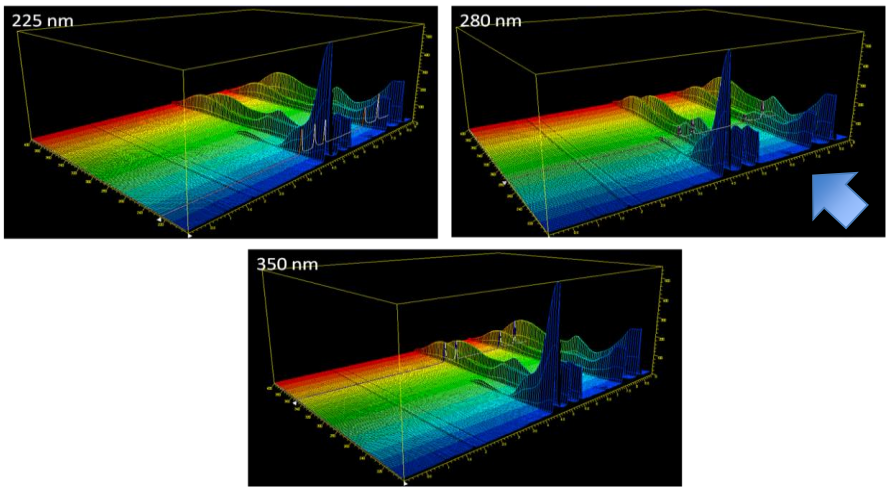


Figure 3.3: 3D plot for the maximum wavelengths of the five analytes.

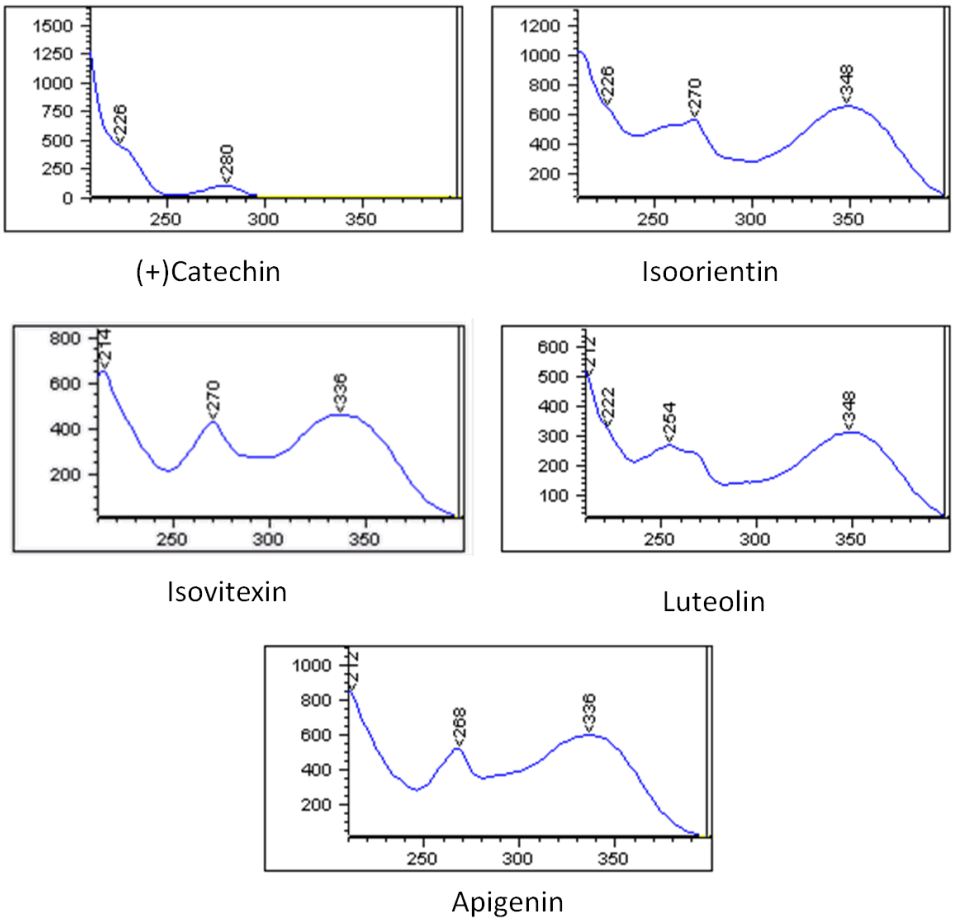


Figure 3.4: Absorption spectra of the five analytes.

3.2.1 Peak Characterization

The aim of peak characterization is to identify each peak in the chromatogram by matching it with individual standards in terms of retention time and peak spectra. The chromatograms given in **Figure 3.5** show the match of retention time of individual standards peaks to those in the chromatogram obtained for the mixture. All of these runs were done under the same chromatographic conditions as in **Table 3.2**. In addition, peak spectra for each peak in the mixture also matched with its corresponding individual peak as shown in **Figure 3.6**.

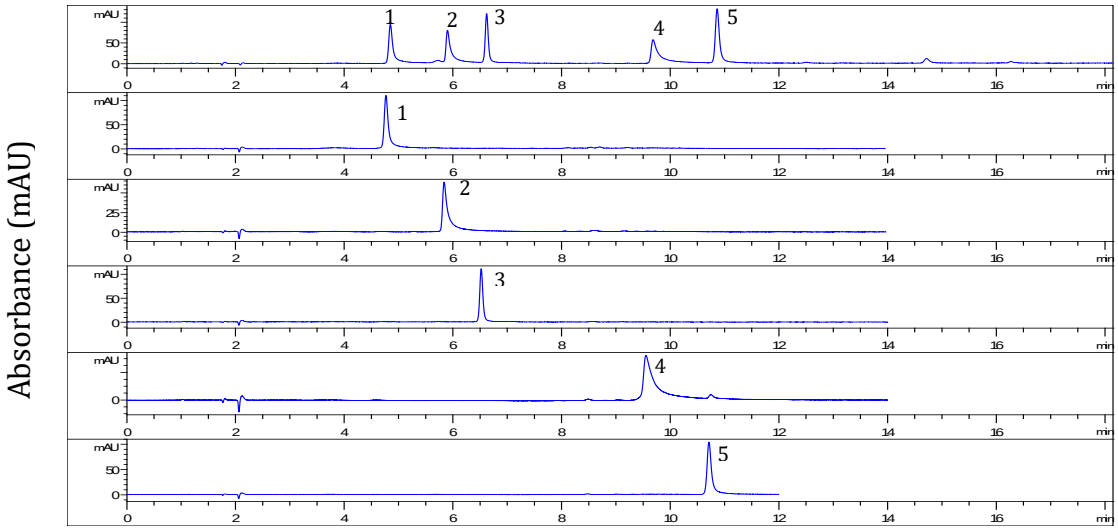


Figure 3.5: Chromatogram of five standards, matching each standard to its retention time as compared with the mixture. Peaks: 1, (+)-catechin; 2, isoorientin, 3, isovitexin, 4, luteolin and 5, apigenin.

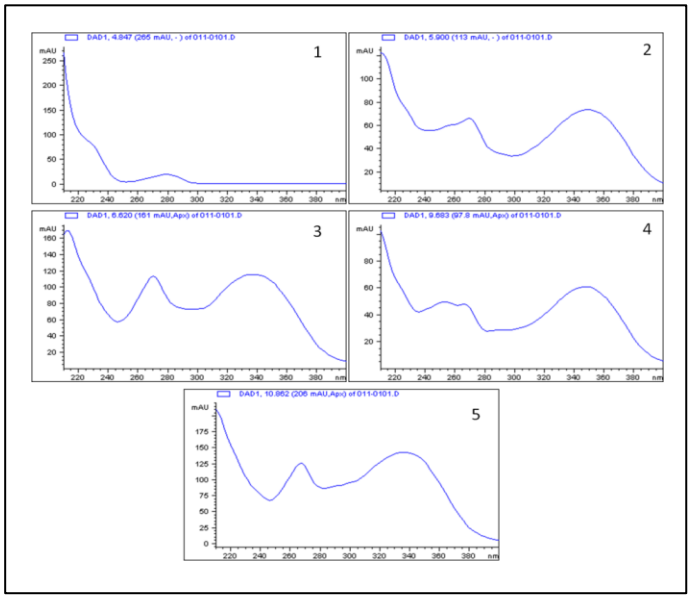


Figure 3.6: UV Spectra of the studied analytes. 1, (+)-catechin; 2, isoorientin, 3, isovitexin, 4, luteolin and 5, apigenin.

3.3 Optimization of HPLC Conditions

Equation (1.4) is a fundamental relationship of different parameters in LC, which guides the chromatographer to control resolution (R_s) by varying k'_{av}, N and α, where k'_{av} is the average of retention factors of a critical pair, N is the number of theoretical plates (efficiency) and α is the selectivity factor. k'_{av}, N and α are virtually independent, so that one term can be optimized first then the other. k'_{av} can be varied by changing the solvent strength, i.e., the ability of a mobile phase to provide large or small k'_{av} values. This parameter can be altered by changing the mobile phase composition, pH of the mobile phase, buffer concentration or the column temperature. α, on the other hand, can be changed through changes in the mobile phase or column type. N can mainly be changed by changing the mobile phase flow rate. However, the column length, diameter or particle size may also affect separation [78]. **Figure 1.1** helps to systematically change these parameters so as to obtain the best resolution.

Experimental design methodology was used to optimize the conditions. Keeping all parameters constant, one parameter was varied at a time to comprehend any change and account for it. Although simple, this approach does not take into consideration the possible interactions between the factors. The experimental design methodology is much more economical, effective and faster than a random optimization procedure. In addition, it minimizes the number of experiments performed to reach the final chromatogram.

3.3.1 Is Isocratic Elution Possible?

Based on the following mathematical estimations shown **Figure 3.7**, since $\Delta t_g > 0.25 \times t_g$, it implies that isocratic elution would not favor the separation of these analytes.

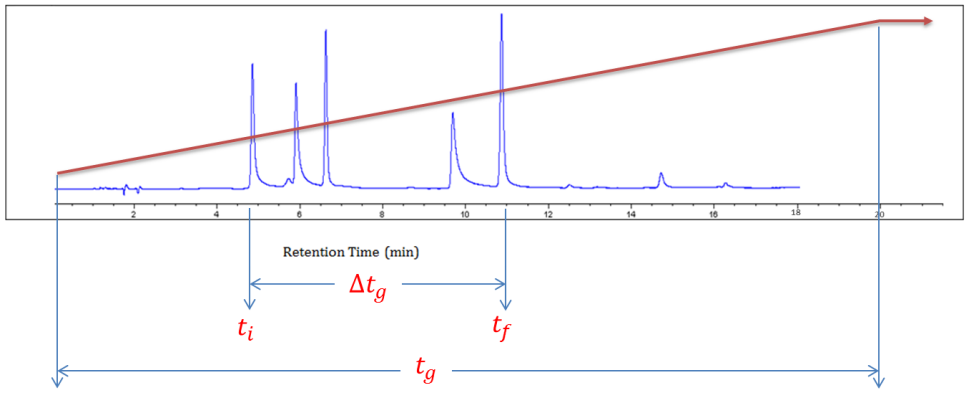


Figure 3.7: Calculations to decide on the elution mode.

$\Delta t_g = t_f - t_i$

$t_g\ (gradient\ time) = 20$

If $\Delta t_g < 0.25 \times t_g$ isocratic is possible

If $\Delta t_g > 0.25 \times t_g$ isocratic is not possible

$\Delta t_g = 10.861 - 4.848 = 6.013$

$0.25 \times t_g = 0.25 \times 20 = 5$

3.3.2 Effect of Gradient Time

Having decided that isocratic elution was not possible and gradient elution would be used, the parameters affecting the latter were optimized. These parameters include the gradient time, initial and final compositions of the mobile phase and the flow rate.

The impact of gradient time was investigated by running the gradient chromatograms for different intervals of time ranging from 12 to 22 min. From the chromatograms given in **Figure 3.8**, it seemed that the composition of the mobile phase did not show a significant effect upon the first three analytes. Conversely, a significant effect on t_R was observed of the two late coming peaks. This shows that the higher the percentage concentration of ACN in the mobile phase, the shorter the gradient time, also

the faster the analytes are eluted, based on a higher volume of %ACN. This indicates that the polarity of H_2O, which is higher than that of ACN, will tend to elute the analytes at a slower rate with a higher $\%H_2O$, because of the polarity of the analytes causing more interaction in the aqueous phase. The optimum gradient time was chosen as 12 min considering a better peak shape with less peak broadening. Analysis time was significantly reduced, also considering k'_{av} which is in the accepted range for determination of 2-10.

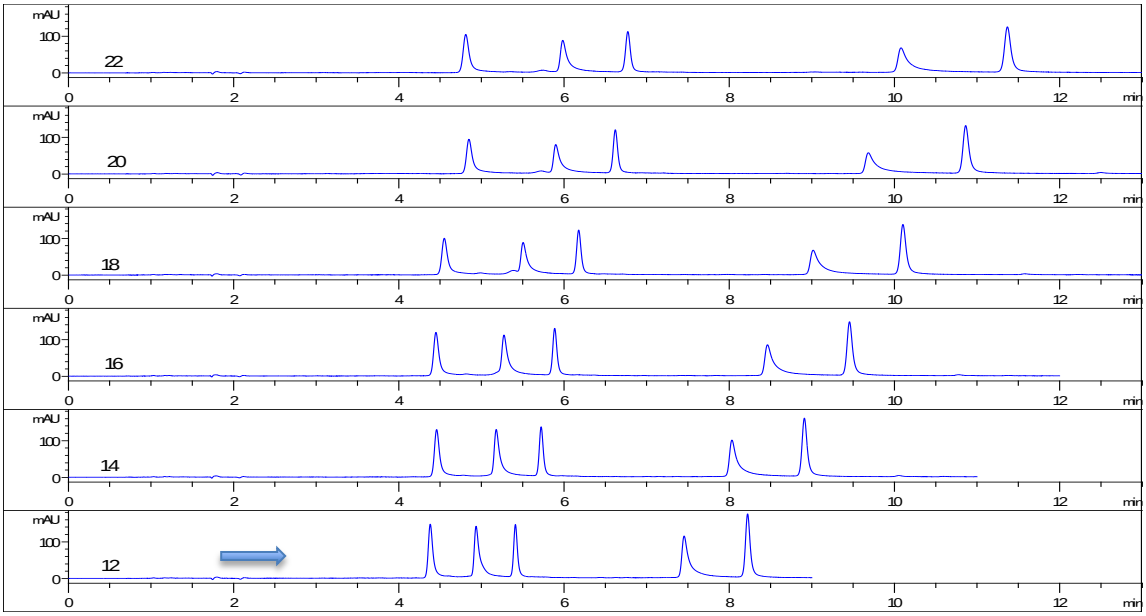


Figure 3.8: Chromatograms showing the effect of gradient time.

3.3.3 Effect of Initial Mobile Phase Composition

The next parameter which was thought to affect resolution was the initial composition of the mobile phase. The initial concentration of ACN in the mobile phase was changed within the range of 5.0 to 25% ACN.

From the chromatograms shown in **Figure 3.9** and the plot of k'_{av} (the average was calculated for the five peaks) versus initial %ACN (**Figure 3.10**), it can be deduced

that k'_{av} decreased as the concentration of ACN increased in the initial mobile phase composition. At higher percentage of ACN, α also started to decrease and the first two peaks started to co-elute. Although at 5% ACN, the analysis time was slightly higher than that it was at 10% ACN, the former was considered optimum because a better resolution would make the proposed method be applied to other matrices with minimum overlapping of other constituents in the samples.

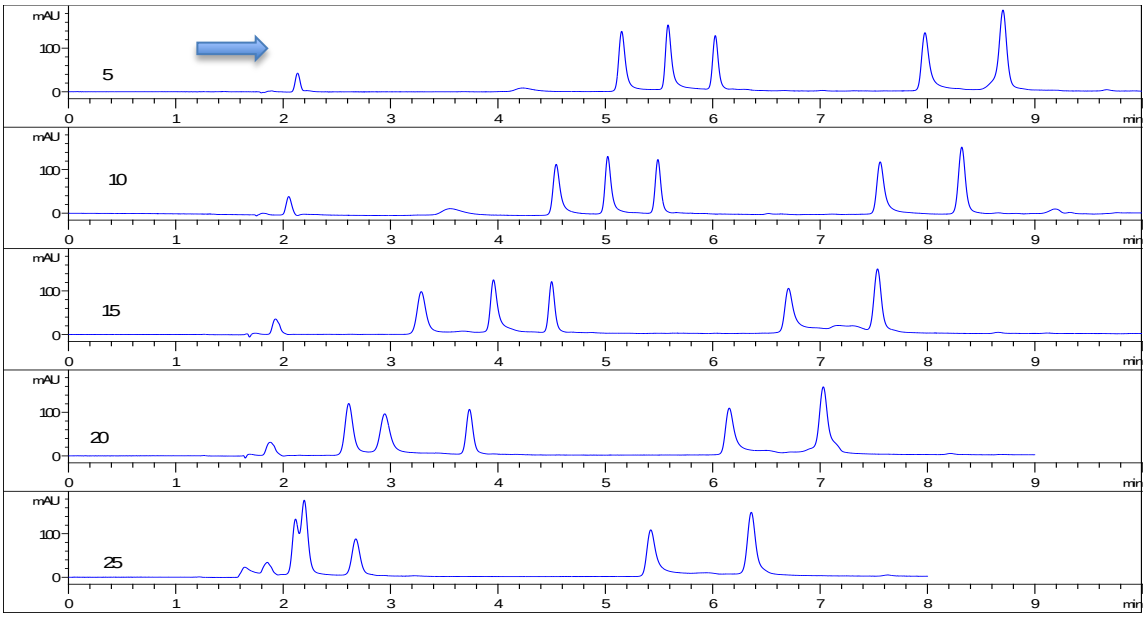


Figure 3.9: Chromatograms obtained by changing the initial composition of %ACN composition.

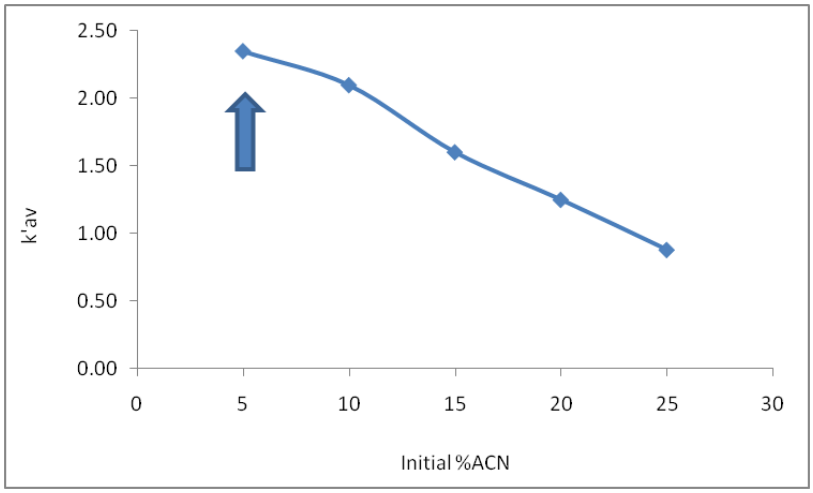


Figure 3.10: Graph of the effect of initial %ACN composition on k'_{av}.

3.3.4 Effect of Final Mobile Phase Composition

Similarly, the final composition of the mobile phase was thought to affect resolution as well. To determine the optimum final composition of the gradient system, the optimum composition for the initial %ACN was kept constant at 5%, while the final composition was varied from 80 to 100% ACN. From the graph shown in **Figure 3.11** and the chromatograms given in **Appendix 3**, it was observed that less significant effect of the final composition on k'_{av} results from changing the final mobile phase composition. A 90% ACN was taken as optimum.

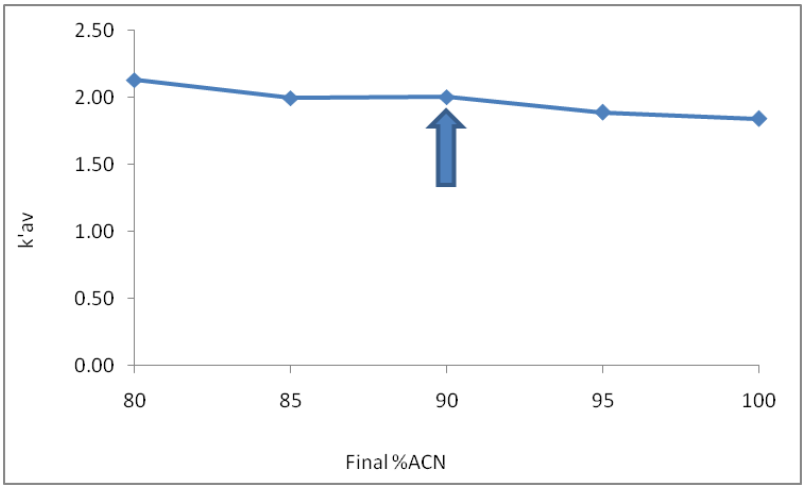


Figure 3.11: A graph of the final composition of % ACN against k'_{av}.

3.3.5 Effect of Flow Rate

The flow rate was studied within the range of 0.6–1.4 mL min^{-1} with all other parameters optimized earlier kept constant. Calculations from the chromatograms given in **Appendix 4** and **Figure 3.12,** which is showing the effect of flow rate on N, it was concluded that there was a slight but insignificant increase in N for a change of flow rate within this range. However, high flow rates are well known to be associated with an increase of the back-pressure in the system, which is the resistance of the column due to higher pressure of the pump. As can be seen from **Figure 3.13**, an increase in the flow rate gave a significant increase of an undesired back-pressure, from 120 to approximately 180 bar. **Figure 3.14** also shows the effect of the flow rate on the retention time of the five analytes in average. Expectedly, higher retention times were obtained at lower flow rates. Considering these factors, 1.0 mL min^{-1} was taken as a compromise of analysis time and back-pressure, as well as considering the peak broadening as the flow rate decreases.

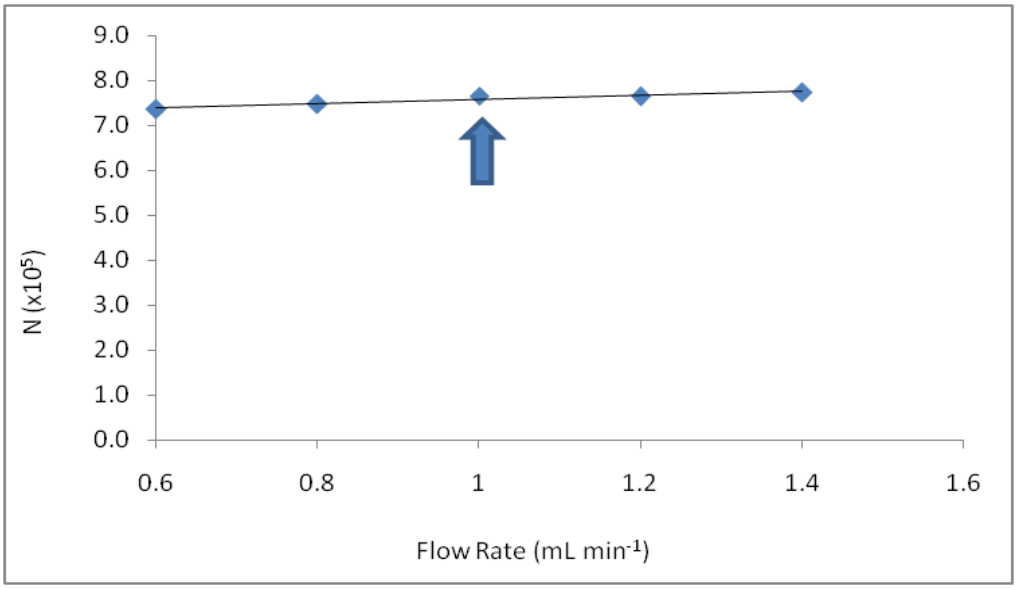


Figure 3.12: Effect of flow rate on N.

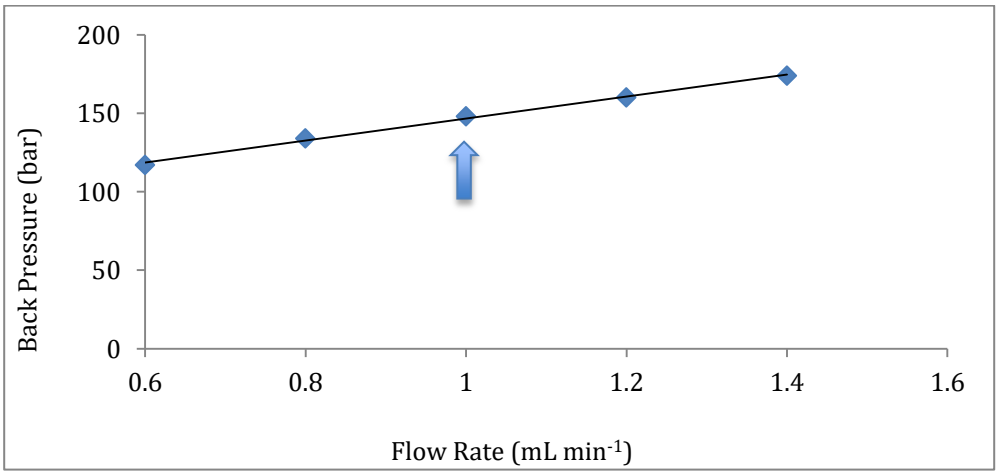


Figure 3.13: Effect of flow rate on back-pressure.

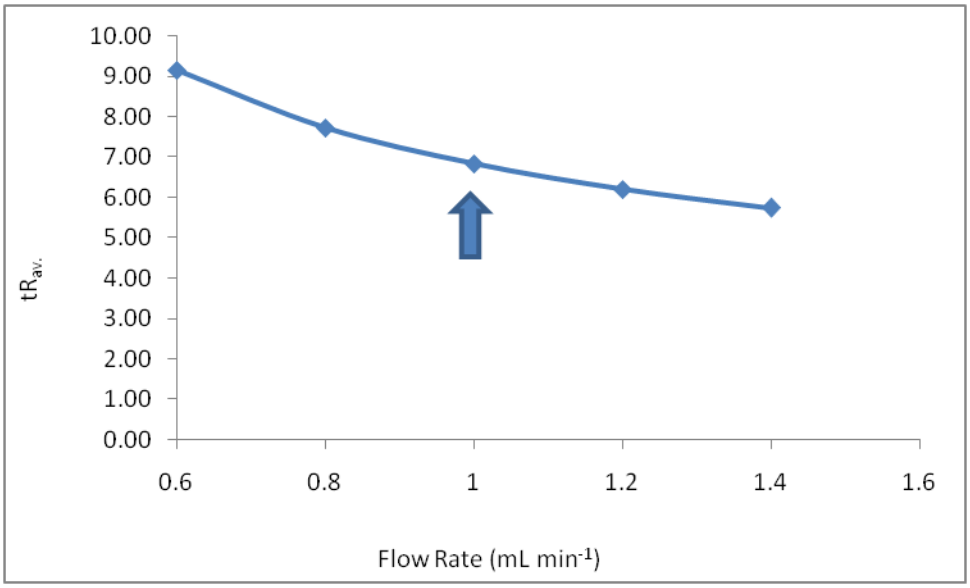


Figure 3.14: Effect of flow rate on analysis time.

3.4 Identification of Isoorientin in *A. ramosus*

The extract obtained after ultrasonic-assisted extraction of the *A. ramosus* leaves (as described in **Section 2.6**) was injected into the HPLC system under the optimized conditions after appropriate dilution with 45/55 (v/v) ACN/H_2O was made. The resulting chromatogram is shown as in **Figure 3.15**. It can be observed from the real sample chromatogram that the peak of isoorientin eluted at the same retention time as that of the standard. In addition, absorption spectra were compared and were find to match more that 99.9% as calculated using the software (Figure Insert in **Figure 3.15** and **Figure 3.4**). Moreover, no interfering peaks from the sample co-eluted with the analyte peak, showing a good selectivity of the optimized method. Isoorientin was found to be the most abundant flavonoid identified in *A. ramosus*.

The peak, being identified as isoorientin, was quantified through calibration with the isoorientin standard.

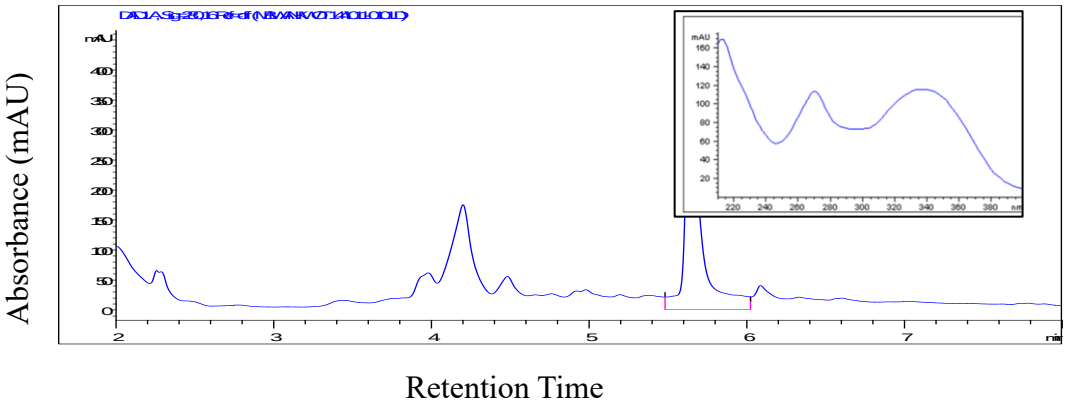


Figure 3.15: A chromatogram of the real sample.

3.5 Analytical Performance and Figures of Merit

3.5.1 External Aqueous Calibration Graph

The calibration curve was established by injecting isoorientin standards prepared in 45:55 (v/v) of ACN/H_2O at varying concentrations within the range of 15 to 75 mg L^{-1}. At least three replicate runs were performed. To obtain a calibration graph, peak areas, giving a better reproducibility than peak height, obtained from each injection was plotted against the analyte concentration. The response was found to be linear throughout this range. The calibration graph is shown in **Figure 3.16**.

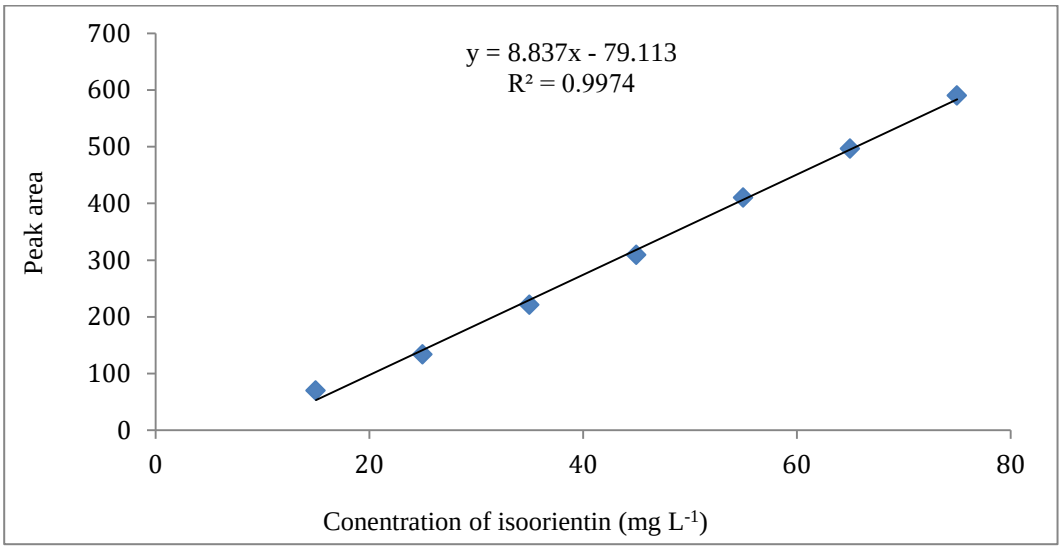


Figure 3.16: Calibration curve obtained with isoorientin standard solutions.

Regression equation, coefficient of determination (R^2), precision in terms of intraday and interday percentage relative standard deviation (%RSD), limit of detection (LOD), limit of quantitation (LOQ), and linear dynamic ranges (LDR) are summarized in **Table 3.3**.

The response was linear over the concentration range from LOQ up to 75.0 mg L^{-1}, with R^2 being 0.9974. LOD and LOQ were 1.65 and 4.5 mg L^{-1}, respectively. Reproducibility of the proposed method was evaluated in terms of intraday and interday precision. An acceptable precision was obtained with %RSD values of 3.3% for intraday and 4.5% for interday experiments.

Table 3.3: Figures of merit of the proposed method.

Analyte	Regression equation[a]	R^2	RSD (%)[b]		LOD[b] (mg L^{-1})	LOQ[c] (mg L^{-1})	LDR[d] (mg L^{-1})
			Intraday	Interday			
Isoorientin	$y = 8.837(\pm 0.13)x - 79.113(\pm 6.39)$	0.9974	3.3	4.5	1.65	4.5	4.5– 75

[a] Peak area = slope (± SD) × concentration (mg L^{-1}) + intercept (± SD)

[b] Percentage relative standard deviation, $n = 7$

[c] Limit of detection

[d] Limit of quantitation

[e] Linear dynamic range

3.6 Quantification of Isoorientin in *A. ramosus* Leaves

Quantification of isoorientin in *A. ramosus* leaves was done using the external calibration obtained with the standards. The sample was injected under the same conditions after being diluted 3, 5 and 10 times with 45/55 (v/v) ACN/H_2O. The results are overlaid with the calibration graph in **Figure 3.17**.

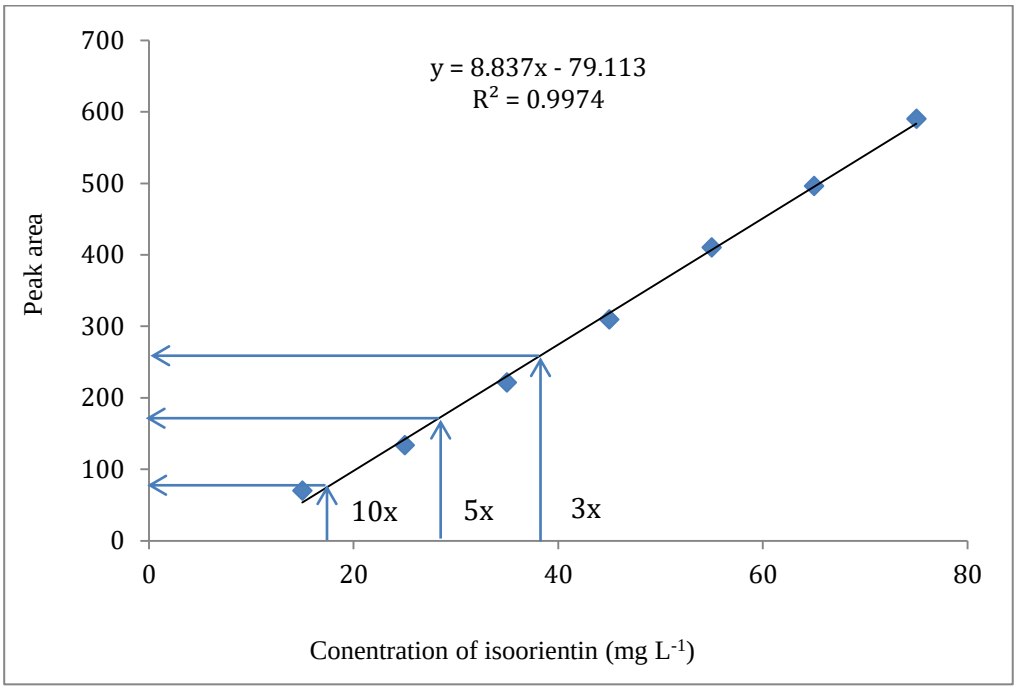


Figure 3.17: Determining the concentration of isoorientin in the sample solution with $3\times$ $5\times$ *and* $10\times$ dilutions.

Therefore, using the external standard calibration curve, the average concentration of isoorientin in the sample solution of the *A. ramosus* leave extract was found to be 781.8 mg L^{-1}, which corresponded to 0.15% of isoorientin in the sample.

CHAPTER 4
CONCLUSIONS AND RECOMMENDATIONS

Reversed-phase high-performance liquid chromatography with a photo-diode array detector (RP-HPLC-DAD) as a reliable instrument was used for the separation of five flavonoids (+)-catechin, isoorientin, isovitexin, luteolin and apigenin and the quantitation of isoorientin in leaves of *A. ramosus*. The conditions optimized showed that this method is fast, efficient, inexhaustible and cheap. Considering the wide range of flavonoids and their similarity in structure, this method was proven to be able to separate these five important flavonoids. The method can be applied to determine these flavonoids in other *Asphodelus* species, which were commonly found in different *Asphodelus* species in the literature. Further studies should focus on extraction methods that would be able to preconcentrate these analytes to make them detectable and quantifiable at lower concentrations in the plants.

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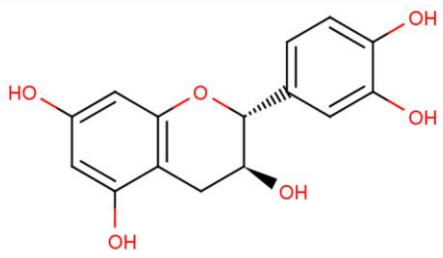
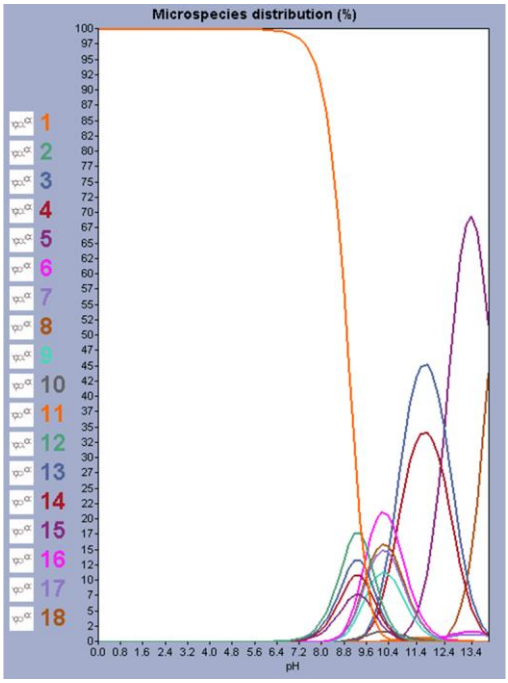
APPENDICES

Appendix 1: Microspecies distribution versus pH of the studied analytes.

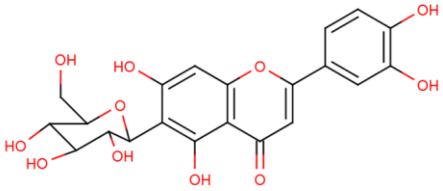
(+)-Catechin



$logP = 1.8$



Isoorientin



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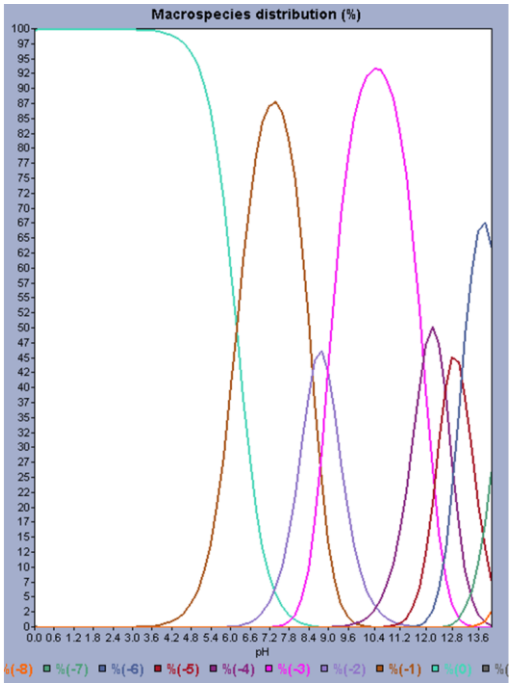
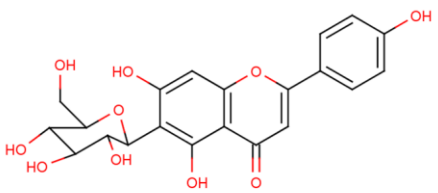
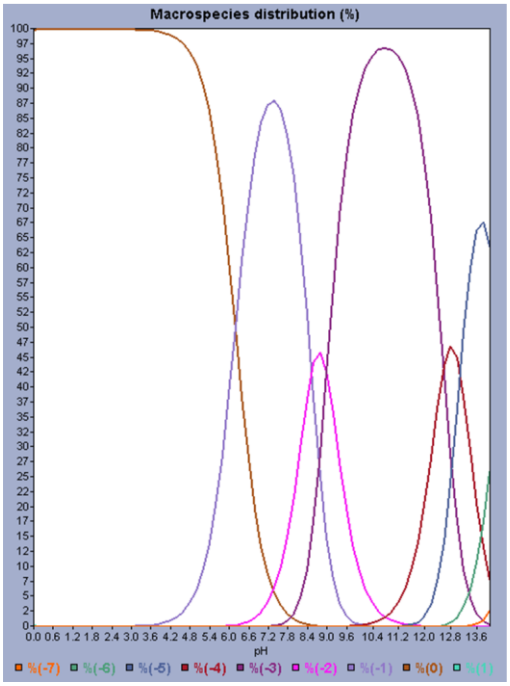


Appendix 1: Cont'd.

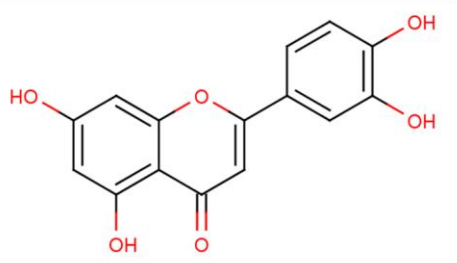
Isovitexin



$logP = -0.05$



Luteolin



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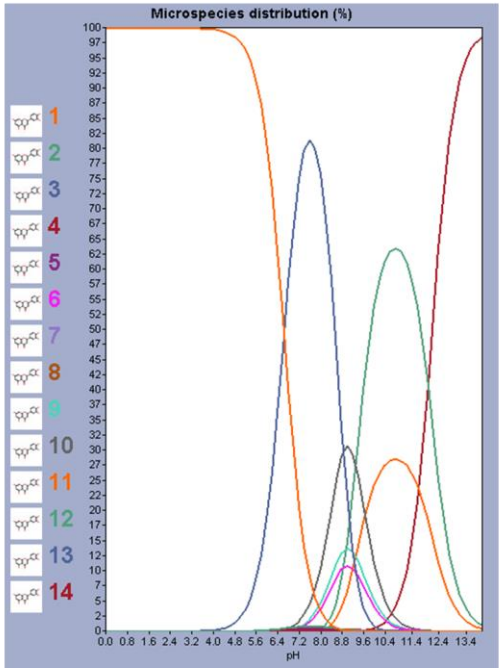
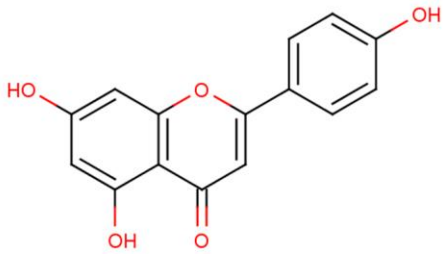
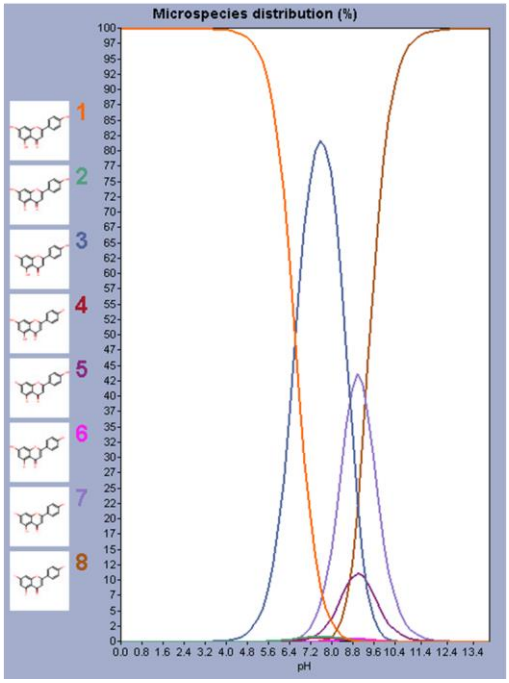


Appendix 1: Cont'd.

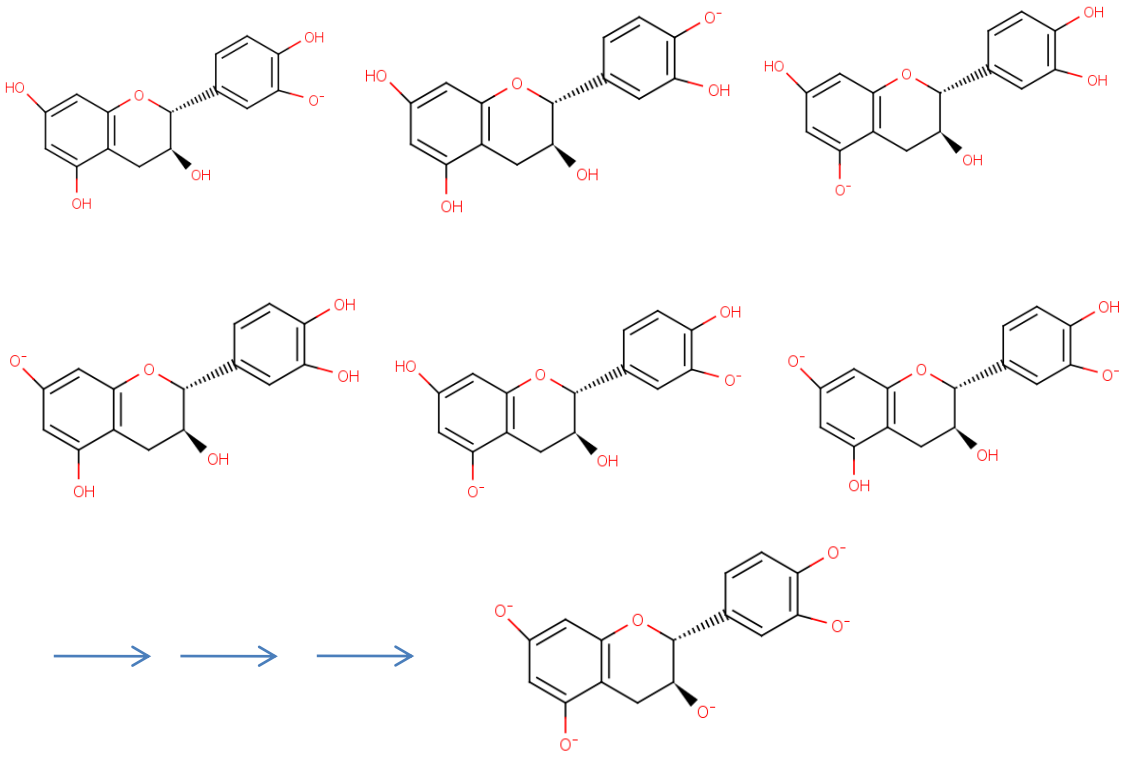
Apigenin



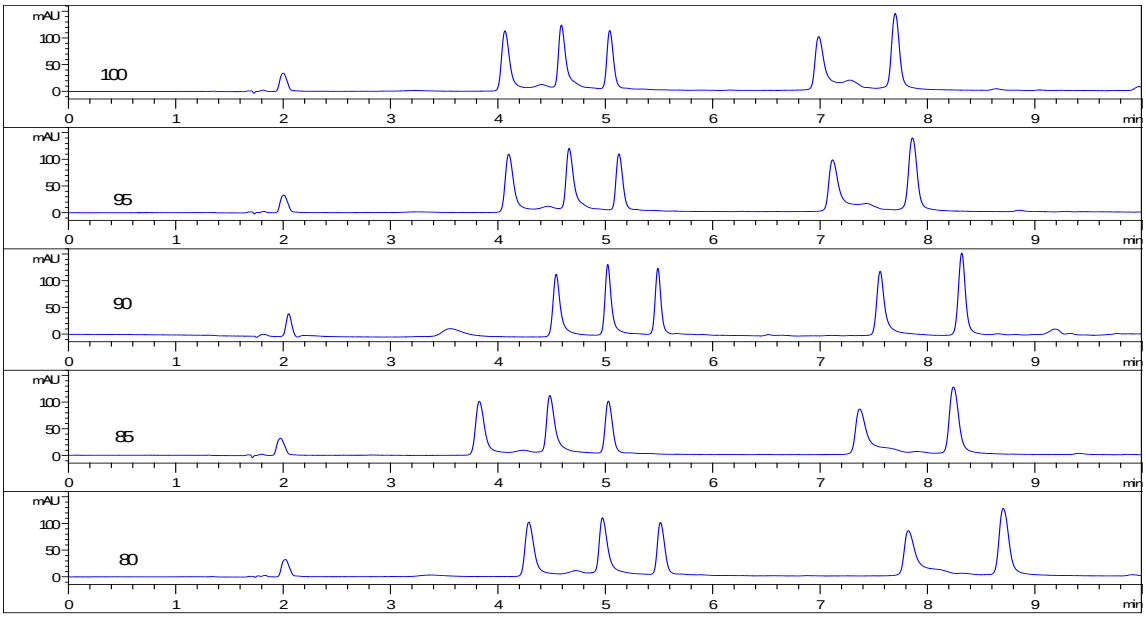
$logP = 2.71$



Appendix 2: Possible ionizations of (+)-catechin with change in pH.



Appendix 3: Chromatograms showing the effect of finial % of ACN.



Appendix 4: Chromatograms showing the effect of flow rate.